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The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

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FRONTIER

Management of obstructive colon cancer: Current status, obstacles, and future directions

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Abstract

Approximately 10%–18% of patients with colon cancer present with obstruction at the initial diagnosis. Despite active screening efforts, the incidence of obstructive colon cancer remains stable. Traditionally, emergency surgery has been indicated to treat patients with obstructive colon cancer. However, compared to patients undergoing elective surgery, the morbidity and mortality rates of patients requiring emergency surgery for obstructive colon cancer are high. With the advancement of colonoscopic techniques and equipment, a self-expandable metal stent (SEMS) was introduced to relieve obstructive symptoms, allowing the patient's general condition to be restored and for them undergo elective surgery. As the use of SEMS placement is growing, controversies about its application in potentially curable diseases have been raised. In this review, the short- and long-term outcomes of different treatment strategies, particularly emergency surgery *vs* SEMS placement followed by elective surgery in resectable, locally advanced obstructive colon cancer, are described based on the location of the obstructive cancer lesion. Controversies regarding each treatment strategy are discussed. To overcome current obstacles, a potential diagnostic method using circulating tumor DNA and further research directions incorporating neoadjuvant chemotherapy are introduced.

Key Words: Colonic neoplasms; Self-expandable metallic stents; Intestinal obstruction; Survival rate; Morbidity; Mortality

Core Tip: The optimal management of obstructive colon cancer remains intricate and

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controversial, particularly now that the use of colonoscopic stents is expanding. Several studies have demonstrated the better short-term outcome achieved by delayed surgery after self-expandable metal stent (SEMS) placement than emergency surgery. However, concerns that SEMS placement diminishes oncologic outcomes have been increasing. This article summarizes the impact of SEMS placement in managing obstructive colon cancer and suggests future research to resolve current problems.

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INTRODUCTION

Obstructive colon cancer requiring emergency surgical or procedural intervention accounts for 10%-18% of patients initially diagnosed with colon cancer[1-3]. With obstructive symptoms of nausea, vomiting, abdominal pain, and severe distension, patients often present with physical deterioration resulting from the catabolic state of muscle wasting and poor oral intake. Occasionally, patients with obstructive colon cancer have concomitant spontaneous colon perforation, a frightening complication associated with high postoperative morbidity and mortality, at either the cancer site or proximal colonic segment[4]. Moreover, the obstructive feature of colon cancer itself is an independent high-risk feature of recurrence, and patients with obstructive colon cancer tend to have an advanced cancer stage with poor prognostic factors[5]. Compared to patients undergoing elective surgery, patients requiring emergency surgery for obstructive colon cancer have worse short-term and long-term oncologic outcomes[1,2,5].

Unfortunately, despite active screening efforts, the incidence of obstructive colon cancer remains stable[3,6]. The management of obstructive colon cancer is complex and challenging due to multiple factors, including the patient's index presentation of a poor general condition, limited information regarding the cancer stage, and the need for immediate intervention in an emergency setting. This article addresses the current treatment optionsr, difficulties, and future directions for managing obstructive colon cancer to achieve an optimal outcome.

LOCATION OF OBSTRUCTIVE CANCER LESION

It is well known that the most common cause of colonic obstruction in adults is colorectal cancer[7]. Abdominopelvic computerized tomography scans are an excellent diagnostic modality that is readily available and accurately localizes obstructive cancer lesions with high sensitivity and specificity[8]. Obstructive colon cancer is usually classified as right-sided or left-sided according to proximal or distal to the splenic flexure. Previous studies indicate that more than half of acute obstructive colon cancer occurs on the left side, most commonly in the sigmoid colon[1,6,8]. The anatomic characteristic of a narrow luminal diameter in the left-sided colon explains the higher incidence of obstructive colon cancer compared to the right-sided colon. on the other hand, a large diameter of the cecum and ascending colon allows a bulky characteristic of the tumor. Indeed, right-sided obstructive colon cancer seems more locally advanced than left-sided[3]. An obstructive cancer lesion in the rectum is the least frequent due to the sizeable luminal diameter of the rectum and the symptoms of bleeding and defecation difficulty caused by rectal cancer itself[3].

The anatomic characteristics of the colon and rectum have led to different treatment approaches depending on the obstructive lesion location. Primary tumor resection with ileocolic anastomosis has been preferred to treat right-sided cancer obstruction [9]. Generally, oncologic resection of the right-sided colon is considered less arduous. Additionally, the relatively low anastomotic complication rate, from 2.8% to 4.6%, supports primary resection and anastomosis[10]. Double-barrel entero-colostomy after primary tumor resection is an alternative for patients with a high risk of anastomotic leakage. Compared to right-sided obstructive colon cancer, left-sided colon cancer has

variable treatment options, primarily comprised of emergency primary tumor resection with or without stoma formation or decompression followed by delayed tumor resection. The choice is usually made based on the patient's general condition, availability of resources, and complete oncologic resection feasibility. In obstructive rectal cancer, optimal oncologic resection with total mesorectal excision is unobtainable. Rectal cancer that causes acute obstruction is usually locally far advanced and highly likely to invade adjacent urogenital organs, large neurovascular structures, and even bony structures. Therefore, for obstructive rectal cancer, decompression to relieve acute symptoms is more desirable than primary tumor resection to avoid serious intraoperative morbidity and suboptimal surgical outcomes. This article discusses the details regarding treatment options and the outcomes of obstructive colon cancer.

TREATMENTS AND THEIR OUTCOMES

Right-sided cancer obstruction

Traditionally, primary oncologic resection *via* right hemicolectomy or extended right hemicolectomy with ileocolic anastomosis has been advocated for right-sided obstructive colon cancer[11]. A systematic review of studies related to treating right-sided obstructive colon cancer demonstrated that 86% of patients underwent emergency resection[12]. Less strenuous surgical techniques for mobilization and resection of the right-sided colon lead many surgeons to prefer primary tumor resection in an emergency setting. Compared to colocolic or colorectal anastomosis, many surgeons pursue primary ileocolic anastomosis, even in frail patients, due to the abundant blood supply and relatively simple manipulation of the dilated proximal bowel with enough length. A recently published multicentered retrospective study on the outcomes of elderly patients treated for obstructive colon cancer revealed that 97% of patients received upfront surgery for proximal colon cancer obstruction[13]. In the study, 54% of patients were over 75 years of age. The rate of resection with primary anastomosis among the elderly patients was not different from that of the younger patients.

Not surprisingly, the short-term outcome after emergency right hemicolectomy with ileocolic anastomosis for obstructive right-sided cancer is worse than that after elective surgery for right-sided colon cancer. Postoperative morbidity after emergency surgery is reported to range from 46% to 54%[13,14]. Compared to the morbidity rate of 30% after elective right hemicolectomy for colon cancer[15], the postoperative morbidity rate after emergency surgery is much higher. The rate of anastomotic leakage after emergency surgery is reported to range from 12% to 16.4%[13,14], which is also higher than the leakage rate of 4.1% after elective surgery[16]. As expected, the postoperative mortality rate after emergency surgery is 14.5%, higher than the rate of 2.6% after elective surgery. Risk factor analysis for anastomotic leakage supports the notion that emergency surgery imposes a greater risk of anastomotic leakage and leakage-related mortality[17]. Such short-term outcomes indicate that oncologic resection with primary anastomosis for right-sided obstructive colon cancer in an emergency setting may not be as uncomplicated as anticipated.

Other surgical treatment options in an emergency setting may include the creation of loop ileostomy after resection and anastomosis, the creation of end ileostomy after resection only, the creation of loop ileostomy without resection, or enterocolic bypass surgery. For resectable right-sided obstructive colon cancer, primary tumor resection may be an optimal treatment option. In cases of unstable hemodynamics or severe intraperitoneal contamination by bowel perforation, loop ileostomy or end ileostomy is unavoidable. However, high output from ileostomy and related morbidities, such as dehydration, electrolyte imbalance, and acute renal failure, frequently occurs. Distressingly, surgeons encounter the dilemma of risking anastomotic leakage or stoma-related morbidity. For unresectable right-sided obstructive colon cancer, the creation of loop ileostomy or enterocolic bypass surgery is recommended to decompress proximal bowel dilatation, alleviating bowel obstruction symptoms[11]. Relevant studies on the treatment options for unresectable right-sided obstructive colon cancer are scarce and limited to the formation of percutaneous cecostomy, which is currently not performed due to malfunctions and complications or is reserved only for a small number of patients with very high morbidity[11,18].

Staged operation after endoscopic placement of the SEMS may offer a possible treatment option for both resectable and unresectable right-sided obstructive colon cancer. However, the technical difficulty seems to hamper the wide application of

colonoscopic stenting for right-sided colonic obstruction. Additionally, due to a lack of evidence, the use of the SEMS as a bridge to elective surgery is currently not recommended, except for patients with high morbidity[11]. Nevertheless, a newly updated systematic review comparing the treatment outcomes of staged operations after SEMS placement in curable right-sided obstructive colon cancer demonstrated that the technical success rate reached 96%, disputing the previous belief of technical difficulty[12]. Furthermore, the postoperative complication rate is approximately 30%, ranging from 7% to 44%[12,14], similar to the morbidity rate after elective surgery. After the staged operation, the rate of anastomotic leakage is estimated to be approximately 5.5%[12], comparable with the rate observed after elective surgery. The mortality rate of 1.2% after the staged operation is comparable to elective surgery[12]. The long-term oncologic outcome of right-sided obstructive colon cancer appears to be equivalent or better in patients who underwent the staged operation after SEMS insertion than in patients who received emergency surgery[19].

Current evidence provides safety and feasibility of the staged operation after SEMS insertion to treat right-sided obstructive colon cancer. However, the studies were mostly designed retrospectively with small sample sizes. Patients with unstable hemodynamics would have been excluded from the staged operation, leading to selection bias. The short-term outcome might have been related to the staged operation. On the other hand, such evidence indicates that the staged operation after SEMS placement can achieve a better short-term outcome in a certain group of patients. The selection criteria of an appropriate patient for the staged operation after SEMS placement can be suggested. Because SEMS insertion is impossible if an obstructing cancer is located in the cecum or ileocolic valve, SEMS insertion could be considered when the obstructive cancer is located beyond the cecum in the abdominopelvic computed tomography (CT) scan. Additionally, experts in colonoscopy seem to handle a long colonic length of the distal segment. Therefore, if an expert in colonoscopy is available and there is no sign of perforation on CT imaging, colonoscopic SEMS insertion may be an option before undergoing emergency surgery. After all, optimal treatment for right-sided obstructive colon cancer should be determined based on the resectability of the tumor, presence of perforation and peritonitis, and hemodynamic stability of the patient. Large-sized prospective comparative studies are necessary to obtain concrete evidence.

Left-sided cancer obstruction

Compared to those for right-sided obstructive colon cancer, treatment options for left-sided obstructive colon cancer are diverse and controversial. Conventionally, primary tumor resection with end stoma formation has been highly preferred to treat obstructive left-sided colon cancer in an emergency setting[20,21]. However, emergency surgery itself has been identified as an independent risk factor for mortality[22]. The postoperative complication rate is higher than the rate after elective surgery[8,23]. A significant number of patients end up with temporary or permanent stoma after emergency surgery. Furthermore, subsequent surgery for stoma reversal is associated with a high morbidity rate of 21% to 36%[24,25]. Up to 71% of patients never undergo surgery for stoma reversal, significantly affecting the quality of life[25,26]. The risk factors related to nonreversal of the stoma include advanced age, a postoperative complication that occurred after emergency surgery, comorbidity, and advanced cancer stage[24,26]. Although the operative approach with primary tumor resection with end stoma is considered the safest option due to the absence of anastomotic complications[20], the two-stage operation is complex and may significantly reduce patient quality of life.

Diverting stoma is another operative procedure for damage control in acute left-sided colonic obstruction. The formation of a diverting stoma is followed by the second-stage operation of primary tumor resection with or without colostomy closure. Stoma closure can take place at a third stage. A diverting stoma may stabilize the patient's general condition and allow bowel preparation and proper staging before oncologic resection. However, patients for whom a three-stage procedure is planned may not undergo subsequent operations, even if they are considered fit. When the morbidity and mortality associated with each surgical stage are considered cumulatively, the staged operation does not provide any advantage. Furthermore, a randomized controlled trial comparing emergency colostomy followed by a staged operation to emergency Hartmann's procedure showed similar morbidity and mortality rates between two procedures; thus, the authors did not support the use of colostomy in frail patients[27]. One advantage of the staged operation is a lower rate of permanent stoma. However, patients who undergo staged operations require a more extended hospital stay for additional surgical procedures following initial colostomy

formation. Nevertheless, due to a lack of evidence, a Cochrane systematic review in 2004 could not conclude which of the two alternative approaches was the most beneficial in acute left-sided obstructive colon cancer[28].

In an emergency setting, the reconstruction of bowel continuity has been avoided in the management of acute left-sided colonic obstruction due to the risk of anastomotic leakage. However, a two-stage operation followed by Hartmann's operation has the significant disadvantage of cumulative morbidity and mortality associated with a second operation and reduced quality of life when the stoma is kept. Nonetheless, some colorectal surgeons attempted to create a primary anastomosis after emergent primary resection and demonstrated its feasibility and safety in selected patients[29,30]. In a systematic review, Breitenstein *et al*[31] evaluated the superiority of the one-stage procedure compared to multistage procedures for left-sided obstructive colon cancer. The authors demonstrated that primary resection and anastomosis were superior to two- or three-stage operations in terms of mortality, with a relative risk difference from -2% to -27%[31]. It appeared that selection bias strongly affected the study result, in which patients with better prognoses were more likely to have a one-stage operation. Previous studies extensively investigated risk factors related to mortality and anastomotic leakage and identified high-risk conditions, such as advanced age, presence of comorbidity, advanced tumor stage, malnutrition, and presence of peritoneal contamination[22,32]. Currently, primary resection and anastomosis are the preferred options for uncomplicated left-sided obstructive colon cancer without risk factors for anastomotic leakage[11].

There are two main methods of resection, and the optimal procedure type is still debated. Segmental colonic resection with intraoperative colonic irrigation is one option, and the other is subtotal or total colectomy. Segmental resection can preserve the proximal colonic segment, but on-table lavage is time-consuming and may result in fecal spillage[33]. Subtotal or total colectomy is advocated for low risk of anastomotic leakage in ileocolic or ileorectal anastomosis[34,35]. It can eliminate the distended proximal colon with ischemic lesions and serosal tears on the cecum, reducing the risk of fecal spillage and contamination. Subtotal or total colectomy can also effectively manage synchronous tumors in the proximal colon[36]. Negative aspects of subtotal or total colectomy include the need for an experienced surgeon or subspecialist, a prolonged operation time, and a decreased bowel function[37]. In terms of morbidity and mortality, a multicenter randomized controlled trial demonstrated no differences when two different surgical procedures – total/subtotal colectomy *vs* segmental colectomy with on-table lavage – were compared[37]. Therefore, the current 2017 WSES guideline states that total colectomy is not preferred to segmental colectomy in the absence of impending perforation in the cecum, evidence of bowel ischemia, or synchronous right colonic cancers[11].

As in right-sided obstructive colon cancer, endoscopic stent placement followed by the staged operation is an alternative option frequently applied in left-sided obstructive colon cancer. Although SEMS was first introduced for palliative treatment in unresectable colorectal cancer[38], its use has been expanded to relieve colonic obstruction to avoid emergency surgery in resectable disease[39]. Its role as a "bridge to surgery" for patients with curable disease has been widely accepted because it allows planned curative resection after the adequate restoration of the patient's general condition and bowel preparation[40]. The use of SEMS appeared to decrease morbidity and mortality and the rate of stoma formation compared to emergency surgery[41]. Ultimately, its efficacy and effectiveness in reducing medical costs and improving quality of life were shown to be associated with shorter hospital stays, fewer stays in the intensive care unit, and fewer surgical procedures[41-43].

However, other studies comparing the SEMS decompression outcome to emergency surgery demonstrated controversial results in terms of morbidity, mortality, and the stoma rate. An observational study by Kavanagh *et al*[44] comparing emergency surgery to SEMS placement reported no difference in postoperative morbidity and mortality and the stoma formation rate, although technical and clinical success with SEMS placement was achieved in 91% and 83% of patients. A systematic review and meta-analysis including a total of 197 patients, 97 with SEMS placement *vs* 100 with emergency surgery, reported a clinical success rate of only 52.5% in the SEMS group in contrast to a rate of 99% in the emergency surgery group[45]. The overall complication rate and 30-d postoperative mortality rates of both groups were similar. The two groups also showed no significant difference in the permanent stoma rate or anastomotic leakage rate. In a systematic review and meta-analysis based on seven different randomized controlled trials, Huang *et al*[43] reported that the mean technical success rate was 77%. In contrast, the permanent stoma rate, primary anastomosis rate, and overall mortality rate were 9%, 67%, and 11%, respectively[43].

It appears that the location and length of an obstructing cancer lesion influence technical and clinical success[46]. The European Society of Gastrointestinal Endoscopy (ESGE) guidelines, updated in 2020, suggests that by an experienced endoscopist or under the supervision of an expert, colonic stenting should be attempted with an individually tailored method to the length of the stenosis and location of the tumor [47].

Despite the controversy, the use of SEMS is endorsed by surgeons pursuing minimally invasive elective surgery with laparoscopy[48,49]. The multimodal approach using endoscopic and laparoscopic procedures demonstrated favorable short-term outcomes in SEMS placement as a bridge to surgery, offering less invasive treatment than multistage open surgery. In a meta-analysis including eight different randomized controlled trials comparing SEMS decompression as a bridge to surgery to emergency surgery from 2009 to 2016, Arezzo *et al*[50] confirmed similar rates of morbidity and mortality in both treatment approaches. Nonetheless, SEMS decompression showed a significantly lower rate of temporary and permanent stoma and a higher success rate of primary anastomosis than emergency surgery[50]. Based on many clinical studies and other randomized controlled trials, the 2017 WSES and ESGE guidelines concluded that SEMS decompression as a bridge to elective surgery offers a better short-term outcome than immediate emergency surgery[11,47].

The long-term oncological outcome remains uncertain and rather suboptimal when SEMS decompression was compared to emergency surgery, as shown in Table 1. Several meta-analyses evaluating the oncologic outcome of patients who underwent SEMS decompression followed by elective surgery demonstrated that disease-free survival and overall survival were not significantly different from those who received emergency surgery[51]. Additionally, in the recent update of the randomized controlled trial by Arezzo *et al*[52], the 3-year overall survival, time to progression, and disease-free survival of the patients with SEMS decompression as a bridge to surgery were not significantly different from those of the patients with emergency surgery. However, the meta-analysis of eight different randomized controlled trials by Yang *et al*[53] showed that patients with SEMS decompression presented a higher tumor recurrence rate than patients with emergency surgery, with an odds ratio of 1.79 (95% confidence interval 1.09–2.93). Another meta-analysis of seven randomized controlled trials by Foo *et al*[54] demonstrated that the overall recurrence rate in the SEMS decompression group was higher than that in the emergency surgery group, at 37.0% and 25.9%, respectively. Although the 3-year overall survival and disease-free survival were not different between the two groups, the risk ratio of systemic recurrence was 1.627 for the SEMS decompression group[54]. Nevertheless, available data are limited, and the sample size is inadequate to draw a firm conclusion on the long-term oncological outcome of SEMS decompression as a bridge to surgery. The ESGE guidelines currently recommend that decision-making for individual patients be influenced by the relative importance of particular endpoints, either short-term outcomes or long-term outcomes[47]. Patients should be informed within a shared decision-making process to use SEMS decompression as a bridge to surgery in potentially curable left-sided obstructive colon cancer regarding a potentially higher risk of recurrence[47].

PITFALLS OF THE SEMS DECOMPRESSION FOLLOWED BY DELAYED OPERATION

Stent-related complications are a vital issue to address. A previous randomized controlled trial comparing SEMS placement to emergency surgery in left-sided obstructive colon cancer was terminated prematurely due to a high technical failure rate of 53.3%[49]. As the most lethal complication, colonic perforation during the procedure was the main reason for the premature closure of the trial. Other complications include stent migration, failure to expand within the colonic lumen, bleeding, and subsequent reobstruction[46]. The rate of colonic perforation related to stent placement was reported to be as high as 12.8%[55]. Stent migration and bleeding occur less frequently at a rate of 0% to less than 5%[55,56]. As expected, colonic perforation during the procedure, failure to self-expand, or bleeding requires emergency surgery, which may lead to mortality directly related to SEMS placement. Currently, the 30-d stent-related mortality is estimated to be 4%[46].

Stent-related perforation has not only an imminent mortality risk but also existing concerns about its implication in worsening oncological outcomes. In a recent systematic review and meta-analysis evaluating the oncological outcome of patients

Table 1 Oncological outcome after self-expandable metal stent placement as a bridge to surgery *vs* emergency surgery in malignant colonic obstruction

Ref.	Year	Study population	Study design	Location of obstructive cancer	Survival outcome
Matsuda *et al* [75]	2015	*n* = 1136: (1) BTS = 432; and (2) ES = 704	Meta-analysis: (1) 2 RCTs; (2) 2 prospective nonrandomized comparative studies; and (3) 7 retrospective comparative studies	Right- and left-sided	(1) No difference in disease-free survival and overall survival; and (2) No difference in recurrence
Ceresoli *et al* [76]	2017	*n* = 1333: (1) BTS = 688; (2) ES = 655	Meta-analysis: (1) 5 RCTs; (2) 3 prospective nonrandomized comparative studies; and (4) 9 retrospective comparative studies	Left-sided	(1) No difference in local recurrence and overall recurrence; (2) No difference in 3-yr and 5-yr recurrence; and (3) No difference in 3-yr and 5-yr mortality
Yang *et al*[53]	2018	*n* = 497: (1) BTS = 251; and (2) ES = 246	Meta-analysis: 8 RCTs	Left-sided	Higher tumor recurrence rate in BTS with an odds ratio of 1.79, 95%CI: 1.09–2.93
Amelung *et al*[51]	2018	*n* = 1919: (1) BTS = 938; and (2) ES = 981	Meta-analysis: (1) 5 RCTs; (2) 4 prospective nonrandomized comparative studies; and (3) 12 retrospective comparative studies	Left-sided	(1) No difference in locoregional recurrence and overall recurrence; (2) No difference in 3-yr and 5-yr disease-free survival; and (3) No difference in 3-yr and 5-yr overall survival
Foo *et al*[54]	2019	*n* = 448: (1) BTS = 222; and (2) ES = 226	Meta-analysis: 7 RCTs	Left-sided	(1) Overall recurrence rate: 37.0% in BTS *vs* 25.9% in ES; (2) The risk ratio of systemic recurrence 1.627 for BTS; and (3) No difference in 3-yr overall survival and disease-free survival
Arezzo *et al* [52] (ESCO trial)	2020	*n* = 115: (1) BTS = 56; and (2) ES = 59	RCT	Left-sided	No difference in 3-yr overall survival, time to progression, and disease-free survival

BTS: Bridge to surgery; ES: Emergency surgery; RCT: Randomized controlled trial; CI: Confidence interval.

who experienced stent-related perforation in an attempt at SEMS decompression, patients with stent-related perforation demonstrated significantly higher rates of global recurrence and locoregional recurrence than patients without perforation, although stent-related perforation did not influence the survival outcome[57]. A significantly increased risk of systemic and locoregional spread of the tumor was also observed in a previous meta-analysis by Foo *et al*[54,57]. Indeed, the current guidelines do not state SEMS decompression as a bridge to surgery as the treatment of choice in a potentially curable disease but consider it only as an alternative to emergency surgery in patients with an increased risk of perioperative morbidity and mortality, despite its better short-term outcome[11,47].

Several background theories explain the increased risk of tumor recurrence. First, the manipulation of a tumor during colonoscopy can cause the dissemination of cancer cells into peripheral circulation by mechanical compression of the guidewire and air insufflation, violating the principle of oncologic treatment[58]. Additionally, an increase in interstitial pressure within the tumor may cause tumor embolism in the lymphatic channels, resulting in lymphatic invasion[59]. Moreover, clinical or silent, stent-related perforation would promote cancer spread inside the peritoneal cavity, leading to locoregional and peritoneal metastasis[60]. Such arguments are supported by clinical correlations with pathologic findings, of which surgical specimens from patients with SEMS decompression presented a higher rate of perineural and lymphatic invasion[61]. In a recent systematic review and meta-analysis by Balciscueta *et al*[60], patients who underwent SEMS decompression had a significantly higher risk of perineural and lymphatic invasion, with odds ratios of 1.98 and 1.45, respectively. The authors claim that stent placement as a bridge to surgery modifies the pathologic characteristics, including perineural and lymphatic invasion, which may worsen the long-term prognosis, raising the risk of global recurrence by 1.7 times the locoregional recurrence and carcinomatosis by 2.4 times[60]. However, pathologic findings of perineural and lymphatic invasion are often observed in obstructive colon cancer. From the available data, the significant influence of pathologic changes on survival outcomes is ambiguous. However, data on survival outcomes still lack a firm conclusion. Further translational research on how disruption in the microenvironment of tumors affects locoregional and systemic metastasis may delineate SEMS decompression's effect on survival outcome.

OPTIMAL INTERVAL BETWEEN SEMS PLACEMENT AND ELECTIVE SURGERY

The time interval between SEMS placement and elective surgery remains uncertain in the current management of obstructive colon cancer. At present, prospective comparative data on how the different intervals affect the short- and long-term outcomes are not available. Two retrospective studies have reported conflicting results regarding resection timing after decompression for postoperative morbidity and oncologic outcomes[62,63]. A retrospective study using the Dutch nationwide cohort demonstrated that surgery within 5–10 d resulted in a longer hospital stay, a lower rate of laparoscopic resection, and a higher rate of stoma creation than surgery after 11 d[63]. Additionally, stent-related complications were most frequently observed in patients who underwent surgery after 17 d[63]. On the other hand, a multicenter retrospective study on the optimal timing of elective surgery after SEMS placement by Kye *et al*[62] supports the concept that early elective surgery within seven days after SEMS placement correlates with better oncological outcomes than elective surgery after seven days. Currently, the ESGE guidelines state only that the time interval for surgery after SEMS placement should balance stent-related adverse events and surgical outcomes[47]. Further investigation is necessary to determine the optimal time interval between SEMS placement and elective surgery.

ROLE OF CIRCULATING TUMOR DNA

The development of genomic sequencing technology and molecular diagnostic testing has allowed the detection of tumor-specific DNA in peripheral blood samples, suggesting the new diagnostic concept of "liquid biopsy"[64]. Circulating cell-free DNA (cfDNA) is derived and released from apoptotic or necrotic cells, and circulating tumor DNA (ctDNA) with tumor-specific DNA from tumor cells undergoing apoptosis or necrosis is released into the systemic circulation[65]. Diagnostic strategies measuring ctDNA are under active investigation for clinical application in screening, diagnosis, and predicting tumor response or resistance to treatment[65]. The concentration of ctDNA in the bloodstream represents the tumor burden in individuals; thus, its use is highly accurate and valued as a biomarker for therapeutic monitoring[65,66].

Applying the concept of therapeutic monitoring, Takahashi *et al*[67] evaluated ctDNA concentration changes after SEMS decompression to test whether SEMS placement disturbs the tumor microenvironment, causing cancer spread. In a prospective observational study, the authors observed that SEMS placement increased the ctDNA concentration in 83% of cases, indicating that SEMS placement inherently induces tumor manipulation and disruption[67]. The authors suggested that stent-induced tumor manipulation may worsen the prognosis of patients with obstructive colon cancer[67]. However, the effect of an increased ctDNA concentration on prognosis is still under investigation. A long-term follow-up study with a larger cohort is required to determine the effect of increased ctDNA on oncological outcomes.

Furthermore, the ctDNA concentration change pattern may help decide the timing and type of subsequent treatment. In the study by Takahashi *et al*[67], the ctDNA concentration changes after SEMS placement showed an increase in concentration over time. This finding implies that the longer time interval between SEMS placement and elective surgery may result in a more unsatisfactory oncological outcome. Since stent-related complications occur mostly within seven days, a short interval after SEMS placement should be considered to minimize its impact on the survival outcome[47]. On the other hand, a long interval may optimize the patient's general condition and reduce the risk of postoperative complications[47]. Serial measurement of the ctDNA concentration may help decide the optimal timing for elective surgery for individual patients considering the long-term outcome.

APPLICATION OF NEOADJUVANT SYSTEMIC CHEMOTHERAPY IN OBSTRUCTIVE COLON CANCER

Neoadjuvant chemoradiotherapy has proven its benefit of downstaging tumors and reducing the local recurrence rate in locally advanced rectal cancer[68]. Recently, there has been a growing body of literature on the use of neoadjuvant chemotherapy before and after neoadjuvant concomitant chemoradiotherapy in locally advanced rectal

cancer for early control of micrometastasis, an increase in the complete response rate, conservative surgery with organ preservation, and an increase in adherence to chemotherapy[69]. In colon cancer, since the mainstay of treatment for the potentially curable disease is complete oncologic resection, it is rare to find studies evaluating the effect of neoadjuvant chemotherapy, and only a few studies have been conducted to evaluate its safety[70-72]. The use of neoadjuvant chemotherapy after SEMS placement in patients with potentially curable obstructive colon cancer is rare. Only one retrospective analysis with a small sample size (n = 9) was found[73]. In this study, the efficacy and safety of SEMS insertion followed by neoadjuvant chemotherapy and elective surgery were evaluated, and the study results revealed relatively low toxicity, high adherence to two to three cycles of neoadjuvant chemotherapy before elective surgery and no evidence of perineural invasion in resected specimens[73]. The authors suggested that neoadjuvant chemotherapy may lower the risk of perineural invasion, possibly improving survival outcomes[73]. In the FOXTROT trial, a randomized controlled trial evaluating the efficacy of neoadjuvant chemotherapy in locally advanced colon cancer, a few patients underwent SEMS placement as a bridge to surgery, and the results showed a significant decrease in the R1 resection rate and a nonsignificant trend toward better oncological outcomes at two years[70]. However, it is difficult to draw meaningful conclusions about the effect of neoadjuvant chemotherapy after SEMS placement from these studies.

In obstructive colon cancer, the ESGE guidelines updated in 2020 recommend that the treatment strategy of SEMS placement followed by neoadjuvant chemotherapy is occasionally used in patients with stage IV disease for the early and safe introduction of systemic chemotherapy[47]. In a review article, Matsuda *et al*[74] suggested that systemic chemotherapy after SEMS placement may be an optimal treatment for patients with unresectable metastatic disease who are unfit for emergency surgery. However, the authors warned of delayed stent-related complications that require strict monitoring[74]. Additionally, the use of bevacizumab should be avoided because bevacizumab is a risk factor for stent-related perforation[74]. Nevertheless, for patients with stage IV obstructive colon cancer, an optimal outcome of upfront treatment for colonic obstruction would require immediate symptomatic control and minimizing complications related to emergent treatment for subsequent treatment for systemic disease. A personalized treatment approach should be implemented to achieve the ultimate result in an individual patient.

Perhaps an observational study utilizing a serial measurement of ctDNA concentration may help determine the efficacy of this treatment approach by decreasing the ctDNA concentration following neoadjuvant chemotherapy. A well-designed, randomized controlled trial evaluating the effect of neoadjuvant chemotherapy on the short- and long-term outcomes of SEMS placement followed by neoadjuvant chemotherapy and elective surgery in potentially resectable colon cancer would provide an ample amount of information regarding the management of obstructive colon cancer.

CONCLUSION

This article summarizes the current treatment strategies for obstructive colon cancer. Clinicians and surgeons often encounter complicated decision-making processes with complex and controversial treatment strategies. Decisions should be made based on the patient's index presentation of the general condition and risk factors that affect the short-term outcome. For the long-term outcome, it would be wise to implement a treatment approach that may induce a better oncological response in individual patients. Nevertheless, a diagnostic method for monitoring treatment response is still lacking. Clinical application of ctDNA analysis may offer new insights into individualized therapeutic strategies. Although several obstacles and preconditions should be resolved, incorporating neoadjuvant chemotherapy may be a viable option for effective systemic control of micrometastasis in selected patients. After all, further investigation on the implementation of neoadjuvant chemotherapy in the treatment of obstructive colon cancer is required.

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REVIEW

Role of endoscopic ultrasound in anticancer therapy: Current evidence and future perspectives

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Abstract

The digestive system is one of the most common sites of malignancies in humans. Since gastrointestinal tumors represent a massive global health burden both in terms of morbidity and health care expenditures, scientists continuously develop novel diagnostic and therapeutic methods to ameliorate the detrimental effects of this group of diseases. Apart from the well-established role of the endoscopic ultrasound (EUS) in the diagnostic course of gastrointestinal and hepatobiliary malignancies, we have recently become acquainted with a vast array of its therapeutic possibilities. A multitude of previously established, evidence-based methods that might now be guided by the EUS emerged: Radiofrequency ablation, brachytherapy, fine needle injection, celiac plexus neurolysis, and endoscopic submucosal dissection. In this review we endeavored to provide a comprehensive overview of the role of these methods in different malignancies of the digestive system, primarily in the treatment and symptom control in pancreatic cancer, and additionally in the management of hepatic, gastrointestinal tumors, and pancreatic cysts.

Key Words: Pancreatic cancer; Endoscopic ultrasound; Endoscopic ultrasound-guided fine needle injection; Pancreatic cyst; Gastrointestinal tumor; Portal vein

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Core Tip: Apart from the well-established role of the endoscopic ultrasound (EUS) along the diagnostic path of gastrointestinal and hepatobiliary malignancies, the EUS recently emerged as a carrier of various therapeutic modalities. In this review we sought to give a comprehensive overview of the role of various established methods that might now be guided by the EUS in different malignancies of the digestive system, primarily regarding the treatment and symptom control in pancreatic cancer, and additionally in the management of hepatic, gastrointestinal tumors, and pancreatic cysts.

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INTRODUCTION

Endoscopic ultrasound (EUS) is one of the principal tools in cancer screening and staging. Apart from its use in the diagnostic course of gastrointestinal and hepatobiliary malignancies, it has an entire range of other therapeutic possibilities (Figure 1). The ability of the EUS to obtain tissue samples using either fine needle aspiration (FNA) or fine needle biopsy makes it a unique method in the screening of pancreatic cystic lesions, as well as in the assessment of regional lymph node involvement in esophageal, gastric, and rectal cancer (Figures 2-4). The EUS is therefore essential in the concurrent pancreatic cancer diagnosis[1]. Over the last few years, the implementation of the EUS has expanded from the diagnostic into the therapeutic field. Endoscopic ultrasound-guided fine needle injection (EUS-FNI) of chemotherapeutics, immunotherapy and gene therapy, tissue ablation, stereotactic radiation therapy, brachytherapy and celiac plexus neurolysis have been thoroughly investigated and steadily introduced into the clinical practice[1,2]. Multiple studies suggest that some of these methods might be crucial in overcoming the problem of drug distribution to tumorous tissue in patients with pancreatic cancer, whereas other methods could be of aid in pain management of the same population. In this review we endeavored to give a comprehensive overview of the role of the EUS in anticancer therapy: primarily in the treatment and symptom control in pancreatic cancer, and, additionally, in the management of hepatic and gastrointestinal tumors.

THE EUS IN TREATMENT OF PANCREATIC CANCER

As hypovascularity and abundant desmoplasia are landmarks of pancreatic cancer, delivery of chemotherapeutic medications to the tumor-affected area is insufficient[3]. Consequently, effects of chemotherapeutics on pancreatic cancer are mitigated, resulting in higher required therapeutic doses which increases the incidence of adverse effects[4]. Therefore, several local strategies that could possibly overcome these issues were developed. Among a number of EUS-based therapeutic interventions, radiofrequency ablation (RFA), brachytherapy, EUS-FNI, and EUS-guided celiac plexus neurolysis (EUS-CPN) emerged as viable strategies for improvement of poor outcomes regarding the pancreatic cancer.

RFA

RFA is an invasive antitumor method which works by generating heat from high-frequency alternating current that induces frictional heating, leading to thermal coagulative necrosis of the target tissue[5]. In addition, several authors demonstrated that RFA may trigger immunomodulatory activity, and in this way, further dampening the cancer development[6-9]. RFA is already a well-established therapeutic modality in management of other cancers, particularly for patients with hepatocellular carcinoma, and has been included in the latest guidelines for management of hepatocellular carcinoma by a major European organization[10].

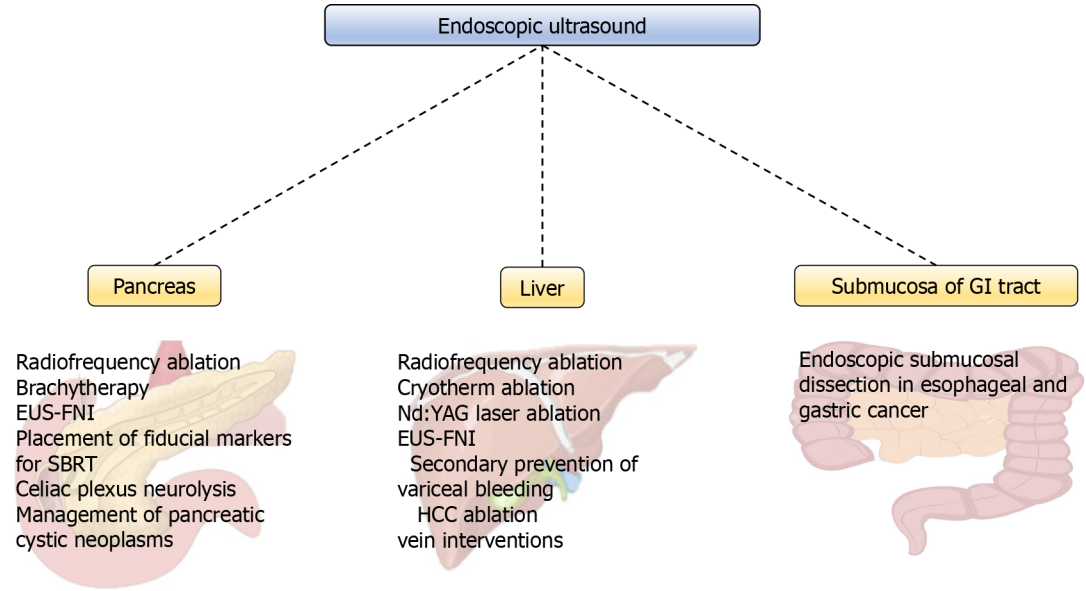

Figure 1 Overview of endoscopic ultrasound-guided methods. EUS-FNI: Endoscopic ultrasound-fine needle injection; HCC: Hepatocellular carcinoma; GI: Gastrointestinal; Nd:YAG: Neodymium-doped yttrium aluminum garnet; SBRT: Stereotactic body radiotherapy.

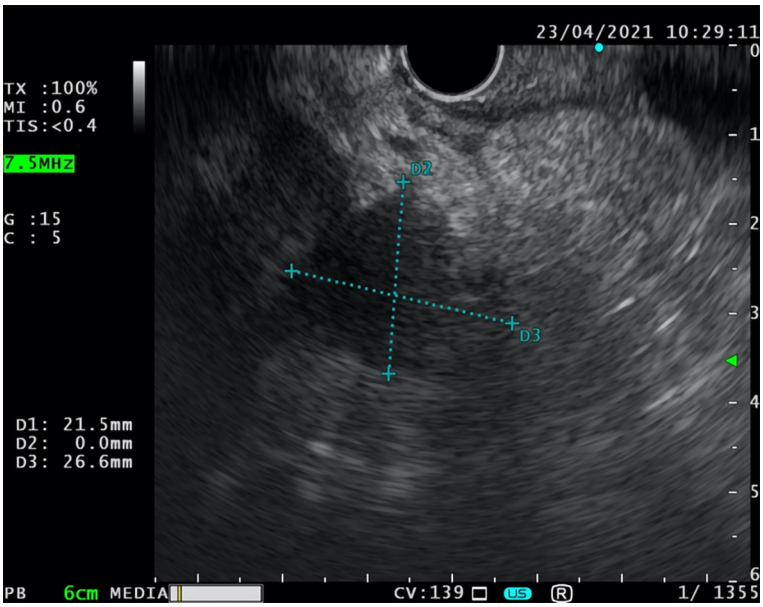

Figure 2 Endoscopic ultrasound-fine needle aspiration. Fine needle aspiration of inhomogeneous oval lesion located on the border between head and corpus of the pancreas (26.6 mm × 21.5 mm).

Owing to the anatomical positioning of the pancreas, suitable approaches for the inclusion of the RFA in pancreatic cancer are multiple, including open surgery, laparoscopic approach, percutaneous approach, and an EUS-guided approach. Initially, safety and effectiveness of EUS-guided RFA of the pancreatic tissue were evaluated in porcine models[11-14]. Although results of the animal studies were promising, clinicians were doubtful, as thermal-induced pancreatitis and thermal injuries of the adjoining structures emerged as adverse effects in the animal studies and in early intraoperative studies[11,15-17]. Early on, surgeons observed that detrimental effects of intraoperative RFA could be reduced by using lower temperatures, maintaining a margin from the major adjacent vessels, and by using stepwise approach in bigger, poorly demarcated lesions[18,19]. Intraoperative studies have also provided an insight into the effectiveness of the RFA. Multiple studies have demonstrated that intraoperative RFA leads to tumor necrosis and a decrease of tumor volume, as well as reduction in CA 19-9 plasma levels, the main pancreatic cancer marker[16,20-25]. Unfortunately, in all of the aforementioned studies, all patients that underwent RFA

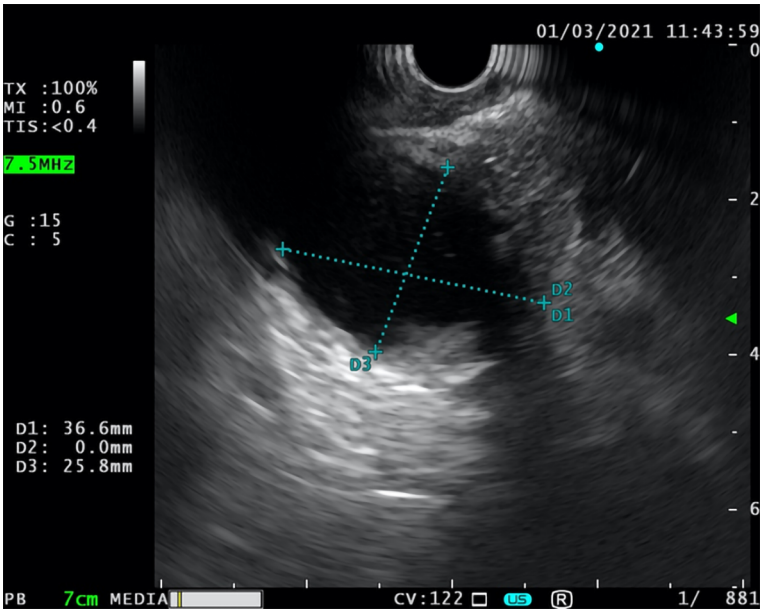

Figure 3 Unsuccessful endoscopic ultrasound-fine needle aspiration. Unsuccessful attempt of fine needle aspiration of the cystic lesion with thick, calcified border (37 mm × 26 mm) located in the head of the pancreas.

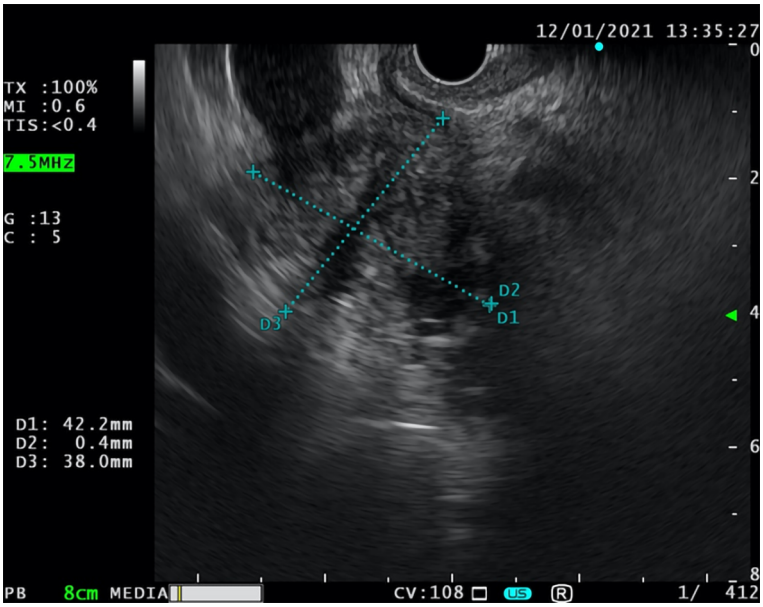

Figure 4 Endoscopic ultrasound-fine needle biopsy. Fine needle biopsy of the focal lesion in the pancreatic head (42 mm × 38 mm).

eventually developed disease progression[16,20-25]. Of note, in a small sample (n = 25) study by Spiliotis *et al*[16], authors have shown a significant prolongation in survival in patients treated with both palliative care and RFA in opposition to patients that received palliative care exclusively, an observation that Matsui *et al*[25] failed to demonstrate. In addition, Cantore *et al*[26] argue that combined multiple-treatment followed by RFA can prolong survival in patients with unresectable pancreatic cancer.

Together, these findings paved the way for the investigation of the EUS-guided RFA. In a pilot study by Song *et al*[27], 6 patients with unresectable pancreatic cancer underwent EUS-guided RFA. Two out of six patients suffered abdominal pain, whereas other patients reported no adverse, effects implying the safety of the procedure. However, as the purpose of the study was to assess the technical feasibility and safety of the procedure, long-term survival of the patients was not studied. Multiple similar studies were performed following this study, and their results with regard to effectiveness and adverse events were summarized in a recent meta-analysis by Dhaliwal *et al*[28]. Meta-analysis showed that technical success, defined as the

successful placement of the needle within the pancreatic lesions with safe margins from the surrounding vital structure, and clinical success, defined as decrease in lesion size and presence of necrosis on CT scan after the procedure, were 100% and 91.5%, respectively. Adverse events were observed in 15% of the patients, with abdominal pain being the most common (10%) and only 2 cases of pancreatitis and 1 perforation in total. Overall, the EUS-guided RFA appears to be safe, but multicenter, randomized control trials are needed in order to clearly define the utility of this method.

EUS-FNI

EUS-FNI is an antitumor agent delivery method in which the EUS serves as a guide for needle placement into target lesions. Aside from injection of various therapeutic agents, EUS-FNI is also suitable for implantation of fiducial markers that enables targeted radiation therapy and for injection of dyes to tattoo the tumor[29,30]. The first human clinical trial (phase I) using the EUS-FNI was performed by Chang *et al*[31] in 2000. Authors used EUS-FNI to deliver allogeneic mixed lymphocyte culture (cytoimplant) into the tumor tissue. The median survival of the 8 patients enrolled in the study was 13.2 mo, with 3 partial responses and no adverse events reported. Since then, the largest conducted trial of this sort was a phase III trial in which effects of TNFerade™ biologic, in combination with fluorouracil and radiotherapy, were assessed on 304 patients with advanced pancreatic cancer[32]. Unfortunately, Herman *et al*[32] failed to demonstrate better progression-free survival or overall survival in comparison to controls. Recently Lee *et al*[33] conducted a phase I trial in which authors combined adenovirus-mediated double-suicide gene therapy with gemcitabine in patients with locally advanced pancreatic cancer. Nonetheless, despite the fact that the trial proved the safety of the procedure, pioneers of EUS-FNI argued in a recent paper that a lot of hurdles have to be overcome in order to develop a clinically functional EUS-FNI method, especially in terms of oncolytic viruses[34]. Among recently conducted trials, we have highlighted the following two: (1) Nishimura *et al*[35] injected a double-stranded RNA oligonucleotide that repressed tumor growth in 6 patients with unresectable pancreatic cancer and reported no adverse effects, as well as reduction in plasma levels of the target molecule and tumor size reduction; and (2) A prospective study by Levy *et al*[36] tested the use of EUS-FNI for the guided delivery of gemcitabine, a chemotherapeutic whose role in treatment of pancreatic cancer has been well-established. Authors administered gemcitabine in 36 patients with various stages of pancreatic cancer (II-IV), and no adverse events were reported. Overall survival of the patients was 10.4 mo, and more importantly, 20% of patients with stage III unresectable disease were down-staged and underwent an R0 resection. In conclusion, as pancreatic cancer is a systemic disease, it is practically impossible to assume that EUS-FNI will fully replace current therapeutic modalities of pancreatic cancer, but, given the safety and feasibility of the method, as well as expansion of translational medicine, it could emerge as a viable adjuvant method in the future.

Brachytherapy

EUS-guided brachytherapy involves implantation of radioactive seeds directly into or adjacent to the tumor-affected tissue. The target tissue is then exposed to the emission of low-energy gamma, X-rays, or beta particles, leading to localized tissue injury and tumor ablation. The main advantage of brachytherapy is its ability to deliver a markedly higher dose of radiation to the tumor mass in comparison to external beam radiation therapy. In the latter, radiation beams pass through other non-tumorous tissues in reaching the target mass, thus resulting in collateral toxicity and more damage to healthy tissue. Although there is abundant evidence to suggest that brachytherapy can deliver a higher dose of radiation and provide local control as well as palliative benefits, currently no brachytherapy device is approved for the treatment of patients with pancreatic cancer[37-42]. There are multiple approaches for implantation of radioactive seeds, one of which is the EUS-guided brachytherapy, and a variety of chemical elements that could be used in this manner (phosphorus-32 (P-32), iodine, gold, iridium, *etc.*)[43,44]. Two pilot trials that tested the potential of EUS-guided brachytherapy in patients with pancreatic cancer had rather disappointing results with no benefits with regard to overall survival rate (27%) and partial response rate (13.6%), respectively[45,46]. Nonetheless, authors of one of the trials argued that such results might be due to an insufficient radiation dose to local lesions[47]. They performed another study in which they implemented a novel computer-aided treatment-planning system (TPS). Under the support of the new TPS, partial remission rate was 80% and expected median survival time of the 42 patients was 9.0 mo (24 patients were in stage IV). At present, there is an ongoing open-label, single-arm pilot study of EUS-guided

brachytherapy with P-32 microparticles in combination with gemcitabine and/or nab-paclitaxel in unresectable locally advanced pancreatic cancer (OncoPaC-1 study)[48]. However, the role of the EUS-guided brachytherapy in pancreatic cancer is yet to be determined. Hopefully, conjunction of brachytherapy and chemotherapeutics will increase the share of patients with pancreatic cancer that convert to resectable and provide us with more durable, local control in comparison to conventional treatments.

EUS-guided celiac plexus neurolysis

Among a multitude of options, the EUS has also found its place in the palliative treatment of patients with pancreatic cancer. Given the fact that most patients present in the advanced stages of the disease, palliative care is often the primary goal of care. Pain, the most significant and most common complication of pancreatic cancer, has traditionally been managed by nonsteroidal and opioid analgesic, often with numerous side effects, including constipation, sedation, nausea, vomiting, and delirium[49,50]. In order to overcome these issues and while trying to improve quality of life, endoscopic ultrasound-guided celiac plexus neurolysis (EUS-CPN) was introduced by Wierseme and Wierseme in 1996 (Table 1)[51].

EUS-CPN is a minimally invasive method for the treatment of pain through the chemical ablation of the celiac plexus under the control of the EUS. The current National Comprehensive Cancer Network guidelines recommend EUS-CPN for treatment of severe pancreatic cancer-associated pain in the case of failure of achieving adequate analgesia and/or intolerable adverse effects[52].

The EUS-guided neurolysis is indicated for patients with chronic abdominal pain caused by an upper GI tract malignancy, mostly pancreatic cancer. Patients with unresectable disease whose pain affects their quality of life are considered good candidates for this approach. Furthermore, there are pivotal studies of the EUS-CPN efficacy in patients with gallbladder carcinoma[53]. However, besides pancreatic carcinoma, the main indication for EUS-CPN still remains chronic pancreatitis[50,54].

The optimal timing in which the EUS neurolysis should be applied is still unclear. However, in randomized, double-blind, controlled trial, Wyse *et al*[55] found that early EUS-CPN, performed during the diagnostic EUS, provided better pain management and prevented an increase in morphine consumption than EUS-CPN later in the course of the disease. However, further studies are needed to define the optimal timing for CPN. CPN consists of injecting a neurolytic agent (absolute alcohol or phenol) into the area around the celiac plexus monitored by an echo endoscope with prior administration of a local anesthetic (bupivacaine or lidocaine). Application of the neurolytic agent can be unilateral (just above the celiac trunk) or bilateral (on both sides of the celiac trunk)[56-58].

Several studies have shown pain improvement after CPN[50,51,54,55]. In the initial study, pain improvement was achieved at 2, 4, 8, and 12 wk after CPN in 79%-88% patients[51]. Additionally, a Cochrane Review of six studies (358 patients), that showed that the EUS-CPN was superior at 4 and 8 wk compared to drug-based management, with significant drug consumption reduction[5]. Whether the application of the EUS-CPN affects survival remains unclear. A study by Fujii-Lau *et al*[59] indicates that the EUS-guided neurolysis is associated with longer survival than the non-EUS guided approach. However, further studies are needed to assess the potential impact of the neurolysis on survival. In order to improve the efficiency of pain treatment, the following EUS-CPN modifications have been presented: EUS-guided direct celiac ganglia neurolysis (EUS-CGN); and EUS-guided broad plexus neurolysis[60-62]. In a randomized study by Doi *et al*[60], authors indicated better pain improvement in CGN over CPN. Levy *et al*[61] have successfully demonstrated the modification of the neurolysis of the celiac plexus in the form of direct injection of the agent into the celiac ganglion, requiring prior visualization of the ganglia itself. Sakamoto *et al*[62] in 2010 described the injection of a neurolytic agent in the area around the origin of the superior mesenteric artery, introducing a method of broad plexus neurolysis, which demonstrated better pain relief compared to the CPN.

A recent review of 20 studies comprising 1142 patients, revealed that complications of EUS-CPN occurred in 21% of 661 EUS-guided CPN interventions[63]. The most frequent complications included diarrhea, transient pain exacerbation, and hypotension. Most of the complications seem to be a consequence of a sympatholytic reaction and are self-limited (< 48 h). However, in 0.2% of cases, major complications were observed, including paraplegia, retroperitoneal abscess, and ischemia with visceral injury[63].

Given the unequivocal beneficial effects of EUS-CPN in the palliative care of the patients with advance pancreatic cancer, we can expect further increase of its usage in routine clinical practice.

Table 1 Overview of the endoscopic ultrasound-guided celiac plexus neurolysis characteristics

Procedure	Chemical ablation of the celiac plexus
Indications	Chronic abdominal pain caused by: Pancreatic cancer; Chronic pancreatitis; Gallbladder carcinoma[1]
Contraindications[57]	Coagulation disorders; INR > 1.5; Platelet count < 50000/μL; Retroperitoneal abscess; Disturbed anatomy (difficulties in visualizing the celiac trunk and ganglia); Malformations of the celiac or superior mesenteric artery
Serious complications (observed in 0.2% of the patients[63])	Paraplegia; Retroperitoneal abscess; Ischemia with visceral injury
Supporting evidence	Kaufman *et al*[50]; Wiersema *et al*[51]; Puli *et al*[54]; Wyse *et al*[55]; Arcidiacono *et al*[58]

[1]Only pivotal studies were so far conducted. INR: International normalized ratio.

EUS IN PANCREATIC CYSTIC NEOPLASMS

Pancreatic cystic neoplasms (PCN) represent a heterogeneous group of pancreatic cysts with significant differences in their pathological and clinical features, the most important one being the difference in malignant potential among subtypes. These differences are important as they determine the approach for both the treatment and the surveillance of PCN[64-66]. A prevalence of all PCN varies from 2%-45% in general population)[67-70], with constant increase in the PCN detection rate. The explanation for the observed increase lies in the improved modalities of non-invasive (computed tomography (CT) scan, magnetic resonance imaging- cholangiopancreatography (MRI-MRCP)) and minimally invasive (endoscopic ultrasound-fine needle aspiration (EUS-FNA)) imaging methods, and their broader use in preventive check-ups, as well as an awareness of true nature of PCN and the necessity for their close follow-up. According to different studies, abdominal ultrasonography detects PCN in 0.21% of individuals [71], CT in 2.6%[72], and MRI-MRCP in 2.4% to 49.1% of tested individuals[65,72-74]. The management of PCN may be quite challenging, with identification of specific PCN type being a crucial step since malignant potential varies significantly between different types of PCN. Timely and correct management of PCN is vital, as it may prevent progression to pancreatic cancer and decrease the need for lifelong follow-up [71].

Due to the challenges in differentiation between the various types of PCN and its implications on therapeutic approach, guidelines on the management of PCN were proposed by expert groups, most notably by the Association of Pancreatology, the American Gastroenterological Association, and by the European Study Group on Cystic Tumors of the Pancreas[65,66,73,75]. Various approaches, including surgical resection, endoscopic techniques, and surveillance, are covered in all of the above-noted guidelines. Surgical resection is the golden standard for management of premalignant and malignant cystic lesions. Indications for resection depend on the presence of symptoms, probability of malignancy, location of the lesion, and surgical risk of the patient. On the other hand, surgery carries a considerable risk, with perioperative complication rates from 20% to 40%, and mortality rates up to 2%[76-79]. Therefore, endoscopic techniques represent an important alternative to surgery, especially in patients with significant comorbidities or in cases of indeterminate cystic lesions.

The main advantage of the EUS in this setting is the fact that EUS-guided pancreatic cyst ablation using ethanol and/or paclitaxel enables organ preservation, leaving endocrine and exocrine function intact[80-83]. However, there is concern about the use of ethanol, as the rate of reported complications was as high as 2%–10%[84]. Another disadvantage of this method is the inability to obtain a sample for histopathological analysis. The long-term effects of ablation and prevention of malignant alteration are yet to be evaluated in future studies[84-87]. The current diagnostic workup of PCN includes CT or MRI with the addition of MRCP and EUS when appropriate[64-67,88]. EUS is indicated in addition to other imaging modalities if there are worrisome clinical or radiological features present (nodules, dilated pancreatic duct, thickened wall), or if there is a need for obtaining the cystic fluid for cytological and/or biochemical analysis.

The reported accuracy of EUS imaging for differentiating mucinous from non-mucinous PCN is relatively low (48%-94%), with sensitivity of 36%-91%, and specificity of 45%-81%[89-93]. Cytopathological analysis of cystic fluid may reveal dysplasia or clear malignancy. Although cytology is highly specific (83%-100%), it is relatively insensitive (27%-48%), resulting in relatively low diagnostic accuracy of this

procedure (8%-59%)[89-92,94]. However, sensitivity could be increased by an additional puncture of the cystic wall. Additional biochemical markers, including carcinoembryonic antigen (CEA), which has been proved to be useful in distinction between mucinous and non-mucinous PCN, and amylase, which strongly suggests a connection between the cyst and the pancreatic ductal system, may also be obtained from cystic fluid[95,96]. Combination of multiple EUS-guided methods, such as EUS morphology, cytology, and cyst fluid CEA, provide us with greater accuracy in detecting malignant PCN than any of the methods individually[97,98]. Recently, DNA testing of pancreatic cyst fluid emerged as a promising additional tool for the differentiation between mucinous and non-mucinous PCN, between mucinous PCN subtypes, and between premalignant PCN and advanced neoplasia[99].

According to the latest recommendations, surgically fit patients with asymptomatic cysts that are presumed to be premalignant (intraductal papillary mucinous neoplasms (IPMN) or mucinous cystic neoplasms(MCN)), but which possess no concerning features, should be monitored, preferably using MRI-MRCP or EUS if MRCP is not available[100,101]. In cases in which any of the worrisome features emerges, the EUS-FNA should be used in the cyst follow-up. For both IPMN and MCN, surveillance should continue as long as the patient is fit for surgery[102]. On the other hand, current guidelines do not address the need for surveillance in asymptomatic patients with serous cystic neoplasms (SCN), since malignant progre-ssion of SCN is very rare[102-104].

EUS-GUIDED PORTAL VEIN INTERVENTIONS

The portal vein (PV) can be accessed *via* trans-gastric or trans-duodenal access under the EUS guidance using an 18G-25G needle with low risk of complications. Multiple studies have demonstrated that EUS is successful in sampling of the PV to reveal circulating tumor cells, as well as obtaining the portal vein thrombus specimen[105], which has an utmost significance in the staging of hepatocellular liver cancer (HCC)[106-108]. The diagnostic yield of the EUS is confirmed by the reported cases of HCC detection using the EUS-FNA of radiologically suspected malignant thrombi without an evident liver mass[108,109].

As a significant step forward, Park *et al*[110] have even demonstrated the technical feasibility of the EUS guided transhepatic PV stenting in porcine models, without any immediate or late complications[110]. However, patients who undergo PV stenting due to malignant thrombosis or stenosis may bear certain procedural risk factors, such as coagulopathy and risk of rapid clinical deterioration if biliary leak or bacteremia occurs.

The EUS-guided PV injection of chemotherapy (EPIC) is another novel therapeutic possibility with a few significant advantages in comparison to the current methods[106]. EPIC uses drug eluting microbeads that eventually lodges in hepatic sinusoids and results in the prolonged hepatic drug exposure[111]. Studies suggest that EPIC achieves appropriate intrahepatic drug levels, while simultaneously bypassing the systemic side effects and avoiding the ischemic bile duct injury that occurs during the transarterial approach[106]. Faigel *et al*[111] compared the EPIC administration of irinotecan loaded liquid chromatography beads with the systemic unloaded irinotecan application in animal models and revealed EPIC-associated higher irinotecan intrahepatic concentration, as well as lower plasma, bone marrow, and skeletal muscle drug concentrations. Two years later, the same group confirmed their findings on a greater number of animal models using irinotecan, doxorubicin, and albumin-bound paclitaxel nanoparticles[112].

Preoperative selective embolization of the PV branch that feeds the tumor-affected liver lobe has been utilized in clinical practice since 1986 using the percutaneous transhepatic approach[106,113]. Embolization leads to the atrophy of the involved liver segment and hypertrophy of the remnant liver parenchyma, thus preventing the postoperative liver failure[113]. Recently, Park *et al*[113] used nine porcine models to successfully prove the efficacy and safety of the EUS guided embolization of the PV using coil and cyanoacrylate. Furthermore, Matthes *et al*[114] demonstrated efficient selective PV embolization using ethylene-vinyl alcohol copolymer, known as EVAL (Enteryx), in an animal model. Unfortunately, to our knowledge, studies including PV interventions in anticancer treatment have been so far limited to animal models. Still, exciting advances in the field are revealed, and prospective studies involving humans are eagerly awaited.

ROLE OF THE EUS IN LIVER TUMOR MANAGEMENT

EUS-guided tumor ablation is a safe and effective treatment modality for tumor lesions of the caudate and left liver lobe. Multiple studies have successfully demonstrated the benefits of ethanol administration *via* EUS-FNI in the treatment of both HCC and liver metastases[115]. Carrara *et al*[116] described the EUS guided trans-gastric bipolar hybrid cryotherm ablation on a porcine model without complications. Varadarajulu *et al*[117] reported RFA in animal models with effective coagulation necrosis of large areas and without damage to the surrounding liver parenchyma. Multiple studies have also demonstrated success of the EUS-guided neodymium-doped yttrium aluminum garnet (Nd:YAG) laser ablation in patients with HCC and colorectal cancer metastases[118,119]. In addition, several other methods, including injection of sclerosants and chemotherapeutics, represent viable future therapeutic options[115].

In the management of the HCC-related complications, one of the most common and disastrous is the variceal bleeding incident. With respect to secondary prevention of this complication in patients with inoperable HCC, Tang *et al*[120] used EUS guided cyanoacrilate injection, which led to reduced rebleeding rates, as well as improved variceal bleeding free survival.

ROLE OF EUS IN ENDOSCOPIC SUBMUCOSAL DISSECTION

The development of gastrointestinal endoscopic tissue resection techniques demands precise diagnostic tools in the preoperative evaluation. Accurate information about the depth of tumor invasion of the gastrointestinal wall and the nodal involvement are necessary for determining the appropriate intervention.

Endoscopic submucosal dissection (ESD), the newest and most invasive method, has become standard of care of precancerous and some early cancer lesions in gastrointestinal tract, allowing curative resection of the lesions[121]. Depending on the proximity of the GI tract wall during the procedure, the EUS enables clear image of the lesion depth and vital surrounding structures, especially of the lymph nodes[121].

Endoscopic resection is indicated in early esophageal cancer with minimal or no lymph node invasion[122]. According to the latest guidelines for the treatment of esophageal cancer of the Japan Esophageal Society, the absolute indication for endoscopic resection is defined as flat lesion (Paris 0-II), with m1 (intraepithelial) - m2 (invading lamina propria) invasion, and circumferential extent of ≤ 2/3[123]. A systematic review and meta-analysis has demonstrated that sensitivity and specificity for T1a staging were 85% and 87%, respectively, and 86% for both sensitivity and specificity for T1b staging[124]. However, the ability of the EUS to predict endoscopic resectability by discrimination between T1 and T2 lesions is still intensively studied. Available data suggests that 15%-25% of cases are under-staged compared with endoscopic mucosal resection staging, while about 4%-12% of cases are over-staged [125,126].

Conventional EUS has limited accuracy in the detection of submucosal invasion in early esophageal cancer[127,128]. It remains questionable whether the EUS should be routinely performed prior to ESD of esophageal superficial lesions. European Society of Gastrointestinal Endoscopy (ESGE) suggests that EUS should be considered in esophageal superficial carcinomas with suspicious features for submucosal invasion or lymph node metastasis[122].

Esophageal submucosal tumors are becoming more common indication for ESD. EUS allows for the evaluation of size, echo pattern, layer of origin, and eventual surrounding nodal involvement[121,129]. The biggest setback in EUS evaluation is still interobserver variation. Notwithstanding, endoscopic ultrasonography has become the most valuable tool in diagnostics of esophageal submucosal tumors[129].

The role of EUS in establishing the feasibility of endoscopic resection of superficial gastric lesions is still controversial. ESD is indicated as the treatment of choice for most gastric superficial neoplastic lesions, including low- or high-grade non-invasive neoplasia and adenocarcinoma with no evidence of deep submucosal invasion[130-133]. Although EUS is considered a reliable method for locoregional staging, endoscopic evaluation is still favored over EUS for predicting endoscopic resectability [134]. We should also highlight different approaches in the use of EUS before intervention. Although favored in the prior planning of endoscopic resection in Western countries, in the Eastern countries (in which the incidence of gastric cancer is notably higher), it is not considered necessary to perform EUS in the preoperative

evaluation prior to the planned intervention[131,135,136].

Since ESD has been achieving similar results compared with surgery in treatment of gastric submucosal tumors (< 50 mm in size), the role of the EUS in preoperative management has recently evolved[137]. Today, it is the main tool in preoperative assessment, including evaluation of size, layer of origin, and echo pattern. Furthermore, the use of EUS has extended to marking the lesion with EUS-assisted injection into the muscularis propria, providing a deeper safety cushion for submucosal dissection procedure[138].

A randomized study by Fernandez-Esparrach *et al*[139] concluded that EUS and MRI have similar accuracy in T and N staging for rectal cancer. The ESGE recommends using one of these methods for staging of rectal cancer, but not for colon cancer[122]. However, the role of EUS and MRI for superficial lesions has been undefined.

A prospective study comparing high frequency EUS *vs* magnifying chromoendoscopy in early colorectal neoplasia showed that high frequency EUS was superior to chromoendoscopy in determining the depth of invasion, showing an accuracy of 93% *vs* 59%[140]. Nevertheless, endoscopic resection will probably remain the best staging tool for superficial rectal lesions, and if the endoscopist feels the lesion is endoscopically resectable, it will probably not require preoperative EUS[122]. On the other hand, according to the ESGE recommendations, the use of EUS or MRI should be considered for rectal lesions with endoscopic features suspected for submucosal invasion, since the finding of suspicious lymph nodes could be an indication for neoadjuvant treatment[122].

To summarize, the role of EUS in ESD is still evolving. The main goal of the endoscopic ultrasonography remains to evaluate a potential submucosal invasion and locoregional staging of the disease. Future research on the role of EUS in ESD should be concentrated in reduction of interobserver variations and alleviating possible complications.

CONCLUSION

Even though recent technological advancements in endoscopic approaches led to an improvement of outcomes of tumors in the abdominal cavity, as indicated by reduced mortality, morbidity, and palliative care, we are still far from having an optimal treatment, particularly for pancreatic cancer. Nonetheless, the novel EUS-guided approaches (RFA, brachytherapy, FNI, *etc.*) for pancreatic cancer might be crucial in overcoming the problem of drug distribution to tumorous tissue and reducing the required therapeutic doses and incidence of adverse effects. In addition, alleviating extreme pain that many patients with pancreatic cancer endure seems to be achievable through celiac plexus neurolysis. Furthermore, EUS is already a part of the algorithm in management of PCN, where EUS-FNA is the method of choice in case of appearance of certain alarming features of cysts.

The utility of EUS in PV interventions is most prominent in relation to the staging of HCC. The role of EUS in ESD with respect to management of precancerous and some early cancer lesions in GI tract, as well as EUS-guided treatment of various hepatic cancers, is yet to be determined, as current data is insufficient to recommend these techniques as standards of care.

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REVIEW

Pancreatic intraductal papillary mucinous neoplasms: Current diagnosis and management

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Abstract

Intraductal papillary mucinous neoplasms (IPMNs) represent approximately 1% of all pancreatic neoplasms and 25% of cystic neoplasms. They are divided into three types: main duct-IPMN (MD-IPPMN), branch duct-IPMN (BD-IPMN), and mixed type-IPMN. In this review, diagnostics, including clinical presentation and radiological investigations, were described. Magnetic resonance imaging is the most useful for most IPMNs. Management depends on the type and radiological features of IPMNs. Surgery is recommended for MD-IPMN. For BD-IPMN, management involves surgery or surveillance depending on the tumor size, cyst growth rate, solid components, main duct dilatation, high-grade dysplasia in cytology, the presence of symptoms (jaundice, new-onset diabetes, pancreatitis), and CA 19.9 serum level. The patient's age and comorbidities should also be taken into consideration. Currently, there are different guidelines regarding the diagnosis and management of IPMNs. In this review, the following guidelines were presented: Sendai International Association of Pancreatology guidelines (2006), American Gastroenterological Association guidelines, revised international consensus Fukuoka guidelines (2012), revised international consensus Fukuoka guidelines (2017), and European evidence-based guidelines according to the European Study Group on Cystic Tumours of the Pancreas (2018). The Verona Evidence-Based Meeting 2020 was also presented and discussed.

Key Words: Pancreatic cyst; Pancreatic cystic neoplasm; Intraductal papillary mucinous neoplasm; Pancreatic cancer; Pancreatectomy; Guidelines

Core Tip: Intraductal papillary mucinous neoplasms (IPMNs) account about 1% of all pancreatic neoplasms and 25% of cystic neoplasms. We can distinguish three IPMN

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types: main duct-IPMN (MD-IPPMN), branch duct-IPMN (BD-IPMN), and mixed type-IPMN. Magnetic resonance imaging is the most useful approach for most IPMNs. Management depends on the type and radiological features of IPMNs. MD-IPMN is recommended for surgery. In BD-IPMN, management involves surgery or surveillance depending on the tumor size, cyst growth rate, solid components, main duct dilatation, high-grade dysplasia in cytology, the presence of symptoms (jaundice, new-onset diabetes, pancreatitis), and CA 19.9 serum level. The patient's age and comorbidities should also be taken into consideration. Currently, there are different guidelines regarding the diagnostics and management of IPMNs: Sendai International Association of Pancreatology guidelines (2006), American Gastroenterological Association guidelines, revised international consensus Fukuoka guidelines (2012), revised international consensus Fukuoka guidelines (2017), and European evidence-based guidelines based on the European Study Group on Cystic Tumors of the Pancreas (2018). The experts of Verona Evidence-Based Meeting 2020 determined the most important further directions regarding guidelines on IPMN management.

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INTRODUCTION

Pancreatic cystic neoplasms represent about 10%-13% of pancreatic cysts, 25% of cystic neoplasms and 1% of pancreatic carcinomas[1,2]. Pancreatic intraductal papillary mucinous neoplasms (IPMNs) are one of the two types of mucin-producing pancreatic cystic tumors (PCTs)[1,2]. According to World Health Organization, IPMNs are neoplasms which grow within the pancreatic ducts and produce mucin. They contain epithelial cells that can create papillary projections[2]. In 1982, Ohhashi *et al*[3], for the first time, reported four cases of mucin-producing pancreatic cancer. The term "intraductal papillary neoplasm" was introduced by Morohoshi[4] in a report of six cases in 1989. It should be added that numerous different terms were used for IPMNs before establishing the current nomenclature. The earlier names used were as follows: mucinous ductal ectasia, ductectatic mucinous cystadenoma and cystadenocarcinoma, intraductal mucin-hypersecreting neoplasm, intraductal papillary adenocarcinoma, intraductal mucin-producing tumor, and mucin-producing tumor[1].

At this time, the number of pancreatic IPMNs has significantly increased, and there are many reports on these tumors. The aim of this study is to review and present most of the current important literature regarding the etiopathogenesis, classification, diagnostics and treatment of pancreatic IPMNs.

ETIOLOGY AND PATHOGENESIS OF IPMNS

The etiology of pancreatic IPMNs is not clear. A main feature of many IPMNs is excessive mucin production. It has been reported that mucin 2 (MUC2) is procuded by most IPMNs, while there is no expression of mucin 1 (MUC1) in IPMNs, except of components of ductal cancer[5,6]. Adsay *et al*[6] noted that invasive ductal adenocarcinomas develop from intraepithelial neoplasms of the pancreas (PanINs) (5-y survival is less than 15%), whereas IPMNs are often associated with colloid carcinoma (5-y survival is better of more than 55%). It is known that an associated invasive carcinoma is reported in approximately 30% of patients with IPMN. Adsay *et al*[6] described an association of mentioned above pancreatic pathologies by investigating the expression of MUC1 and MUC2 glycoproteins as "aggressive" and "indolent" phenotypes in pancreatic carcinoma, respectively. In fact, MUC1 (mammary-type mucin) and MUC2 (intestinal-type mucin) have been reported as markers of "aggressive" and "indolent" phenotypes in pancreatic cancer, respectively. IPMN and colloid (mucinous noncystic) carcinoma form a distinct pathway of carcinogenesis in the pancreas, and MUC2 may

be the marker of this pathway. Furthermore, ordinary ductal carcinoma of the pancreas was found to lack expression of this marker but showed MUC1 expression instead[6]. In conclusion, the results of this study supported a dichotomial nature of the dysplasia-carcinoma in situ (CIS) sequence in the pancreas. Authors analyzed 2 routes leading to different types of invasive cancers. They noted that MUC2 is a marker of the "indolent" pathway (IPMN and colloid cancer), and MUC1 is a marker of the "aggressive" pathway (PanIN to ductal adenocarcinoma)[6].

In IPMNs, a classic "adenoma-carcinoma sequence" is observed. The duration of developing invasive carcinoma from low-grade dysplasia is approximately from 4 to 6 years. Various somatic mutations in the oncogenes KRAS and GNAS are reported in up to 90% of IPMNs. Other mutated genes are as follows: CDKN2A/p16, TP53, SMAD4, and less commonly STK11, BRAF, PIK3CA, PTEN. It has been noted that inactivated CDKN2A/p16, absent SMAD4 and mutation in TP53 are associated with progression from IPMN to carcinoma. They are almost exclusively reported in malignant IPMNs[7].

CLASSIFICATION OF IPMNS

IPMN is an exocrine neoplasm of the pancreas consisting of epithelial cells growing within the pancreatic ducts [main pancreatic duct (MPD) or its major branches and producing mucin[1]. There is no ovarian-type stroma in IPMNs in contrast to mucinous cystic neoplasms[2]. According to the revised international consensus Fukuoka guidelines (2017)[8], IPMNs are divided into the following three types: MD-IPMN, BD-IPMN, and MT-IPMN diagnosed in radiological/histological investigations. In MD-IPMN, MPD segmental or diffuse dilation of > 5 mm without other obstruction reasons is noted. Although MPD dilation of 5-9 mm is not an absolute indication for surgery, it is one of the "worrisome features". MPD diameter ≥ 10 mm is one of the "high-risk stigmata". BD-IPMNs are cystic lesions of the pancreas measuring > 5 mm which communicate with MPD. They need differential diagnosis with pseudocysts in patients followingacute pancreatitis. In MT-IPMN, the features of both MD-IPMN and BD-IPMN are present[7].

HISTOPATHOLOGY OF IPMNS

Histologically, pancreatic IPMNs are noninvasive epithelial neoplasms arising from cells which produce mucin located within the MPD or its branches[9]. According to the degree of cytological atypia and abnormal crowding of the epithelium, low-grade, intermediate-grade and high-grade dysplasia IPMNs are distinguished[10]. The four histopathological IPMN types are distinguished such as gastric type (49%-63%), intestinal type (18%-36%), pancreaticobiliary type (7%-18%), and oncocytic type (1%-8%). The gastric type is observed the most commonly. It is typically of low grade, rarely leading to cancer. Pancreatic cancer developing from this IPMN type is usually of the tubular type and is similar to ordinary pancreatic ductal adenocarcinoma. The intestinal type is reported in numerous MD-IPMNs. The pancreaticobiliary type is not well characterized and is uncommon. According to some authors, it is a high-grade dysplasia variation of the IPMN gastric type. Ductal and aggressive invasive cancer is commonly related to this IPMN type. The oncocytic type is the less frequent variant consisting of complex aborising papillae with delicate cores, oncocytic cells, and intraepithelial lumina formation. These lesions are uncommon and have limited invasion capability. Histological types correlate with the immunohistochemical phenotype of IPMN. This correlation was presented in Table 1[7,9].

DIAGNOSTICS OF IPMNS

Diagnostics of IPMNs involve analysis of clinical presentation, radiological imaging, and laboratory investigations, including biochemical and cytological tests.

Clinical presentation

The following clinical symptoms have been reported in patients with IPMNs: Epigastric discomfort or pain (70%-80%), loss of weight (20%-40%), nausea and vomiting (11%-21%), backache (10%), diabetes, and jaundice[5]. The mucin, which is

Table 1 Histological types and immunohistochemical profiles of intraductal papillary mucinous neoplasms[7,9]

Type	Percentage	Immunohistochemical profile			
		MUC1	MUC2	MUC5AC	MUC6
Gastric	49-63	(-)	(-)	(+)	(+)
Intestinal	18-36	(-)	(+)	(+)	(±)
Pancreatobiliary	7-18	(+)	(-)	(+)	(±)
Oncocytis	1-8	(+)	(-)	(±)	(+)

MUC: Mucin.

hyperproduced, can obstruct normal secretion in the pancreas, that is a reason of meals-related pain. In this case, a patient does not eat to avoid pain. In advanced tumors, loss of appetite is related to neoplastic cachexia. Jaundice is a consequence of obstruction of the common bile duct by viscid mucin, mural nodules, or direct compression due to the size of the IPMN. Persistent occlusion of the MPD with mucin can lead to exocrine and/or endocrine pancreatic insufficiency, and persistent hyperamylasemia[5]. Regarding clinical presentation, an association between IPMNs and recurrent acute pancreatitis (AP) should be emphasized. According to Venkatesh *et al* [10], the AP is reported in 12%-67% of IPMN patients. Both MD-IPMN and BD-IPMN may lead to AP, with a similar risk. AP in IPMN patients is usually mild and does not need treatment. There is no difference in AP occurence between benign and malignant IPMNs. AP occurs more frequently in IPMN patients compared to cancer patients, possibly because of obstruction of the MPD by mucin. It is important to remember the above mentioned association in patients with recurrent AP. Frequently, in patients following AP, pancreatic pseudocysts or fluid collections are diagnosed and IPMNs are less frequently considered in the differential diagnosis. In our opinion, oncological vigilance is very important in patients with pancreatic cystic lesions and recurrent pancreatitis in medical history because the prognosis and management of patients with IPMNs and pancreatic pseudocysts are different[10]. Jang *et al*[11] analyzed IPMN patients with AP or acute recurrent pancreatitis (ARP) (AP/ARP) treated in the period of 2000-2008 in a single tertiary referral center. IPMN-associated AP/ARP was noted 34 (7%) of 488 IPMN patients, and the MD/MT-IPMN more frequently was associated with AP/ARP compared to the BD-type (14% *vs* 5%; $P = 0.002$). The mild AP was diagnosed in analyzed patients. Histological findings of 24 surgically treated tumors were as follows: Adenomas ($n = 4$) (17%), borderline malignancies ($n = 17$) (71%), CIS ($n = 2$) (8%), and invasive carcinoma ($n = 1$) (4%). There was no AP/ARP recurrence in any patients during the follow-up period (median 52 mo, range 38-115 mo). The authors concluded that, though uncommon, AP/ARP could be an initial clinical IPMN manifestation, which is helpful in the diagnostic process[11].

Regarding ARP as a clinical IPMN manifestation, Bernardoni *et al*[12], in their preliminary report, assessed the efficacy of pancreatic sphincterotomy (PS) in patients with IPMN-associated ARP. A prerequisite for treatment was the fact that IPMN-associated ARP may lead to a lower quality of life and chronic pancreatitis. In IPMN manifested as AP, a higher cancer risk is reported. According to Fukuoka consensus [13], pancreatitis may be an indication for surgery despite of no signs of malignancy in radiological and cytological investigations[12,13]. However, pancreatic surgery is associated with an increased morbidity and mortality risk even when performed at high volume surgical centers. Higher surgical risks are reported in old patients with numerous comorbidities. According to the IPMN-associated AP pathophysiology, the hypothesis regarding the falicitated mucin outflow into the duodenum by PS has developed. According to this theory, reduction of intraductal pressure could lead to reduction of AP episodes[12]. The authors retrospectively analyzed patients with ARP and IPMN undergoing PS in 2010-2015. Patients were divided into two different groups: (1) MD/MT-IPMN; and (2) BD-IPMN with or without worrisome features/high-risk stigmata. In this study, complete, partial (reduction of pancreatitis episodes > 50%), and no response were reported in 11 (68.7%), 3 (18.7%), and 2 (12.5%) patients, respectively. In 1 (6.25%) patient, mild pancreatitis was observed following endoscopic retrograde cholangiopancreatography (ERCP). There was no cancer in resected patients. Additionally, during follow-up, there were no worrisome features/high-risk stigmata[12]. The authors concluded that PS was effective for reduction of the number of AP and it should be taking into condideration as a treatment option in

selected IPMN patients. It is important that systematic follow-up should be performed in this patients' group due to the malignant IPMN potential[12].

Apart from typical IPMN clinical presentation regarding abdominal symptoms and jaundice, skin lesions named pancreatic panniculitis have been reported. Yamashita *et al*[14] described a case of a 68-year-old man presenting pancreatic panniculitis on his trunk coexisted with IPMN-associated AP. A skin biopsy of the lesion histologically showed lobular panniculitis with characteristic "ghost cells" (pancreatic panniculitis). The authors concluded that clinicians should take into account IPMN in patients with in orderto avoid a missed or delayed diagnosis[14]. Similar cases of AP and IPMN-related panniculitis have also been reported by other authors[15,16]. Furthermore, IPMN-related panniculitis has been reported[17,18]. Therefore, we also recommend oncological vigilance in patients with panniculitis.

Infrequently, IPMN can form a fistula into the adjoining organs, including the stomach, duodenum, common bile duct, large and small bowel. The fistula may be related to benign IPMN (low-grade dysplasia). This fistula may occur as a consequence of mechanical penetration as a result of pressure by the mucin-filled ducts or due to inflammation or autodigestion by enzyme-rich fluids, or it could be a result of direct invasion due to malignancy, as in malignant IPMN (high-grade dysplasia)[5,9].

Some clinical symptoms, such as jaundice and new-onset diabetes, are more frequently associated with IPMN malignancy[5,19]. Additionally, according to Weisenauer *et al*[19], new-onset diabetes mellitus and jaundice suggest malignant IPMN. The authors noted that the absence of these features did not predict benign disease[19].

Imaging diagnostics of IPMNs

Currently, there are several different guidelines on diagnostic and therapeutic management in IPMN, including Sendai International consensus guidelines for the management of pancreatic IPMNs and mucinous cystic neoplasms according to the International Association of Pancreatology (IAP) (2006)[20], American Gastroenterological Association Institute guidelines on the diagnosis and management of asymptomatic neoplastic pancreatic cysts according to the American Gastroenterological Association (AGA) (2015)[21], revised international consensus Fukuoka guidelines for the management of IPMN of the pancreas (2012)[13], revised international consensus Fukuoka guidelines for the management of IPMN of the pancreas (2017)[8], and European evidence-based guidelines on pancreatic cystic neoplasms according to the European Study Group on Cystic Tumors of the Pancreas (2018)[22]. Diagnostic investigations are performed to select IPMN patients indicated for surgical resection. Therefore, diagnostic investigations should show alarming symptoms for malignant transformation in IPMN. As such, indications for surgery according to different guidelines should be known. They are presented in Table 2[5,8,13,20-22,41].

Computed tomography of the abdominal cavity

According to the most recent European guidelines for pancreatic cystic neoplasms (PCNs) (2018)[22], the accuracy of abdominal CT for identifying the specific PCN type is 40%-81%[22]. Multidetector row computed tomography (MDCT) for IPMN diagnosis should be performed according to a special standardized protocol[22]. Takeshita *et al*[23] evaluated predictive factors for discriminating benign from malignant pancreatic IPMN on MDCT. The study included 53 patients. Tumors were classified as MD-type (n = 7) and BD-type (n = 46). All MD-IPMNs weremalignant, while 8 of 46 BD-IPMNs were malignant, and 38 were benign. In additionn, MPD dilatation and mural nodules or large cystic diameter combined were significant risk factors of malignancy in BD-IPMN. According to the authors, MD-IPMN is strongly associated with malignancy[24]. Nakagawa *et al*[24] retrospectively evaluated the utylity of MDCT with multiplanar reformations and curved planar reformations in diagnosis of protruding lesions in IPMNs compared to single-detector CT (SDCT) and endoscopic ultrasonography (EUS). This study showed that MDCT was more useful than SDCT and similar to EUS in diagnosis ofprotruding lesions in IPMNs[24]. Tan *et al*[25] also retrospectively evaluated the imaging features of IPMNs in MDCT. Comparison with the pathological diagnosis revealed that the sensitivity, specificity, and accuracy of MDCT in assessing the IPMN were 100%, 87.5% and 95%, respectively. Thus, MDCT can be used to predict the IPMN malignancy[26]. Murayama *et al*[26], compared CT and MRI in assessment of IPMN malignancy. There was a statistical difference in MPD diameter (P = 0.017) and intraductal volume (P = 0.0013) inadenoma, CIS, and invasive cancer. This study showed that intraductal volume (≥ 10 cm) was helpful in the malignant IPMN diagnosis[26].

Table 2 Indications for surgery in intraductal papillary mucinous neoplasms according to the International, European and American Gastroenterological Association guidelines[5,8,13,20-22,41]

Guidelines	Indications for surgery
IAP (2006)	Symptoms; Cyst size ≥ 3 cm; Mural nodule; MPD ≥ 5 mm; Positive cytology
AGA (2015)	High risk features: Cyst size ≥ 3 cm; Presence of solid component; Dilated MPD
	HGD or cancer on cytology
IAP (2017)	High risk stigmata: Jaundice; Enhancing mural nodule ≥ 5 mm; MPD ≥ 10 mm
	HGD or cancer on cytology
	Worrisome features: Cyst size ≥ 3 cm; Acute pancreatitis (due to IPMN)
	Enhancing mural nodule ≥ 5 mm; Thickened and enhancing cyst wall
	MPD dilation 5-9 mm; Abrupt change of MPD caliber with distal pancreatic atrophy; Presence of lymphadenopathy; Elevated serum CA 19-9; Cyst growth rate > 5 mm/2 yr
European (2018)	Absolute indications: Jaundice; Enhancing mural nodule ≥ 5 mm; MPD ≥ 10 mm; HGD or cancer on cytology; Solid mass
	Relative indications: Cyst size ≥ 4 cm; Enhancing mural nodule ≥ 5 mm/years; Acute pancreatitis (due to IPMN); New onset of diabetes; Rapidly increasing cyst size; Elevated serum levels of CA19-9

IPMN: Intraductal papillary mucinous neoplasm; IAP: International Association of Pancreatology; AGA: American Gastroenterological Association; MPD: Main pancreatic duct; HGD: High grade dysplasia.

Monnings *et al*[27] analyzed preoperative CT scans in IPMN patients. Benign (bIPMN; n = 28) and malignant (mIPMN; n = 19) tumors were compared. The MPD diameter was greater in patients with mIPMN (P < 0.0001). Obstruction of the bile duct, solid tumor components, contrast enhancement in walls of the cyst, peripancreatic lymph nodes, and abrupt MPD diameter changes were observed in more mIPMN patients (P < 0.01). In addition, in mIPMN, the CT cyst density was higher (P = 0.0063). The summary diagnostic accuracy was higher than all single CT parameters [27].

Apart from the numerous above mentioned benefits, CT also has a disadvantage, which is most important in IPMN patients requiring systematic control imaging diagnostics. It has been reported that repeated exposure to ionizing radiation following CT increases the cancer risk[22,28]. Sodicson *et al*[28] estimated the cumulative radiation exposure and lifetime attributable risk (LAR) of radiation-induced cancer from CT scanning of adult patients at a tertiary care academic medical center. The analysis showed that 33% of patients had ≥ 5 lifetime CT investigations, and 5% had 22-132 examinations. Cumulative effective doses > 100 mSv in 15%, and 250-1375 mSv in 4% of patients, respectively, were reported. In 7 % of patients, LAR > 1% was noted. It should be added that assigned effective doses per CT examination are as follows: for CT of the abdomen (without pelvis), 7.5 mSv, and for CT of the abdomen and pelvis, 15 mSv[28].

MRI and magnetic resonance cholangiopancreatography

According to European guidelines for PCNs[22], the accuracy of MRI/magnetic resonance cholangiopancreatography (MRCP) for identifying the special PCN type is 40%-95%. These guidelines recommend MRI as the preferred method for the investigation of patients with PCN. The higher sensivity of MRI/MRCP compared to CT for detection of communication between a PCN and the pancreatic ducts and presence of mural nodules or internal septations has been noted. MRI/MRCP is also good in the differential diagnosis of single and multiple PCNs, including multifocal BD-IPMN. Moreover, IPMN patients frequently require long-life control investigations, and MRI is less invasive than CT[24]. According to the same guidelines, MDCT is helpful for diagnosis of calcification, tumor staging assessment, or for diagnosing postoperative recurrent disease[22].

Min *et al*[29] retrospectively analyzedpatients undergoing surgery for IPMN followingpreoperative CT and MRI in 2009-2019. There were 88 (50.3%) malignant IPMNs in this study. All 3 high-risk stigmata (MPD ≥ 10 mm, mural nodule ≥ 5 mm, and obstructive jaundice) and 2 worrisome features (MPD 5-9 mm and increased level of CA 19.9) were related to malignant IPMN on CT and MRI (P < 0.05). A mural nodule < 5 mm on MRI was also related to malignant IPMN (P < 0.01). This study showed that CT and MRI were comparable for diagnosis of high risk stigmata (73.7% *vs* 75.4%; P =

0.505). In addition MRI was superior to CT for diagnosis of mural nodules, and similar to CT for differentiation ofmalignant from benign IPMNs[29].

Liu *et al*[30], in a meta-analysis, assessed the diagnostic properties of CT, PET/CT, MRI/MRCP, DWI, and EUS in differential IPMN diagnosis (benign *vs* malignant tumors). Twenty eight studies were included. This study showed the highest diagnostic accuracy results for PET/CT, and the use of MRI/MRCP, PET/CT was recommended as a first-line investigation in the diagnosis of malignant IPMN, and DWI, EUS and CT were additional for MRI/MRCP in IPMN diagnosis[30].

Jeon *et al*[31] investigated the MRI utility to predict the malignant IPMN potential. In this study, enhancing mural nodule size ≥ 5 mm, MPD ≥ 10 mm / MPD of 5-9 mm, and MPD abrupt changes significantly predicted to malignant IPMNs ($P < 0.05$). In multivariate analysis, enhancing mural nodules ≥ 5 mm, MPDs ≥ 10 mm or MPDs of 5-9 mm, larger entropy, smaller compactness were significant predictors for malignant IPMNs ($P < 0.05$)[31].

Boraschi *et al*[32] retrospectively in their retrospective study, showed the MRI utility in the diagnosis of worrisome features and high-risk stigmata in patients with BD-IPMNs during 10 years of observation from the tumor diagnosis[32].

Endoscopic ultrasound

According to European guidelines[22], EUS is recommended as additional to other radiological investigations. It is helpful for diagnosing PCN indicated for surgery. Similar to MRI and CT, EUS is not perfect in diagnosis of the exact PCN type of EUS is recommended in patients with PCNs with concern clinical or radiological features[22].

Contrast harmonic enhanced EUS (CH-EUS) is recommended for assessment of mural nodules. CH-EUS is also useful in assessment of presence of vessels and septations within the cyst. Hyperenhancement of a mural nodule, solid mass, or septations on CH-EUS predict malignancy, that is indication for EUS-fine needle aspiration (FNA) of the tumor[22].

Choi *et al*[33] compared EUS, CT and MR in the diagnosis of IPMN malignant transformation. All compared investigations were similar in this analysis. In the multivariable analysis, enhanced solid components on contrast-enhanced CT and MRI and mural nodules on EUS, MPD diameters ≥ 10 mm, MPD diameters of 5-9 mm and thickened septa or walls were significant ($P < 0.05$). Thus, the diagnostic performance of CT, MRI, and EUS for predictiion of malignant IPMNs was comparable[33].

The diagnostic accuracy of EUS increases if biopsy is performed and pancreatic cyst fluid is collected for analysis during EUS. EUS-FNA increases diagnostic accuracy for differentail diagnosis of mucinous from nonmucinous PCN and malignant from benign PCN in patients in whom CT or MRI are unclear. A combined analysis of cyst fluid CEA, lipase levels, and cytology has the highest accuracy for differential diagnosis of mucinous from nonmucinous PCNs. It is important that EUS-FNA is recommended only when the results can modify management and EUS-FNA should not be performed if the diagnosis is already made using radiological investigations and in patients with clear indications for surgical treatment. Relative contraindications for this investigation are as follows: A distance of > 10 mm between the cyst and the transducer, a high hemorrhage risk, and the use of dual antiplatelet drugs[22]. Assessment of cyst fluid CEA, combined with cytology, or KRAS/GNAS mutation analyses may be considered for differentiating an IPMN or MCN from other PCNs[22].

Mc Carty *et al*[34] published a systematic review and meta-analysis including 6 studies (785 tumors) to asses the diagnostic utility of K-ras and G-nas mutations in EUS-acquired pancreatic cyst fluid for the diagnosis of IPMNs and mucinous cystic lesions. It should be added that molecular cyst fluid diagnostics are not yet a standard. There was a significantly higher accuracy of combined K-ras + G-nas compared to K-ras alone and G-nas alone in the differential diagnosis ($P < 0.001$). The pooled sensitivity, specificity, and diagnostic accuracy of K-ras + G-nas mutations in the IPMN diagnosis were 94%, 91% and 97%, respectively. They were significantly higher compared to CEA alone (all $P < 0.001$)[34].

Kadayifci *et al*[35] investigated the value of GNAS investigation in addition to KRAS and CEA tests of pancreatic cystic fluid (PCF) for the IPMN diagnosis. There were 108 IPMN and 89 non-IPMN patients in the analyzed group. GNAS was noted in 51 (47.2%) IPMN patients, and a KRAS mutation was noted in 42 (82.3%) patients. The diagnostic accuracy increased from 76.6% to 79.1% ($P > 0.05$), when GNAS to KRAS was added and from 66.4% to 80.7% ($P < 0.05$) when GNAS to CEA was added. It should be noted that the diagnostic accuracy of the combined all tests was significantly higher compared to all single investigations ($P < 0.05$)[35].

Lee *et al*[36] published a meta-analysis to analyze KRAS and GNAS mutations in pancreatic cystic lesions. In this study, KRAS and GNAS mutations were more common in IPMNs compared to mucinous and serous cystic neoplasms, respectively. KRAS and GNAS mutations were frequently reported in the gastric ($P < 0.001$) and intestinal ($P < 0.001$) types, respectively. KRAS mutation was not common in high-grade dysplasia IPMNs ($P = 0.032$). This meta-analysis confirmed that KRAS and GNAS mutations are useful for diagnostic tools for IPMN[36].

Gillis *et al*[37], in their meta-analysis, noted 42% sensitivity and 99% specificity of PCF cytological analysis for differential diagnosis of mucinous *vs* nonmucinous PCNs [22]. According to most authors, a cyst fluid CEA cutoff level of ≥ 192 ng/mL can differentiate mucinous cysts from nonmucinous cysts, with a sensitivity of 52%-78% and specificity of 63%-91%[22].

Indications for EUS-FNA are different depending on International Consensus Guidelines (ICG), AGA, and European guidelines. According to ICG, this investigation is indicated in patients with pancreatitis, tumor diameter > 30 mm, thickened or enhanced wall of the cyst, MPD 5-9 mm, nonenhancing mural nodules, abrupt tapering of the pancreatic duct and atrophy of the distal tail. AGA recommends EUS-FNA in the presence of two of the following risk factors: cyst diameter > 30 mm, the presence of a solid component in the cyst, and MPD dilatation. The European guidelines recommend the use of EUS as part of a multimodality diagnostic assessment[38,39].

ERCP and/or pancreatoscopy

The role of ERCP in IPMN diagnostics is limited. According to European guidelines [22], pancreatoscopy may be used in selected patients to assess the MD-IPMN location and extent and can help to differentiate MD-IPMN from chronic pancreatitis. The diagnostic accuracy of pancreatoscopy was higher in MD-IPMN (88%) compared to BD-IPMN (67%). Intraoperative MPD pancreatoscopy made with frozen sections of intraductal biopsies may be used in assessment of the IPMN extent and MPD involvement, which is important for surgeons' decisions regarding the extent of surgical resection[22].

Blood tests

The role of blood tests in IPMN diagnostics is also limited. According to current guidelines on IPMNs[22], molecular blood tests are not used in PCNs diagnostics. Only serum cancer antigen CA 19.9 can be useful in IPMN in patients with malignant transformation suspected[22].

MANAGEMENT OF IPMNS

Indications for surgery

Management of IPMNs is still controversial because of different recommendations of the ICG, AGA, and European guidelines. The earliest (2006) Sendai ICG guidelines were the most restrictive. In 2006, Tanaka *et al*[20] recommended resecting all MD- and MT-IPMNs as long as the patient is a good candidate for surgery. Patients with BD-IPMNs, with no symptoms, require surgery not only to relief the signs but also due to a n increased risk of malignant transformation. Moreover, according to these guidelines, BD IPMNs > 30 mm in diameter and without MPD dilation or mural nodules should be assessed if all BD-IPMNs > 30 mm in diameter require surgery immediately. The Sendai recommendations have resulted in a high rate of "unnecessary" pancreatic surgeries. This is important because pancreatectomy is a complex procedure associated with relatively high morbidity and mortality rates[38]. The original Sendai group published revised ICG, commonly known as the Fukuoka guidelines in 2012. According to the IAP Fukuoka 2012 guidelines, revised in 2017, surgery is strongly recommended for all MD-IPMNs with a MPD of diameter > 10 mm or with "high-risk stigmata" (HR), such as an enhancing solid component or jaundice. Dilatation of the MPD 5-9 mm is considered a "worrisome feature," and it is not recommended for immediate resection but requiring further assessment using EUS[8, 13]. In 2015, AGA recommended surgical treatment for patients, with no symptoms, only in the presence of two of three "concerning features" (presence of nodule, diameter > 30 m, or duct dilation) and malignant transformation in EUS-FNA[21].

Authors of the European guidelines[22] recommended surgery in IPMNs with jaundice, an enhancing mural nodule (≥ 5 mm) or a solid component, positive cytology, or MPD diameter ≥ 10 mm. Surgical management was also recommended for

IPMNs with MPD dilatation 5-9.9 mm, cystic growth rate ≥ 5 mm/year, elevated serum CA 19.9 concentration (> 37 U/mL), signs, enhancing mural nodules, and IPMNs > 40 mm regardless of the presence of other high-risk factors[22]. In BD-IPMNs, jaundice, high-grade dysplasia or cancer in cytology, a contrast-enhancing mural nodule (≥ 5 mm) or solid mass are absolute indications for surgery. The relative indications for surgery are the following: Growth rate ≥ 5 mm/year, elevated serum CA 19.9 concentration (in the absence of jaundice), MPD diameter 5-9.9 mm, IPMN size ≥ 40 mm, clinical manifestation (new-onset diabetes mellitus or AP), and contrast-enhancing mural nodules[22].

In conclusion, according to all current guidelines, surgical treatment is recommended in all IPMNs involving the MPD, but there is still no consensus regarding MPD dilation. In the absence of other "high-risk stigmata", MPD dilatation alone is considered as a risk of misdiagnosis and possible overtreatment. Therefore, some authors suggested radiologic surveillance in patients with no symptoms and with "worrisome" MPD dilatation (5-9 mm) and without other HR stigmata[40]. All guidelines regarding current management in IPMN patients are presented in Table 3 [5,8,13,20-22,41].

Extent of surgical resection

According to Sendai guidelines[21], pancreatectomy with lymphadenectomy is necessary when invasive cancer is suspected. The type and extent of surgery depend on the IPMN location and extent[22]. The pancreatic head is the most frequent IPMN location. Therefore, pancreaticoduodenectomy (PD) is recommended in IPMNs located within the pancreatic head, uncinate process, and neck. Distal pancreatectomy (DP) is indicated for IPMNs located within the pancreatic body and tail. Total pancreatectomy (TP) is performed in exceptional cases when IPMN diffusely involves the whole pancreas or when a proximal IPMN extends through the distal pancreas. It is associated with the long-term consequences of TP, such as exocrine and endocrine pancreatic insufficiency requiring supplementation of pancreatic enzymes and diabetes treatment with insulin use. In each partial pancreatic resection, an assessment of the margin by frozen section is needed to confirm R0 resection with negative margins, and the resection should be extended in cases with cancer-positive surgical margins[5].

According to the revised Fukuoka guidelines[8], PD, DP, or TP according to the IPMN location and extent with lymphadenectomy should be the standard surgical treatment. Limited resections or even focal nonanatomic resections (excision, enucleation, uncinatectomy) can be performed in BD-IPMN not suspected for invasive cancer[8]. The authors added that nonanatomic resections could be associated with infrequent but possible mucin leakage followed by peritoneal pseudomyxoma, a higher risk of postoperative pancreatic fistula and a risk of neoplasm recurrence. Standard pancreatectomy and lymphadenectomy should be performed if the cancer possibility is present[8]. We recommend using the European guidelines in decision making regarding the extent of IPMN surgery. According to the European guidelines [22], PD with frozen section investigations of the resection margins is recommended for patients with MPD dilatation comprising the entire pancreas. TP can be taken into consideration in patients with mural nodules within the MPD, and a higher cancer risk (familial pancreatic cancer). For BD-IPMNs, the authors recommend oncological resection with standard lymphadenectomy. It should be emphasized that parenchyma-sparing pancreatectomy is not an oncological procedure that can be performed only in lesions with a very low malignancy probability-for example, in patients without risk factors strongly wishing to be surgically treated. Due to a high malignancy risk, oncologic resection including standard lymphadenectomy is the recommended for IPMN with an absolute indication for resection. In multifocal BD-IPMN, each tumor should be assessed individually for the presence of malignancy-associated features. Patients with IPMNs with no concerning features can be observed[22].

Surveillance in IPMN patients

Patients with IPMNs lacking HRS/absolute indications should undergo nonoperative management. The surveillance strategies according to different guidelines are presented in Table 4[5,8,13,20-22,41].

According to the revised Fukuoka guidelines, surveillance is determined by IPMN diameter. The revised guidelines are more restrictive compared to the Fukuoka (2012) and Sendai guidelines (2006) and recommend initial surveillance performed at a shorter interval (within 6 mo for cysts < 20 mm and within 3-6 mo for cysts 2-3 cm). Following initial risk stratification, cysts < 10 mm should be radiologically monitored every 2 years in cysts with no changes. Cysts 10-20 mm should also be controlled

Table 3 Management of intraductal papillary mucinous neoplasm patients regarding indications for surgery according to the International, European and American Gastroenterological Association guidelines[5,8,13,20-22,41]

Guidelines	Management
IAP (2006)	Indications: Surgery
AGA (2015)	Indications: Surgery
IAP (2017)	High risk stigmata: Surgery
	Worrisome features: Surgery versus close surveillance based on: Patients' age/comorbidities: More aggressive management (surgery) in young patients
	EUS findings: Surgery indicated in clear MPD involvement and/or high-risk features
European (2018)	Absolute indications: Surgery
	Relative indications: Surgery according to criteria count, depending on comorbidities
	In fit patients: surgery for 1 criterion
	In patients with significant comorbidities: surgery for 2 criteria

IAP: International Association of Pancreatology; AGA: American Gastroenterological Association; EUS: Endoscopic ultrasonography; MPD: Main pancreatic duct.

Table 4 Surveillance in intraductal papillary mucinous neoplasm patients regarding indications for surgery according to the International, European and American Gastroenterological Association guidelines[5,8,13,20-22,41]

Guidelines	Indications	Investigations	Algorithm of follow-up
IAP (2006)	BD-IPMNs ≤ 30 mm; Without: Symptoms, mural nodules, positive cytology	MRI/MRCP or CT	Size ≤ 20 mm: every 6-12 mo; Size 20-30 mm: every 3-6 mo; The interval can be longer after 2 yr without changes
AGA (2015)	BD-IPMNs ≤ 30 mm; Without: Solid component, dilated MPD, HGD/cancer	MRI	Years 1, 2, 5 from initial diagnosis; It can be considered to discontinue; If there is no changes after years
IAP (2017)	No HRS/WF	MRI/MRCP, CT	Size < 10 mm: At 6 mo from diagnosis every 2 yr (if no change)
	No HRS/WF	MRI/MRCP, CT	Size 10-20 mm: At 6 mo from diagnosis yearly per 2 yr
	No HRS/WF	MRI/MRCP, EUS	Size 20-30 mm: EUS in 3-6 mo, yearly EUS or MRI
	No HRS, WF present and size < 30 mm	MRI/MRCPEUS	Every 3-6 mo EUS or MRI
European (2018)	No AI	MRI/MRCP or EUS, CA 19.9	Every 6 mo for the first year; Yearly after first year
	No AI, 1 RI in patient, with comorbidities	MRI/MRCP or EUS, CA 19.9	Every 6 mo

IPMN: Intraductal papillary mucinous neoplasm; IAP: International Association of Pancreatology; AGA: American Gastroenterological Association, MRI: Magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct; HGD: High grade dysplasia; EUS: Endoscopic ultrasonography; HRS: High risk stigmata; WF: Worrisome features; AI: Absolute indications for surgery; RI: Relative indications for surgery.

radiologically every 2 years, EUS or MRI should be performed every 1 year in cysts 20-30 mm. A diameter change alone (≥ 5 mm growth in 2 years), in addition to the presence of any worrisome features, is sufficient to recommend systematic EUS[8,13,20,41].

The AGA guidelines[21] recommend surveillance for patients with BD-IPMNs < 30 mm, with no a solid component, dilated MPD, HGD or cancer in cytologic findings. In these patients, MRI should be performed in years 1, 2, and 5 from initial diagnosis. If no significant change occurs, surveillance discontinuation should be considered. Other patients should be referred to surgery[8,21].

The authors for the European guidelines recommend a 6-mo follow-up (using MRI/MRCP and/or EUS and serum CA 19.9) in the first year and then yearly follow-up, in patients with a suspected IPMN that does not meet the indication for surgery.

The guidelines recommend to continue observation as long as the patient remains surgically fit[8,22,39].

Follow-up after surgery

According to the revised Fukuoka guidelines[8], all IPMN patients, including those with noninvasive IPMNs with negative surgical margins, need follow-up after surgery to diagnose a new IPMN requiring surgery or pancreatic cancer. Tanaka *et al*[8] recommend continuing surveillance as long as the patient remains fit. In patients with higher risks, such as a family history of pancreatic cancer, HGD in surgical margins, and nonintestinal IPMN histological type, radiological investigations at least twice a year are recommended, and in others investigations every 6-12 mo should be performed. The follow-up of invasive IPMN should be the same as in pancreatic cancer [8].

The European guidelines are similar, and according to them[22], lifelong follow-up is recommended after IPMN resection as long as the patient is fit for surgery. Patients with IPMN-associated invasive cancer should be followed up in the same manner as those with resected pancreatic cancer. In HGD IPMN and MD-IPMN, follow-up every 6 mo for the first 2 years, followed by yearly surveillance is recommended. LGD IPMN should be observed in the same manner as nonresected IPMN. Patients with IPMN in the remnant pancreas with no HGD or MD-IPMN should be observed as nonresected BD-IPMN. In a postoperative observation, MRI or EUS are recommended[22].

The AGA guidelines are very liberal. The authors recommend postoperative surveillance only for patients following surgery due to invasive IPMN. According to the AGA guidelines, patients with invasive cancer or dysplasia in the cyst after surgery should undergo MRI every 2 years. Moreover, the AGA did not recommend routine follow-up of IPMNs with no HGD or malignancy in the surgical specimen[21].

The clinical utility of the current guidelines regarding the management of IPMNs

Hsiao *et al*[42] evaluated the utility of the 2006 Sendai and 2012 Fukuoka guidelines in the differential diagnosis malignant and benign IPMNs. The study included 138 IPMN patients operated on between January 2000 and March 2015. Patients were "Sendai positive" if the tumor diameter was ≥ 30 mm, with no symptoms, with mural nodules or a thickened wall, or with a dilated MPD of ≥ 6 mm. Patients without above mentioned criteria were classified as "Sendai negative". Patients were characterized as "Fukuoka high risk" in the presence of: obstructive jaundice, or enhancing solid component, or MPD of ≥ 10 mm. "Fukuoka worrisome" were IPMNs with the presence of any worrisome features (pancreatitis, a tumor diamater of ≥ 30 mm, a thickened/enhancing cyst wall, nonenhancing mural nodules, an abrupt MPD diamater change with distal pancreatic atrophy, and an MPD of 5-9 mm). The positive predictive value (PPV) and negative predictive value (NPV) of the Sendai and Fukuoka guidelines for HGD/IC were 35.1%, 43.3%, 100%, and 85.4%, respectively. According to the multivariate analysis, jaundice, tumors of ≥ 30 mm, presence of mural nodules, and age < 65 years were associated with HGD/invasive cancer in IPMN patients. There was a better NPV in the Sendai guidelines, but a better PPV in the Fukuoka guidelines. In the authors' opinion, a more aggressive management in patients with Fukuoka worrisome features couled be considered. The study showed that IPMNs of ≥ 30 mm, but not pancreatitis, are associated with malignancy[42].

Pérez-Cuadrado-Robles *et al*[43] assessed the accuracy of the European guidelines in BD-IPMN patients indicated for surgery in a multicenter, observational, retrospective study including 91 patients with absolute (n = 21), relative (n = 60), or no formal indications (n = 10) for surgery. There were 60 patients with one (n = 35) or ≥ 2 relative indications (n = 25) for surgery in this study. The global advanced tumor and invasive cancer rates were 40% and 13.3%, respectively. There were not risk factors for GHD or invasive cancer. A lower risk of invasive cancer was reported in patients with one relative indication compared to patients with ≥ 2 relative indications (5.7% *vs* 24%, respectively; P = 0.048). The advanced IPMN incidences were similar in the compared groups (37.1% *vs* 44%; P = 0.593)[43].

Jan *et al*[44] also validated the European guidelines for the management of IPMNs. The study included 158 patients with resected IPMNs between January 1994 and December 2016. All patients were stratified into three groups according to the European guidelines: Absolute, relative indications, and conservative approach. The missed rate for HGD/IC by the European guidelines was 1.9% (3 of 158). The sensitivity, specificity, positive and negative predictive values, and accuracy of the absolute or relative indications for resecting IPMN according to these criteria were 94.1%, 28.0%, 38.4%, 90.9%, and 49.4%, respectively. Jaundice, enhancing mural nodules < 5 mm, cyst diameter > 40 mm, elevated serum CA 19.9 concentration, new-

onset diabetes, and MPD dilation were associated with HGD/IC. Thus, the missed rate for HGD/IC was low using the European guidelines. Increased serum CA 19.9 and new-onset diabetes in European recommendations were verified as indications for the surgical resection of IPMNs[44].

Correa-Gallego *et al*[45] analyzed two independent nomograms to predict the findings of adenoma, high-grade dysplasia (HGD-CIS), and invasive carcinoma separately in both MD- and BD-IPMN. This study involved 219 patients including 56% of BD-IPMN in resected specimens. The significantly higher proportion of HGD-CIS was reported in MD-IPMN (33%) compared to BD-IPMN (15%) ($P = 0.003$). Invasive cancer was significantly more frequent in MD-IPMNs (41%) compared to BD-IPMNs (15%) ($P < 0.001$). In addition patient sex, history of prior malignancy, presence of a solid component, and weight loss were significantly associated with the ordinal outcome for MD-IPMN patients and were included in the nomogram (concordance index 0.74). For BD-IPMN patients, weight loss, solid component, and lesion diameter were associated with the outcome (concordance index 0.74)[45].

Capurso *et al*[46] investigated patient- and cyst-related factors associated with progression into WF or HRS categories of BD-IPMNs. This study included 540 patients diagnosed from 2009 to 2018 with at least 12 mo of surveillance until February 28, 2020. The revised Fukuoka criteria were used. Disease progression was noted in 130 (24.1%) patients. The probability of progression was 3.7% during 1 year, 23.4% during 5 years, and 43.3% during 10 years. Surgical treatment was performed in 15 (2.8%) patients. In 7 (1.3%) patients, cancer was found, and 3 (0.56%) patients died of pancreatic-associated disease. Initial cyst size > 15 mm, body mass index > 26.4 and heavy smoking were independent progression risk factors. The authors analyzed the association between AB0 blood group and progression risk. The higher association of AA group compared to 00 group with progression was also associated. The authors concluded that IPMN diameter alone is not a sufficient for the assessment of progression risk; however, it is useful in correlation with correlated with other features in observation of BD-IPMN patients[46].

Kwon *et al*[47] validated the current guidelines on BD-IPMNs in a meta-analysis including 40 studies (6301 patients). In this meta-analysis, HGD or pancreatic cancer was significantly associated with clinical manifestation, cyst diameter ≥ 30 mm, thickening of the cystic wall, mural nodules, MPD dilatation, abrupt MPD diameter changes, lymphadenopathy, increased CA 19.9 and increased CEA[47].

Srinivasan *et al*[48] published a systematic review to assess the clinical utility of the Sendai Consensus Guidelines and Fukuoka Consensus Guidelines for IPMNs. This review included 10 studies assessing the Fukuoka guidelines, 8 assessing the Sendai criteria and 4 assessing both guidelines. Pooled analysis showed that 751 of 1801 (42%) Fukuoka-positive neoplasms were malignant, and 599 of 697 (86%) Fukuoka-negative neoplasms were benign. The PPVs of the high-risk and worrisome-risk groups were 465/986 (47%) and 239/520 (46%), respectively, while 265 of 802 (33%) Sendai-positive neoplasms were malignant and 238 of 266 Sendai-negative (90%) neoplasms were benign. In conclusion, a higher PPV was noted in the Fukuoka compared to the Sendai criteria. However, the NPV of the Fukuoka guidelines was slightly lower compared to the Sendai guidelines. A higher PPV and lower NPV was reported in the Fukuoka compared to the Sendai criteria. Thus, malignant and even invasive IPMNs may be missed using both guidelines[48].

The participants of the Verona Evidence-Based Meeting on IPMN[49] assessed and compared the dissemination, use in clinical practice, and reliability of current guidelines for the management of PCNs. PCN classification as well as clinical and radiologic features were based on the IAP, European guidelines, and AGA recommendations. The answers to 47 questions were collected from 259 international responders, including participants from Europe (86%), Asia (8%), and the United States (6%). Among the responders, 58% were surgeons and 38% were gastroenterologists. The European guidelines were the best-known (79%), followed by IAP (69%) and AGA (61%) recommendations. The diagnostic investigations (MRI, CT, EUS, and cyst fluid analysis) were known by all participants; however, contrast-enhanced EUS was available only for 41% of responders. The analysis showed that guidelines were the most widely disseminated among surgeons and gastroenterologists, but the clinical application was decreased by the limited availability of diagnostic examinations. For example, contrast-enhanced EUS examination is not available for > 50% of physicians. Although enhancing mural nodules ≥ 5 mm, considered high-risk stigmata, are absolute indications for surgery, according to >30% of physicians, this feature was not a sufficient indication for surgery. Therefore, according to Verona EBM experts, some questions (including the role of mural nodes in patients during follow-up, the correlation between imaging and histopathological findings, the optimal diameter

cutoff for the optimal assessment of the risk malignancy, and the most accurate imaging for optimal diagnosis) should be resolved. Despite of knowledge of the increased rate of malignant transformation in resected IPMNs with an MPD of diameter 5.0-9.9 mm, according to > 80% of responders, this feature was not a sufficient indication for surgery. Without prospective observational data on the observed IPMN, moderate MPD dilatation alone was not associated with an increased perception of cancer risk by clinicians. According to > 60% of responders, IPMN diameter and cyst growth rate were not enough indications for surgery. According to Verona EBM participants, further studies regarding IPMN-related symptoms as indications for surgery are needed. The guidelines should be more detailed to identify patients requiring surgery due to clinical presentation to avoid unnecessary surgery. The length of follow-up is also questionable. According to the AGA guidelines, surveillance should be discontinued after 5 years in patients with a stable pancreatic cystic neoplasm. Only 18% of responders would consider to discontinue observation after 5 years, but according to 54% of them, there is not enough evidence to recommend lifetime observation. Therefore, further studies assessing the most cost-effective surveillance protocols and identifying the most suitable population for surveillance discontinuation are required. In addition, further studies, including randomized controlled trials, should identify patients requiring adjuvant treatment after surgery for invasive IPMNs. The authors of Verona EBM pointed to three levels of discrepancies regarding recommendations in pancreatic cystic neoplasms: among the 3 existing guidelines themselves, between guidelines and available evidence, and between guidelines and clinical practice. The role of MPD dilatation, mural nodules, tumor diameter and growth rate, tumor-associated clinical signs, and discontinuation of observation are the most important issues. According to experts, the current guidelines should be updated and unified to facilitate their use in clinical practice. The goal of Verona EBM participants was to define future research directions to increase the level of available evidence[49].

Prognosis of IPMN patients following surgery

The overall 5-year survival is reported to be 36%-77%. It depends on tumor advancement and the presence of malignant transformation in the resected tumor. The best prognosis is in benign IPMNs. The 5-year survival following surgery for non-invasive IPMN is 77%-100%. In malignant IPMNs, the prognosis is poorer. The 5-year survival rate following surgery for IPMN with invasive cancer is 27%-60%[5].

CONCLUSION

Clinical decision making for patients with pancreatic IPMNs is still challenging. While the management of MD-IPMN does not raise doubts and all guidelines require resection due to the high risk of malignant transformation, the management of BD-IPMN is controversial. The most important is the correct selection of patients requiring surgery at the right time, without unnecessarily exposing patients who do not require surgical treatment to complications related to pancreatic resection. It is known that pancreatectomy performed even in the most experienced centers is associated with the risk of complications. The correct algorithm of observation of patients not qualified for resection is also important. This review of the literature showed that the current guidelines are indeed useful in managing patients with IPMNs but are not ideal. Further prospective multicenter studies are needed to optimally select surgical candidates so that only those patients who need surgery are operated on and that treatment is avoided for the remaining patients who can be safely monitored.

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REVIEW

Combined treatments in hepatocellular carcinoma: Time to put them in the guidelines?

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Abstract

The time for battling cancer has never been more suitable than nowadays and fortunately against hepatocellular carcinoma (HCC) we do have a far-reaching arsenal. Moreover, because liver cancer comprises a plethora of stages-from very early to advanced disease and with many treatment options–from surgery to immunotherapy trials–it leaves the clinician a wide range of options. The scope of our review is to throw light on combination treatments that seem to be beyond guidelines and to highlight these using evidence-based analysis of the most frequently used combination therapies, discussing their advantages and flaws in comparison to the current standard of care. One particular combination therapy seems to be in the forefront: Transarterial chemoembolization plus ablation for medium-size non-resectable HCC (3-5 cm), which is currently at the frontier between Barcelona Clinic Liver Cancer classification A and B. Not only does it improve the outcome in contrast to each individual therapy, but it also seems to have similar results to surgery. Also, the abundance of immune checkpoint inhibitors that have appeared lately in clinical trials are bringing promising results against HCC. Although the path of combination therapies in HCC is still filled with uncertainty and caveats, in the following years the hepatology and oncology

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fields could witness an HCC guideline revolution.

Key Words: Hepatocellular carcinoma; Transarterial chemoembolization; Radiofrequency ablation; Microwave ablation; Systemic therapy; Immunotherapy combined treatments

Core Tip: The field of hepatocellular carcinoma has become highly interesting in recent years, given the emergence of a large amount of data on immunotherapy and combination treatment strategies. In this light, the current clinical practice guidelines may appear restrictive, especially in borderline cases, which have become a significant challenge in tumor boards across the world. The current review is designed to provide an exhaustive analysis of the most notable advances in the field, focusing on combination therapies and their role in the therapeutic algorithm, with the ultimate goal of aiding clinicians to navigate the Barcelona clinic liver cancer classification maze.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, ranking sixth overall among malignancies in incidence and, disproportionately, fourth in mortality[1]. It is a multifaceted disease, atypical among cancers due to its intricate and non-linear prognostic indicators, as survival is closely intertwined with the tumor extension, the severity of the underlying liver disease, and overall fitness. To this point, there is a wide array of available techniques in the therapeutic arsenal against HCC. The options range from curative-intent solutions such as surgery or transplantation to local ablation, interventional radiology, and systemic therapies[2,3]. However, despite recent advances and potentially game-changing developments, HCC is still associated with a poor prognosis, with an incidence to mortality ratio dismally approaching number one[4].

Currently, the most frequently employed algorithm for standardizing care is the Barcelona Clinic Liver Cancer Classification (BCLC) and its subsequent updates[5]. Arguably the best algorithm to this point and backed by extensive validation, it still poses significant clinical dilemmas, especially for cases that do not fit perfectly in its boxes. In such scenarios, the comfort of evidence-based guideline recommendations tends to fade, leaving both the clinicians and the patients in uncharted waters, seeking the best path forward.

Consequently, multiple approaches have been attempted, combining available techniques in various shapes and forms with the ultimate goal of improving the overall outcome. These combinations try to negate the individual deficiencies of each method while augmenting their strengths, hoping to provide a perfect match for any specific clinical scenario. Ranging from an early tumor in an advanced, decompensated liver disease, to an advanced tumor in an otherwise relatively normal liver and anything in-between, the severity spectrum of HCC leaves room for epistemic, data-based improvisation. However, as the range of therapies is ever-increasing, there is a thin line between being too conservative and overtreating, as both extremes could lead to additional harm and cost. The current review aims to provide an evidence-based analysis of the most frequently used combination therapies, discussing their advantages and caveats in comparison to the current standard of care.

COMBINED TREATMENT TO FACILITATE CURE

Hepatic resection plus intraoperative ablation

Although surgical resection (SR) still represents the ideal and best option as treatment, having curative potential, unfortunately for patients diagnosed with liver malignancies, this treatment is feasible in only 10%-20% of cases[6]. The remaining 80%-90% of patients, which are not suitable for radical intervention, include patients with multiple tumors, located in both hepatic lobes with insufficient hepatic reserve[7]. In the case of HCC, according to the guidelines used in clinical practice (BCLC staging system), the patients with unresectable multicentric neoplasia and preserved liver function fall into stage B, being candidates only for transarterial chemoembolization (TACE)[8]. However, the results of various studies from the literature are beginning to support the use of combined techniques for this category of patients[9]. According to the literature, there are several radical options for bilobar localization, including two-stage resections, ALPPS technique (associating liver partition and portal vein ligation for staged hepatectomy), and combined techniques: Hepatic resections and ablative techniques[9].

Thereby, this complex treatment, which involves the combination of hepatectomies with simultaneous tumor ablations, has the role of increasing the proportion of patients who can become candidates for a radical, potentially curative treatment. However, although there are numerous reports in the literature underlining the clinical outcomes, a standard conduit and a therapeutic consensus in this field have not been yet established[6,10].

General indications for combined therapy are patients with multicentric, bilobar, unresectable neoplasms who are not candidates for curative resection but present with a compensated liver disease[11,12]. Other indications include inoperable tumors due to proximity to major vascular structures and/or the presence of liver cirrhosis with functional liver parenchyma, but not being able to tolerate a major resection[6,10]. Ablative techniques are indicated even if the tumors are in the proximity of a main portal branch, hepatic vein, or inferior vena cava[12].

The extrahepatic presence of neoplasia represents a contraindication for combined treatment, although there are authors who advocate for this treatment in the case of the associated resectable lung tumors or local invasion from liver tumors (in the diaphragm or adrenal gland)[9]. The association of ablation is not indicated when the tumor involves the right or left liver duct[12]. Incontestably, patients with decompensated liver disease, refractory ascites, coagulation disorders, and/or low-performance status (PS) cannot benefit from the combined resection-ablation treatment.

The intraoperative technique involves performing simultaneously, under general anesthesia and by laparotomy, both liver resections, and HCC ablation sessions. Most authors recommend performing resection first, followed by ultrasound-guided ablation, most commonly by tissue destruction by radiofrequency ablation (RFA)[6]. Microwave ablation (MWA) comes with some advantages, these being cited by some authors, but with a lower usage than RFA[9]. Although feasible, with comparable outcomes with "conventional" open surgery, the combined treatment performed by a laparoscopic approach is not a standardized technique, with only a few reports being found in the literature[13,14].

To exclude extrahepatic neoplasia it is mandatory to do a complete exploration of the entire abdominal cavity and intraoperative hepatic ultrasound (IOUS) to assess the topography and tumor relationships[15,16]. Anatomical resections are preferred whenever possible[17]. The indication for atypical, minor (1-2 segments), or major (> 3 segments) hepatectomy is determined by the preoperative assessment of the liver function, of the patient status, and by tumor extent. Also, to prevent blood loss, intermittent clamping of the afferent hepatic pedicle (Pringle maneuver) may be necessary. According to Qiu *et al*[6], it is required in less than 50% of cases[6]. Another advantage of this maneuver is the reduction of the cooling effect induced by the proximity of tumors to main vascular branches ("heat sink"), the rate of achieving a complete ablation being higher[18]. Evaluation of the necrotic area and the efficacy of RFA can be performed immediately by contrast-enhanced-IOUS (CE-IOUS), making possible repeated ablation sessions immediately[19].

Doing a literature survey guided by the words "hepatocellular carcinoma", "combined", "liver resection" and "ablative treatment", using the PubMed database for titles in English published from 2010 to 2020, we found that the mortality reported for the surgery-ablative combined technique is around 1%-2% with a general incidence of complications in the range 22%-70%[6,20-25] (Table 1).

Table 1 Morbidity, mortality, recurrence and survival after hepatic resection plus intraoperative ablation

Ref.	Patients, *n* (%)	MO, *n* (%)	Mo, *n* (%)	Recurrence	Survival rates (1 yr/3 yr/5 yr)
Qiu *et al*[6], 2014	112	2	22.3	72.3%	67.5%/32.5%/12.5%
Hou *et al*[23], 2016	51	0	70.6	54.9%	88.2%/66.7%/52.9%
Zhang *et al*[24], 2014	114	0.9	-	-	34.4%/70.7%/40.7%
Huang *et al*[25], 2020	51	-	-	-	86.3%/66.6%/34.2%

MO: Mortality; Mo: Morbidity.

In general, the literature supports the combined technique as feasible and safe, although the cited recurrence rate is high[6,20,23] (Table 1). The rate of major complications is reported to be around 15%, with a rate of acute liver failure of 1.8% and postoperative bleeding of 0.9%[6,21]. Other specific complications are biliary leaks (8.9%), postoperative ascites (11.6%), perihepatic abscesses (1.8%). 0.9% of patients with post-operative complications may require reinterventions[6,21]. No significant differences were reported between the rates of complications after combined techniques and conventional liver resections. Doing a literature survey guided by the words "hepatocellular carcinoma", "combined", "liver resection" and "ablative treatment", using the PubMed database for titles in English published from 2010 to 2020, we found that the mortality reported for the surgery-ablative combined technique is around 1%-2% with a general incidence of complications in the range 22%-70%[6,20,21].

In regards to long-term survival, we found a range between 12% and 88%, depending on 1-, 3- and 5-year survival reports (Table 1). As can be seen, there is a wide range of results, most likely explained by the lack of standardization of the combined procedures and by the contribution of the case selection bias.

Most of the authors concluded that whenever resection can be performed, it must be chosen instead of ablation. However, RFA remains a feasible alternative in cases that are not suitable for resection, except for large tumors, over 5 cm[22]. Moreover, whenever possible, combined therapy should be indicated to the detriment of TACE, the latter being followed by a shorter 5-years survival: 52.9% (combined treatment) *vs* 9.8% (TACE)[23].

The results are more optimistic when comparing combined treatment with resections only, with overall survival (OS) at 1-, 3- and 5-years being comparable between the two groups: 86.3%, 66.6% and 34.2% *vs* 92.8%, 67% and 37%, respectively ($P = 0.4$)[6,21,24,26].

Supported by the data mentioned in the literature above, the combined treatment (resection plus ablation) is a feasible alternative to the therapeutic options already existing in current guidelines. The fact that more and more studies highlight the increase in the number of curative resections by using these techniques will certainly support their integration into the standard conducting algorithm for HCC.

Combination of locoregional therapies for early and intermediate stage including comparison with other therapies

Locoregional therapies (LRT) include TACE and local ablation techniques. Local ablation including percutaneous ethanol injections, RFA, MWA, laser- and cryoablation (CA) are considered alternative curative methods for early-stage HCC[27,28]. As 75% of HCCs nodules are inoperable at the time of the diagnosis, TACE plays an important role in the management of unresectable HCC and is considered the first-line therapy for BCLC stage B HCC based on Barcelona Clinic Liver Cancer guidelines[27,28].

Limits of LRT and rationale for combination therapy: For some patients undergoing TACE procedure the tumor necrosis rate is low with consequent frequent tumoral residue and high intrahepatic recurrence[27,29]. Along these lines there are several reasons for this limited efficacy: (1) The difficulty to embolize all the feeding arteries of the tumor; (2) Recanalization and angiogenesis which may occur after TACE with consequent tumor recurrence and metastasis; and (3) The re-establish of collateral circulation[27]. Ablations techniques like RFA present a high performance in tumors below 2-3 cm where complete necrosis may be achieved in up to 90% of cases[21]. However, RFA is less effective in medium-sized (3-5 cm) or large tumors (5-9 cm)

where the efficacy dismally drops to 61% and 24% respectively[21]. When RFA was performed for lesions located near major vessels a heat sink effect was reported leading to an increased recurrence rate as well.

In this regard, the location of the lesion is a crucial factor when considering ablation on the grounds that some lesions cannot be successfully treated with thermal ablation without damaging adjacent structures (bile ducts, colon, diaphragm)[30]. Thereby, one intention-to-treat analysis found that 9% of small HCCs were not amenable to percutaneous ablation because of their location[30].

The rationale for combining TACE with ablation is to maximize the percentage of complete tumor response rate and thereby to reduce local recurrence rate due to incomplete or inadequate treatment of the adjacent hepatic parenchyma[9]. As follows, the synergy of the therapies leads to larger volumes of destructed tumoral tissue with consequent efficient treatment of presumed microsatellite nodules and microvascular invasion[9,27]. The sequencing of combined therapies is controversial, with most authors preferring TACE followed by RFA, although some prefer RFA followed by TACE or both techniques in the same session[9,27]. The theoretical advantages of performing TACE before ablation include (1) TACE reduces hepatic artery blood flow, thus diminishing heat sink effects and maximizing the size of the ablation zone; and (2) TACE can detect satellite lesions not seen on cross-sectional imaging[9,27].

TACE combined with RFA *vs* TACE: There are several papers published on this combination with different clinical scenarios. The most important ones are presented in Table 2.

By analyzing these studies, it was discovered that combination therapy might be beneficial compared to TACE alone, or RFA alone in several clinical scenarios.

BCLC-A patients: Song *et al*[31] have compared the results of 71 patients with HCC within Milan criteria treated by TACE to 87 and 43 patients treated by TACE + RFA and respectively RFA. The combination therapy yielded a significantly higher complete response rate according to mRECIST criteria in comparison to TACE (96.5% *vs* 81.6%, $P = 0.019$) and a lower rate of local tumor progression at 1, 3 and 5 years (6%, 33%, and 45% *vs* 17%, 58% and 78%). Nonetheless, what is surprising is the fact that OS was significantly higher for TACE + RFA *vs* TACE or RFA alone for lesions below 3 cm but not for lesions larger than 3 cm[31]. Similar results were reported as well by Lee *et al*[32] with lower recurrence rate at 1 and 3 years 7.3% and 16.5% *vs* 12.5% and 31% but no difference in OS[32].

BCLC-B patients: Liu *et al*[33] have compared a B1 HCC population treated by TACE (195 pts) *vs* TACE + RFA (209 pts) and found a significant difference in favor of combination therapy regarding progression-free survival (PFS) and OS[33]. Also, the same results [significantly higher median OS-840 *vs* 2466 d and median time-to-tumor progression (TTP)-140 *vs* 1148 d] were communicated by Hirooka *et al*[34] although on a small sample of patients[34]. Likewise, Ren *et al*[35] have investigated a large BCLC-A and B population treated either by TACE (271 pts) or TACE + RFA (128 pts) and concluded by all means that the combination therapy is significantly superior to TACE in terms of PFS and both median and cumulative OS[35]. The superiority of TACE + RFA over TACE in terms of longer OS and PFS was demonstrated also for medium and large nonresectable HCCs in 2 large recent trials[36,37].

The combination therapy was assessed also in patients with small lesions not feasible for RFA and lesions in unreachable locations (*e.g.*, near the hepatic hilum, subdiaphragmatic, subcapsular). TACE procedures resolve this dilemma due to intratumoral accumulation of radio-opaque iodized oil used and give radiographic contrast to a small tumor either of poor conspicuity or even ultrasound (US) blind and also difficult to target spots such as hepatic dome[27]. An undoubtedly higher complete response rate (100% *vs* 54%, $P < 0.01$) and lower TTP rate were revealed by Hyun *et al*[38] in a series of patients with HCC not feasible for US-guided RFA treated by TACE (54 pts) or TACE + RFA (37 pts)[38]. However, Yang *et al*[39] compared the efficacy of combination therapy in 37 patients with HCC in special locations to 85 patients with HCC in convenient locations and found no differences in PFS and OS[39]. Three meta-analyses concerning the comparison of TACE + RFA *vs* TACE have been already published[40-42]. The last one issued in 2017 found that for intermediate-stage HCC, TACE plus RFA attained higher tumor response rates (OR = 6.08, 95%CI: 4.00-9.26, $P < 0.00001$), achieved longer recurrence-free survival (RFS) rates (ORRFS = 3.78, 95%CI: 2.38-6.02, $P < 0.00001$) and OS rates (OR1-year = 3.92, 95%CI: 2.41–6.39, $P < 0.00001$; OR3-year = 2.56; 95%CI: 1.81–3.60; $P < 0.00001$; OR5-year = 2.78; 95%CI: 1.77–4.38; $P < 0.0001$) when comparing to TACE alone. Unfortunately, as expected the number of

Table 2 Comparison of transarterial chemoembolization plus radiofrequency ablation to transarterial chemoembolization

Ref.	Treatment type, *n* (%)	Clinical scenario	Response rate mRECIST	Outcome
Morimoto *et al*[111], 2013	TACE + RFA (132)	HCC 1-5 cm, subcapsular	98.5% CR	LTP (3 yr) 9.7%. OS (3, 5, 7 yr): 79.3%, 60.6%, 50.9%
Song *et al* [31], 2016	TACE (71) *vs* TACE + RFA (87) *vs* RFA (43)	HCC within Milan	81.6% *vs* 96.5% *vs* 97.6% (TACE *vs* TACE + RFA P = 0.019)	LTR (1, 3, 5 yr): 17%, 58%, 78% *vs* 6%, 33%, 54% *vs* 10%, 31%, 48% (TACE + RFA *vs* TACE P = 0.015; RFA *vs* TACE P = 0.005). OS (1, 3, 5 yr): 98%, 90%, 83% *vs* 98%, 95%, 90% *vs* 94%, 84%, 71% OS significantly higher (P = 0.019) for TACE + RFA *vs* TACE or RFA for lesions < 3 cm but not for lesions > 3 cm
Lee *et al*[32], 2018	TACE (85) *vs* TACE + RFA (n = 82)	HCC BCLC 0 or A invisible for ultrasound	97.6% *vs* 100% (CR)	LTP (1, 3, 5, 7 yr): 12.5%, 31%, 37% *vs* 7.3%, 16.5%, 16.5% (P = 0.013). Median TTP: 18 mo *vs* 24 mo (P = 0.037). OS (1, 3, 5 yr): 100%, 93.2%, 87.7% *vs* 100%, 96.6%, 87.4% (P = 0.686)
Liu *et al*[33], 2019	TACE (195) *vs* TACE + RFA (209)	HCC B1	N/A	Median PFS: 14 mo *vs* 20 mo. PFS (1, 3, 5 yr): 59.1%, 11.0%, 2.2% *vs* 71.8%, 26.6%, 13.0% (P < 0.001). OS (1, 3, 5 yr): 80.7%, 26.4%, 6.7% *vs* 83.7%, 45.8%, 24.8% (P = 0.003)
Hiraoka *et al* [73], 2017	TACE (32) *vs* TACE + RFA (32)	HCC BCLC B1 + B2	N/A	Median OS: 840 d *vs* 2466 d. OS (1, 3, 5 yr): 86.3%, 43.5%, 15.8% *vs* 100%, 78.6%, 62.3% (P < 0.001). Median TTP: 140 d *vs* 1148 d (P < 0.0001)
Ren *et al* [35], 2019	TACE (271) *vs* TACE + RFA (128)	HCC BCLB A and B	44.7% *vs* 85.9% (CR)	Median OS: 16 mo *vs* 59 mo (P < 0.001). Median PFS: 4 mo *vs* 45 mo. OS (1, 3, 5, 8 yr): 64.5%, 15.1%, 10.8%, 10.8% *vs* 90.6%, 76.6%, 68.0%, 68.0%
Chu *et al* [36], 2019	TACE (314) *vs* TACE + RFA (109) *vs* RFA (115)	HCC 3.1-10 cm	84.7% *vs* 95.4% *vs* 94.8% (CR)	RFS (5, 10, 15 yr): 59.1%, 11.0%, 2.2% *vs* 25.5%, 13.3%, 7.9% *vs* 9.2%, 2.9%, and 2.9% (P = 0.002). OS (5, 10, 15 yr): 16.2%, 10.9%, 7.7% *vs* 57.8%, 41.8%, 30.9% *vs* 35.2%, 11.9%, 11.9% (P = 0.022)
Liu *et al*[37], 2020	TACE (124) *vs* TACE + RFA (77)	HCC 3-10 cm	N/A	Median PFS: 4 mo *vs* 9.13 mo (P < 0.001). PFS (1, 3, 5 yr): 11.9%, 0%, 0% *vs* 43%, 18%, 9.5%. Median OS: 12 mo *vs* 27.57 mo (P < 0.001). OS (1, 3, 5 yr): 48%, 6.5%, 0% *vs* 76.2%, 37.1%, 16.4%
Hyun *et al* [38], 2016	TACE (54) *vs* TACE + RFA (37)	HCC not feasible for RFA	57% *vs* 100% P < 0.01 (CR)	Median TTP: 29.7 mo *vs* 34.9 mo (P = 0.014). OS (1, 2, 3 yr): 91%, 79%, 71% *vs* 100%, 97%, 93%
Yang *et al* [39], 2020	TACE + RFA special location (n = 37) *vs* TACE + RFA conventional location (n = 85)	HCC special locations	91.9% *vs* 85.9% (CR) (NS)	Median PFS: 14 mo *vs* 17 mo (NS). Median OS: 32 mo *vs* 28 mo (NS). OS (1, 2 yr): 96.3%, 65% *vs* 89.9%, 63.3% (NS)
Hyun *et al* [112], 2016	TACE + RFA (14)	HCC < 2 cm caudate lobe	90.9% CR	LTP (1, 3, 5 yr): 0%, 12.5%, 12.5%. PFS (1, 3, 5 yr): 81.8%, 51.9%, 26%. OS (1, 3, 5 yr): 100%, 80.8%, 80.8%
Hyun *et al* [113], 2018	TACE +RFA (69)	HCC < 3 cm not feasible for RFA	100% CR	LTP (1, 3, 5, 7 yr): 4.4%, 6.8%, 8.2%, 9.5%, 9.5%. OS (1, 3, 5, 7 yr): 100%, 95%, 89%, 80%, 80%
Yan *et al* [114], 2018	TACE + RFA single session (87)	HCC < 7 cm not resectable	87.4% CR	LTP (1, 3, 5 yr): 0%, 29.9%, 55.2%. Median OS: 39 mo. OS (1, 3, 5 yr): 100%, 65.5%, 47.5%
Kim *et al* [115], 2019	TACE + RFA (67)	BCLC A, non-surgical	N/A	PFS (1, 3, 5 yr): 86.8%, 55.9%, 29.7%. OS (1, 3, 5 yr): 100%, 93.4%, 83.5%
Duan *et al* [116], 2020	TACE + RFA, one session (46)	HCC > 8 cm	N/A	PFS (2, 3 yr): 9.4 mo and 10.2 mo. OS (2, 3 yr): 18.4 mo and 26.4 mo
Zhang *et al* [117], 2020	TACE + RFA (1) naive (40); (2) recurrent (36); and (3) hepatectomy	1 tumor < 7 cm, up to 3 tumors < 3 cm, Child A or B	62.5% *vs* 70% (CR + PR)	OS (1, 2, 3 yr): 97.5%, 84%, 66% (A) *vs* 90%, 82%, 66% (B) *vs* 90%, 79%, 63% (C) (A *vs* B *vs* C NS). DFS: 75%, 51%, 35% (A) *vs* 50%, 31%, 17% (B) *vs* 80%, 59%, 40% (C) (A *vs* B P = 0.013)
Wang *et al* [118], 2018	TACE (13) *vs* TACE + RFA (13)	HCC with hepatic vein thrombus	0% + 92.3% *vs* 46.2% + 53.7% (CR + PR)	Median OS: 6.5 mo *vs* 18 mo (P = 0.02)
Song *et al* [119], 2020	TACE (63) *vs* TACE + RFA (96)	Recurrent HCC < 5 cm after HR	N/A	DFS (1, 3, 5 yr): 41.1%, 9.9%, 4.9% *vs* 55.1%, 22.5%, 9.7%. OS (1, 3, 5 yr): 75.9%, 30.7%, 11.3% *vs* 82.3%, 42.7%, 16.5% (NS)

P value less than 0.05 (typically ≤ 0.05) is statistically significant. HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization; OS: Overall survival; PFS: Progression free survival; RFS: Recurrence free survival; TTP: Time to progression; LTP: Local tumor progression; LTR: Local tumor recurrence; DFS: Disease free survival; TR: Tumor recurrence; CR: Complete response.

complications were higher in the TACE plus RFA group than in the TACE alone group (OR = 2.74, 95%CI: 1.07-7.07, P = 0.04)[40].

The combination therapy TACE + RFA was also compared to RFA in several other meta-analyses[43-46]. The last one including 8 trials and 648 patients showed that RFA

plus TACE is associated with a significant advantage in RFS [hazard ratio (HR) = 0.58; 95%CI: 0.42-0.80, P = 0.001], and OS (HR = 0.60; 95%CI: 0.47-0.76, P < 0.001). The authors concluded that TACE combined with RFA is more competent and appealing than RFA alone, especially for intermediate and large-size hepatic tumors or younger patients with HCC[47].

TACE combined with RFA *vs* hepatic resection: According to the BCLC guidelines published in 2018, the mainstay of curative therapy for early HCC is RFA or surgery-either HR or liver transplantation. As mentioned earlier, even though HR is a cornerstone treatment in non-metastatic disease, some patients are considered inappropriate candidates due to underlying liver disease[48]. We have emphasized in the previous lines that the combination therapy of TACE and RFA is superior and beneficial for the treatment of early and intermediate HCC *vs* the therapies used individually. Along these lines, the comparison of the outcome of TACE + RFA *vs* HR is of paramount importance as some of the patients are not ideal surgical candidates. Several comparative studies are presented in Table 3.

For small single HCC (2-3 cm) TACE + RFA have similar outcomes to HR in terms of disease free survival (DFS) and OS but with significantly lower complications rate and hospital stay[49]. However, as expected for medium size and larger lesions tumor recurrence (75% *vs* 35.4%, P = 0.005) and local tumor progression (LTP) (55.7% *vs* 16%, P = 0.013) are significantly lower for HR[49]. Moreover, it seems that for patients fitting the up-to seven criteria both treatment options provide similar DFS and OS. However for patients outside the Milan criteria but within up to seven criteria there was an increased median OS when HR was performed[50]. For patients within the Milan criteria, Takuma and colleague reported similar DFS and OS, whereas Liu *et al* [51] found a significantly increased DFS and OS for patients who had undergone HR [51]. In a recent study, Lin *et al*[52] demonstrated a significantly higher 5 years OS (61.2% *vs* 38.2%, P = 0.009) for HR in patients with HCC BCLC-B[52].

Beyond a shadow of a doubt, the association of TACE with RFA has proved its benefit in terms of controlling much more suitably early and intermediate primary liver cancer compared to the therapies used alone and we consider that it has gained its place in the treatment of HCC. Moreover, combination therapy is also an important alternative when surgery is not feasible.

TACE combined with MWA: MWA is a dielectric heating technique that generates an electromagnetic field surrounding the needle tip, consequently producing the coagulation necrosis of the target area. Along with RFA, MWA is an established thermal ablation method, best-suited for treating early-stage HCC with curative intent. Being non-inferior to RFA in the standard guideline setting, MWA boosts some theoretical advantages: Higher efficacy in larger nodules, quicker heating, lower procedural time, and higher heating temperatures[53]. Therefore, like RFA, MWA has often been combined with TACE in a multitude of clinical scenarios. These situations either fit outside the standardized BCLC boxes or aim to improve the relatively dim prognosis of the well-established path in intermediate or advanced HCC.

As addressed by Renzulli *et al*[54], intermediate-and advanced-stage HCC often leads the clinicians in the uncertain waters of an imperfect solution[54]. The typical case resembles the following: Curative intent solution off the board, unsatisfactory gold-standard (TACE), and technically treatable nodules within ablative reach. This has led to a recent surge of interest with regards to combining TACE and MWA, with numerous papers published on this topic in the past decade. The most important articles are comprised in Table 4, which will provide the cornerstone for the upcoming discussion.

As shown in Table 4, there is a wide array of clinical scenarios in which the TACE + MWA approach was tested. However, the large majority of the data comes from the most heterogeneous class: Intermediate-stage, BCLC-B. However, most of the HCC disease spectrum stood for trial, ranging from small, solitary unresectable nodules, up to 10 nodules and more advanced BCLC-C tumors[55-58]. The overall results were promising, if not always definitely positive. Therefore, both the empirical and the epistemological conclusions suggest that TACE + MWA can be safely employed whenever it is technically feasible with regards to tumor characteristics, vascularization, and percutaneous approach.

Three key aspects define therapeutic efficacy in HCC: Treatment response according to the mRECIST criteria, PFS, and OS. In this regard, the TACE + MWA combination appeared to exceed the standard of care in most of the clinical scenarios, in all three aspects (Table 4). Of course, the precise survival data varies widely, as the study designs were extremely heterogeneous, even within the same BCLC class. In

Table 3 Comparison transarterial chemoembolization + radiofrequency ablation to other curative therapies

Ref.	Treatment type, *n* (%)	Clinical scenario	Response rate mRECIST	Outcome
Saviano *et al* [49], 2017	TACE + RFA (*n* = 25) *vs* HR (*n* = 29)	HCC 3.0-8.8 cm, solitary HCC 3-5 cm	N/A	OS (1, 3 yr): 89.4%, 48.2% *vs* 91.8%, 79.3% (*P* = 0.117). TR (1, 3 yr): 42.4%, 76.0% *vs* 29.5%, 45.0% (*P* = 0.034); LTP (3 yr): 58.1% *vs* 21.8% (*P* = 0.005). TR: 75.1% *vs* 35.4% (*P* = 0.016); LTP: 55.7% *vs* 16.0% *P* = 0.013)
Pan *et al* [50], 2017	TACE + RFA (*n* = 154) *vs* HR (*n* = 176)	Within Up-To Seven criteria	N/A	Median OS: 56 mo *vs* 58 mo (NS). OS (1, 3, 5 yr): 96.1%, 76.7%, 41.3% *vs* 96.1%, 86.4%, 46.2% (*P* = 0.138). Median OS (beyond Milan): 52 mo *vs* 45 mo (*P* = 0.023)
Liu *et al*[51], 2016	TACE + RFA (*n* = 100) *vs* HR (*n* = 100)	Within Milan	N/A	OS (1, 3, 5 yr): 96%, 67.2%, 45.7% *vs* 97%, 83.7%, 61.9% (*P* = 0.007). RFS (1, 3, 5 yr): 83%, 44.9%, 35.5% *vs* 94%, 68.2%, 48.4% (*P* = 0.026). Complications rate: 11% *vs* 23%, *P* = 0.024)
Lin *et al*[52], 2020	TACE (*n* = 231) *vs* TACE + RFA (*n* = 57) *vs* HR (*n* = 140)	BCLC-B	N/A	OS (1, 3, 5 yr): 69.5%, 37.0%, 15.2% *vs* 86.0%, 57.9%, 38.2% *vs* 89.2%, 69.4%, 61.2%. OS higher HR *vs* TACE + RFA (*P* = 0.009), HR *vs* TACE (*P* < 0.001) and TACE + RFA *vs* TACE (*P* = 0.004)
Wei *et al* [63], 2020	TACE + RFA (*n* = 107) *vs* HR (*n* = 79)	Recurrent HCC < 5 cm after HR	N/A	DFS (1, 3, 5 yr): 58.2%, 35.2%, 29.6% *vs* 64.8%, 41.6%, 38.3% (*P* = 0.258). OS (1, 3, 5 yr): 84.6%, 66.9%, 49.1% *vs* 84.8%, 60.2%, 51.9% (*P* = 0.871). Lower major complication rates (*P* = 0.009) and shorter hospital stay (*P* < 0.001) for TACE + RFA
Sheta *et al* [64], 2016	TACE (*n* = 20) *vs* TACE + RFA (*n* = 20) *vs* TACE + MWA (*n* = 10)	Non resectable single lesion HCC > 4 cm	50% *vs* 70% *vs* 80% (CR at 6 mo)	LTR (1, 3, 6 mo): 30% *vs* 5% *vs* 0% (*P* = 0.027); 14.3% *vs* 15.8% *vs* 10% (NS); 16.7% *vs* 12.5% *vs* 11.1% (NS). Complications rate: 40% *vs* 10% *vs* 10%
Yuan *et al* [65], 2019	TACE + RFA (*n* = 41) *vs* TACE + MWA (*n* = 34)	HCC > 3 cm. HCC 3-5 cm. HCC > 5 cm	68.3 *vs* 85.3% (NS). 73.5 *vs* 88.5% (NS). 42.9 *vs* 75% (*P* = 0.041)	DFS (1, 2, 3 yr): 53%, 29%, 12% *vs* 58%, 38%, 29% (*P* = 0.07). OS (1, 2, 3 yr): 68%, 36%, 14% *vs* 79%, 53%, 38% (*P* = 0.393)
Thornton *et al*[120], 2017	TAE/TACE + RFA (*n* = 15) *vs* TAE/TACE + MWA (*n* = 20)	BCLC 0 and A	80% *vs* 95% (NS)	LTR: 30% *vs* 0%
Vasnani *et al* [67], 2016	TACE + RFA (*n* = 11) *vs* TACE + MWA (*n* = 31)	HCC within Milan	91% *vs* 67% (CR) 45% *vs* 35% (rates of complete tumor coagulation on pathology)	

mRECIST: Modified RECIST; n: Number of patients; BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; MWA: Microwave ablation; HR: Hepatic resection; OS: Overall survival; PFS: Progression free survival; TTP: Time to progression; LTP: Local tumor progression; DFS: Disease free survival; TR: Tumor recurrence; CR: Complete response; NS: Not significative; N/A: Not applicable.

intermediate-stage HCC, OS ranged from 17.1 mo[57] to 35 mo[59] as it reliably extended OS compared to TACE alone by 4 to 12 mo[56,57,60,61]. To objectivize the apparent benefit, a meta-analysis was recently performed by Liu *et al*[62], comparing TACE with TACE + MWA in single and up to three HCC nodules exceeding 5 cm[62]. The analysis included over 1700 patients, all from Chinese-conducted studies, and showed a significantly higher OS for the latter (1-year OS rate: RR = 1.36, 95%CI: 1.28–1.44; 2-year OS rate: RR = 1.56, 95%CI: 1.40–1.74, and 3-year OS rate: RR = 2.07, 95%CI: 1.67–2.57, *P* < 0.001). However, when compared to other non-standard therapies, such as radiation segmentectomy[55] or CA[63] the differences in outcome were not statistically significant, which might further suggest the rather suboptimal standard of care for intermediate HCC. Other clinical scenarios have shown a benefit of combination therapy, as TACE + MWA + Sorafenib outperformed TACE + Sorafenib in off-guideline advanced HCC[58]. However, it is unclear whether the available reports provide sufficient grounding for altering the current time-tested recommendations since no major randomized controlled trials are available. Furthermore, the overall quality of the data can be improved, as most studies are retrospective.

There are only a few small sample studies directly comparing MWA to RFA in combination with TACE, with no significant differences in major outcomes such as PFS and OS[64-66]. However, subgroup analysis suggests a higher response rate in larger tumors for MWA.

Per available data, TACE + MWA appears to laterally exceed the BCLC-B stage. On one hand, it might secure substantial survival benefits for nodules not amenable to curative intent solutions. One such scenario might be large, borderline BCLC-A nodules, unfit for resection due to portal hypertension, yet fit for transplantation, but

Table 4 Available studies on the transarterial chemoembolization plus microwave ablation

Ref.	Treatment type, *n* (%)	Clinical scenario	Response rate (CR + PR) mRECIST	Outcome
Ni *et al*[59], 2020	TACE + MWA (546)	BCLC B	N/A	Median PFS: 6.5 mo. Median OS: 35 mo
Ni *et al*[121], 2019	TACE + MWA (349)	Up to 3 nodules, 5-8 cm diameter	77.1%	Median PFS: 4.8 mo. Median OS: 28 mo
Chen *et al*[47], 2017	TACE (96) *vs* TACE + MWA (48)	HCC ≤ 5 cm	46.3% *vs* 92.1%	2-yr PFS: 57.3% *vs* 10.4%; 2-yr OS: NS
Smolock *et al*[56], 2018	TACE (16) *vs* TACE + MWA (22)	HCC 3-5 cm	76% *vs* 95% (NS)	Median PFS: 4.2 mo *vs* 22.3 mo. Median OS: 14.8 mo *vs* 18.5 mo. 3-yr OS: 42.1% *vs* 79%
Zheng *et al*[57], 2018	TACE (166) *vs* TACE + MWA (92)	Solitary HCC > 5 cm; 2-3 nodules > 3 cm; 4-10 nodules regardless of size	55.4% *vs* 81.5%	Median PFS: 12.5 mo *vs* 26.6 mo. Median OS: 6.7 mo *vs* 17.1 mo. 3-yr OS: 11.4% *vs* 32.6%
Zhang *et al*[60], 2018	TACE (100) *vs* TACE + MWA (50)	BCLC-B	55% *vs* 74%. At 6-mo, including stable disease	Median PFS: 6.1 mo *vs* 10.1 mo. Median OS: 14.4 mo *vs* 18.5 mo. 3-yr OS: 42.1% *vs* 79%. 5-yr OS: 21% *vs* 67.7%
Wang *et al*[61], 2020	TACE (111) *vs* TACE + MWA (72)	Recurrent (post-surgery) BCLC-B	N/A	Median PFS: N/A. Median OS: 14.4 mo *vs* 26.7 mo. 5-yr PFS: 13.0% *vs* 21.7%. 5-yr OS: 27.9% *vs* 43.3%
Li *et al*[29], 2020	MWA (88) *vs* TACE + MWA (62)	BCLC-B	N/A	3-yr PFS: 34.5% *vs* 32.5% (NS). 3-yr OS: 47.6% *vs* 49.2% (NS)
Biederman *et al* [55], 2017	TACE + MWA (80) *vs* Radiation segmentectomy (41)	Unresectable, solitary, ≤ 3 cm	CR 82.5% *vs* 82.9% (NS)	Median PFS: 12.1 mo *vs* 11.1 mo (NS). 90-d mortality: 0% all groups. Median OS: N/A
Ni *et al*[59], 2020	TACE + Sorafenib (*n* = 75) *vs* TACE + Sorafenib + MWA (77)	BCLC C	12% *vs* 46.7%	Median PFS: 3 mo *vs* 6 mo. Median OS: 13 mo *vs* 19 mo
Sheta *et al*[64], 2016	TACE (20) *vs* TACE + RFA (20) *vs* TACE + MWA (10)	Unrsesectable, solitary	6-mo CR–50% *vs* 70% *vs* 80%	Median PFS: N/A. Median OS: N/A
Wei *et al*[63], 2020	TACE + MWA (48) *vs* TACE + Cryoablation (60)	BCLC B	73.3% *vs* 33.4%	Median PFS: 8.8 mo *vs* 9.3 mo (NS). Median OS: 20.9 *vs* 13 mo (NS)

TACE: Transarterial chemoembolization; MWA: Microwave ablation; RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer classification system; CR: Complete response; PR: Partial response according to the mRECIST criteria; PFS: Progression-free survival; OS: Overall survival; NS: Non-significant; N/A: Not available.

living in a low-transplantation rate medical system. On the other hand, for BCLC-C patients, TACE + MWA appears to bring survival benefits, especially when adding sorafenib into the mix. The further one navigates beyond BCLC-B, the lesser the strength of the data. Within BCLC-B though, TACE + MWA shines the brightest, appearing to add a substantial survival benefit[67].

TACE combined with other local therapies: In a RCT comparing CA + drug-eluting bead (DEB)-TACE to CA monotherapy in large tumors (mean: 7.2 ± 4.5 cm and 6.5 ± 3.8 cm, respectively), the combination group demonstrated superior OS [16.8 mo *vs* 13.4 mo, respectively (P = 0.0493)] and significantly increased PFS [8.1 mo *vs* 6.0 mo, respectively (P = 0.0089)]. Interstitial laser therapy (ILT) uses optical fibers to create cytotoxic temperatures *via* the conversion of absorbed infrared light to heat with consequent coagulative necrosis[68]. TACE followed by ILT was used in patients with tumors up to 8 cm that has obtained a median OS of 36 mo (95%CI: 29.3–42.6)[69].

Stereotactic body radiation therapy (SBRT) can provide satisfactory local control with a low incidence of radiation-induced liver disease in patients with unresectable HCC that are not amenable to thermal ablation[68]. *Via* radiosensitization of tissue TACE/TAE may serve as targeting fiducials for SBRT. TACE + SBRT was compared to SBRT in a retrospective study including patients with tumors with a median size of 8.5 cm. An increased 5-year OS was reported in the combination arm *vs* SBRT arm (46.9 *vs* 32.9%, P = 0.047)[70]. In addition to cytotoxic properties, SBRT seems to be a potent activator of the adaptive immune system, and thus might stand as an interesting player in the field of immunotherapy[68].

COMBINED TREATMENT TO ENHANCE PALLIATION AND INCREASE SURVIVAL

TACE + systemic therapies–the downfall of an era

As mentioned previously, current guidelines recommend local ablative therapies (RFA/MWA) for patients with early-stage HCC (BCLC-0) and TACE for patients with intermediate-stage BCLC-B or BCLC-A patients not suitable for resection or ablation [2]. Unfortunately, many of these patients experience a progression of the disease and a worsening of the liver function after repeated sessions of percutaneous ablation or TACE[71]. The OPTIMIS study, which assessed the outcomes of HCC patients treated with TACE alone, or with TACE followed by sorafenib found that the proportion of patients with a progressive disease increases with each subsequent TACE, while the objective response rates decline as the number of TACE sessions increased (first TACE: 40%; second TACE: 26%; third TACE: 24%; and fourth TACE: 25%)[72]. Moreover, up to 30% of these patients experienced a deterioration of liver function. Similarly, Hiraoka *et al*[73] reported that the liver function deteriorated with repeated TACE[73]. These findings emphasize the importance of the appropriate timing of switching from local therapy to systemic therapies to obtain the maximal benefits of other therapies and consequently improve the OS.

As a result of an exclusively arterial vascularization of HCC tumors and comprising the fact that the normal surrounding liver parenchyma is vascularized from branches of the portal vein, TACE and other image-guided transcatheter treatments were born to destruct arterial tumoral vessels and hence inducing tumor necrosis[74]. TACE procedure is based on an intra-arterial infusion of a chemotherapy agent such as doxorubicin or cisplatin, frequently embedded in lipiodol as a vehicle to increase the availability of the drug. Furthermore, the tumoral blood vessels could be embolized with different agents such as gelatine sponge particles, metallic coils, polyvinyl alcohol, starch microspheres and autologous blood clots leading to an increased tumoricidal and ischemic effect[27]. According to the European Association for the Study of the Liver (EASL), the median survival for untreated patients at an intermediate-stage [BCLC-B–multinodular disease, good PS, without vascular invasion or extrahepatic spread] is around 16 mo and in rigorously selected candidates TACE can increase the survival up to 3 years[2].

TACE causes local hypoxia in the tumor, building up an expression of hypoxia response genes in tumor cells regulated by hypoxia-inducible factor-1 alpha (HIF-1α). The response triggers vascular endothelial growth factor (VEGF) expression and thus leading to the formation of neovascularization, and thereby forming a vicious cycle leading to tumor recurrence and metastasis[75]. In this regard, studies have conceded that dynamic changes in serum HIF-1α and VEGF levels occur after TACE in HCC patients[76-78]. Along these lines, Jia *et al*[76] investigated the expression levels of serum HIF-1α and VEGF before and after TACE and analyzed the correlations between prognosis factors and serum HIF-1α as well as VEGF levels[76]. The serum HIF-1α and VEGF levels of HCC patients pre-TACE, 1 d, 1-wk, 1-mo post-TACE were analyzed using ELISA and compared with that of 20 healthy volunteers[76]. The study revealed that the expression levels of serum HIF-1α and VEGF in HCC patients were significantly higher than those in the control group. One day after TACE, both serum HIF-1α and VEGF levels reached the peak values. One-week post-TACE, expression levels of them were decreased, but still significantly higher than those before TACE. The levels of both HIF-1α and VEGF incomplete response group 1-mo post-TACE were significantly lower than those in partial response, stable disease, or progressive disease groups. Thus, HIF-1α and VEGF might be important predictors of TACE efficacy.

Along these lines, hepatologists and oncologists hypothesized whether the combination of systemic therapy and TACE might be beneficial in terms of survival in HCC patients. In an effort to address this problem, several trials with TACE and anti-angiogenic therapies have emerged. Nevertheless, some challenges have arisen. Firstly, TACE is addressed to BCLC-B class patients which is a heterogeneous group due to the wide range of liver function (Child-Pugh A or B cirrhosis) and variable lesion number and dimension. Secondly, the use of chemotherapy, degree of selectivity and management of adverse effects have to be considered. The GIDEON trial, the first observational trial of more than 3000 patients with HCC BCLC A to C treated with sorafenib or in combination with TACE reported that sorafenib could be safely associated or used sequentially with TACE[79]. Thereby, taking this assumption into account, several randomized controlled trials have been reported, as seen in Table 5.

Table 5 Chemoembolization plus systemic therapies

Trial	Experimental arms, *n* (%)	Outcomes
TACE + sorafenib		
SPACE trial (Lencioni *et al*[80], 2016)	DEB-TACE plus sorafenib (154) *vs* DEB-TACE plus placebo (153)	5.6 mo *vs* 5.5 mo; HR: 0.797 (95%CI: 0.588–1.080); *P* = 0.072
TACE 2 trial (Meyer *et al*[81], 2017)	DEB-TACE plus sorafenib (157) *vs* DEB-TACE plus placebo (156)	7.8 mo *vs* 7.7 mo; HR: 1.03 (95%CI: 0.75–1.42); *P* = 0.85
STAH trial (Park *et al*[82], 2019)	cTACE plus sorafenib (170) *vs* sorafenib (169)	12.8 mo *vs* 10.8 mo; HR: 0.91 (95%CI: 0.69–1.21); *P* = 0.290
TACTICS trial (Kudo *et al*[83], 2020)	cTACE plus sorafenib (80) *vs* cTACE (76)	25.2 mo *vs* 13.5 mo; HR: 0.59 (95%CI: 0.41–0.87); *P* = 0.006
TACE + other therapies		
BRISK-TA trial (Kudo *et al*[85], 2014)	cTACE or DEB-TACE plus brivanib (249) *vs* cTACE plus placebo (253)	26.4 mo *vs* 26.1 mo; HR: 0.90 (95%CI: 0.66-1.23); *P* = 0.53
ORIENTAL trial (Kudo *et al*[86], 2018)	cTACE plus orantinib (445) *vs* cTACE plus placebo (444)	31.1 mo *vs* 32.3 mo; HR: 1.090 (95%CI: 0.878–1.352); *P* = 0.435
TACE combined with celecoxib and lanreotide (Tong *et al*[89], 2017)	TACE (*n* = 35) *vs* TACE + C + L (36)	7.5 mo *vs* 15.0 mo; HR: 0.534 (95%CI: 0.321-0.888); *P* = 0.016
TACE combined with thalidomide (Wu *et al*[87], 2014)	TACE + thalidomide (56)	21 mo (95%CI: 16–28 mo)
TACE plus bevacizumab (Pinter *et al*[88], 2015)	TACE + bevacizumab (20) *vs* TACE + placebo (20)	5.3 mo *vs* 13.7 mo; HR: 1.7 (95%CI: 0.8-3.6); *P* = 0.195

HR: Hepatic resection; TACE: Transarterial chemoembolization.

SPACE prospective randomized phase II trial included 307 patients with BCLC-B HCC randomly allocated to DEB-TACE with sorafenib 400 mg twice daily and DEB-TACE with placebo. Unfortunately, there was no difference in TTP between the 2 arms (169 d *vs* 166 d in the sorafenib and placebo arms, respectively, *P* = 0.072) and no impact on OS (*P* = 0.29) was observed[80].

A year later a phase III trial of TACE with sorafenib (TACE-2) from the United Kingdom emerged and included 313 patients randomized to sorafenib or placebo with DEB-TACE 2–5 wk later and additional TACE on demand[81]. The study aimed to reduce the adverse effects induced by combination treatment and to increase the prospect of continuing the drug at the time of the TACE procedure. Sadly, as in the SPACE trial, this RCT was also negative with a median PFS of 7.9 *vs* only 7.8 mo in the sorafenib and placebo arms, respectively (*P* = 0.94), and median OS of 21.1 and 19.7 mo in the sorafenib and placebo groups, respectively (*P* = 0.57). Moreover, discouraging results were also reported by the STAH trial in 2019 when comparing the combined treatment with sorafenib alone. The authors justify their results due to delays in starting sorafenib after TACE and/or low daily sorafenib doses[82].

TACTICS trial was the only phase II RCT that attested to the benefits of TACE-sorafenib synergy and met its primary endpoint for the treatment of intermediate stage HCC[83]. The authors reported a median PFS significantly longer in the TACE plus sorafenib group *vs* TACE alone group (25.2 *vs* 13.5 mo; *P* = 0.006). Moreover, the innovation of the trial stands in the modification of PFS, defined as time-to-unTACEable progression (TTUP), characterized as untreatable tumor progression, transient deterioration to Child-Pugh C, or appearance of vascular invasion/extrahepatic spread. Patients in the combination group received sorafenib 400 mg once daily for 2–3 wk before TACE, followed by 800 mg once daily during on-demand conventional TACE sessions until time to untreatable TTUP. Howbeit, a further analysis conceded that the TACTICS trial did not show an improved OS in the combination group as compared with TACE alone although significantly better PFS was consistently observed. However, the OS in TACE plus sorafenib arm showed the longest OS (36.2 mo) with the longest ΔOS (5.4 mo) as compared with the previous TACE combination trials[84]. The authors explain that the major reason for the negative OS result was due to many post-trial active treatments (other systemic treatments and immunotherapy agents) performed in the sorafenib group (76.3%), which implies that the OS endpoint in the TACE combination trial may not be feasible anymore in the current era of personalized medicine and immunotherapy.

It seems likely that other TKIs (brivanib, orantinib) and thalidomide derivatives as well combined with conventional TACE failed to meet the primary endpoint of OS[85-87]. Moreover, the addition of bevacizumab to TACE raised some safety concerns related to sepsis and vascular complications of the combination treatment[88]. Nevertheless, encouraging results were published by Tong *et al*[89] which have compared TACE alone with TACE combined with the selective COX-2 inhibitor, celecoxib and the somatostatin analog, lanreotide in advanced HCC[89]. The patients receiving the combination therapy had a median OS of 15 mo compared to 7.5 mo for those receiving TACE alone, and a subgroup analysis of advanced patients demonstrated an OS of 13 mo for the combination and 4.5 mo for TACE alone (P = 0.013). Likewise, encouraging results published in 2020 confirmed that the use of lenvatinib-TACE sequential treatment after progression during lenvatinib therapy was associated with better post-progression survival (HR = 0.08; 95%CI: 0.01–0.71; P = 0.023)[90].

Overall, up to this point, the literature has failed to support the use of multi-kinase inhibitors in combination with TACE. However, with the emergence of immunotherapy, combined strategies are encouraged. Moreover, because intermediate stage liver cancer is very heterogeneous a personalized approach is the key to a better outcome for patients.

TACE + immunotherapy–a new beginning?

It has been noted for several years that LRTs result in the release of tumor antigens, which are taken up by antigen-presenting cells (mainly dendritic cells) and which have been shown to activate a tumor-specific immune response a[91,92]. This evidence suggests that l LRTs may boost the response to immune-oncology drugs. Preliminary results of the phase I/II PETAL clinical trial (NCT03397654) showed good tolerability of pembrolizumab after TACE without cumulative side effects. Additionally, several clinical trials testing immune checkpoint inhibitors (ICI) as neoadjuvant or adjuvant therapy in patients treated with LRTs are currently running (Table 6).

However, despite the strong antitumor response induced by ICI, not all patients experienced an objective response[93]. Currently there is no biomarker to predict response or resistance to immunotherapy in HCC. Emerging evidence revealed that VEGF is not only a proangiogenic factor but that VEGF also plays an important role in the development of the immunosuppressive tumor microenvironment (*e.g.* inhibition of dendritic cell maturation, accumulation of dendritic cell maturation, accumulation of myeloid-derived suppressor cells and induction of T reg cells). Voron *et al*[94] showed that targeting VEGF-A can decrease the VEGF-induced expression of inhibitory receptors mediating CD8+T cell exhaustion[94]. Given these results, the association of anti-angiogenic therapy [*e.g.* inhibitors of VEGF (bevacizumab) or TKIs (lenvatinib)] with ICI seems to overcome tumor-intrinsic resistance to immune checkpoint blockade.

LRTs are minimally invasive therapies; however, it has become clear that the treatment regimens adopted 15 years ago will change in the next few years. In the light of new evidence, three groups might benefit from the combined therapy (locoregional therapy and immunotherapy), including (1) Patients with a high risk of recurrence after a complete response by local ablation; (2) Patients who progressed under TACE; and (3) Patients with poor predictors of response to immunotherapy, when such predictors will be validated, possibly including NAFLD as underlying liver disease.

Currently, there are several trials underway evaluating different combinations (ICI ± anti-angiogenic therapy) as options for patients treated with LRTs (Table 6). It is important to remember that immunotherapies represent a two-edged sword, thus we must find the right timing, dose and combination of immunotherapy for a robust response and minimal side effects.

Ablation + other treatments

HCC is an attractive target for immunotherapy due to several reasons: (1) Usually, HCC develops on a background of chronic inflammation (cirrhosis or chronic hepatitis); (2) In the context of cirrhosis there is an immunosuppressive environment; and (3) Immune evasion was described in patients with liver cancer[95].

Combining thermal ablation with immunotherapy is a very appealing approach. Thermally-induced necrosis can act as a permanent source of tumor antigens, the sub-lethal zone around the necrotic zone can generate inflammatory cytokines, and the thermal stress is capable of making HCC cells more sensitive to immune therapies[48,96,97]. The field of immunotherapy in HCC (different from other cancer entities) was only recently unraveled. However, some preliminary studies with RFA and immunotherapy combinations (immune-ablation) have been already published. When used in a palliative setting, tremelimumab (anti-CTLA4) in combination with RFA or TACE in

Table 6 Summary of ongoing clinical trials evaluating combination therapy of immune checkpoint inhibitors with locoregional therapies

BCLC stage	Estimated/included patients	Clinical trial identifier	Phase	Arm
0	530	NCT03383458	III	Arm 1: RFA/MWA/curative resection + nivolumab (neoadjuvant) *vs* Arm 2: RFA/MWA/curative resection
B	26	NCT03397654 (PETAL)	Ib	Single arm: TACE followed by pembrolizumab
B	950	NCT04246177 LEAP-012	III	Arm 1: TACE + lenvatinib + pembrolizumab *vs* Arm 2: TACE
B	49	NCT03572582 (IMMUTACE)	II	Single arm: TACE + Nivolumab
B	522	NCT04268888 TACE-3	II/III	Arm 1: DEB-TACE + Nivolumab *vs* Arm 2: DEB-TACE
B	765	NCT04340193, CheckMate 74W	III	Arm 1: TACE + nivolumab + ipilimumab *vs* Arm 2: TACE + nivolumab + placebo
A	50	NCT03939975	II	Single arm: Pembrolizumab or nivolumab or toripalimab. For participants with stable disease or atypical progression to immunotherapy therapy, RFA or MWA is performed additionally
B	130	NCT03864211	I/II	Single arm: RFA or MWA followed by Toripalimab
B	61	NCT01853618	I/II	Single arm: Tremelimumab + RFA or TACE
B	30	NCT03638141	II	Single arm: Initial DEB-TACE followed by Durvalumab + tremelimumab
B	22	NCT03937830	II	Single arm: Durvalumab and bevacizumab + TACE
B/C	600	NCT03778957 EMERALD-1	III	Arm 1: TACE + durvalumab *vs* Arm 2: TACE +bevacizumab + durvalumab
A/B	662	NCT04102098 IMbrave050	III	Atezolizumab plus bevacizumab in HCC patients at high risk of recurrence after surgical resection or ablation *vs* Active surveillance in HCC patients at high risk of recurrence after surgical resection or ablation

BCLC: Barcelona Clinic Liver Cancer Classification; RFA: Radiofrequency ablation; MWA: Microwave ablation; HCC: Hepatocellular carcinoma.

32 HCC patients showed indeed interesting results. The patients received a total of six doses of tremelimumab at a 4-wk interval followed by an intentionally incomplete RFA or TACE to induce anti-tumor response at the ablation tumor junction. Patients with clinical response had an increase in CD8+ T cells in tumor biopsies obtained 6 wk after treatment. More interestingly, some patients experienced tumor responses in untreated lesions[98]. In another study, Ma *et al*[99] injected RetroNectin activated killer cells 14 d after RFA in patients with an HCC less than 4 cm[99]. They reported no severe adverse events, recurrences, or deaths during a seven-month follow-up. Using a similar approach, Cui *et al*[100] studied the combination of RFA and cellular therapy. Mononuclear cells from 30-HCC patients (early, intermediate and advanced stage) were harvested and induced into natural killer cells, γδT cells and cytokine-induced killer (CIK) cells which were subsequently infused back into RFA-treated patients for three or six courses. The combination improved PFS and reduced HCC recurrence compared to RFA alone[100].

Adjuvant treatment for HCC patients is an unsolved medical need. Using cellular-based immunotherapy Lee *et al*[101] studied the use of CIK cells injected after RFA (n = 69), ethanol injection (n = 13), or surgery (32) in patients with early-stage HCC[101,102]. They reported a better OS and cancer-specific survival in patients treated with a combined approach *vs* those treated with RFA, percutaneous ethanol injection (PEI), or surgery alone (P = 0.006 and P = 0.02). Similar findings were also reported in one multicentre randomized open-label phase 3 trial of adjuvant immunotherapy with CIK cells. The study included 230 patients with HCC treated by SR, RFA, or PEI. Patients were assigned randomly to receive immunotherapy or no adjuvant therapy. Adjuvant CIK cell therapy increased both recurrence-free survival and OS[103].

The use of monoclonal antibodies in combination with RFA was also studied in HCC. Either injected during RFA (131I-chTNT) or after RFA (131I metuximab) in an adjuvant setting both combinations showed improved PFS or OS[103,104]. More data about studies investigating the combination of RFA with different immunotherapy strategies are depicted in Table 7.

Table 7 Radiofrequency ablation combined with immunotherapy

Ref.	BCLC, *n* (%)	Treatment, *n* (%)	Results	Level of evidence
Cui *et al* [100], 2014	A (10); B (10); C (10)	RFA and cellular immunotherapy 8-11 d after RFA *vs* RFA alone	Higher PFS ($P < 0.001$). Six courses had better survival prognosis than three courses	III
Ma *et al* [99], 2010	A (7)	RFA and autologous RAK cells 14 d after RFA	No severe adverse events, recurrences or deaths during a seven month follow-up	IV
Duffy *et al* [98], 2017	C (21)	Tremelimumab every 4 wk and subtotal RFA on day 36	Median OS-12.3 mo. Median time to progression–7.4 mo. A significant increase of CD3+ and CD8+ immune cells infiltrates in lesions not treated by RFA	III
Lee *et al* [102], 2015	A (114)	PEI (13); RFA (69); Surgery (32) and adjuvant CIK cells *vs* PEI, RFA or Surgery alone	OS was significantly longer in the immunotherapy group than in control group ($P = 0.006$). CSS was significantly longer in the immunotherapy group ($P = 0.02$)	II
Tu *et al* [103], 2014	A and B	RFA and monoclonal antibody (131I-chTNT) injection during ablation *vs* RFA alone	Increased OS. Improved progression-free survival. Increased circulating white blood cells	IV
Bian *et al* [104], 2014	0 + A (94); B (33)	RFA and adjuvant 131I metuximab *vs* RFA alone	Prevention of tumor recurrence	II
Lee *et al* [101], 2019	0 and A (239)	RFA or PEI or Surgery plus CIK *vs* RFA or PEI or surgery alone	Increased recurrence-free survival and OS	I

BCLC: Barcelona Clinic Liver Cancer Classification; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; CIK: Cytokine-induced Killer; OS: Overall survival; CSS: Cyberchondria severity scale; PFS: Progression-free survival.

Nevertheless, the treatment of HCC will change over the following years. Not much has changed in the last 20 years but the era of immunotherapy has started and we will probably witness groundbreaking changes in the years that will come. New treatments, new guidelines, from single option to multiple options and from RFA to "immune-ablation" the burden has moved from scientist to clinicians: It is an interesting world out there.

Assuming similar suppositions with the TACE procedure, RFA was also combined with VEGF inhibitors. One study showed that Bevacizumab is useful in preventing the rapid progression of residual HCC following RFA in a rat model[105,106]. EMERALD-2 is an ongoing A Phase III, multicenter study of Durvalumab monotherapy or in combination with Bevacizumab as adjuvant therapy in patients with HCC who are at high risk of recurrence after resection or RFA (NCT03847428). Last but not least, nanoparticle-mediated drug delivery systems have also gained ground in oncology. The lyso-thermosensitive liposomal doxorubicin (LTLD) treatment aims to deliver doxorubicin at the peripheral thermal ablation zone, where the thermal elevation is suboptimal. When heated to 40 °C, LTLD releases a 25-fold greater concentration of Doxorubicin. The HEAT study is a global randomized, double-blind, dummy-controlled trial comparing RFA plus LTLD *vs* RFA alone that enrolled 701 patients with ≤ 4 unresectable up to seven HCC lesions. No differences in PFS and OS were found. The subgroup post hoc analysis showed improved efficacy when the thermal ablation indwell time for a solitary lesion was ≥ 45 min and increased treatment time *per* tumor volume was associated with better OS in the RFA + LTLD group[107,108]. The subsequent phase III OPTIMA study (NCT02112656) was halted in the interim analysis for futility reasons.

An exhaustive report about the combination of different tyrosine kinase inhibitors, distinctive ICI, or even their combo in the advanced liver cancer setting is beyond the scope of our review. However, we consider it far-reaching to mention one of 2020's revolutions–the association of bevacizumab (VEGF inhibitor) with atezolizumab (PD-L1 inhibitor) that brings encouraging data for unresectable HCC patients[109].

Although the combination of TACE and TKI's seemed promising in terms of inhibiting hypoxia-activated tumoral growth factors, studies do not appear to benefit any amalgam therapy compared to TACE monotherapy. To such a degree, one might say that the association of TACE and TKI's might have seen its downfall. Hence, the attention of the hepatology and oncology community was diverted to a new star-immunotherapy. In both the association with TACE and ablation, ICI have quietly demonstrated their benefit in trials. Although this path is still filled with uncertainty and caveats, in the following years we will witness an HCC guideline revolution.

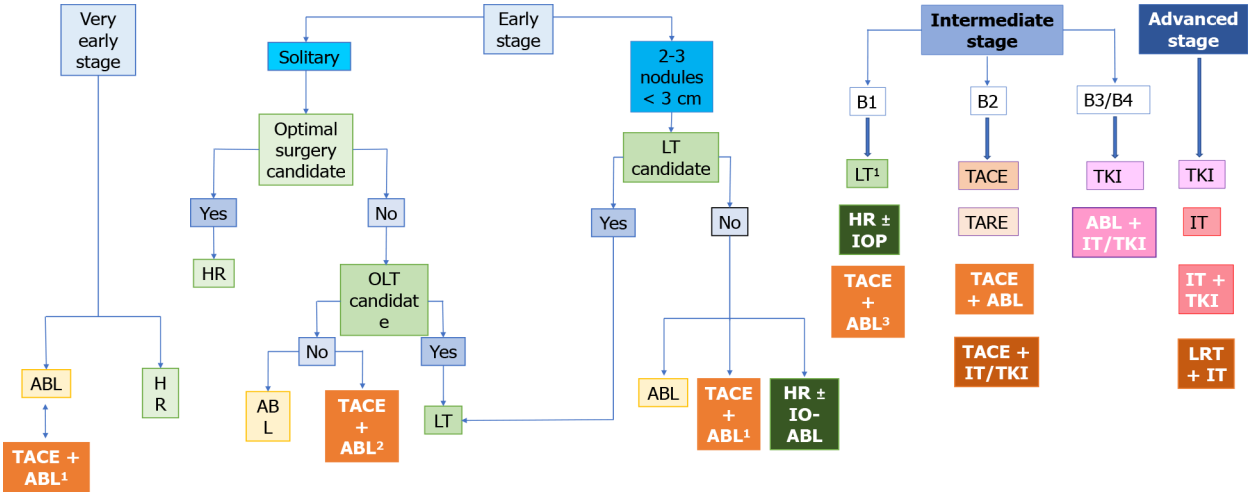

Figure 1 The place of combined therapy in the Barcelona clinic liver cancer classification algorithm. [1]Lesion not seen at ultrasound or in inappropiate positions; [2]Lesion > 3 cm; [3]Within Up-to-seven criteria. ABL: Ablation; HR: Hepatic resection; IOP: Intraoperatiove ablation; IT: Immunotherapy; LT: Liver transplantation; LRT: Locoregional therapy; OLT: Orthotopic liver transplantation; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; TKI: Tyrosine kinase inhibitors.

THE PLACE OF COMBINED THERAPY IN THE BCLC/EASL-HCC GUIDELINES

In tumor boards across the world, the most debatable section of the BCLC classification appears to be ranging from non-resectable BCLC-A to the less severe spectrum of the BCLC-C class, which is, by excellence, the appanage of interventional therapies [110]. Conventionally, the available treatments are dichotomized in curative intent and, possibly mislabelled, palliative therapies. The first group comprises RFA, MWA, PEI, CA, irreversible electroporation, and the latter includes bland trans-arterial embolization, conventional TACE, DEB-TACE, and endovascular radiotherapy-selective internal radiation therapy[2,3].

However, the basis for the aforementioned dichotomy might be fading, as research published in the past decade has shown that combination therapy is at least technically feasible, with the most relevant results being discussed in the previous sections. This has prompted a discussion with regards to the place of combination therapy in the therapeutic algorithm, as some of the approaches might be suited for second-, or even first-line choices for a select group of patients. On the other hand, it might be important to recognize that over-complicating a relatively straightforward algorithm could lead to disputable therapeutic choices and widespread heterogeneous interpretation, rendering data collection difficult.

As discussed earlier, one particular combination therapy appears to stand-out among other approaches: TACE-ablation for small, non-resectable HCC (3-5 cm), which is currently at the threshold between BCLC-A and -B. Not only does it improve outcomes in comparison to each individual therapy alone, but, according to limited data, it also appears to generate outcomes similar to surgery, which otherwise would have not been available[30]. A proposed alteration of the BCLC classification, which speculates on the potential role of combination therapies based on the available data previously discussed, is shown in Figure 1.

CONCLUSION

Of course, our proposal is based on the best available data and still needs further consensus validation, but might provide a foundation for future recommendations, as well as hinting towards potential areas of future development. There is a great need for well-designed, large-scale randomized controlled trials to adequately assess the benefits of combination therapy. Moreover, there are multiple nuances open for debate. Which is the best radiological method for combination therapy: Bland TAE, TACE, or DEB-TACE? Which ablative technique has the most benefits? Should treatments be applied in the same session or sequential? Which is the best sequence? The authors strongly believe that methodically addressing these questions could

ultimately lead to a truly personalized approach, hoping to improve the quality of life and OS of HCC patients.

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REVIEW

Unique situation of hepatocellular carcinoma in Egypt: A review of epidemiology and control measures

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Abstract

Hepatocellular carcinoma (HCC) is the sixth most common primary malignancy worldwide, and the third most common cause of death among cancers worldwide. HCC occurs in several pre-existing conditions, including hepatitis C, hepatitis B virus, and non-alcoholic cirrhosis. Egypt used to be the country with the heaviest hepatitis C virus (HCV) burden. The relationship between HCV and HCC is an important research area. In Egypt, HCC is a significant public health problem. A possible cause for the increasing rates of detection of HCC in Egypt is the mass screening program that was carried by the government for detecting and treating HCV. A multidisciplinary approach is now widely applied to HCC management in health centers all over Egypt. Different treatment modalities are available in Egypt, with success rates comparable to global rates. The Egyptian health authorities have made the elimination of HCV from Egypt a special priority, and this approach should lead to a decrease in number of HCC cases in the near future. In this article we review the current situation of HCC in Egypt, including epidemiological aspects, relevant risk factors for HCC development, strategies, and efforts established by health authorities for the screening and prevention of both HCV and HCC in Egypt. We highlight the different modalities for HCC treatment.

Key Words: Hepatocellular carcinoma; Liver cancer; Hepatitis C virus; Hepatitis B virus; Screening; Egypt

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Core Tip: Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, and the fourth most common in Egypt. Many risk factors may lead to the development of HCC, and the relationship between hepatitis C virus (HCV) and HCC in Egypt is an important research area. Major screening programs for HCV in Egypt, such as the national initiative for screening 65 million citizens, have produced high success rates on the way for eliminating the main risk factor for HCC in the country. It is now an appropriate time for principled guidance and screening programs for HCC in Egypt.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common primary malignancy worldwide[1] with higher incidence and prevalence in Africa and Asia[2]. The incidence of HCC worldwide has changed over the past few years, with some areas showing decreased rates, and others showing the opposite[3].

In Egypt, the relation between hepatitis C virus (HCV) and HCC is an important research area. Firstly, Egypt has a high recorded HCV transmission rate, with around 416000 new infections each year[4]. Secondly, there is known to be a relationship between HCV and HCC development. Thirdly, the programmed screening and follow up that was initiated by the government increased the number of known cases of individuals having both diseases. According to a study carried out by Ziada *et al*[5], 108 out of 514 patients diagnosed with HCV infection (21%) had focal lesions detected by ultrasound. In another study carried out by Abd-Elsalam *et al*[6], HCC occurred more frequently in patients with HCV than in those with hepatitis B virus (HBV) infection. These results may indicate the main predisposing factor for the development of HCC in Egypt.

A possible cause for the increase in detection of HCC in Egypt is the mass screening program that was implemented by the government for detecting and treating HCV. Due to this program, many patients were diagnosed and treated for HCC. According to a study carried out by Shaker *et al*[7], 75% of identified HCC cases came from rural areas in Egypt, with 45.7% of individuals ranging in age between 51-60 years.

According to the global cancer observatory, liver cancer represented 19% of all newly diagnosed cases in all ages and both sexes in 2018, with an incidence rate of 32% and a mortality rate of 31%[8].

RISK FACTORS FOR HCC IN EGYPT

HCV

HCV protein expression in infected hepatic cells causes mutation and malignant transformation leading to the development of HCC[9-11]. Repeated inflammation, damage and regeneration are believed to be the main cause of malignant transformation[12]. HCV infection increases the risk of HCC development up to 20-fold[13]. About 0.5%10% of HCV-related cirrhosis leads to HCC annually[14]. There are other factors that increase the risk of developing HCC with HCV, such as male gender, smoking, obesity, diabetes, and HBV or human immunodeficiency virus co-infection [14,15]. During the era of interferon-based therapy, sustained virologic response (SVR) and HCV eradication was associated with decreased incidence of HCC[16]. This outcome was hoped for from the direct-acting antiviral agents (DAAs), but researchers could not reach an agreement on that point. Reig *et al*[17] found an early recurrence of HCC in patients receiving DAA (27.6%). This was not the case in other studies, that found no increase in recurrence after DAA therapy[18-21].

Another review was published by Reig *et al*[17], debating about revising the published data, and they concluded that no solid evidence could be reached about the relationship between HCC recurrence and DAA therapy[22]. El Kassas *et al*[23] concluded that there is a possible role of DAAs in HCC recurrence.

Egypt recorded the highest prevalence of HCV worldwide, as a consequence of unsafe IV treatment of schistosomiasis in 1950s until the 1980s[24]. A decline was recorded in the prevalence of HCV infection from 14.7% in 2008 to 10% in 2015. This was attributed to the aging of the group who received antischistosomal treatment[25, 26].

In Egypt, genotype 4 is the main genotype, occurring in up to 92.5% of infected patients, followed by genotype 1 (3.6%)[27-30]. A study demonstrated that at least in Egypt, the lymphotoxin alpha gene mutation may have a role in susceptibility to HCV infection, and the subsequent development of clinical manifestations[31].

HBV

DNA viruses can be incorporated into a host genome[32], inducing malignant transformation by downregulating tumor suppressor genes and activating oncogenes[9]. The annual incidence of HCC is 0.42%[33] which differs according to the presence of HBV infection or cirrhosis[34], with the lifetime risk of HCC development among HBV carriers being from 10% to 25%[32]. Antiviral treatment for HBV can decrease HBV-DNA levels[35], with improved liver function and histology. There is increasing evidence that nucleos(t)ide analogs (NAs) decrease, but do not eliminate, the risk of HCC development[36,37].

In Egypt, the population prevalence of HBV was 1.4%, with an HBV-HCV co-infection rate of 0.06%[38]. The nationwide vaccination program has decreased the prevalence of HBV infection considerably[39,40]. The HBeAg negative variant was found to be highly prevalent in Egypt, and represents a late phase of HBV infection with persistent viral replication. This situation will lead to early development of cirrhosis[41]. However, 16% of patients with HCV have an occult B infection[42] A study carried out by Fouad *et al*[43] found that 81.9% of their chronic HBV cohort were HBeAg negative.

Of patients with liver cirrhosis, 3%-5% develop HCC annually[44]. In Egypt, HCC represents nearly 70% of all liver tumors[45].The increased incidence in Egypt may be related to the increased screening carried by the government. and a greater focus on HBV and HCV as predisposing factors in the past few years[46].

Environmental toxins

The liver is the main organ involved in the metabolism of chemical agents[47]. It has a characteristic blood supply, and is involved in many metabolic and excretory processes. This causes damage to the liver ranging from fatty liver, hepatocellular injury, cirrhosis, and HCC.

In Egypt, nearly 26% of the population works in agriculture[48], and thus have a high risk of exposure to pesticides. A study carried out by Abou El Azm *et al*[49] found that 13.87 % of the total HCC in Egypt was associated with risk factors other than HVB or HCV, predominantly pesticides, and superphosphate and ammonium sulfate fertilizers (94.87%, $P < 0.001$) with significant exposure occurring in industry, farming, and residences. The HCC in these cases had specific criteria, being solitary, of smaller size, and having lower alpha fetoprotein (AFP) titers[49].

Aflatoxins are known to have a major role in the development of HCC in Egypt. They are known carcinogenic metabolites of molds, mainly *Aspergillus flavus*, and parasites that contaminate many agricultural products, such as peanuts, maize, and cotton seed[50].

Beside molds, a study conducted on desserts in Egypt showed that aflatoxin B1 (AFB1) was detected at above the acceptable limits of 2 ppb in 70% of samples of one of the dairy desserts, and Aflatoxin M1 exceeded the limits in 10% of each type of sample[51]. High serum levels were detected in Egyptians with HCC by a study that was carried out by Dilber *et al*[52]. AFB1 is the main metabolite produced, and is the most carcinogenic, teratogenic, and mutagenic metabolite[53]. It was present in high levels in those presenting with multiple hepatic focal lesions over 5 cm in diameter [54]. Anwar *et al*[55] found that presence of Aflatoxins and HCV is connected to hepatic disease progression to G3S3 which indicates HCC. Aflatoxin levels were found to be significantly higher in HCC patients than in cirrhotic individuals and controls in a study conducted by Sharaf-Eldin *et al*[56].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) produces abnormal fat accumulation in the liver, without significant alcohol ingestion. NAFLD includes a broad spectrum of liver conditions ranging from steatosis and reaching up to cirrhosis. It is considered to be the most common liver disease related to obesity[57], and is a condition that can progress to HCC[58]. HCC development is related to disease progression from NAFLD to non-alcoholic steatohepatitis (NASH). NAFLD can accelerate the disease burden of HCV in terms of morbidity and mortality[59]. A study that was carried out on school children in Egypt, fatty liver was prevalent in 15.8% of the study group, and increased significantly with age (P = 0.004)[60]. NAFLD (56.8%) was a predominant feature among the study population in a study that was conducted by Abd El-Wahab EW *et al*[61] on 190 adults seeking health check-ups at the outpatient clinic of a tertiary care hospital in Alexandria, Egypt. Fatty liver was detected in 47 (65.3%) children, and in 52 (62.7%) adults in another Egyptian study by Wafaa *et al*[59]. A study concluded that NASH is present in 5.3% of Egyptian patients presenting with HCC[62]. This finding reflects the high prevalence of the condition in Egypt, and the subsequent increased risk of HCC transformation. Screening and early detection of the condition indicates the importance of avoiding further burdens on public health, as in the campaign carried out by the Egyptian government last year with respect to the detection of obesity, diabetes, and hypertension as predisposing causes for NAFLD.

Lifestyle factors (alcohol consumption, smoking, and dietary factors)

Excessive alcohol consumption is a well-known risk factor for developing HCC[62]. In the European Union, 60%-80% of liver-related mortality is caused by excessive drinking[63] and alcohol-related chronic disease is considered to be the second most common indication for liver transplantation, accounting for approximately 40% of all primary liver transplants[64]. In Egypt, this risk is low[48,65-67]. Heavy alcohol consumption increases the risk of HCC by up to 16%[68]. The risk is increased by 5- to 7-fold with heavy ethanol consumption for more than 10 years[69,70].

Smoking is another factor that may lead to HCC, due to the tobacco[71]. A Korean study reported a connection between primary liver cancer and smoking with the risk increased by up to 50% compared with non-smokers[72]. Bakir and Ali-Eldin[73] concluded that 64% of Egyptian patients with HCC are smokers. Abou El Azm *et al*[49] reported that heavy smoking is one of the primary risk factors for non-B non-C HCC in Egypt. Another study mentioned smoking as one of the main causative agents for HCC in Egypt[5]. Another Egyptian study documented an increased risk of HCC development in patients with a smoking pattern of 20 cigarettes per day for more than 29 years[74].

Obesity

Around 1.9 billion people around the world are overweight, and 600 million are suffering from obesity[75]. Obesity is related to the development of many metabolic disorders, including diabetes mellitus and hypertension, with an increased burden of HCC development. Premorbid obesity is associated with up to a two-fold risk of HCC related mortality[75]. It has been suggested that for every 5 unit increase in body mass index (BMI), there is a 39% increased risk of HCC[76]. In another study, carried out by Calle *et al*[77], the HCC related mortality in obese men (BMI, 30-34.9 kg/m^2) was 1.9 times the number in men with normal BMI (BMI 18.5-24.9 kg/m^2).

In Egypt, a study on primary school students showed that the overall prevalence of obesity and overweight was 13.9% and 16.2% respectively[78]. In adults, it is estimated to be present in 61%70% of the whole population aged 20 and above, with a prevalence of 18%22% in men and 39%48% in women[79]. Aitsi-Selmi *et al*[80] investigated the relationship between wealth, education, and obesity among 49058 Egyptian women using the Demographic and Health Surveys' datasets. Obesity was mainly recorded among women with a primary education or less, and whether they are poor or wealthy. A survey of young people in Egypt[81] found that consuming more white bread and carbonated drinks is directly related to their economic state.

Genetic factors

Some hereditary liver diseases with genetic mutations are believed to carry a risk for HCC development. These diseases are Wilson disease, hemochromatosis, alpha-1 antitrypsin deficiency, tyrosinemia, glycogen storage diseases, and porphyrias. The same is true for polymorphisms with increased risk for HCC. Polymorphisms in *UGT1A7*, *MnSOD*, and *IL-1B* were reported to be significantly associated with risk [82]. HCV and HBV infection are reported to increase the risk of gene mutation,

leading to the development of HCC[83-87].

In an Egyptian study, the TNF-α-308 G > A polymorphism was associated with increased HCC risk in an Egyptian population, but no significant difference was found for cytokines interleukin (IL)-1β and IL-10[88]. In another study on Egyptian patients, XRCC1 G28152A (rs25487) and XRCC7 G6721T (rs7003908) polymorphisms were found to have a role in susceptibility to HCC in the Egyptian population[89].

Epidermal growth factor gene polymorphism 61*G was found to be positively associated with HCC risk in Egyptians. Uncreased concentration of EGF was associated with the G/G genotype[90].

The prevalence of hereditary hemochromatosis in Egypt is reported to be 0.5%[50]. This indicates that hereditary disorders are not a major cause of HCC.

Preventive measures: HCV control in Egypt

In 2015, 10% of the population tested positive for HCV antibodies, which would amount to around 5.5 million persons at that time[91]. As a major cause of HCC in Egypt, after the World Health Assembly's decision in 2016 to eliminate HCV, the Egyptian government decided to begin a nationwide campaign for the detection and treatment of HCV in Egypt[92]. More than two million individuals were treated by the year 2018 under the umbrella of this campaign, with cure rates reaching 90%. Disease elimination was achieved mostly by the decrease in the cost of direct-acting antiviral drugs implemented by the Egyptian government. This process was first applied to identified chronic patients. The government then began mass screening of the population, to facilitate rapid and effective elimination of the disease. Screening was done in all of the hospitals related to universities, military hospitals, rural health units, and police hospitals. This was achieved by moving teams to other areas, using gathering spaces, factories, and open places to aid in the screening. Finger prick rapid diagnostic tests were used. Patients reported positive were scheduled for evaluation and treatment plans. Between October 2018 and April 2019, 79.4% of the targeted population participated spontaneously in the screening, with higher female than male participation (84.5% *vs* 74.6%). By the end of September 2019, 1148346 (76.5%) of screened individuals were reported to have viremia, and treatment was started in 91.8% of them. Out of these people, 465992 reached 12-wk follow up after ending treatment. At this stage, 386103 (82.9%) had a known treatment outcome, and 381491 (98.8%) of those with a known outcome had a SVR. Of the 93651 patients with viremia who did not show up for treatment, 53445 who were reached reported having treatment in private[93].

There have been no screening programs for HCC in Egypt until now. Because HCC in Egypt is mostly diagnosed early, as more patients are diagnosed under surveillance, the survival duration is longer than in other African countries[94]. The effect of treatment itself is controversial. A study carried by El Kassas *et al*[23] reported: "Our data point to a high (*i.e.*, almost 4 times) increased rate of recurrence after DAA treatment for patients with a history of successfully treated HCC, when compared to similar patients who were not given DAAs".

After HCV elimination, decreased rates of HCC were expected, but Reig *et al*[17] found exactly the opposite tendency after using direct-acting antiviral drugs for HCV treatment.

This work was followed by a paper that emphasized the early occurrence of HCC in patients receiving DAAs for HCV[18]. Another study produced different results, in which no difference in the cumulative incidence was found in developing *de novo* HCC in patients with HCV and those treated by DAAs or interferon-based therapy[95]. Similar results were reached by Cabibbo *et al*[96]. A study on patients with HCV-related cirrhosis treated with DAAs and subsequently developing HCC reported a relation between age, Child-Pugh classification, liver stiffness, history of HCC, and the development of HCC[18]. In 2019, a study on 7344 patients concluded that DAAs decrease the risk of developing HCC[97].

An Egyptian study concluded that DAAs do not increase the risk of HCC recurrence, but still did not recommend abolishing it, rather implementing close follow up[98]. Another study denied the occurrence of HCC after DAAs although a high incidence of recurrence was still found. This study also suggested that high AFP before treatment is a good predictor for developing HCC[99].

Immunization for HBV and protection against HCC was discussed in a study on an analysis of 1509 patients with HCC in Taiwan. The study concluded that risk reduction of HCC is obvious after immunization of infants against HBV[100]. The HBV vaccination program in Egypt began in 1992 with a schedule of 2, 4, and 6 mo of age. This program was not associated with simultaneous screening for pregnant women [101]. A multicenter study was carried on 3600 children aging from 9 mo to 16 years

old to assess the effectiveness of the Egyptian vaccination program. The study concluded that the vaccination is protective from 1 years to 16 years post vaccination [40]. Another study assessed the benefit of follow up post vaccination response and seroprotection persistence, to determine the importance of booster doses in healthy subjects. A protective level of HBsAb was found (> 10 IU/mL) among 66.7% of all individuals studied[102]. The risk of HCC danger is escalated by co-infection with occult HBV in HCV patients[103].

HCC SCREENING

Screening programs gain value when the benefits from screening are greater than the expected harm. A large randomized controlled trial showed benefits for screening noncirrhotic HBV patients for the development of HCC, leading to improved early detection, better treatment, and better survival rates[104]. An association between screening for HCC and improvement in three-year survival rates is well established [105]. A study observed the difference between the survival rates of HCC in Japan and in Hong Kong. Japan has an intensive screening program unlike Hong Kong. The survival rate was 52 *vs* 17.8 mo[106]. In spite of the psychological or financial harm that could result from screening for HCC, the benefits overweigh the harm. Cirrhotic patients show an annual risk of 2%-4% of developing HCC which makes screening highly recommended in all cirrhotic patients whatever the etiology[107,108].

The risk of progression to HCC in non-cirrhotic patients has ranges from 7% to 54%, varying according to etiology and geographic distribution[109]. The most common etiological factors for this condition are obesity, aflatoxins, NAFLD, genetic mutations, smoking, inherited diseases, and sex hormones[107,109-112]. Non-cirrhotic liver HCC has a better prognosis and better results following surgical intervention than cirrhotic liver HCC[113]. In the European Association for the Study of the Liver 2018 report, a risk stratification model was recommended for non-cirrhotic HCC patients, namely PAGE-B (platelet, age, gender, hepatitis B), that is currently used in non-cirrhotic HBV patients[114,115].

The risk of HCC development in cirrhotic patients is from 2% to 4% annually. This high risk makes screening an obligation for all cirrhotic patients, whatever their etiology[107,111]. Screening is mainly to be done for compensated cirrhosis with Child-Pugh class A and B, while class C is to be offered liver transplantation[116].

Screening methods

Ultrasound is the most widely used imaging technique for regular screening for HCC. It has many advantages, being easy, readily available, non-invasive, and inexpensive. The sensitivity of ultrasound in detecting HCC is not more than 45%[117], especially in lesions less than 1 cm in diameter[118]. It is affected by the operator, the patient ability to hold their breath during examination, and the nodularity of the liver, which makes the detection of new lesions difficult, with some areas unreachable, like the dome of the liver. Obesity and NASH renders examination difficult which, decreasing the efficacy of the procedure[119]. In such cases, magnetic resonance imaging (MRI) and computed tomography (CT) scanning can replace ultrasound[120,121], but they are not cost effective, so they are not considered as first-line screening methods for HCC[119, 121].

Biomarkers

AFP is the biomarker most widely used in screening for HCC[122]. Although it is readily available, inexpensive, and easy to perform, its addition in the guidelines along with ultrasound was debatable. The American Association for the Study of Liver Diseases recommends using ultrasound, with the use of AFP to be judged by the clinician according to the patient's condition[107]. However, European guidelines recommend using ultrasound with no AFP needed[123].

In Egypt, HCV is the main etiological factor for liver cirrhosis, followed by HCC. Liver elastography is a documented method for assessing liver stiffness. A study investigated its role in the early detection of HCC in HCV cirrhotic patients. It recorded cutoff value of 24 kPa for diagnostic prediction of HCC produced sensitivity 100%, specificity 83.3%, PPV 94.5%, NPV 77.3%, and AUC 89%[124]. Another study discussed the superiority of an abbreviated MRI protocol over AFP and ultrasound in detecting small hepatic focal lesions in post HCV cirrhotic patients[125].

A scoring system was suggested by Abdelaziz *et al*[126]. The HCC Multidisciplinary Clinic-Cairo University (HMC-CU) score (Logit probability of HCC = -2.524 + 0.152 ×

age -0.121 × Hb -0.696 × INR -1.059 × Alb + 0.022 × AFP + 0.976 × Sex. Male = 1, Female = 0), with a cutoff of 0.559 was superior to other scores for predicting HCC, having a sensitivity of 90% and a specificity of 80.6%. In 2010, El-Zayadi *et al*[127] investigated the effect of surveillance of HCC on tumor staging and treatment options in Egypt. The study divided the patients into two groups: (1) For those who followed screening regularly; and (2) Who were diagnosed as HCC as first presentation with no screening program followed. They produced variable results three months after interval screening was suggested, as the doubling time of the tumor size is from 1 mo to 19 mo, and as HCV is the main predisposing factor in Egypt. The study reported that surveillance increased the detection of small lesions in the absence of vascular invasion.

A prospective study carried out by Gomaa *et al*[46] on 2000 patients diagnosed with HCC reported that BCLC has the best prognostic stratification for Egyptians with HCC. Salama *et al*[128] suggested adding leptin to AFP for HCC screening in Egyptians. All of these studies were trials from separate centers to detect and screen for HCC in Egypt.

TREATMENT OF HCC IN EGYPT

HCC is a disease with different modalities of treatment. Surgical resection comes in the first place, followed by liver transplantation. Ablative techniques come next, including ethanol (percutaneous ethanol injection), microwave (MWA) or radiofrequency (RFA), catheter-directed trans arterial chemoembolization (TACE) or radioembolization (TARE). Last comes external beam radiation therapy in the form of stereotactic body radiation therapy or proton beam therapy, systemic targeted small molecule tyrosine kinase inhibitors (TKIs), check-point inhibitor immunotherapy, and investigational agents.

A multidisciplinary approach has been now widely recognized and is the mainstay in managing HCC in different health centers all over Egypt. This approach includes a scientific committee with the patient of HCC presented to it, and through discussion is performed, along with counseling the patient with different treatment options.

Surgical intervention

Surgery for HCC includes tumor resection or liver transplantation. Liver transplantation is the best choice, as the whole organ is replaced by a new one, and the underlying pathology is ended forever. However, this is not possible in all cases. Milan criteria were developed to diagnose a patient's suitability as a candidate for liver transplantation[129]. When it is inconvenient to do transplantation, surgical resection of HCC comes next. In non-cirrhotic patients, tumors less than 5 cm are best offered resection as the best treatment modality from an oncological point of view [130]. However, partial resection carries the risk of tumor recurrence[131]. Tumor size is not a contraindication for partial hepatectomy, but other factors such as extrahepatic metastasis, vascular invasion, main bile duct affection and portal hypertension may affect the decision[132]. In an Egyptian study carried out by Zakaria *et al*[132], the researchers concluded that total tumor volume is an appropriate prognostic measure to evaluate the tumor burden in HCC patients. Assessment of the hepatic function and future remnant liver are cornerstones in the liver resection decision[133]. A study carried out by Senbel *et al*[134] concluded that hepatic resection is an effective treatment for Child-Pugh A patient candidates for liver transplant. A study at Assuit university hospital reported 28 cases that underwent hepatic resection for HCC from 2013–2017. The study defined low serum albumin, high MELD score and high Child score to be risk factors for developing post-resection liver failure[135] in 268 patients who had undergone hepatic resection between the years 2010 and 2019 in Mansoura University, Egypt[136].

In Arab countries, 3804 liver transplants were done between the period 1990–2013, of which living donor liver transplantation (LDLT) was 80%, and deceased donor liver transplantation was 20%. Fifty-six percent of the reported cases were in Egypt[137]. In Egypt, the only source for a liver graft is from a living donor. From 2001 to 2019, 1230 cases of liver transplantation were reported from three transplantation centers in Egypt. Of them, 394 cases were HCC transplanted patients. In a retrospective study done by the surgical team in Dar ALfouad, Egypt, 60 patients with HCC who had undergone liver transplant within and beyond the Milan criteria were investigated for their clinical outcome. The results were as follows: "Overall 1-, 3-, and 5-year survival rates were 98.3%, 93.5%, and 71.4%. Overall disease-free survival rates at 1, 3, and 5

years were 96.6%, 93.5%, and 64.2%. There was no statistically significant difference in overall survival time between patients within and beyond the Milan criteria. Factors affecting recurrence were the tumor grade, lobar distribution, size of the largest nodule, and the total tumor burden in the explanted liver"[138]. In a study done by Galal *et al*[139], the researchers concluded that AFP may predict HCC recurrence after LDLT (area under the curve = 0.806) at cutoff values of more than 66 ng/mL, with 60% sensitivity, 94.3% specificity, 42.9% positive predictive value, and 97.1% negative predictive value.

We in Egypt have certain constants regarding liver transplantation as an option for HCC treatment, the major issue being the high cost of the operation, and the difficulty of finding a proper matched donor, as only living donor transplant is allowed in Egypt. Nevertheless, the success rate of liver transplant in Egypt is comparable to international results. So, it became of importance to allow health insurance coverage for liver transplantation operations in public health centers as a better treatment option for Egyptian HCC patients.

Local ablation techniques

Using thermal ablation for hepatic focal lesions has many advantages, such as the ability to repeating the maneuver, low morbidity and very few complications[140]. MWA ablation provides better results in areas with high blood flow, or near vessels, because it is not affected by the heat sink effect[141]. An Egyptian study carried out by Soliman *et al*[142] aimed to investigate the efficacy of MWA ablation in risky areas adjacent to other organs, near the diaphragm, and near blood vessels. In the study group, MWA reached ablation rates of 100%, 75%, and 87.5% for lesions close to the gall bladder, perivascular lesions, and subcapsular lesions, respectively. Another study done at Menoufia university, Egypt, compared single local ablative and combined techniques in HCC. The combined locoregional method provided better results[143]. However, Kamal *et al*[144] found no difference between MWA ablation and RFA ablation in treating HCC. Due to the high incidence of HCC related HCV in Egypt, the high risk of recurrence in those patients was investigated by Sharaf-Eldin *et al*[145]. The study concluded that in those patients, the presence of hepatomegaly, heterogenous liver, and splenomegaly, a sign of portal hypertension, together with tumor factors such as large size, bilobar affliction, and lesions near the liver capsule, showed a significant association with tumor recurrence.

TACE

TACE is the treatment of choice for patients with intermediate stage HCC, according to BCLC[123]. It is also the standard treatment in non-resectable HCC[107]. It is considered to be a palliative treatment, with positive impacts on survival and quality of life[146]. Since Seldinger described his technique in 1953, many intravascular procedures have been used[147]. This was followed by percutaneous selective angiography and arterial infusion of vasopressin by catheterization for controlling gastrointestinal bleeding. For more than a decade there was a debate about the use of chemotherapy to support TACE over trans arterial embolization[148]. Many studies supported TACE for providing both embolectomy and chemotherapy, and for keeping a good hepatic reserve for better survival[149-151]. TACE is not used only in non-resectable HCC, but also for downstaging before liver transplantation[152], and has good outcomes and overall survival[153]. Farouk Ahmed *et al*[154] found that the main etiology for HCC in Egypt is HCV. Patients who were inappropriate for transplantation, being outside of the Milan criteria, were chosen for downstaging by TACE before transplant. The study showed that good selection of patients for downstaging by TACE has good outcomes on liver transplantation. The patients' quality of life post TACE was evaluated by Fouad *et al*[155] in a study on 99 patients with HCC. The study showed improved quality of life after three months. In another Egyptian pilot study, RFA ablation showed better results with respect to quality of life than TACE [156].

TARE

Guidelines recommend TACE as the standard line of treatment for BCLC-B, but the results are still not very satisfactory[157]. Radiation from external beams to the liver is not effective in delivering lethal doses, as HCC is radio-resistant[158]. Radioembolization with Yttrium-90 microspheres is a recently used catheter-based treatment for HCC. It can be performed safely in patients with portal vein thrombosis, due to its low embolic effect[159]. TARE has the advantages of short hospital stay[160], prolonged time until progression[148], and long progression free survival[161].

Hamed *et al*[162] investigated the efficacy of Yttrium-90 on 20 Egyptian patients with intermediate and advanced HCC, with good outcomes even in the presence of compromised liver functions. Similar results were produced by Hetta *et al*[163], in a study in which TARE was investigated in advanced HCC with or without portal vein thrombosis. TARE Y90 showed the best results, especially in advanced stage disease, when compared to TACE in a study on 86 Egyptian patients with intermediate HCC [164].

Systemic therapies

Treatment for advanced HCC is now based on systemic therapy relying on TKIs, anti-angiogenesis agents, and immunotherapy[123]. Before the development of sorafenib, no drug was available that could provide this improved overall survival in such patients[165]. Sorafenib is an oral multi-kinase inhibitor with anti-proliferative and anti-angiogenic properties. It acts by inhibiting vascular endothelial growth factor receptor (VEGFR) -2 and -3 tyrosine kinases, platelet-derived growth factor receptor (PDGFR)-β tyrosine kinases, and rapidly accelerated fibrosarcoma kinases[166]. Sorafenib was first used in cases with well-preserved liver function, but results from the Global Investigation of Therapeutic Decisions in Hepatocellular Carcinoma and of its treatment with sorafenib (GIDEON) found a similar safety profile, irrespective of Child-Pugh staging[167]. Routine use of sorafenib in patients with underlying liver dysfunction is not recommended.

Lenvatinib (Lenvima, Eisai) is an oral TKI of fibroblast growth factor receptor (FGFR), VEGFR, PDGFR-α, rearranged during transfection, and KIT. It has been accepted as a first-line therapy for unresectable HCC since August 2018[168]. Regorafenib (Stivarga, Bayer) came next. It is a potent oral inhibitor of angiopoietin-1 receptor (Tie2), VEGFR, PDGFR, and FGFR, and was studied by Bruix and colleagues in patients who did not respond to sorafenib. It was approved by the FDA based on this multinational study[169].

A study on sorafenib in Egypt claimed that it cannot be used except in patients with Child A and low disease burden[170]. The same recommendation was made by Abdel-Rahman *et al*[171]. When sorafenib was studied in Egyptian patients with advanced HCC, it gave better outcomes, overall survival, and progression free survival when compared to no treatment[172]. It is, however, considered to be a costly treatment for the Egyptian patients, as was found in a study carried out by Hamdy Elsisi *et al*[173], in which they concluded that "sorafenib does offer increased survival and quality of life at an increased cost but at an incremental cost effective ratio that exceeds the nationally accepted cost-effectiveness threshold". Hanafy showed that a combination of sorafenib and low dose capecitabine is effective in advanced HCC in an Egyptian population[174].

A comprehensive summary of studies discussing the results of different treatment modalities for HCC in Egypt is presented in Table 1.

NATIONAL POPULATION-BASED CANCER REGISTRY PROGRAM

The Egyptian National Cancer Registry Program (NCRP) was launched in 2008 to represent a source for cancer incidence figures in Egypt[175]. NCRP stratified Egypt into 3 geographical areas: lower, middle, and upper. Data are regularly collected from specialized cancer treatment centers that are scattered all over the country map. Results of NCRP showed that HCC was the first among the most frequently observed cancers in lower and middle Egypt and the 2nd in upper Egypt (Figure 1).

HCC SCREENING AFTER HCV TREATMENT WITH DAAS

A major breakthrough was noted after the national campaigns of fighting and screening HCV, in which all of the population was screened for HCV, and basic laboratory results and ultrasonography were performed[93,176]. Many HCC patients were discovered and provided with treatment options. Despite the high safety profile of DAAs therapy, which enabled treatment of advanced cases and with expected lower incidence rate of HCC post-treatment, there were some contradictory reports on HCC incidence rates post SVR[177].

The major drawback in our campaign in Egypt was lack of a program after achieving SVR for continued screening for HCC after cure of HCV, with a resultant faulty impression of the patient that they were completely cured, with no need for

Table 1 Summary of studies discussing the results of different treatment modalities for hepatocellular carcinoma in Egypt

Treatment modality	Ref.	Design	Sample size	Summary of the most important results
Resection	Senbel *et al* [134]	Retrospective	84	Median OS was 50 mo
	Zakaria *et al* [132]	Retrospective	204	Predictors of decreased survival: serum AFP level > 400 ng/mL, TTV > 65.5 cm^3, microvascular invasion, postoperative decompensation
	Makhlouf *et al*[135]	Retrospective	28	Predictors for developing post-resection liver failure: low serum albumin-higher child score
Liver transplant	Kamal *et al* [144]	Retrospective	60	Overall disease-free survival rates at 1, 3, and 5 yr were 96.6%, 93.5%, and 64.2%; Overall, 1-, 3-, and 5-yr survival rates were 98.3%, 93.5%, and 71.4%. Factors affecting recurrence were the tumor grade, lobar distribution, size of the largest nodule, and the total tumor burden in the explanted liver
	Galal *et al* [139]	Retrospective	75	AFP may predict HCC recurrence after LDLT (area under the curve = 0.806) at cutoff values of more than 66 ng/mL
MWA	Soliman *et al* [142]	Prospective	88	MWA reached ablation rates of 100%, 75%, and 87.5% for lesions close to the GB, perivascular lesions, and subcapsular lesions, respectively
Radio frequency	Sharaf-Eldin *et al*[145]	Retrospective	45	Hepatomegaly, heterogenous liver, and splenomegaly, a sign of portal hypertension, together with tumor factors such as large size, bilobar affliction, and lesions near the liver capsule, showed a significant association with tumor recurrence
	Nouh *et al* [143]	Prospective	60	Combined techniques (RFA and percutaneous ethanol injection) give the best results for management of HCCs in comparison with individual techniques
TACE	Farouk *et al* [154]	Retrospective	27	Successful TACE for down-staging of HCC can be achieved in the majority of carefully selected patients and is associated with excellent post transplantation outcome
	Fouad *et al* [155]	Prospective	99	Improved quality of life after three months of TACE
TARE	Hamed *et al* [162]	Prospective	20	The complete response, partial response, stable disease and disease progression rates for the study sample after 3 mo using the conventional RECIST criteria was 0%, 55%, 30% and 10%, while after 6 mo it became 0, 50%, 20% and 25% respectively
	Hetta *et al* [163]	Prospective	40	The overall response (complete or partial response) was exhibited by 9% of patients, stable disease exhibited by 80% of patients, progressive disease seen in 11% of patients after one month of TARE
	El Fouly *et al* [164]	Prospective	86	The median OS (TACE: 18 mo *vs* TARE Y-90: 16.4 mo) and the median TTP (TACE: 6.8 mo *vs* TARE Y-90: 13.3 mo) were not statistically different between TACE and TARE group
Systemic therapy	Nada *et al* [170]	Retrospective	130	The median overall survival of patients with HCC treated with sorafenib was 5 mo (CI: 4.166-5.834), and progression free survival was 4 mo (CI: 3.479-4.521)
	El Baghdady *et al*[172]	Prospective	55	The one-year OS was 0.0% *vs* 75.5% (P = 0.008) in control and sorafenib respectively. Median PFS was 5 mo *vs* 12 mo in control group and sorafenib respectively (P = 0.008). Sorafenib treatment showed a better outcome OS, PFS and QOL as compared to no-treatment in Egyptian patients with advanced Hepatocellular Carcinoma

HCC: Hepatocellular carcinoma; LDLT: Living donor liver transplantation; MWA: Microwave; OS: Overall survival; RFA: Radiofrequency; TACE: Trans arterial chemoembolization; TARE: Trans arterial radioembolization; RECIST: Response Evaluation Criteria in Solid Tumors; CI: Confidence interval; PFS: Progression-free survival; QOL: Quality of life; AFP: Alpha fetoprotein; TTV: Total tumor volume; TTP: Time to progression.

lifetime follow up and screening for HCC.

This is why it is important to highlight the importance of screening for HCC for all individuals with SVR for cirrhotic features for life. Increasing public awareness of the importance of the screening is warranted just as in the national screening campaign for breast cancer in Egypt 2020[178].

Major screening programs in Egypt, like the National Initiative of 100 Million Healthy Individuals and Breast Cancer 2020 have produced high success rates[178]. Now it is time for proper guidance and screening programs for HCC in Egypt.

CONCLUSION

HCC is a disease posing a rising burden in Egyptian society. HCV is the main etiology in our country, with an expected decline following the decline in HCV incidence. HBV is the second most important etiology in Egypt. Mass vaccination campaigns are the

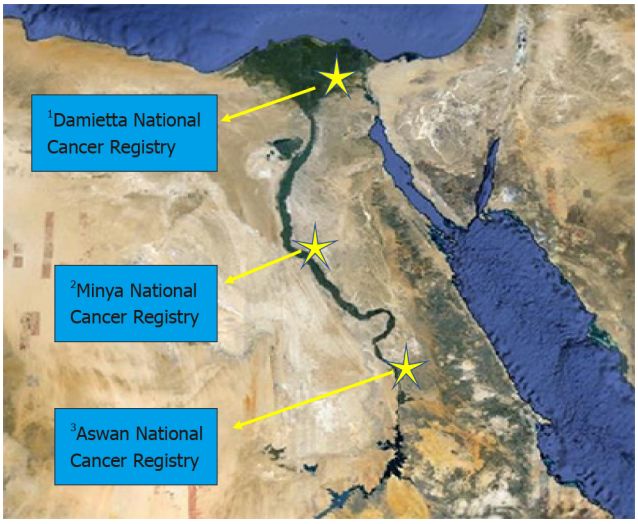

Figure 1 Proportion and age standardized rate of liver cancer in lower, middle, and upper Egypt (results of the National Population-Based Cancer Registry Program). [1]Lower Egypt: Damietta National Cancer Registry [liver cancer has the highest proportion among the most frequently observed cancers (29.6%)]. Males: Proportion and age standardized rate (ASR): 41.7% and 81.0/100000. Females: Proportion and ASR:16.3%and32.6/100000. [2]Middle Egypt: Minya National Cancer Registry [Liver cancer has the highest proportion among the most frequently observed cancers (15.2%)]. Male: Proportion and ASR: 20.4% and 37.6/100000. Females: Proportion and ASR:8.9%and13.7/100000. [3]Upper Egypt: Aswan National Cancer Registry [Liver cancer has the 2nd highest proportion among the most frequently observed cancers (8.2%)]. Male: Proportion and ASR: 11.8% and 17.5/100000. Females: Proportion and ASR: 5.1% and 8.7/100000.

only way to stop the disease and ameliorate its effects. A registry of the different modalities for management for HCC is still lacking in Egypt, and will require a more systematized effort between different centers. A national campaign is crucial for early diagnosis and management.

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REVIEW

Moving forward in the treatment of cholangiocarcinoma

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Abstract

Despite being the second most frequent primary liver tumor in humans, early diagnosis and treatment of cholangiocarcinoma (CCA) are still unsatisfactory. In fact, survival after 5 years is expected in less than one fourth of patients diagnosed with this disease. Rare incidence, late appearance of symptoms and heterogeneous biology are all factors contributing to our limited knowledge of this cancer and determining its poor prognosis in the clinical setting. Several efforts have been made in the last decades in order to achieve an improved classification/understanding with regard to the diverse CCA forms. Location within the biliary tree has helped to distinguish between intrahepatic, perihilar and distal CCA types. Sequence analysis contributed to identifying several characteristic genetic aberrations in CCA that may also serve as possible targets for therapy. Novel findings are expected to significantly improve the management of this malignancy in the near future. In this changing scenario our review focuses on the current and future strategies for CCA treatment. Both systemic and surgical treatments are discussed in detail. The results of the main studies in this field are reported, together with the ongoing trials. The current findings suggest that an integrated multidisciplinary approach to this malignancy would be helpful to improve its outcome.

Key Words: Cholangiocarcinoma; Treatment; Genetic aberration; Immunotherapy; Liver resection; Liver transplantation

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Core Tip: Cholangiocarcinoma is a lethal malignancy characterized by a poor survival. In this review we discuss in detail the actual treatment and the future therapeutic perspectives for this cancer. Systemic and surgical strategies are reported with the corresponding results. Improved knowledge of this malignancy and a multidisciplinary therapeutic approach are likely to improve the cholangiocarcinoma outcome in the future.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a primary malignancy of the biliary system and represents the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC), constituting around 15% of primary liver tumors and 3% of gastrointestinal malignancies[1,2]. It is a rare tumor with a global incidence of 0.3-6 per 100000 inhabitants per year, displaying an increasing trend in the last decades[1]. However, in some Asian countries, such as Thailand, Cambodia and Laos, rates can be as high as 85 per 100000 due to infection with liver flukes[2].

Distinction into subgroups of CCA is anatomical: intrahepatic CCA (iCCA) arises in the liver above the second order bile ducts; perihilar CCA (pCCA), also known as Klatskin tumor, arises in the first order or main bile duct above the junction with the cystic duct; and distal CCA (dCCA) originates distally to the cystic duct (Figure 1). This classification is crucial as each subtype has distinct clinical characteristics and therapeutic strategies. pCCA accounts for the majority of diagnoses (50%-60%), with dCCA (20%-30%) and iCCA (10-20%) being less frequent[3]. iCCA can be further classified on the basis of the cells of origin as large and small duct types, with chronic biliary inflammation and chronic hepatis as risk factors, respectively[4]. On top of this, a recent interesting study involved the epigenomic and transcriptomic analysis of CCAs from 10 different countries in order to further understand and classify the genetic basis of CCA. The authors performed the analysis on CCA samples associated with liver flukes (mainly *Opisthorchis viverrine* and *Clonorchis sinensis*) and non-fluke cases. Four CCA clusters were likely driven by distinct etiologies, with separate genetic, epigenetic and clinical features found, highlighting how distinct cancer subtypes in the same organ may arise through different carcinogenic pathways[5].

Unfortunately, symptoms often appear when the disease is already advanced, resulting in a poor prognosis. In fact, this malignancy has an overall survival rate at 5 years of 5%-20%[1,3]. Nonetheless, many promising new approaches are currently under investigation.

Several issues have been encountered in the pursuit of a curative treatment for CCA in humans. Despite the evidence of different biological and epidemiological risk factors and genetic aberrations between diverse types of CCAs, these tumors are still frequently pooled together (also with gallbladder cancer) or misclassified in studies focusing on natural history or treatment[6,7]. On the other hand, histological classification (in particular for iCCA forms) remains suboptimal and also relies on heterogeneous genetic aberrations identified in this cancer[2]. The difficulties in CCA classification and in the comprehension of its biology therefore affect both clinical and basic research in this field. For instance, despite next generation models now attempting the construction of complex 3D CCA systems in culture (such as organoids or spheroids), an adequate reproduction of this tumor remains difficult in the preclinical experimental setting[8].

From the clinical side, CCA symptoms are generally not specific and share similarities with inflammatory diseases of the biliary tract. Moreover, general biomarkers used in medical practice, such as carbohydrate antigen 19-9 exhibit a sensitivity and specificity lower than 70%, underscoring the importance of the identification of possible novel genomic or proteomic biomarkers[9]. Also the appropriate surveillance of CCA-predisposing conditions, such as primary sclerosing cholangitis,

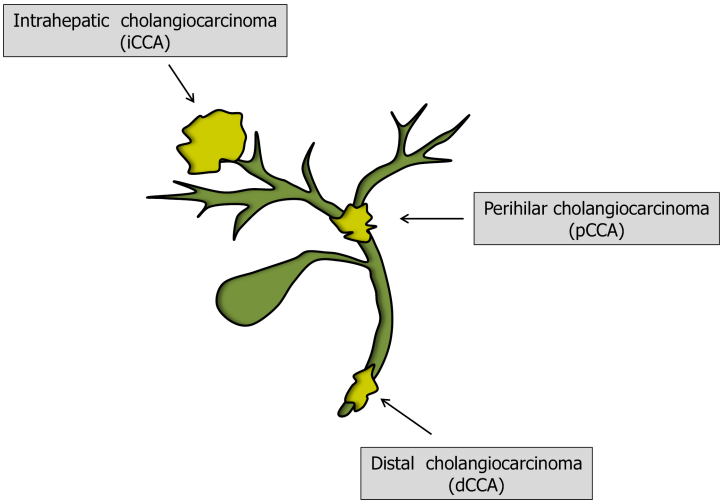

Figure 1 The anatomical location of intrahepatic, perihilar and distal cholangiocarcinoma is depicted.

remains undefined, leading to disappointing late-stage tumor identification in the majority of patients[10].

Furthermore, CCA remains an infrequent cancer in the majority of countries, several cases arise in the absence of recognized risk factors, and when some intraductal papillary or tubular forms are excluded[11], there is usually a short-term poor prognosis. Due to all of the above, clinical investigations and trials remain complicated and of partial impact. Framed in this perspective, this paper summarizes and critically reviews existing therapeutic strategies (both drug-based and surgical) for CCA and provides an overview of future perspectives in the treatment of this malignancy.

CCA PHARMACOLOGICAL TREATMENT: THE PRESENT

As described in detail in the dedicated paragraphs, the opportunity for a complete CCA cure should be offered in rare cases just employing surgical techniques. On the other hand, despite the fact that current drug therapy for this cancer is unsatisfactory, the pharmacological approach may present a larger margin of improvement in the future in comparison with operative methods.

Palliative treatments

At present, in subjects with unresectable, advanced disease, the best option is represented by cisplatin/gemcitabine first-line treatment. Confirmation of the utility of this treatment was obtained by a large study comparing this association with gemcitabine alone[12]. Two-hundred and four patients affected by biliary tumor (nearly one third with gallbladder cancer) treated with the gemcitabine/cisplatin regimen had an increased tumor response (81.4% *vs* 71.8%, $P = 0.049$) and median survival (11.7 *vs* 8.1 mo, $P < 0.001$) in comparison with a similar group treated with gemcitabine alone. In the absence of an adequate second-line treatment, a recent Phase 3, open-label, randomized trial (ABC 06) was published on patients with CCA progression under gemcitabine/cisplatin comparing folinic acid/fluorouracil/oxaliplatin therapy to active symptom control[13]. Overall survival was longer in the folinic acid/fluorouracil/oxaliplatin group (6.2 *vs* 5.3 mo, $P = 0.03$), thus demonstrating the possible feasibility of second level therapy and possibly changing our clinical approach to these patients in the near future.

Adjuvant treatments

With regard to adjuvant therapy in subjects amenable to surgical resection, the major indication came from the BILCAP trial[14]. In this study, patients undergoing surgical treatment of biliary cancer ($n = 447$) were allocated to receive capecitabine or just observation after a macroscopically complete tumor resection. Capecitabine increased survival by almost one third. This difference was statistically significant in the per-protocol (53 mo *vs* 36 mo, $P = 0.02$) but not in the intention-to-treat analysis. Serious adverse events occurred in the two groups at a similar rate. A randomized Phase 3 clinical trial conducted with adjuvant gemcitabine chemotherapy did not show

significant improvement in overall survival or relapse-free survival in comparison with untreated control[15]. An attempt was also conducted with adjuvant gemcitabine/oxaliplatin in the PRODIGE 12 study[16], and again no improvements were observed in comparison with supportive care.

In conclusion, excluding the modest, above-described, therapeutic options, physicians and patients are lacking any further pharmacological strategy. Also, radiation therapy gave inconclusive results in this setting[17], meaning that current national guidelines are not able to give an unequivocal indication on this approach[18]. In conclusion, the scarce results of systemic therapy have prompted extensive research in recent decades in order to find a more satisfactory pharmacological approach for this cancer. Current preclinical models and results together with ongoing trials are reported in the following paragraph.

CCA PHARMACOLOGICAL TREATMENT: THE FUTURE

The possible evolution of systemic therapy for CCA is largely dependent on the resolution of some issues with regard to this cancer[19]. First, scientists are still searching for an appropriate preclinical model of CCA[20]. CCA cell culture and tumor xenotransplantation in nude mice are the most commonly used strategies, but they do not adequately reproduce the neoplastic microenvironment[21]. From the clinical experimental side, the rarity of this neoplasm and competition between new molecules do not facilitate the performance of trials with an adequate number and homogeneous type of CCAs. While exploring this undefined horizon, research efforts are oriented in some main fronts, as reported in the following subparagraphs.

Trying to overcome chemoresistance

One of the main issues greatly limiting chemotherapy effectiveness in CCA is represented by chemoresistance[22]. Chemoresistance describes the capacity of cancer cells to escape or attenuate therapeutic drug effects[23]. Several mechanisms have been identified as the basis of chemoresistance, some opposing drug uptake or increasing its extracellular export and others reducing cellular necrosis/apoptosis or stimulating tumoral phenotypic changes. For instance, the reduced expression of organic cation transporter 1, as observed in both CCA and HCC, has been related to a poor response to tyrosine kinase inhibitors such as sorafenib[24]. On the other hand, the phenotypic CCA evolution from an epithelial to a mesenchymal trait (so-called epithelial-mesenchymal-transition) not only counteracts chemotherapy effects but also seems to favor metastatic progression[25]. Several strategies have been attempted in preclinical experimental studies to improve therapeutic response to chemotherapy, such as drug transporter induction or export pump inhibition in CCA cells or targeting cells with specific organic molecules such as bile acids or vesicles. With regard to human trials, a gemcitabine analogue (NUC-1031)[26] not requiring nucleoside cellular transport or intracellular kinase activation is currently being tested in a Phase 3 trial (NCT 04163900).

Targeting genetic aberrations

Several genetic aberrations have been identified in CCA, with a different distribution among intrahepatic, perihilar or distal CCA[27]. Kirsten rat sarcoma gene mutations are frequently encountered, ranging from 9%-40% of cases according to CCA location within the biliary tract[28]. A specific molecule (AMG 510) targeting the Kirsten rat sarcoma/G12C mutation is currently being tested in a Phase1/2 trial (NCT03600883); however, downstream pathway suppression, obtained by kinase inhibition (such as those of the Raf or MEK family) also may be attempted. In this perspective, the dual suppression of BRAF and MEK, obtained with dabrafenib and trametinib, gave excellent results in anecdotal cases[29], thus stimulating the Phase 2 ROAR study in patients with the BRAFV600E solid tumor mutation (NCT02034110). In an interim analysis of this trial of 43 patients with biliary tract cancer, the overall response rate (after external data review) accounted for 20% of cases[30].

The fibroblast growth factor (FGF) family comprises a group of proteins that react with their specific receptors (FGFRs) to stimulate several developmental and proliferative processes, also involving stem cell differentiation[31]. FGFR (subtype 2) genetic alterations, characterized by fusion with other genes, have been observed in nearly 15% of iCCAs, so FGF/FGFR signaling has emerged as a possible target to cure this cancer[32]. Among the FGFR inhibitors, infigratinib and pemigatinib have been evaluated in Phase 2 trials (NCT02150967 and NCT02924376, respectively) on advanced

iCCA harboring FGFR aberrations[33,34]. Progression-free survival was slightly better with pemigatinib, accounting for 62% at 6 mo for patients with an FGFR2 mutation. On the basis of these results, this drug was approved by the FDA in April 2020 for the treatment of advanced iCCA harboring this genetic aberration. Other molecules, such as derazantinib, futibatinib and Debio 1347, have been registered for evaluation in clinical trials (NCT03230318, NCT04093362 and NCT03834220), but the results are not yet available.

Since isocitrate dehydrogenase 1 mutations have been identified in approximately 13% of iCCA and 0.8% of other CCAs and the impairment of this enzyme may lead to the accumulation of the pro-oncogenic metabolite D-2-hydroxyglutarate, isocitrate dehydrogenase 1 inhibitors have been suggested for treatment of this cancer. The ClarIDHy phase 3 trial tested the isocitrate dehydrogenase 1 inhibitor ivosidenib in CCAs with a mutation of this enzyme and refractory to previous systemic therapy[35]. Six-month progression-free survival was 32% in the ivosidenib group in comparison with 0% in the placebo group. Other inhibitors are currently being examined in different trials, as summarized in a recent review on this issue[36]. Other genetic aberrations, such as those involving the ERRB family and proto-oncogene tyrosine-protein kinase 1, represent possible targets for CCA therapy; some drugs are under evaluation[37].

Immune checkpoint targeting

The activation of immune checkpoint (IC) pathways seems to be involved, under normal conditions, in tolerance and the prevention of autoimmune diseases[38]; however, tumor-mediated stimulation, hindering immune surveillance, may favor cancer proliferation and spread[39]. In this perspective, IC inhibitors have recently gained major importance with regard to cancer therapy, achieving a complete response in 20% of melanoma patients[40]. Among diverse IC pathways, the cytotoxic T-lymphocyte antigen 4 and programmed cell death protein 1/programmed cell death protein ligand 1 are those that are mainly recognized and targeted in oncology. In another study, 22 patients harboring CCA characterized by microsatellite instability and mismatch repair reduced protein (findings related to IC upregulation) were treated with the programmed cell death protein ligand 1 inhibitor pembrolizumab, obtaining a median progression free survival of 4.2 mo and a median overall survival of 24.3 mo[41]. However, these results might be improved with careful patient selection since an increased response has been observed as a function of programmed cell death protein ligand 1 expression[42]. Several trials with IC inhibitors alone or in combination and including CCA patients are ongoing.

Newly identified pathways as possible targets for therapy

The neuroendocrine regulation of CCA expansion (as shown by preclinical experimental studies) might be an important factor to consider while searching for a therapy for this cancer[43]. Secretin, somatostatin and melatonin have all been demonstrated to decrease CCA growth, as observed in cancer cell lines or in animal models such as tumor xenotransplantation in nude mice[44-46]. At present, however, no clinical data are available with either secretin or melatonin for CCA treatment, while a trial with somatostatin gave negative results[47]. Also, angiogenic factors such as vascular endothelial growth factor are considered possible targets for CCA therapy. Vascular endothelial growth factor in fact seems to be increased in half of human biliary tract cancers[48]. A trial using the anti- vascular endothelial growth factor antibody bevacizumab, in association with standard chemotherapy (gemcitabine, oxaliplatin), however, gave modest results[49].

SURGICAL TREATMENT: THE PRESENT

Surgery remains the best treatment option for long-term patient survival in CCA, and it is recommended to undertake surgical treatments in highly specialized centers to minimize morbidity and mortality[50].

Preoperative considerations

Preoperative workup and biliary drainage have been widely discussed in recent decades. The current consensus is that preoperative biliary drainage is required in cases of concomitant cholangitis, need for neoadjuvant therapy, malnutrition, hepatic or renal failure and need for portal vein embolization (PVE)[1]. When jaundice is the only indication, need for decompression is still a matter of debate. Asian guidelines

recommend preoperative drainage because of the higher risk of patients with cholangitis[51,52]. Furthermore, drainage may help restore liver function, decreasing the chance of postoperative liver failure[52]. On the other hand some studies have shown that, while biliary drainage is beneficial by reducing morbidity and mortality in patients with small future liver remnant (FLR), it is equally detrimental when FLR is large enough[53,54]. In Western countries, many centers prefer to use selective biliary drainage when FLR is less than 30%-40%[55]. When stenting is required, both endoscopic and percutaneous methods are used. Percutaneous transhepatic biliary drainage has some advantages, such as reducing the need for re-intervention, reducing the time to achieving a therapeutic effect and fewer procedural risks. However, a recent randomized trial of percutaneous *vs* endoscopic stenting was terminated early due to excess mortality in the percutaneous group (41% *vs* 11%), mandating further prospective studies and a reconsideration of drainage strategies[55]. Alternatively, nasobiliary drainage may be a valid option, showing good success rates and low morbidity despite greater patient discomfort[56,57]. The optimal timing of surgery in drained patients is currently unknown. A recent study identified a preoperative bilirubin level of < 75 μmol/L (2.9 mg/dL) to be correlated with fewer complications, less mortality and longer 5-year overall survival[58].

Surgical considerations for iCCA

Patients are considered eligible for surgery whenever complete resection of the tumor with negative margins (R0) can be achieved, providing sufficient FLR. Bilateral multifocal or multicentric disease is associated in many studies to a significantly shorter overall survival (OS)[59,60]. In practice, only 32% of iCCAs satisfy resectability criteria at presentation. On top of this, around 30% of iCCAs will be deemed inoperable on the operating table. Staging laparoscopy can detect unresectable disease in around 36% of patients with minimal costs[61] and is advocated by current guidelines[62].

Principles: The established principles of surgery for iCCA are to achieve R0 resections and to provide adequate staging with hilar lymphadenectomy, sparing at the same time as much parenchyma as possible to avoid post-hepatectomy liver failure. Margin status is the primary objective in iCCA surgery. Evidence mainly derives from large single center and multicenter studies, which have demonstrated a significant survival impact of R0 resection. Overall survival at 5 years for R0, R1 and R2 resections are reported to be 28.7%, 13.9% and 0%, respectively[63], with an increased survival benefit for > 5 mm margins[64].

Lymphadenectomy and nodal disease: Nodal disease is recognized as the most important prognostic factor in most studies[59,63-66]. In fact, some authors have reported that margin status may have limited impact in the presence of nodal metastases[64]. Most guidelines suggest routine consideration of regional lymphadenectomy and a minimum of six lymph nodes are needed for accurate staging[2,62,67]. Nonetheless, the role of lymphadenectomy remains controversial in Western countries, where the practice is not widespread, and almost 50% of patients have no lymph nodes examined[68]. Regional lymph nodes include cystic, bile duct, hepatic artery and portal vein. Right and left hemi-livers have distinct lymphatic drainage: for right liver iCCA, the retropancreatic nodes along the common bile duct are considered regional nodes and should be removed, while for left liver iCCAs, the same considerations are true for the lesser curvature and inferior phrenic nodes. Lymph nodes may be positive in as many as 30% of cases, but with current adjuvant therapy, survival is acceptable, and this should not refrain the surgeon from resection[68]. On the contrary, distant nodes such as celiac, superior mesenteric, paraaortic or caval should be considered as distant metastatic disease and contraindicate extensive surgery as patients are unlikely to gain any benefit[2,62,67].

Extended procedures: Given the poor prognosis (0% 5-year OS) of unresectable disease or R2 resection[63,65], in recent years, some groups have explored the benefits of major vascular resections to obtain R0 resection, resulting in up to 84% of patients [66] with morbidity and mortality rates comparable to standard resection[69]. Overall survival of these patients is also comparable to patients who did not undergo vascular resection[66,69,70]. In general, all patients with localized iCCA should be considered for resection even if this implies major hepatectomy or vascular resection[62]. In recent decades, based on the principles of liver regeneration, some authors have pushed the boundaries for resectability in liver surgery by introducing the concept of two-stage hepatectomies, namely portal vein ligation and PVE. The latter can enhance the resectability rates of liver tumors, allowing extensive resection with adequate FLR and are

usually well-tolerated by the patient. However, a major drawback is the long waiting time for the second stage procedure, which can take up to several weeks, carrying the risk of tumor progression. To solve these problems, a German group of authors developed a new technique, known as associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), which was found to allow rapid growth of the FLR, with a median period of 9 d[71]. Another study investigated benchmark outcomes in ALPPS, demonstrating that it has a comparable standard outcome as other types of major liver surgery[72]. ALPPS for iCCA has been evaluated in an international multicenter study in which 102 patients underwent first-stage ALPPS; 99 completed the second procedure, and R0 resection was obtained in 85% of cases with 29% major morbidity and 7% mortality[60]. When disease is considered unresectable, neoadjuvant chemotherapy can convert as many as 53% of cases to secondary resectable disease[73].

Recurrent disease: Recurrence of iCCA is frequent. Most recurrences are intrahepatic and therefore potentially amenable to re-resection[74], with satisfactory outcomes when repeated resections are undertaken. These results lead to the recommendation that the same principles for resectability should be applied in consideration of primary and secondary resection[75].

Surgical considerations for pCCA

pCCA represents a surgical challenge due to its intrinsic anatomical location. Nonetheless, its higher prevalence (50% of CCAs) has translated into more extensive literature and pioneering advances in surgical treatment.

In pCCA, the main criteria that define surgical unresectability are inadequate FLR, absence of a suitable field for biliary reconstruction (*i.e.* bilateral segmental ductal extension) and major vascular infiltration[51]. Growth of FLR may be induced with two-stage hepatectomy techniques, broadening indications for resection. Nonetheless, 20%-50% of patients are deemed to be unresectable upon surgical exploration, making explorative laparoscopy a useful tool to avoid unnecessary laparotomies.

Principles: Surgery for pCCA routinely involves en bloc hemi-hepatectomy and bile duct resection to achieve negative biliary and parenchymal margins, with additional resection of the caudate lobe, regional lymphadenectomy[51,76] and biliary reconstruction. Negative margins are paramount. The caudate lobe usually drains directly into the biliary confluence, hence the necessity of its resection for curative intent is advised by current guidelines as it improves OS[51,77]. A number of studies have demonstrated that intraoperative additional resection to achieve R0 confers a significant survival advantage with few complications and a prognosis comparable to primary R0[78,79]. In particular, aggressive approaches such as pancreaticoduodenectomy seem to offer improved results[78]. Lately, a new concept of isolated circumferential margin has been introduced for pCCA. Stremitzer *et al*[80] identified a group of patients who, despite being classified as R1, did not have distal or proximal margin positivity but only focal circumferential positivity. These patients had better survival than those with surgical resection margin positivity, although inferior when compared to their R0 counterparts. Finally, a recent study has challenged these surgical dogmas, arguing that with current adjuvant therapies R1 patients may have similar survival to R0[77].

Lymphadenectomy and nodal disease: European guidelines affirm that lymphadenectomy should be considered the standard of care, but there is no consensus on the extent of lymphadenectomy for pCCA[81]. A recent systematic review identified a minimum of seven lymph nodes to convey sufficient information avoiding understaging, with no benefit coming from higher lymph node counts (≥ 15) which could only be achieved with extended lymphadenectomy[82]. The regional nodes for pCCA are cystic, biliary, hepatic artery, portal vein and retropancreatic. The impact of extended lymphadenectomy of N2 nodes (dissection of celiac, superior mesenteric and paraaortic nodes) on survival has not been established, but trials are ongoing[83,84]. For known N2 positive disease, current expert consensus suggests no benefit of resection[2].

Extended procedures: Extended resections have been explored for pCCA, including two-stage hepatectomies such as portal vein ligation/PVE and ALPPS with acceptable outcomes. The first reports of 29 ALPPS procedures for this indication featured a strikingly high mortality rate, although statistically comparable to results of 29 matched patients who underwent non-ALPPS resection[85]. These poor initial results

have dramatically improved for most ALPPS indications with better patient selection and inter-stage management and will hopefully improve for hCCA as well[86]. As of 2020, ALPPS should only be considered in highly experienced institutions. Hepatopancreaticoduodenectomy entails resection of the entire extrahepatic biliary tree, thus necessitating resection of the pancreatic head and duodenum. It is used for tumors with concomitant distal bile duct spread. This procedure is associated with high major morbidity rates of up to 37%. Nonetheless, the latest reports from highly specialized centers have been encouraging and suggest that hepatopancreaticoduodenectomy could be considered in young, fit patients when it represents the only chance of a cure [87]. Vascular resection can be adopted to increase R0 rates. Long-term oncological results are in the range of 25%-45%[88,89].

Surgical considerations for dCCA

dCCA affects the third portion of the extrahepatic biliary duct, which lies in a retro/intra pancreatic position. This particular anatomical configuration translates into a completely different surgical approach compared to iCCA and pCCA. In particular, resection involves pancreaticoduodenectomy, as for cancer of the pancreatic head. Negative margin status is imperative, as positive margins increase anastomotic recurrence rates and herald poor survival. An aggressive approach is justified in cases with vascular infiltration. Resection of the superior mesenteric or portal vein and reconstruction to obtain R0 obtains survival comparable to patients without vascular resection with no additional morbidity and mortality[90]. Data on arterial resection is more limited[91]. Specific for dCCA is the need to resect the bile duct high in the liver hilum as well as a lymphadenectomy of the porta hepatis and gastroduodenal ligament[76]. Unfortunately, dCCA diagnosis is not always defined preoperatively, and these steps may be omitted, increasing the chance of R1 if the tumor has prominent intraductal spread.

SURGICAL TREATMENT: FUTURE PERSPECTIVES

Progress in the field of surgery for CCA has been limited for many years, yet the coming decade harbors great promise, with numerous innovations on the horizon. The main ongoing surgical trials are reported in Table 1.

Preoperative care

Resectability for CCA is limited mainly by inadequate FLR, especially when extensive resections are required. Portal vein ligation, PVE and ALPPS are compelling procedures for enhancing resectability with adequate FLR. On top of this, recently Guiu *et al*[92] described an interesting new technique, named liver venous deprivation, which involves PVE with simultaneous embolization of one or two hepatic veins. In a subsequent study, the same group demonstrated that liver venous deprivation permits a significantly greater increase in both FLR volume and function compared to PVE [93]. A randomized trial is ongoing with the aim of establishing the superiority of this technique (NCT03841305). Liver venous deprivation could represent an important advancement in liver surgery, combining the low morbidity of PVE with the greater efficacy and rapidity of ALPPS.

Minimally invasive surgery and enhanced recovery protocols

Minimally invasive approaches have developed slowly in liver surgery. Few studies specifically address the use of minimally invasive surgery for CCA with comparable outcomes, although no benefit has been clearly demonstrated so far[94]. For pCCA, the literature is discordant, but nevertheless it is possible that it could develop further in the near future[94].

Liver transplantation for unresectable CCA

Liver transplantation (LT) for hCCA has been investigated for many years, but the practice was abandoned due to very poor results compared to other indications, in the setting of the ongoing organ shortage. Initial experiences featured 5-year OS survival rates of 23%-38%, mainly due to early recurrence[95].

In the early 2000s, the idea of LT for unresectable iCCA changed thanks to the work of Vreede *et al*[96] at the Mayo Clinic. They developed a very rigorous protocol to optimize the selection of patients who were most likely to benefit from LT. In particular, patients with a diagnosis of unresectable, non-metastatic hCCA were

Table 1 Surgical ongoing trials for cholangiocarcinoma

CCA Type	Domain	Trial name	Summary
iCCA/pCCA	Hepatic venous deprivation	NCT03841305	Randomized trial of portal vein embolization *vs* hepatic venous deprivation. Primary endpoint: future liver remnant at 3 wk
iCCA	Liver transplantation	NCT02878473	Liver transplantation for early (< 3 cm) iCCA. Single group assignment
iCCA	Liver transplantation	NCT04556214	Liver transplantation for stable (> 6 mo), advanced (unresectable) iCCA. Single group assignment
iCCA	Liver transplantation	NCT04195503	Liver transplantation for stable (> 6 mo), advanced (unresectable) iCCA. Single group assignment
pCCA	Lymphadenectomy	ChiCTR1800015688	Randomized trial of extended *vs* regional lymphadenectomy for resectable pCCA. Primary endpoint: overall survival
pCCA	Liver transplantation	NCT02232932	Randomized trial of liver transplantation *vs* resection for resectable pCCA (< 3 cm). Primary endpoint: overall survival at 5 yr

iCCA: Intrahepatic cholangiocarcinoma; pCCA: Perihilar cholangiocarcinoma.

treated with external beam radiotherapy (4500 cGy in 30 fractions) with concomitant intravenous 5 fluorouracil followed 3 wk later by transcatheter brachytherapy with an iridium-193 wire and finally maintenance oral capecitabine (as tolerated) until transplantation. Before LT, patients underwent staging laparotomy to exclude any intra-abdominal disease, including distant lymph node sampling. With this protocol, they reported a 5-year survival of 82% for patients undergoing LT[97]. Of note, almost half of the patients who enrolled in the protocol were not transplanted due to death or disease progression. Surgical exploration resulted in findings that precluded transplantation in 23% of cases.

Sahai *et al*[98] reported similar efficacy with a different neoadjuvant protocol consisting of higher brachytherapy doses and the omission of external beam radiotherapy. These successful experiences have been replicated in other studies[99-101]. Other studies investigated risk factors for drop-out or recurrence. Elevation of carbohydrate antigen 19-9 above 500 U/mL, a mass larger than 3 cm and Model End-stage Liver Disease score above 20 points predicted protocol drop-out before LT[102]. On the other hand, predictors for recurrence were elevated carbohydrate antigen 19-9, portal vein encasement and incomplete response to neoadjuvant therapy defined as residual tumor on the hepatectomy specimen as well as pathologic stage and perineural and perivascular invasion[99,100,102,103].

Notably, patients with hCCA developing in the setting of primary sclerosing cholangitis had a significantly better outlook than sporadic hCCA[104]. Given the technical and management complexity of this surgery, outcomes are influenced by center experience, with centers having performed at least six procedures providing the best results[104,105]. These experiences have led neoadjuvant therapy followed by LT to become the current standard of care for locally advanced non-metastatic unresectable hCCA, with both cadaveric and living donor programs active in highly specialized centers worldwide.

To date, iCCA is generally considered a contraindication to LT due to poor results in initial experiences[106]. Vilchez analyzed 440 patients with iCCA from the UNOS database and reported a significantly reduced OS with respect to HCC patients undergoing LT[107]. Yet, it may not be correct to generalize these poor results as analysis of the National Cancer Data Base revealed that only 2.2% of patients with iCCA underwent LT[107]. Furthermore, none of the studies cited so far have investigated the benefits of preoperative neoadjuvant therapy. The success and implementation of LT programs for pCCA compels consideration of this strategy for iCCA. Lunsford *et al*[108] in 2018 first reported results of their single center LT program for iCCA involving neoadjuvant chemotherapy[108]. Twelve patients were enrolled in the program, and six were transplanted. OS and disease-free survival were 83% and 50% at 5 years, respectively. Two large randomized trials are currently evaluating this approach (NCT04556214 and NCT04195503).

Whether mixed HCC-iCCA should be considered for LT is also debated. The literature is conflicting, with some studies reporting outcomes similar to HCC and others to iCCA[107,109].

Table 2 The new systemic, surgical and combined approaches to cholangiocarcinoma

	Approaches
Systemic therapy	(1) Overcoming chemoresistance; (2) Genetic aberration targeted therapy; (3) Immune checkpoint inhibitors; and (4) Neuroendocrine modulation of cancer growth
Surgical therapy	(1) Liver venous deprivation; (2) Minimally invasive surgery; and (3) Liver transplantation
Combined therapy	Liver transplantation or surgical resection after radiotherapy and/or neoadjuvant treatment

Liver transplantation for resectable CCA

Successful results of LT after neoadjuvant therapy have induced investigators to compare them with conventional resection for resectable hCCA. Rea *et al*[97] reported a significantly improved OS at 5 years for patients undergoing LT compared to those undergoing resection. Ethun *et al*[110] showed similar results in an intention-to-treat analysis as well. They also went further and analyzed results for a subgroup of patients that were selected to be more comparable to patients in the resection group (*i.e.* hCCA not associated with primary sclerosing cholangitis, < 3 cm and lymph node negative). Even in this case, results were significantly better in the LT group. The authors suggest that the available data should prompt consideration of LT for hCCA patients with resectable disease. Indeed, this may be the new frontier in hCCA surgery. Nonetheless, some obstacles remain before the implementation of this strategy becomes widespread, the main one being the scarcity of allograft availability. In fact, critics of this approach argue that the benefit of LT (14% 5-year survival increase) is too little compared to the minimum benefit commonly applied to LT (50% at 5 years) and does not justify use of a deceased or living donor allograft. Better identification of patients who would benefit most from LT (*e.g.*, patients who are less likely to undergo an R0 resection) could maximize the benefit and justify an LT program. In any case, a randomized trial is currently ongoing (NCT02232932).

Regarding resectable iCCA, Facciuto *et al*[111] recently published a small series of patients transplanted for HCC or iCCA. Their analysis showed that when iCCA features were within the Milan Criteria survival was comparable to that achieved for HCC. Further insights have come in recent years. Sapisochin *et al*[109] reported that the subgroup of patients transplanted for small iCCA (< 2 cm) had similar survival to HCC. In two subsequent studies, these results were confirmed with OS being significantly different between small (< 2 cm) and large tumors (> 2 cm)[71,112], 65%-73% *vs* 40%-45% respectively. Trials of LT for small iCCA are currently ongoing (NCT02878473).

Neoadjuvant therapy for resectable CCA

Experience with neoadjuvant therapy followed by LT has shown that disease can be stabilized in more than 50% of patients and that 57% of patients who ultimately undergo LT benefit from a complete response[97,103]. While LT seems to offer superior survival compared to resection, it is unknown to what extent neoadjuvant therapy or strict selection criteria contribute to the effect[113]. Neoadjuvant therapy may therefore prove useful in cases of resectable disease as well to increase chances of R0 resection. Consideration should be given to the risk of disease progression and loss of chance of resection. To date, there is little data available on this possible approach [114].

CONCLUSION

Poor CCA prognosis requires important therapeutic improvements in the next few years. Table 2 summarizes the new approaches in CCA therapy. Several attempts are being made or hypothesized at present, as described above in this review, with regard to systemic and/or surgical treatment for this cancer. The heterogeneity and rare occurrence of this tumor, however, impede the design of large trials with homogeneous patients. An increased understanding of the genetic changes occurring in CCA and the institution of collaborative international studies may improve this picture. The results of these efforts would be the possible definition of a model integrating different resources (diagnostic, radiological, surgical and chemotherapeutic) in order to achieve an early diagnosis and the best outcome, according to patient and tumor hallmarks. This integrated model should be implemented over time,

maintaining a strict relationship with new findings on CCA in order to adopt best practice for this lethal cancer.

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REVIEW

Solid extraintestinal malignancies in patients with inflammatory bowel disease

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Abstract

Malignancies constitute the second cause of death in patients with inflammatory bowel diseases (IBD), after cardiovascular diseases. Although it has been postulated that IBD patients are at greater risk of colorectal cancer compared to the general population, lately there has been evidence supporting that this risk is diminishing over time as a result of better surveillance, while the incidence of extraintestinal cancers (EICs) is increasing. This could be attributed either to systemic inflammation caused by IBD or to long-lasting immunosuppression due to IBD treatments. It seems that the overall risk of EICs is higher for Crohn's disease patients and it is mainly driven by skin cancers, and liver-biliary cancers in patients with IBD and primary sclerosing cholangitis. The aims of this review were first to evaluate the prevalence, characteristics, and risk factors of EICs in patients with IBD and second to raise awareness regarding a proper surveillance program resulting in early diagnosis, better prognosis and survival, especially in the era of new IBD treatments that are on the way.

Key Words: Extraintestinal malignancies; Crohn's disease; Ulcerative colitis; Thiopurines; Anti-tumor necrosis factor

Core Tip: Patients with inflammatory bowel disease (IBD) are at risk of malignancies. The incidence of colorectal cancer is decreasing over time as a result of screening surveillance and better endoscopic techniques. Although IBD patients are not at high risk of overall extraintestinal malignancies compared to the general population, they

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have an increased risk of certain cancers. This should be kept in mind in the management of IBD patients especially in the context of immunosuppressive treatment that constitutes the cornerstone of treatment. Guidelines should incorporate all the preventive measures and be applied to everyday clinical practice for early diagnosis and better prognosis.

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INTRODUCTION

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are chronic diseases of unknown etiology. It has been proposed that in genetically predisposed patients, several environmental factors and altered gut microbiota interact, resulting in immune system dysregulation and finally to chronic intestinal inflammation[1]. IBD mainly affects the gastrointestinal tract but not exclusively, as 50% of patients experience at least one extraintestinal manifestation. In Western countries, IBD is not uncommon as the prevalence of CD is 246.7/100000 and UC is 286.3/100000[2].

Cancer is the second cause of death in IBD patients, after cardiovascular diseases[3] and 30% of IBD patients are diagnosed with malignancy at some point during the course of IBD[4]. A multicenter European study showed that the prevalence of cancer in IBD patients, during a 15-year follow-up period, was 9.1%, the majority of them were extraintestinal cancers (EICs) and the overall cancer frequency was equal to the background population[5].

However, it was recognized that patients with IBD are at increased risk of certain EICs and was also shown in other reports[6,7].

The risk of colorectal cancer (CRC) in IBD patients is 1.7-fold higher than that in the general population[3]. Lately, a reduction in the incidence of CRC in IBD has been reported that could be attributed not only to successful inflammation control due to new IBD treatments, but also to better surveillance strategies, colonoscopy techniques and implementation of guidelines supporting colectomy for high-grade dysplasia[3,4,8]. On the other hand, there is evidence that IBD patients have a higher risk of certain EICs[9-11]. EICs in IBD patients could be related either to chronic inflammation or to longstanding immunosuppressive treatment. As the incidence of EICs is increasing among IBD patients, awareness should be raised for more scrupulous surveillance for early diagnosis and curative treatment.

The aim of this review was to evaluate the prevalence, characteristics, and risk factors of EICs in patients with IBD, and to provide possible measures for prevention and early diagnosis.

METHODOLOGY

A review of the literature in PubMed using the MESH terms Inflammatory Bowel Disease AND neoplasms NOT colorectal cancer NOT intestinal neoplasms was conducted. Only studies in English language involving humans were included.

HEAD AND NECK CANCERS

Head and neck cancers (HNCs) include mainly squamous cancers of the oral cavity (OSCC), oropharynx (OPSCC), nasopharynx, hypopharynx and larynx (LSCC). Approximately 75% of HNCs are caused by alcohol and tobacco use and the remaining are mainly human papilloma virus (HPV) 16 related (HPV + HNCs)[12-17].

Only a few studies on HNCs and IBD have been published[18-21]. Danish cohorts showed no increased risk for OSCC and OPSCC[22,23], while a recent US retrospective cohort by Katsanos *et al*[18] showed for the first time an increased risk for OSCC [standardized incidence ratio (SIR) 9.77 (95% confidence interval (CI): 5.14-16.98)] in IBD patients. The risk was more pronounced in females (12-fold increase) compared to males (8-fold increase) and for tongue cancer (22-fold and 17-fold increase in females and males, respectively). A population-based cohort study by Mosher *et al*[24] also showed an increased 20-year risk of OSCC and OPSCC [20 yr relative risk (RR) 2.00 (0.95-4.22)] but not of LSCC [20 yr RR 0.48 (0.07-3.43)]. Indeed, a retrospective case-control Dutch study showed that IBD patients had a higher rate of HPV + HNCs (52.2%) compared to the general population and an impaired OSCC survival (P = 0.018), not related to immunosuppression[19]. Older age and UC diagnosis were risk factors for OSCC and OPSCC. A recent Dutch study showed that older age at IBD diagnosis (P < 0.001) as well as male sex (P < 0.001) were risk factors for LSCC in UC patients. On the other hand, tobacco use (P < 0.001), structuring (P = 0.006) and penetrating (P = 0.008) disease were identified as risk factors in CD patients who developed LSCC, while immunosuppressive medication did not influence survival [20]. Currently, studies on the prevalence of HNCs, risk factors and outcome in IBD patients are limited. Education on modifiable risk factors, such as smoking cessation, and safe sexual behavior, is the cornerstone of prevention of HNCs. In addition, given that IBD patients may have reduced immunosurveillance resulting in persisting HPV infections and cancer, oral screening and prophylactic HPV vaccines could be considered.

THYROID CANCER

Thyroid cancer (TC) is the most common endocrine cancer, and is more common in Caucasians, and young adults, predominantly females. Thyroid dysfunction, radiation exposure at a young age and heredity are known risk factors. Over 80% of all cases are of the papillary type[25].

A recent prospective multicenter Italian study showed that the prevalence of TC in IBD patients was 5.2%[26]. Several studies, have shown that TC risk was not significantly higher in IBD patients compared with the healthy population[27-29]. On the other hand, two studies reported an increased risk of TC in UC patients, the first in both sexes and the latter only in males[30,31]. A recent meta-analysis of 8 case-control studies with 334015 patients, confirmed that patients with UC had an increased risk of TC, while patients with CD did not, independent of sex and race[32]. On the contrary, a case-control study in Ohio, United States with 289935 IBD patients[33] and an Italian cohort study of 3664 IBD patients[9], reported a higher risk for TC only in CD patients [odds ratio (OR) 2.3 (1.06-5.1), P = 0.034 and SIR 5.58 (95%CI: 2.41-11.00), respectively].

Regarding the risk of immunosuppressive treatment for the development of TC in IBD, data are conflicting, with two studies reporting an increased risk in patients treated with immunosuppressants[29,31] and another two studies did not observe this relationship[28,32].

Most of the evidence is based on population-based studies[9,30-32] with many potential confounding factors, and limited studies are focused especially on the risk of TC in IBD patients[29,32,33]. There is not enough evidence to show that IBD is an independent risk factor for TC, but IBD might provide an appropriate inflammatory environment and promote development of the cancer. Further well-adjusted and population-based studies should be conducted to confirm this speculation, in order that screening strategies for TC can be recommended.

LUNG CANCER

Lung cancer is one of the most common cancers and the major cause of mortality worldwide. Several population-based studies[10,34-36] and a recent meta-analysis by Lo in 2020 showed an increased risk [incidence rate ratio (IRR), 1.53 (95%CI: 1.23-1.91), P = 0.00][37] of lung cancer in CD patients. Masala *et al*[38] also reported increased mortality only in CD patients [SMR 4.00 (95%CI: 1.60-8.24)]. On the contrary, in UC patients no significant risk was found in any study[31,34,36,37,39] and actually a significantly lower risk of lung cancers was reported in two studies[29,40]. The difference between CD and UC could be explained by the different association of the two diseases with smoking. In conclusion, further studies focused on lung cancer are

needed and efforts should be made to guide patients to quit smoking, an important risk factor for both lung cancer and CD.

CHOLANGIOCARCINOMA

Cholangiocarcinoma (CCA) is a relatively rare EIC that mainly affects IBD patients in the context of primary sclerosing cholangitis (PSC)[41]. PSC is a chronic inflammatory, immune mediated, liver disease causing fibrosis of the intrahepatic and extrahepatic bile ducts finally leading to strictures[42]. The mechanisms of carcinogenesis in PSC are not well understood but it seems that CCA is the result of DNA damage caused by chronic biliary inflammation and bile acids in IBD patients with altered DNA repair ability[39].

Several studies have shown that IBD patients have an increased risk of biliary cancer (Table 1). A meta-analysis that included 17052 IBD patients showed that CD patients had a borderline significant increased risk of liver-biliary cancer (SIR 2.47, 95%CI: 0.95-6.46), while UC patients are at significantly increased risk (SIR 2.58, 95%CI: 1.58-4.22) and this is attributed to the occurrence of PSC in patients with IBD [10]. The incidence of CCA in PSC patients ranges between 0.5-1.5/100 person-years [42,45,46]. The risk of CCA is increased by 160-fold and PSC patients have a cumulative life-time risk of 5%-10%[44,47]. Approximately 30%-50% of CCAs are diagnosed within the first year of PSC diagnosis[45,46,48]. In another meta-analysis, it was shown that IBD patients were at increased risk of CCA (RR 2.63; 95%CI: 1.47-4.72, CD 2.69, 95%CI: 1.59-4.55 and UC 3.40, 95%CI: 2.50-4.62). In addition, further analyses concerning the site of CCA revealed that IBD patients are notably at increased risk of intrahepatic (RR 2.61, 95%CI: 1.72-3.95) and to a lesser extent extrahepatic CCA (RR 1.47, 95%CI: 1.10-1.97)[49].

As for the risk factors of developing CCA in patients with PSC, those with prolonged IBD duration and those who underwent colectomy due to CRC or colonic dysplasia have an increased risk of CCA[50-52]. On the other hand, in a Scandinavian cohort, although IBD duration was associated with increased risk of CCA, colectomy and CRC were not[48]. CCA develops in chronic inflammation in patients with PSC-IBD and particularly in those with dominant biliary stenosis, something that seems to be a predisposing factor[41]. The definition of a dominant stenosis (DS) in PSC patients is a stricture less than 1.5 mm in diameter in the common bile duct or less than 1 mm in the left or right main hepatic duct[53]. Approximately 10%-62% of PSC patients develop a DS at some point during their disease course[54,55]. In a 25-year study of 128 PSC patients in the United Kingdom, the mean survival of the patients with DS was worst (13.7 years) than for those without a DS (23 years) and this difference was related to a 26% risk of CCA, which developed only in patients with DS. In 50% of patients with CCA, the diagnosis of CCA was made within 4 mo of the diagnosis of PSC[54]. A German study of 171 PSC patients, who were followed prospectively for 20 years, confirmed that the presence of DS in PSC is associated with a worse prognosis due to increased risk of CCA and CRC. Furthermore, this risk was directly related to the presence of underlying IBD. In total, 97 patients had DS, 20 at entry and 77 developed DS over the follow-up period. In patients with DS without IBD, no CCA developed and the survival free of transplantation was 77.8% at 18 years. On the contrary, the 18-year survival was only 23% in the PSC with a DS and IBD. On the other hand, the presence of IBD had no impact on survival in those without a DS[56, 57]. The finding that the risk of CCA is related to the presence of IBD was not confirmed in a cohort of 241 Dutch PSC patients followed for a mean of 6 years[58]. As far as small duct PSC is concerned, it has not been associated with an increased risk of both malignancies (CRC, CCA)[59].

CCA is considered to result in a poor prognosis and the surveillance strategy has not been proven to be beneficial. However, it has been suggested that patients with IBD and PSC should undergo magnetic resonance cholangiopancreatography (MRCP) annually, serum CA 19-9 testing periodically and annual colonoscopy as they also have a higher risk of CRC.

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) mostly occurs in IBD on the background of PSC liver cirrhosis[60] or other established chronic liver diseases such as viral hepatitis. The annual incidence of HCC in cirrhotic patients is 5%, and justifies the 6-mo surveillance

Table 1 Studies on cholangiocarcinoma in patients with inflammatory bowel disease

Ref.	Country	Type of study	Patients	Follow up time	Results	Limitations
Jussila *et al*[30], 2013	Finland	Population-based study	21964 IBD *vs* general population	Mean 10.8 yr	Biliary Ca UC (SIR 7.26, 95%CI: 4.37-11.1); CD (SIR 4.93, 95%CI: 1.02-14.4)	Patients diagnosed in 1987-1993 and 2000-2007 were only included
Kappelman *et al* [35], 2014	Denmark	Population-based cohort	13756 CD, 35152 UC *vs* general population	CD 7.6 yr, UC 7.8 yr	CD: GBC-biliary Ca (SIR 2.4, 95%CI: 1.1-4.5); UC: liver Ca (SIR 1.6, 95%CI: 1.1-2.2) GBC (SIR 2.5, 95%CI: 1.8-3.5), IBD-PSC: Liver Ca 80.0 (95%CI: 32.1-164.8), GBC 129.1 (95%CI: 47.4-281.5), IBD-non-PSC: Liver Ca 1.3 (95%CI: 0.9-1.9), GBC 2.1 (1.4-3.0)	Exclusion of patients with very mild disease, no inpatient encounters prior to 1995
Ananthakrishnan *et al*[43], 2014	United States	Multi-institutional IBD cohort of IBD	5506 CD 5522 UC 224 IBD-PSC	NA	CCA in IBD-PSC patient (OR 55.31, 95%CI: 22.20-137.80) compared to IBD non-PSC patients	PSC diagnosis was predicted with a model. Did not separately examine the risk of large-duct over small-duct PSC
Bernstein *et al*[39], 2001	Canada	Population-based study	2857 CD and 2672 UC patients *vs* with non-IBD (1:10)	14 yr	Liver and biliary Ca: CD (IRR 5.22; 95%CI: 0.96-28.5, $P = 0.06$), UC (IRR 3.96; 95%CI: 1.05-14.9)	Low percentage of the patients received IMMs
Sørensen *et al*[25], 2018	Denmark	Population-based study	222 PSC-IBD patients *vs* 8.231 IBD controls	PSC-IBD 7.4 yr, non-PSC IBD 8.4 yr	CCA PSC-IBD (HR; 190; 95%CI: 54.8-660)	Small number of PSC-IBD patients. Small duct PSC (8%) were included
Scharl *et al*[7], 2019		Cohort study (IBD-Ca *vs* IBD-no Ca)	3119 IBD patients	5 yr	IBD Biliary Ca (SIR 6.3, 95%CI: 1.27-18.41)	Did not compute multivariate regression for Ca subtypes, IBD phenotypes

IBD: Inflammatory bowel disease; Ca: Cancer; UC: Ulcerative colitis; CD: Crohn's disease; SIR: Standardized incidence ratio; CI: Confidence interval; GBC: Gallbladder cancer; PSC: Primary sclerosing cholangitis; CCA: Cholangiocarcinoma; IRR: Incidence rate ratio; HR: Hazard ratio; IMMs: Immunomodulators.

with liver ultrasound ± alpha-fetoprotein. Nevertheless, there have been sporadic case reports of HCC in non-cirrhotic IBD patients, mostly in CD patients treated with azathioprine[61]. Furthermore, it is of note that HCC develops more frequently in PSC-IBD patients compared to IBD non-PSC [hazard ratio (HR), 21.00][62]. However, most population-based studies address the risk of liver cancer without separating the histologic type (HCC or CCA) under the term liver or liver-biliary cancers.

GALLBLADDER CANCER

The risk of gallbladder cancer (GBC) mostly affects IBD patients in the context of PSC. Many studies have confirmed that PSC patients are at increased risk of GBC[62-70], and the prevalence is 3%-14% *vs* 0.35% in the general population[63]. The 10-year cumulative risk was 3% (95%CI: 1-7) in PSC-IBD compared to 0% in non-PSC IBD patients[45]. Furthermore, a study that evaluated 72 gallbladders from 100 liver explants-o (OLT) due to PSC showed that GBC was associated with intrahepatic bile duct dysplasia ($P = 0.001$), CCA ($P = 0.023$), and IBD ($P = 0.03$) and gallbladder dysplasia was associated with hilar/intrahepatic bile duct dysplasia ($P = 0.0006$), CCA ($P = 0.028$), IBD ($P = 0.0014$), and older age at OLT ($P = 0.007$)[71].

A Danish study showed that CD patients had a significant increased risk of liver-GBC and UC patients had a more notable risk (SIR 2.5, 95%CI: 1.8-3.5) compared to the general population. Furthermore, this was profound for UC-PSC (SIR 129.1; 95%CI: 47.4-281.5) than in those without PSC (SIR 2.1; 95%CI: 1.4-3.0)[35]. This indicates the need for annual surveillance with ultrasound of the gallbladder in PSC-IBD patients.

PANCREATIC CANCER

Pancreatic cancer (PC) is the seventh leading cause of cancer death worldwide with increasing incidence and mortality, and is considered to have an unfavorable outcome and prognosis.

In a recent large Scandinavian study with 161926 IBD patients, 442 (0.27%) were diagnosed with PC compared with 3386 (0.21%) of the 1599024 IBD-free individuals. The 20-year cumulative incidence was 0.34% (95%CI: 0.30-0.38) *vs* 0.29% (95%CI: 0.28-0.30), while the IR was 22.1 (20.1-24.2)/100000 person-years in IBD patients. The overall HR was 1.43 (1.30-1.58) [CD 1.44 (1.18-1.74), UC 1.35 (1.19-1.53), IBD unclassified was 1.99 (1.50-2.64)], whereas in IBD-PSC it was 7.55 (4.94-11.5)[72]. In a Korean study, the risk was only increased in females with CD (SIR 8.58; CI: 1.04-31.00)[30]. On the contrary, a previous meta-analysis and several population-based cohort studies have shown that the risk of PC is non-significantly increased in IBD patients [pooled SIR 0.51 (0.06-4.57) and 0.75 (0.30-1.87) accordingly][7-10]. IBD-PSC patients should be referred separately since they are at a higher risk of PC[62]. A Swedish study, which included PSC patients (79% had concurrent IBD), showed that they had a 14-fold greater risk of PC than the background population[46]. In another study of 224 PSC-IBD patients, a significant increased risk was confirmed (OR 11.22, 95%CI: 4.11-30.62) compared to IBD non-PSC patients[43]. Given that higher CCA risk in PSC is well established, we could assume that the increased PC risk might be due to misdiagnosis of periampullary cancer.

GASTRIC MALIGNANCIES

The cause of gastric cancer (GC) in IBD patients is uncertain. GC usually develops on the background of intestinal metaplasia and dysplasia due to *Helicobacter pylori* (*H. pylori*) infection and chronic inflammation[73]. Nevertheless, the prevalence of *H. pylori* in IBD patients is low[74], while a possible causative relation could be attributed to the upper gastrointestinal involvement of CD. A previous review and meta-analysis study, found that CD patients had a significantly increased risk of cancer of the upper gastrointestinal tract (SIR 2.87, 95%CI: 1.66-4.96) and of the stomach *per se* (SIR 2.05, 95%CI: 1.06-3.97)[10]. However, most recent population-based studies[7,11,30,35,39,75] did not confirm this finding. With regard to the risk factors and survival of IBD patients with GC, a study that included 59 GC cases in IBD individuals, showed that UC was more frequent among these patients (69.5% *vs* 51.4%; $P < 0.01$) compared with 177 IBD controls, and IBD patients with GC showed reduced survival ($P = 0.035$; HR 1.385, CI: 1.023-1.875) compared with 1534 individuals of the general population with the same malignancy[76]. Studies on GC in IBD patients are limited and no secure conclusions can be drawn.

BREAST CANCER

Breast cancer (BC) is the second most common malignancy diagnosed worldwide and its mortality is decreasing over time due to screening protocols and improved therapy. Increasing age, prolonged estrogen exposure, obesity in postmenopausal women, reproductive and genetic factors, western life-style, smoking and alcohol are known risk factors[77].

Limited data are available on the pathogenesis of BC in IBD[78]. IBD patients seem to have a shorter period of estrogen exposure than the general population, with later onset of menarche and earlier menopause, that could be related to a lower BC risk[78,79].

No studies primarily investigating the risk of BC in IBD are available. Data, generally not adjusted for confounding factors, can be derived mainly from population-based cohort studies investigating the general and type-specific risk of malignancies[6,15,30,36,39,75,80-88]. In 2012, Hemminki *et al*[84] showed a decreased risk for BC only for CD patients (SIR 0.85, 95%CI: 0.75-0.97) and a decreased mortality rate (HR 0.75, 95%CI: 0.58-0.98), and similar results were seen in the Dutch IBD-SL cohort ($n = 1157$; SIR 0.11 (95%CI: 0.00-0.64)[15] and the TREAT registry cohort study [$n = 2975$; SIR 0.28 (95%CI: 0.08-0.72)][86]. On the other hand, a Swedish cohort study conducted in 21788 CD patients showed a 30% higher BC risk in hospitalized patients between 45 and 64 years, possibly reflecting the advanced risk at older age or the exposure to IBD treatment or unregistered confounders[83]. Similarly, a retrospective

Taiwanese cohort study by Tsai in 2014 indicated that IBD is not associated with increased BC risk (aHR 0.95 (95%CI: 0.66-1.36); however, there was an association between the frequency of IBD-related hospitalizations and BC risk in patients less than 65 years old (aHR 8.45; 95%CI: 4.64-15.4)[87]. An increased risk of BC in both UC and CD was reported by the IBSEN study in 2016[88]. A large cohort study[36], a Finish register study[30], and two meta-analyses, by Pedersen *et al*[10] and by Lo *et al*[37], showed no difference in the occurrence of BC in both UC, CD and the general population, but data on treatment modalities were lacking.

No association between the use of thiopurines and BC has been reported by cohort studies and small series[22,75,89-93]. The correlation between BC and the use of biologics in IBD patients has been addressed in several reports[94-98]. A large nationwide Danish register-based cohort study with 56146 IBD patients and a long follow-up, showed no increased BC risk for patients treated with infliximab, adalimumab or certolizumab[94]. In the TREAT Registry cohort study consisting of CD patients, a decrease in the occurrence of BC was observed in both patients exposed and non-exposed to infliximab [SIR 0.50 (95%CI: 0.24-0.92) and SIR 0.32 (95%CI: 0.12-0.70), respectively], compared to the SEER database of the general United States population [86]. No association for vedolizumab was also reported in a study involving 2830 IBD patients with a follow-up period up to 5 years[95]. In addition, no increased risk of BC in IBD patients exposed to adalimumab monotherapy or combination therapy with thiopurine or methotrexate was suggested in a pooled analysis of 1594 CD patients [96]. Similar results were shown in a pooled analysis of 5 small studies and 5 landmark trials comprising 2385 IBD patients[99] and by a meta-analysis, involving 22 randomized controlled trials comparing anti-tumor necrosis factor (TNF) therapy *vs* placebo, even if only 4 trials had a low risk of bias[100].

In conclusion, the risk of BC is probably decreased in CD patients and seems to be similar to the background population in UC patients. IBD and anti-TNF therapy do not seem to affect BC incidence; however, for thiopurines and combination therapy, this is still uncertain. Future studies on the pathophysiological association between BC and IBD may help identify populations at high risk for BC, who might therefore benefit from close surveillance.

CERVICAL NEOPLASIA

Cervical neoplasia (CN) consists of dysplasia or cervical intra-epithelial neoplasia (CIN) and invasive cervical cancer (CC)[101]. High-risk HPV infection (types 16, 18, 45, 31) is considered the causal agent of CN, whereas smoking, sexual, socioeconomic, and immunologic co-factors may contribute to persistent infection[102]. Most low-grade lesions regress spontaneously but most high-grade dysplasia and CIN 2/3 do not[103, 104]. While HPV vaccines and Papanicolaou (Pap) smear have substantially reduced CC incidence and mortality, these are still high in developing countries and CC remains the second most common cancer in women worldwide[105-107].

The risk of CN in patients with IBD remains controversial and the role of immunosuppressants, a known risk factor for cervical dysplasia in other immune-mediated diseases, is not clear[108,109]. It may be hypothesized that the underlying immunologic changes in IBD or immunosuppressive drugs may reactivate HPV from a latent status or decrease HPV clearance and CIN regression or make HPV vaccination less effective.

Data on the association between IBD, treatment and CN are conflicting[31,75,91,110-119] (Table 2). Also, non-conclusive are the results of two meta-analyses. Allegretti in 2015, in a meta-analysis of 8 studies with 77116 IBD patients but also heterogeneity, found that IBD patients had an increased risk of high-grade dysplasia/cancer compared to healthy controls (OR 1.34, 95%CI: 1.23-1.46) and the risk was greater with the use of immunomodulators, corticosteroids and 5-aminosalicylic acids, but not with anti-TNF[120]. A recent meta-analysis by Lo *et al*[37] in 2020 reported no statistically increased risk for CC in IBD patients.

Regarding screening adherence some studies indicated that this is influenced by various factors such as the state system, increased age and use of immunosuppressants and there is no significant difference with non-IBD patients[121-123]. Singh showed that women with IBD in Canada had a low (54%) screening adherence and older age, lower socioeconomic status, lower intensity of healthcare utilization, CD and exposure to immunosuppressant medications were independent predictors of lower use of Pap testing[121]. In the United States, Long *et al*[122] and Xu *et al*[123] showed higher adherence (70%).

Table 2 Studies on cervical neoplasia in patients with inflammatory bowel disease

Ref.	Country	Type of study	Patients	Results	Limitations
Connell *et al*[91], 1994	United Kingdom	Single centre-registry study, Prospective (1962-1991)	755 IBD patients taking AZA	CC: SIR 4.00	Small power of the study to detect an increased risk (expected 0.5). No variables analysed
Bhatia *et al* [110], 2006	United States	Single centre-cohort study, Retrospective	116 IBD patients 116 age-matched healthy controls	Abnormal Pap smears: 18% *vs* 5%; P = 0.004. Non association with IBD type or treatment exposure	Inter-observer variability in the smear interpretation. Recall bias (questionnaires)
Kane *et al* [111], 2008	United States	Single centre-cohort study (2004-2005)	8 UC; 32 CD. 120 controls (age, race, parity-matched). 58% exposed to ≥ 1 treatment (prednisone, AZA, 6-MP and/or IFX)	Abnormal Pap smear 42.5% IBD *vs* 7% controls; OR 3.4 (95%CI: 1.7-12.1, [a]P < 0.001), low-grade lesions OR 2.2 (95%CI: 1.7-4.4, [a]P < 0.001), high-grade lesions OR 3.1 (95%CI: 1.3-8.7, [a]P < 0.001). Exposed *vs* non-exposed: Abnormal Pap smear OR 1.5 (95%CI: 1.2-7.1, [b]P = 0.02), High-grade lesions OR 6.5 (95%CI: 1.43-30.1, [b]P < 0.05), IMMs > 6 mo OR 1.9 (1.1-12.1, [b]P < 0.001)	Small population study. Level of immune suppression not assessed
Hutfless *et al*[112], 2008	United States	Nested case-control study (1996-2006)	UC 778; CD 476; Controls 12124. TP monotherapy (AZA, 6-MP, MTX)	CC: aOR 1.45 (95%CI: 0.74-2.84), ASA: OR 1.65 (0.34-7.98), corticosteroids: OR 2.79 (0.71-11.00), IMMs: OR 3.45 (0.82-14.45)	Missing data for race, ethnicity, smoking status. No adjustment for therapy or disease severity. Diagnostic or screening Pap smears are not distinguishable
Marehbian *et al*[113], 2009	United States	Insurance claims-based United States population (2002-2005)	CD 22310; controls 111550	IBD *vs* controls: Cervical dysplasia/HPV: RR 1.35 (1.28, 1.43). Exposed *vs* non exposed to treatment: aHR Steroids 1.11 (0.59, 2.09), IS 1.77 (1.26, 2.49), anti-TNF 1.30 (0.68, 2.51), Combination 1.81 (1.10, 3.10)	Disease severity is a possible confounding factor. Limited amount of person-time on various treatments
Lees *et al* [114], 2009	Scotland	Tertiary centre, case-control study Retrospective	UC 178; CD 184; Healthy controls 1448	Abnormal Pap smear: IBD patients OR 0.82 (95%CI: 0.59-1.1.3), IBD patients current smokers *vs* ex or no smokers OR 2.95 (95%CI: 1.55-5.50); P = 0.001, Women < 20 yr at IBD diagnosis *vs* 20-39 yr *vs* > 40 yr: 27% *vs* 13.4% *vs* 5.0% (P = 0.001). No effect of IS therapy	No data on HPV status or exposure to corticosteroids. Small number of patients on MTX or anti-TNF. Median time exposed to IS 2.4 yr. Smoking may be a confounding factor
Singh *et al* [115], 2009	Canada	Population-nested case-control (2002-2006)	UC 233; CD 292; Controls 57898	Cervical abnormalities: OR 1.41 (1.09-1.81), IS+ steroids 1.41 (1.09-1.81). High risk lesions: IS monotherapy aOR 1.23 (0.57-2.63), IS + steroids aOR 1.28 (0.77-2.12), CD patients exposed to > 10 prescriptions of oral contraceptives OR, 1.66 (1.08-2.54), UC OR 1.03 (0.77-1.38)	Administrative databases. Possible bias is smoking, parity and sexual factors. Small number of patients exposed to IMMs alone. No data on HPV infection. CN confirmed histologically in 19% of cases
Jess *et al* [75], 2013	Denmark	Population-nested case-control (2002-2006)	1437 UC; 774 CD. UC median F.U. 15 yr (0-33); CD 14 yr (0-33). 1978-2010 Population-based cohort study (1978-2010). 25176 IBD patients; UC 1437; CD 774(UC median 15yrs/22 582 pt-yrsCD median 14yrs/11 261 pt-yrs)TP (18% of UC pts,45% of CD pts everused TP)	Cervical dysplasia/CC: SIR 1.65 (95%CI: 1.10-2.37), CD diagnosed at age 0-19 yr: SIR 2.52 (95%CI: 1.26-4.51), Smokers: SIR, 2.15 (95%CI: 1.27-3.40), 5-ASA: SIR, 1.69 (95%CI: 1.08-2.51), Thiopurines: SIR, 2.47; (95%CI: 1.54-3.73)	No detailed pharmaco-epidemiological analyses. Cervical dysplasia was analysed along with CC resulting in higher CC incidence
Rungoe *et al*[116], 2015	Denmark	Nationwide population-based cohort (1979-2011)	27408 IBD patients (UC 18691; CD 8717), controls 1508334, median F.U: UC. 7.8 yr; CD 8.3 yr	CD: CC IRR 1.53 (95%CI: 1.04-2.27), High grade lesions IRR, 1.28 (95%CI: 1.13-1.45), low grade lesions IRR, 1.26 (95%CI: 1.07-1.48), CN significantly higher risk in CD patients diagnosed at young age and treated with AZA. UC: CC IRR 0.78 (0.53-1.13), High-grade lesions IRR 1.12 (95%CI: 1.01-1.25), Low-grade lesions: IRR 1.15 (95%CI: 1.00-1.32)	Data for smoking not available. Possible confounding factor is disease severity. Vaccination policies and screening may influence risk estimation
Kim *et al* [119], 2015	United States	Cohort U.S. insurance data (2001-2008 and 2003-2012)	133333 SID patients, including 25176 IBD	High grade dysplasia/CC: aHR 1.72 (0.66-4.45). IS: aHR 1.72 (95%CI: 0.66-4.45)	Confounding factors (race, ethnicity, socioeconomic status, sexual behavioural, gynaecologic history). Study not designed to determine the comparative effect of IS drugs. Short follow-up (mean 2.1 yr)

Jung *et al* [31], 2017	Korea	National Health Insurance claims (2011-2014)	IBD (5595 CD and 10049 UC)	CC: UC 5.65 (2.44-11.13)	Did not focus on cancer occurred during IBD treatment. Missing data (disease diagnosis, phenotype). Short follow-up
Segal *et al* [117], 2021	United Kingdom	Hospital Episode Statistics database (1997-2012)	837 with IBD, 61648 control patients	IBD *vs* controls: CC: 5.2 of 100000 *vs* 4.6 of 100000; $P = 0.042$	Other possible mechanism of carcinogenesis (other than HPV) not evaluated. Database accuracy. HPV-related cancers were not considered separately in CD and UC
Li *et al* [118], 2019	China	Prospective study (2014-2017)	124 IBD patients and 372 controls	HPV 16/18 infection OR 29.035 (3.64-210.988) $P = 0.001$, HPV-infection rate: MTX OR 4.76 (1.471-15.402) $P = 0.005$, > 2 IS OR 3.64 (1.255-10.562) $P = 0.013$, CIN prevalence: 3.2 *vs* 0.0%, $P = 0.004$	No data on sexual behaviour in control group

[a]*P*: Compared to controls.

[b]*P*: Compared to non-exposed.

IBD: Inflammatory bowel disease; AZA: Azathioprine; Pap: Papanicolaou; CC: Cervical cancer; SIR: Standardized incidence ratio; IFX: Infliximab; 6-MP: 6-Mercaptopurine; UC: Ulcerative colitis; CD: Crohn's disease; OR: Odds ratio; aOR: Adjusted odds ratio; CI: Confidence interval; IS: Immunosuppressant; TP: Thiopurine; MTX: Methotrexate; ASA: Amino-salicylate; IMMs: Immunomodulators; anti-TNF: Anti-tumour necrosis factor; SID: Systemic inflammatory disease; HPV: Human papilloma virus; HR: Hazard ratio; IRR: Incidence rate ratio; CIN: Cervical intraepithelial neoplasia.

In many health care systems, it is not clear who has the primary responsibility for CC prevention. As incidence and mortality of CN are highly dependent on screening and treatment of precursor lesions, an inter-specialist cooperation is suggested to guide IBD patients to follow all preventive measures such as HPV vaccination, between 9 and 26 years and prior to the initiation of the sexual activity, in both men and women, annual screening testing for chronically immunocompromised patients and smokers starting at age 21, safe sexual practices and smoking cessation[124].

OTHER GYNAECOLOGICAL CANCERS

No studies primarily investigating the incidence and mortality risk of endometrial, ovarian, or vulvar-vaginal carcinoma in IBD have been identified. Data extracted from large cohort studies investigating the general risk of malignancies, an older meta-analysis and a recent meta-analysis in 2020 showed no increased risk of these cancers even when immunosuppressive medication was used[10,29,31,34,35,37,39,40,80-86,125-127].

URINARY TRACT CANCERS

The urinary tract cancers (UTC) include bladder cancer, that occurs at advanced age, predominantly in males, smokers or in association with chronic inflammation, and renal cell carcinoma (RCC), that is related to smoking, obesity, and hypertension[128-131]. Elevated levels of TNF, a key mediator of cancer-related inflammation, have been reported in the early stages of RCC due to loss of the Von Hippel-Lindau tumor-suppressor gene[132,133].

The prevalence of UTC in IBD patients in a recent, multicenter, prospective Italian study was 9.6% (6.3% in CD and 13% in UC) with main risk factors being the disease duration (in UC) and the use of immunomodulators[134,135]. There are some reports (Table 3) and a recent meta-analysis of 15 studies by Lo *et al*[37] of the non-association between IBD and UTC [Urological: CD IRR 1.34 (0.91-1.98) $P = 0.14$, UC IRR 1.01 (0.82-1.25) $P = 0.92$; RCC: CD IRR 1.93 (0.80-4.65) $P = 0.14$, UC IRR 0.84 (0.33-2.10) $P = 0.71$] [30,35,37,39,75]. However, a case-control study conducted in veteran patients in Texas found an increased risk of RCC in IBD patients[24]. Young IBD patients with a severe disease course also had an increased risk of RCC in a large Dutch cohort study[136]. These patients had a better outcome compared to the general population, probably related to incidental RCC diagnosis at lower stage and younger age due to frequent abdominal imaging. Another key finding of this study was that the use of immunosuppressive or anti-TNFα therapy did not adversely affect RCC disease-free

Table 3 Studies on urinary tract cancer in patients with inflammatory bowel disease

Ref.	Country	Type of study	Patients	Results	Limitations
Bernstein *et al* [39], 2001	Canada	Population-based-cohort study (1984-1997)	5529 IBD patients; Median F.U. 7.9 yr (3.5-12)	Bladder Ca: CD IRR 1.30 (0.51-3.30) UC IRR 0.67 (0.24-1.85) IBD IRR 0.92 (0.47-1.82). RCC: CD IRR 1.02 (0.31-3.34) UC IRR O.8 (0.25-2.58) IBD IRR 0.89 (0.39-2.06)	Possible confounding factor is socioeconomic status. Maximum F.U 12 yr. Data not analysed by extent of disease
Pasternak *et al*[22], 2013	Denmark	Cohort study (1997-2008)	45986 patients	AZA: Current users RR 2.8 (1.24-6.51), former users: RR 1.73 (0.70-4.24)	Possibly confounded by indication
Jess *et al*[75], 2013	Denmark	Population-nested case-control (2002-2006)	1437 UC; 774 CD; Median F.U. UC 15 yr (0-33); CD 14 yr (0-33)	CD SIR 1.69 (0.68-3.49); UC SIR 1.08 (0.56-1.89)	No detailed pharmaco-epidemiological analyses
Jussila *et al* [30], 2013	Finland	Cohort study (1987-1993 and 2000-2007 and followed up to 2010)	21964 patients with IBD	Urological CD IRR 1.56 (0.58-4.21), RCC CD IRR 1.61 (0.62-4.17)	Possibility of misclassification of IBD, CD, UC, and Ca
Kappelman *et al*[35], 2014	Denmark	Population-based-cohort study (1978-2010)	48908 IBD patients	Bladder Ca CD SIR 1.1 (0.8-1.6), RCC CD SIR 0.98 (0.77-1.23)	Not age-and sex specific estimates of absolute Ca risk. Detection bias. Data possible lacking. No inpatient encounters prior to 1995
Algaba *et al* [85], 2015	Spain	Prospective-cohort study (2005-2011)	590 IBD patients; Controls 222219	Bladder Ca RR 5.23 (1.95-13.87)	Small number of cases and limited period of follow-up
Nyboe Andersen *et al*[94], 2014	Denmark	Cohort study (1999-2012)	56146 IBD patients; Median F.U. 9.3 yr (4.2-14)	Anti-TNF: aHR 1.60 (0.61-4.19)	Confounding by indication, smoking, missing data. Short median F.U. of anti-TNF exposed (3.7 yr). Small number of Ca did not permit subgroup analysis
Bourrier *et al* [138], 2016 (CESAME study)	France	Prospective-cohort study (2004-2005)	19486 IBD patients (30.1% receiving TP)	Bladder Ca SIR 1.20 (0.44-2.61); RCC SIR 2.05 (0.98-3.77), AZA > 65 yr *vs* < 50 yr HR 13.26 (3.52-50.03, P = 0.0001), Current users: SIR 3.40 (1.47-6.71; P = 0.006), Ex-users: SIR 0.64 (0.01-3.56)	Smoking is a possible confounding factor. Risk of anti-TNFs not assessed. Short follow-up
Wauters *et al* [139], 2017	Belgium	Retrospective case–control study (1990-2014)	RCC; Exposed to anti-TNF: 2083 IBD patients (952 men and 1131 women); Un-exposed to anti-TNF: 1952 (977 men and 975 women)	Un-exposed to anti-TNF males SIR 5.4 (2.9-9.2), females SIR 8.5 (3.7-16.8) Exposed to anti-TNF males SIR 7.1(2.3-16.5), females SIR 4.8 (0.6-17.3)	Potential confounding factors were not adjusted. Disease type, severity, and drug exposure of hospitalized patients may not be comparable with the global patient population. Different agents and dose-response for anti-TNF were not studied
Mosher *et al* [24], 2018	United States	Case-control study Veteran population (1996-2015)	2080 patients with IBD; 271898 without IBD	Bladder Ca 20 yr RR 1.72 (0.86-3.45); RCC 20 yr RR 2.90 (1.46-5.84)	Administrative data. Possible underestimation of Ca incidence (newly diagnosed Ca treated in other centres)
Derikx *et al* [136], 2015	Holland	Case-control study (1991-2013)	Case control study A: 180 IBD patients with RCC *vs* 1800 IBD patients; Case control study B: 180 IBD patients with RCC *vs* 4388 patients with RCC in the general population	Case control A: Montreal E3 UC OR 1.8-2.5 (95%CI: 1.0-5.3), penetrating IBD-CD OR 2.8 (95%CI: 1.3-5.8), IBD related surgery OR 3.7-4.5 (95%CI: 1.6-8.2), male gender OR 3.2-5.0 (95%CI: 1.7-13.2). Case control B: lower age at diagnosis RCC (P < 0.001), lower N-stage (P = 0.025), lower M-stage (P = 0.020), more frequent surgical treatment for RCC (P < 0.001), better survival (P = 0.026; HR 0.7)	Retrospective data collection. Selection bias (different registries and databases)
Biancone *et al* [26], 2020	Italy	Multi-centre nested case-control study prospective (2011-2017)	403 IBD patients; 806 IBD controls	UC: OR 3.79 (1.27-16.2)	Referral IBD centres included more severe patients compared with community-based centres

IBD: Inflammatory bowel disease; CD: Crohn's disease; IRR: Incidence rate ratio; CI: Confidence interval; F.U. : Follow-up; Ca: Cancer; UC: Ulcerative colitis; RR: Relative risk; SIR: Standardized incidence ratio; UTC: Urinary tract cancer; RCC: Renal cell carcinoma; aHR: Adjusted hazard ratio; AZA: Azathioprine; TP: Thiopurines; anti-TNF; Anti-tumour necrosis factor; TNF-: Not exposed to anti-TNF; TNF+: Exposed to TNF; N-stage: Lymph node stage; M-stage: Metastatic stage.

survival and overall survival[136].

With regard to bladder cancer, an increased risk in IBD patients was clearly demonstrated by a meta-analysis of eight population-based cohort studies with 17052 IBD patients in 2010 (SIR 2.03, 1.14-3.63)[10] and a prospective Spanish cohort study [85]. Moreover, long-standing IBD and use of immunosuppressive medication were found to be associated with the development of bladder cancer[137]. It is noteworthy that a reversible increased risk of UTC in patients treated with azathioprine has been reported by two large studies[22,138].

Cancer risk assessment in patients under anti-TNF therapy is problematic due to frequent combined treatment with thiopurines. Anti-TNF monotherapy does not increase either UTC risk, according to the results of a population-based prospective cohort nationwide Danish study of more than 56000 IBD patients[94] or RCC risk, independent of diagnosis age, type, and duration of IBD or coexistence of known RCC risk factors[139].

There are currently no screening guidelines for UTC in any population, while incidental detection of RCC has been identified as a positive prognostic index[140, 141]. Whether patients with a long IBD duration may require different surveillance programs for preventing UTC needs to be confirmed, but all CD patients should be encouraged to quit smoking as tobacco could be a key risk factor[128,142,143]. Limited evidence is available on the role of immunosuppressive therapy in the development of UTC; however, elderly men on thiopurine therapy, should be closely evaluated for UTC.

PROSTATE CANCER

Prostate cancer (PC) is the second most common cancer in men worldwide, with screening tools available for early diagnosis[144]. The most important risk factors are age, African American ethnicity, genetic and possibly dietary factors.

Some studies have indicated an increased risk of PC in IBD, especially in UC, while others did not confirm this[24,29-31,35,36,39,40,75,83,84,144-147] (Table 4). Intensive surveillance in IBD patients with digital rectal and prostate examination may be a reason for the high incidence of PC, but also potential pathophysiological mechanisms of IBD and PC association have been recognized. In principle, the distinct microbiome level between UC and CD may be related to the elevated risk of PC in UC[148-150]. In addition, simultaneous elevation of IBD-related inflammatory markers, such as C-reactive protein, and of prostate-specific antigen (PSA), known prostatic inflammation or PC index has been reported[151-155]. Receptors of pro-inflammatory cytokines, such as interleukin 6 (IL-6)[156-158], and folate hydrolase 1 (FOLH1)/prostate specific membrane antigen (PSMA) are up-regulated in both IBD and PC and in fact FOLH1/PSMA appears overexpressed in cases of biochemical recurrence with an increase of PSA and metastatic disease[159-163].

A meta-analysis of 9 studies involving 17052 IBD patients by Pedersen *et al*[10] in 2010 showed no association between IBD and PC [IBD: SIR 1.16 (0.88-1.52), UC: SIR 1.14 (0.85-1.42) CD: SIR 0.77 (0.41-1.45)]. These results were not confirmed by recent studies and on the other hand, an increased risk of PC in patients with UC was reported (Table 4). Actually, an increased risk worldwide only for UC patients was shown in another meta-analysis of nine population-based studies by Ge *et al*[164] in 2020, with a large sample and a long follow-up of patients [Cohort studies: IBD RR 1.33 (1.03-1.71); UC RR 1.58 (1.08-2.30); CD RR 1.12 (0.97-1.31); Case control studies: RR 1.81 (1.43-2.29)]. In addition, a recent meta-analysis of eight studies by Chen *et al*[165] in 2020 reported two interesting results. For the first time a higher risk was also found for CD patients [IBD RR 1.78 (1.32-2.41); UC RR 1.76 (1.06-2.91); CD RR 1.29 (1.04-1.61)] and a higher risk in Asian patients with IBD compared to Caucasians was shown [RR 3.02 (95%CI: 2.03-4.48) and 1.6 (95%CI: 1.17-2.19), respectively], in contrast with the known lower risk of PC in the Asian general population[165]. It is noteworthy that two other meta-analyses in 2020, by Carli *et al*[166] [IBD RR 1.71 (1.16-2.51) $P = 0.007$; UC RR 1.21 (0.98-1.51) $P = 0.07$; CD RR 1.10 (0.98-1.25) $P = 0.12$] and by Lo *et al*[37] [UC SIR 1.08 (0.9-1.3); CD SIR 1.04 (0.8-1.35)] did not demonstrate a higher risk of PC in the subgroup analysis of UC or CD.

In conclusion, data for PC risk in IBD patients are conflicting. Further prospective studies are needed to evaluate the impact of concomitant medications on the risk of developing PC and the possible utility of screening programs for early diagnosis of PC in patients with IBD.

Table 4 Studies on prostate cancer in patients with inflammatory bowel disease

Ref.	Country	Type of study	Patients	Results	Limitations
Karlén *et al* [40], 1999	Sweden	Cohort (1955-1989)	1547 UC	UC 7/1547; SIR 0.7 (0.3-1.5)	Missing data. Closer monitoring of UC patients may lead to higher frequency and early detection
Bernstein *et al*[39], 2001	Canada	Cohort (1984 -1997)	5529 IBD-1151000 controls	IBD 26/5529 SIR 0.86 (0.59-1.26), 6293/1151000 controls	Possible confounding factor is socioeconomic status. Maximum F.U 14 yr. Data not analyzed by extend of disease
Winther *et al* [145], 2004	Denmark	Cohort (1962-1997)	1160 UC patients; F.U. median 19 yr	4/1160 UC; UC SMR 0.74 (0.20-1.88)	The treatment principles remained unchanged during the entire follow-up period
Hemminki *et al*[84], 2012	Sweden	Cohort (1964-2004)	27606 UC patients	UC 277/27606 SIR 1.14 (1.01-1.28), All + 1; SIR 1.08 (0.95-1.22)	Possible incidental finding of PC in older UC patients
Hemminki *et al*[83], 2009	Sweden	Cohort (1964-2004)	21788 CD patients	CD 152/21788; SIR 1.19 (1.01-1.4), All + 1 SIR 1.12 (0.94-1.32)	The sparseness of individual cancers did not allow conclusions about the trends
Jess *et al*[75], 2013	Denmark	Cohort (1978-2010)	1437 UC; 774 CD	UC SIR 1.82 (1.17-2.71)	No detailed pharmaco-epidemiological analysis
Jussila *et al* [30], 2013	Finland	Cohort (1987-1993 and 2000-2007)	21964 IBD (16649 UC; 5315 CD); 5351000 controls	IBD 176/21964; 51045/5351000 controls; IBD SIR 0.84 (0.73-0.97) UC 150/16649 SIR 0.85 (0.72-0.99) $P < 0.05$; CD 26/5315 SIR 0.79 (0.52-1.16)	Possibility of mis-classification of IBD, CD, UC, and Ca. Patients diagnosed 1987-1993 and 2000-2007 were only included
Kappelman *et al*[35], 2014	Denmark	Cohort (1978-2010)	42717 IBD (35152 UC; 13756 CD); 5554844 controls. F.U. CD for 7.6 yr, UC for 7.8 yr	IBD 316/42717; controls 33960/5554844. IBD SIR 1.21 (1.08-1.35); UC 258/35152 SIR 1.2 (1.1-1.4); CD 58/13756 SIR 1.2 (0.9-1.6)	No age-estimates of absolute cancer risk. Detection bias. Data possibly missing. No inpatient encounters prior to 1995
Wilson *et al* [36], 2016	Switzerland	Case-control (1995-2012)	19647 IBD (7850 CD; 11797 UC); 19647 controls	IBD 79/19647; 67/19647 controls. IBD aHR 1.19 (0.86-1.65); CD 17/7850; 16/7850 controls; CD aHR 1.08 (0.54-2.15); UC 62/11797; 51/11797 controls UC aHR 1.22 (0.84-1.77)	Exposure misclassification. Potential bias in multivariate analysis (smoking, alcohol, BMI)
Jung *et al* [31], 2017	Korea	Cohort (2011-2014)	9785 UC; 5506 CD; 50750000 controls	19/15291 IBD; 20607/50750000 controls. IBD SIR 3.5 (2.1-5.5); UC SIR 3.47 (2.06-5.48); CD SIR O.99 (0.03-5.54)	The study did not focus on IBD treatment. Data for disease diagnosis, phenotype not available. Short follow-up
So *et al*[29], 2017	China	Cohort (1990-2016)	2621 IBD; 1603 UC; 7392000 controls. Median F.U. 8 yr CD, 10 yr UC	8/2621 IBD; 11115/7392000 controls. IBD SIR 2.03 (1.03-4.06); 8/1603 UC; UC SIR 2.47 (1.24-4.95)	The 25% of the cohort was followed up for < 5 yr. Small size of PC cases. Lead-time and detection bias. Exposure not evaluated
Mosher *et al* [24], 2018	United States	Case-control study Veteran population (1996-2015)	2080 IBD patients; 271898 without IBD	574/2080 IBD; 337/271898 IBD free; 20 yr RR 1.70 (1.28-2.27)	Administrative data. Heterogeneity of the Ca types. Ca incidence rates may be underestimated
Burns *et al* [146], 2019	United States	Cohort (1996-2017)	1033 IBD; 9306 IBD free	IBD 30/1033; IBD free 29/9306, 10 yr HR 4.44 (2.98-6.62) $P < 0.001$; clinically significant PC: 10 yr HR 3.72 (2.15-6.42) $P < 0.001$; RR 9.32 (5.62-15.46)	Variables for IBD missing. Academic medical centre. PC morbidity, mortality, IBD treatments and healthcare utilization not assessed
Meyers *et al* [147], 2020	United States	Prospective Population-based United Kingdom Biobank cohort (2006 and 2010, with follow-up through mid-2015)	2311 IBD (1488 UC; 643 CD); 215773 IBD free; Men aged 40 to 69 at study entry	UC 49/1488; aHR 1.47 (1.11-1.95) $P = 0.0070$, ≤ 20 yr: 1.29 (0.89, 1.85), > 20 yr: 1.87 (1.21-2.91) $P = 0.0052$, BMI ≤ 30: 1.48 (1.07, 2.03), BM1 > 30: 1.35 (0.72, 2.51); CD 14/643 aHR 1.06 (0.63-1.80), ≤ 20 yr: 1.11 (0.58, 2.14), > 20 yr: 0.98 (0.41, 2.37), BMI ≤ 30: 0.83 (0.43, 1.59), BMI > 30: 2.25 (0.93, 5.41)	Number of prior PSA test, DRE, PC morbidity and mortality and IBD treatments were not reported. No data for PC grade or stage. Selection bias

IBD: Inflammatory bowel disease; Ca: Cancer; PC: Prostate cancer; UC: Ulcerative colitis; SIR: Standardized incidence ratio; CD: Crohn's disease; SMR: Standardized mortality ratio; All + 1: Diagnosis of cancer later than the first year of IBD diagnosis; F.U.: Follow-up; AHR: Adjusted hazard ratio; DRE: Digital rectal examination; BMI: Body mass index; PSA: Prostate-specific antigen.

SKIN MALIGNANCIES

Several population-based studies have shown an increased prevalence of skin malignancies among patients with IBD[10,167-169]. Furthermore, the incidence of skin cancer among those receiving immunosuppressants is rising over time[170]. On the contrary, other population-based studies showed that there is no *per se* increased risk of skin cancer in IBD patients[34,39,75]. The skin cancers include melanoma (MSC) and non-melanoma skin cancers (NMSC) with the latter further divided into squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). It is well established that anti-TNF exposure is associated with the development of MSC[169], whereas NMSC is associated with thiopurines exposure (azathioprine, 6-mercaptopurine)[171]. With regard to the mechanism by which thiopurines may predispose patients to skin cancer the most prominent theory supports that thiopurines increase the vulnerability to UVA radiation. In detail, they cause the accumulation of 6-thioguanine in the DNA of patients and further UVA radiation causes production of reactive oxygen species that leads to DNA mutations and oxidative stress, resulting in oncogenesis[172]. On the other hand, MSC is an immunogenic aggressive tumor, in which suppression of the immune response allows these tumors to grow and metastasize[173]. For this reason, immunosuppressants may cause down-regulation of the tumor surveillance mechanisms, increased susceptibility to infection with oncogenic viruses (*e.g.*, melanoma associated retroviruses), or direct pharmacologic effects of these medications on DNA metabolism[174].

There is evidence from several studies of an increased risk of NMSC in IBD associated with thiopurines use (Table 5). These findings were not confirmed by two other studies that found no increased NMSC risk among IBD thiopurine users[89,175]. A meta-analysis that pooled 60351 IBD patients to assess the risk of NMSC in those treated with thiopurines, showed that the risk was only modestly elevated (aHR 2.28; 95%CI: 1.50-3.45)[184]. There is also evidence of an increased risk of MSC in IBD and this risk is associated with anti-TNF use (Table 5). In a review and meta-analysis that included 172837 IBD patients, 179 MSC cases were reported (pooled crude IR 27.5/100000 person-years; 95%CI: 19.9-37.0) and a 37% increased risk of MSC, independent of biologic therapy[185]. On the contrary, a Danish Cochrane review and meta-analysis (56146 IBD patients 8.1% anti-TNF exposed) did not show increased risk of MSC in those treated with anti-TNF (HR 0.62; CI: 0.08-4.57)[94], which was further confirmed by a recent systematic review and meta-analysis (comprising 34029 biologic-treated and 135370 biologic-naive patients), where biologics did not significantly increase the risk of MSC[186]. In a recent study that included IBD patients with MSC, IBD extent was found to be a risk factor, both in UC (pancolitis OR: 3.09; 95%CI: 1.670-5.727) and CD (ileocolonic disease: OR: 1.98; 95%CI: 1.009-3.882), corticosteroids were risk factors in UC (OR: 1.41-3.72) whereas anti-TNFs were protective factors in UC (OR: 0.15-0.88) and CD (0.27-0.92), but this was attributed to *in-situ* melanoma only. Moreover, no association between survival of IBD-MSC patients and anti-TNF or immunosuppressants after MSC diagnosis was found[181].

The known risk factors for the development of skin cancers include smoking, older age, male sex, fair skin type, cumulative sun exposure (mostly for NMSC), sun-burn (mostly for MSC), family history of skin cancer, Caucasian race, geographical area and certain genetic factors (*e.g.*, p53 polymorphisms). All these factors should be taken into account when immunosuppressant therapy is being considered for a patient with IBD. Given these findings, drugs other than anti-TNFs might be safer for use in patients after a melanoma diagnosis or in those at high risk for these tumors, whereas thiopurines should also be avoided in patients with a history of SCC, multiple BCCs, or premalignant skin lesions (*e.g.*, solar keratosis)[187].

With regard to the maintenance of immunosuppressive treatment after the initial diagnosis of skin cancer, the results from various studies have been controversial. A large cohort study showed that thiopurines did not increase the risk of second NMSC when used in combination treatment compared with anti-TNF monotherapy (HR 0.79, 95%CI: 0.30-2.08)[180]. In addition, a previous study by Beaugerie concluded that exposure to immunosuppressants did not increase the risk of new or recurrent malignancy in individuals with previous cancer[188]. On the contrary, another recent cohort study with 54919 IBD patients, of whom 518 developed BCC and maintained their treatment, it was shown that repeated BCC occurrences were associated with active thiopurine use[183]. In line with this, a large meta-analysis that included 3706 IBD patients with 10332 person-years of follow-up after a cancer diagnosis showed, in a subgroup on skin cancer alone, that the risk was significantly higher in immunosuppressant-users (71.6 per 1000 person-year, 95%CI: 58.9-84.2, P = 0.035) compared to those not receiving immunosuppressants (50.8 per 1000 person-year, 95%CI: 43.7-57.8)

Table 5 Studies on skin cancer in patients with inflammatory bowel disease

Ref.	Type of study	Country	Patients	Follow up time	Results	Limitations
Armstrong *et al*[89], 2010	Nested case control	United Kingdom	16663 IBD patients; 392 developed Ca *vs* 1914 IBD controls	6.4 yr	NMSC with AZA use (OR 0.99, CI: 0.35-2.81)	AZA users were included but not 6MP
Long *et al* [167], 2010	Retrospective cohort; nested case control	United States	53377 IBD patients *vs* 160037 non-IBD; 742 IBD NMSC cases *vs* 2968 IBD controls	1.32 yr	NMSC (IRR, 1.64; 95%CI: 1.51-1.78), NMSC recent TP use (OR, 3.56; 95%CI: 2.81-4.50), recent biologics in CD (OR, 2.07; 95%CI: 1.28-3.33), persistent TP use (OR, 4.27; 95%CI: 3.08-5.92), persistent biologic use in CD (OR, 2.18; 95%CI: 1.07-4.46)	Patients aged < 64 yr, no exposure dose, short follow-up
Singh *et al* [168], 2011	Retrospective cohort; case control	Canada	9618 IBD patients *vs* 91378 non-IBD; 237 IBD NMSC cases *vs* 948 IBD controls	11.7 yr	BCC (HR, 1.20; 95%CI: 1.03-1.40). TP use SCC (HR, 5.40; 95%CI: 2.00-14.56) BCC (HR, 1.12; CI 0.68-1.85). Case-control: TP use SCC (OR, 20.52; 95%CI: 2.42-173.81), BCC (OR: 2.07; 95%CI: 1.10-3.87)	Do not include use of IMMs before 1995
Peyrin-Biroulet *et al* [171], 2011	Prospective observational cohort study (CESAME)	France	19486 IBD patients	2.55 yr	NMSC (SIR 2.89, 95%CI: 1.98-4.08) MSC (SIR 0.64, 95%CI: 0.17-1.63). NMSC: ongoing TP use (HR, 5.9; 95%CI: 2.1-16.4; $P = 0.0006$), past TP use (HR, 3.9; 95%CI: 1.3-12.1; $P = 0.02$), age per 1-yr increase (HR, 1.08; 95%CI: 1.05-1.11; $P < 0.0001$)	Younger patients
van Schaik *et al*[175], 2011	Retrospective cohort	Holland	2887 IBD patients	6.46 yr	NMSC AZA use (HR 0.85, 95%CI: 0.51-1.41)	Small study sample size
Long *et al* [169], 2012	Retrospective cohort; nested case-control	United States	108579 IBD *vs* 434 233 non-IBD controls; 209 MSC cases *vs* 823 IBD non-MSC controls, 3288 NMSC cases *vs* 12945 IBD non-NMSC controls	2 yr	MSC (HR, 1.15; 95%CI: 0.97-1.36) NMSC (HR, 1.34; 95%CI: 1.28-1.40). MSC anti-TNF (OR, 1.88; 95%CI: 1.08-3.29), long-term *vs* non-long-term use (OR 3.93, 95%CI: 1.82-8.50), no association with TP or 5-ASA. NMSC any TP use (OR, 1.85; 95%CI: 1.66-2.05), anti-TNF (OR, 1.14; 95%CI: 0.95-1.36), combination treatment (OR, 3.89; 95%CI: 2.33-6.46)	Study population aged < 64 yr, no dose information about treatments, short mean follow-up
Peyrin-Biroulet *et al* [176], 2012	Prospective observational cohort study (CESAME)	France	19486 IBD patients	2.55 yr	MSC previously TP treated (SIR: 0; 95%CI: 0-3.11), current TP users (SIR: 1.09; 95%CI: 0.13-3.94)	Younger patient population
Abbas *et al* [177], 2014	Retrospective cohort; nested case control	United States	14527 patients; 421 NMSC and 45 MSC cases	8.1 yr	NMSC current AZA use (HR 2.1, 95%CI: 1.6-2.6), previous AZA use (HR 0.7, 95%CI: 0.5-1.0). MSC current AZA use (HR 1.5, 95%CI: 0.6-3.4), previous AZA use (HR 0.5, 95%CI: 90.1-1.8)	Patient population limited to VA health care system (older, white, male)
McKenna *et al*[178], 2014	Database inquiry (AE-(FAERS)	United States	315 skin Ca	NA	PRR, increased odds of MSC and NMSC for anti-TNF ($P = 0.035$ and 0.03, respectively) and combination treatment ($P < 0.001$ and $P < 0.001$)	AE database (reporting bias) skewed towards CD
Kopylov *et al*[179], 2015	Nested case control	Canada	19582 patients; (MSC 102 *vs* IBD Controls 1014) (NMSC 474 IBD *vs* Controls 4684)	No reported mean	NMSC: TP treatment ≥ 3 yr (OR 1.41; 95%CI: 1.11-1.79), TP treatment ≥ 5 yr (OR: 2.07; 95%CI: 1.36-3.7), combination treatment (OR: 3.11; 95%CI: 1.33-7.27). After stopping TP, OR: 1.04 (0.69-1.55). IMMs-anti-TNF were not associated with MSC	Younger, employed patients are underrepresented, not mentioned disease severity
Scott *et al* [180], 2016	Retrospective cohort	United States	2788 IBD patients	2.24 yr	Second NMSC with short-term TP treatment (HR 1.53, 95%CI: 0.87-2.70), with > 1 yr of TP therapy (HR 1.49, 95%CI: 0.98-2.27)	Older patient population
Nissen *et al* [181], 2017	2 Retrospective case-control studies	The Netherlands	304 IBD patients with MSC, 1800 IBD controls, 8177 MSC non-IBD controls		MSC: UC (pancolitis OR 3.09; 95%CI: 1.670-5.727), CD (ileocolonic disease: OR 1.98; 95%CI: 1.009-3.882). Corticosteroids (OR 1.41-3.72), anti-TNF UC (OR 0.15-0.88), CD (0.27-0.92). (only attributed to the *in situ* MSC). Survival with anti-TNF (HR 0.32; 95%CI: 0.08-1.27) and TP (HR 0.72; 95%CI: 0.37-1.31). Survival after MSC diagnosis anti-TNF (HR 0.16, 95%CI: 0.02-1.21) and TP (HR 0.55, 95%CI: 0.25-1.23)	Medication of patients after 1990 was included. Not informed about skin type, number of sun burns
Clowry *et al* [182], 2017	Retrospective cohort	Ireland	2053 patients with IBD	9.8 yr	NMSC under IMMs SIR 1.8 (95%CI: 1.0-2.7), TP exposure (OR: 5.26, 95%CI: 2.15-12.93, $P <$	Small sample size, hospital database mostly

					0.001), TP and/or anti-TNF (OR: 6.45, 95%CI: 2.69-15.95, *P* <0.001)	severe IBD
Khan *et al* [183], 2020	Retrospective cohort	United States	54919 patients with IBD; VAHS 518 patients with BCC	5.71 yr	Repeated BCC occurrences, compared with 5-ASA, under active TP use (HR 1.65, 95%CI: 1.24-2.19, *P* = 0.0005), 6 mo after TP discontinuation (HR 1.22, 95%CI: 0.86-1.74, *P* = 0.26), for anti-TNF use (HR 1.27, 95%CI: 0.84-1.90, *P* = 0.26), for combination treatment (HR 1.37, 95%CI: 0.90-2.08, *P* = 0.14)	Study population mostly males. Prescriptions outside VAHS not included

IBD: Inflammatory bowel disease; Ca: Cancer; NMSC: Non-melanoma skin cancer; AZA: Azathioprine; OR: Odds ratio; CI: Confidence interval; 6MP: 6-Mercaptopurine; IRR: Incidence rate ratio; TP: Thiopurine; CD: Crohn's disease; BCC: Basal cell cancer ; SCC: Squamous cell carcinoma; HR: Hazard ratio; IMMs: Immunomodulators; MSC: Melanoma skin cancer; SIR: Standardized incidence ratio; anti-TNF: Anti-tumor necrosis factor; NA: Not applicable; PRR: Projection pursuit regression; AE: Adverse events; 5-ASA: 5-Aminosalicylic acid; VAHS: Veterans affairs healthcare system.

and numerically higher than anti-TNFα therapy (55.5 per 1000 person-year, 95%CI: 44.7-66.3, *P* = 0.22)[189].

With regard to prevention, a recent study showed that only a small proportion of IBD patients (8.3% during the 7-year study period) were seeking dermatologic care [190].

In conclusion, it seems that there is an increased risk of NMSC and a slightly lower risk of MSC in IBD patients, which justifies the recommendations for routine screening for skin malignancies. Although there is some evidence of second NMSC after an index diagnosis with azathioprine, overall data are conflicting regarding maintaining immunosuppressive treatment after the diagnosis of a skin malignancy and the risk of recurrence or a second skin malignancy, so the decision should be individualized. Conclusively, primary prevention measures, such as sun-protection, annual dermatologic examination and smoking abstinence should be encouraged in all IBD patients, regardless of thiopurine and/or anti-TNFα use.

CONCLUSION

Cancer is the second cause of death in patients with IBD, after cardiovascular disease. Lately there has been evidence that the incidence of intestinal cancers is decreasing over time in IBD patients, as a result of the implementation of a tight screening strategy. Although recent data supporting that there is no excess overall risk of extraintestinal cancers, it seems that IBD patients are in higher risk of certain extraintestinal cancers. This indicates the need for close monitoring, as it seems that chronic systemic inflammation but also other factors such as older age, smoking, specific virus infections (*e.g.*, HPV) and concurrent diseases (PSC) play a role in carcinogenesis. The IBD treatments, for both CD and UC, are aimed at clinical improvement, endoscopic remission and sustained mucosal healing. Although immunosuppressants, including azathioprine, methotrexate and anti-TNFs, constitute the cornerstone of IBD treatment, these drugs might have a carcinogenic effect either by directly altering cellular DNA or reducing tumor immunosurveillance. Lately there are more treatment choices available, such as anti-integrins, anti-IL-12/23 and JAK inhibitors. Although these new treatments are considered to have a better safety profile as far as cancer is concerned, data are limited and future studies are required.

Specialists who deal with the management of IBD patients must bear in mind the possibility of extraintestinal malignancies, especially in those with long standing immunosuppressive therapies and persistent systemic or local inflammation. Guidelines and recommendations should incorporate all the preventative measures [sun protection, dermatologic examination, vaccinations (HPV, hepatitis B virus), annual Pap smear, mammography, PSA testing, annual MRCP where appropriate] and applied in everyday clinical practice to diminish the risk of IBD-related extraintestinal malignancies, achieve an early diagnosis and improve the prognosis and overall outcome.

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REVIEW

Mesenchymal stem cell-derived exosomes for gastrointestinal cancer

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Abstract

Gastrointestinal (GI) malignancies, a series of malignant conditions originating from the digestive system, include gastric cancer, hepatocellular carcinoma, pancreatic cancer, and colorectal cancer. GI cancers have been regarded as the leading cancer-related cause of death in recent years. Therefore, it is essential to develop effective treatment strategies for GI malignancies. Mesenchymal stem cells (MSCs), a type of distinct non-hematopoietic stem cells and an important component of the tumor microenvironment, play important roles in regulating GI cancer development and progression through multiple mechanisms, such as secreting cytokines and direct interactions. Currently, studies are focusing on the anti-cancer effect of MSCs on GI malignancies. However, the effects and functional mechanisms of MSC-derived exosomes on GI cancer are less studied. MSC-derived exosomes can regulate GI tumor growth, drug response, metastasis, and invasion through transplanting proteins and miRNA to tumor cells to activate the specific signal pathway. Besides, the MSC-derived exosomes are also seen as an important drug delivery system and have shown potential in anti-cancer treatment. This study aims to summarize the effect and biological functions of MSC-derived exosomes on the development of GI cancers and discuss their possible clinical applications for the treatment of GI malignancies.

Key Words: Mesenchymal stem cells; Exosomes; Gastrointestinal cancer; Cancer treatment; Drug delivery system; Transplanting miRNA

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Core Tip: Mesenchymal stem cells (MSCs) have shown potential for anti-cancer therapy. As an important content of MSCs, MSC-derived exosomes are attracting more and more researchers for anti-cancer studies. We herein summarize the effect of MSC-derived exosomes on gastrointestinal malignancies and discuss their therapeutic potential.

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INTRODUCTION

Gastrointestinal (GI) cancer is one of the most common malignancies in the digestive system, such as the stomach, liver, pancreas, and colorectum[1]. Based on the latest global epidemiological data, GI cancer accounts for 26% of all kinds of cancers and 35% of cancer patients died from GI cancer with approximately 4.8 million new cases and 3.4 million deaths each year[2,3]. Current therapeutic strategies for GI malignancies mainly include surgery, endoscopy, radiotherapy, chemotherapy, targeted therapy, and immunotherapy[4-6]. Despite that treatment strategies are becoming mature and diverse, the prognosis of GI cancer is still very poor due to the fact that most patients miss the therapeutic window[7]. If detected at an early stage, GI cancers are highly curable with traditional treatment methods[8]. However, the early diagnosis of GI cancer is a significant challenge. Therefore, more promising treatment strategies are needed to cut down the mortality of GI malignancies.

Mesenchymal stem cells (MSCs), a type of distinct non-hematopoietic stem cells, possess the capacity of self-renewal and multipotentiality differentiation[9,10]. It has been shown that MSCs are involved in tumor development, including tumorigenesis, tumor growth and metastasis, as well as regulation of tumor microenvironment[11-13]. Therefore, MSCs have been commonly used in anti-cancer studies. However, the underlying mechanisms of how MSCs affect tumor development are still controversial [14]. A series of literature has reported that MSCs are capable of promoting tumor progression through secreting pro-tumorigenic factors[15] and differentiating into cancer-associated fibroblasts[16,17]. Nevertheless, other evidence suggests that MSCs could suppress tumor proliferation *via* secreting cycle inhibitor P21 and anti-tumorigenic factors such as interleukins, IFN-γ, Dkk-1, and promote tumor cell apoptosis through secreting apoptotic executor caspase 3[18,19]. Although the underlying mechanisms of how MSCs regulate tumor cells are still unclear, there is no doubt that MSC-derived exosomes play a key role in the interaction between MSCs and tumor cells[20].

Exosomes, a distinct population of extracellular vesicles, are vital for cell-to-cell communication[21]. Exosomes can be derived from mesenchymal cells, immune cells, and tumor cells and the effects of exosomes of different sources are distinct[22]. MSC-derived exosomes show many similar effects with MSCs, and have also been seen as an important component of the tumor microenvironment[13,23]. More importantly, compared to MSCs, MSC-derived exosomes are safer and show better penetrability, biocompatibility, and stability during the interaction with tumor cells[24,25]. In recent years, the functions of MSC-derived exosomes in GI treatment have been studied with *in vitro* experiments and animal models[21]. However, a systematic review is rare. This paper summarizes the features of exosomes and the effects of MSC-derived exosomes on GI cancers. Besides, the therapeutic potential of MSC-derived exosomes on GI malignancies is highlighted and future research opportunities related to MSC-derived exosomes are also proposed.

MESENCHYMAL STEM CELLS

MSCs, with anticancer, angiogenic, anti-apoptotic, and multi-differentiation capacity, have been commonly used in oncotherapy, and tissue regeneration and restoration[6,26]. Based on the sources, MSCs can be divided into bone marrow-derived MSCs,

embryo-derived MSCs, human umbilical cord-derived MSCs, adipose tissue-derived MSCs, dental MSCs, and menstrual blood-derived MSCs[27-29]. It has also been shown that MSCs are key mediators of inflammation and the tumor microenvironment [30]. Based on these discoveries, a new clinical treatment strategy called cell therapy has been developed, which works *via* transplanting MSCs into human bodies to treat related diseases. At present, cell therapy based on MSCs is still in the clinical trial phase. Although MSC transplantation has shown huge potential in clinical application, more and more side effects and limitations have been found. For example, it has been proposed that MSC transplantation could increase the risk of tumorigenicity and cell death[31]. Besides, MSCs are limited by the lung barrier[32]. To solve these problems, researchers have proposed to replace MSCs with MSC-derived exosomes for cell therapies, because these exosomes show many similar functions with MSCs, are safer and more stable, and can be used as a vehicle to deliver anti-tumor drugs and bioactive factors[33]. In addition, it has been discovered that the MSC-derived exosomes could regulate tumor progression *via* changing the microenvironment of tumors[34].

EXOSOMES

Exosomes, cell-derived membranous structures, originate from the invagination of the endosomal system or segregation of the plasma membrane[35]. Abundant biomolecules such as biomarker proteins, regulatory RNA and DNA, functional cytokines, growth factors, *etc.*[36,37], are included in exosomes. To date, it has been discovered that MSC-derived exosomes contain more than 300 miRNAs and at least 730 proteins[38,39]. The sizes of exosomes are from 50 nm to 200 nm, which play a central role in cell-to-cell signaling networks[36,40]. One study has reported that exosomes are capable of regulating the pathway of downstream signals of recipient cells *via* releasing a variety of biomolecules and transporting the genetic material to downstream cells[28]. Interestingly, exosomes can play a dual role in tumorigenesis, both anti-tumor and pro-tumor, which may be because the exosomes can be derived from different tissues[41]. For instance, one study has proposed that normal tissue MSC-derived exosomes are capable of suppressing tumor development through blocking carcinogenic reprogramming signaling pathways. In contrast, tumor cell-derived exosomes can drive recipient cells to establish malignancy, resulting in tumorigenesis[42]. The main sources of exosomes include MSCs, immune cells, tumor cells, *etc.*[22]. MSC-derived exosomes have been commonly used in the studies of cancer development. Yang *et al*[43] have proposed that MSC-derived exosomes could promote tumor growth through secreting matrix metalloproteinase-2 (MMP-2) or MMP-2 enzyme to alter the tumor microenvironment and cellular functionalities. It has also been found that MSC-derived exosomes are capable of supporting tumor growth *via* transporting tumor-supportive factors such as proteins, miRNA, and metabolites to recipient tumor cells[44]. Besides, MSC-derived exosomes can suppress tumor growth by carrying tumor-inhibiting factors into tumor cells and decreasing the expression of vascular endothelial growth factor (VEGF)[45].

Because of the special properties and biological functions, exosomes have been used as natural nanocarriers to transport drugs and specific factors to tumor sites[46]. For example, one study has indicated that MSC-derived exosomes could reach a higher cell-target specificity by delivering paclitaxel (PTX) to tumor sites[47]. In addition, the glioma-associated MSC-derived exosomes could deliver miR-1587 to recipient glioma stem-like cells, increasing the proliferation and aggressiveness of glioblastoma through down-regulating the expression of the tumor-suppressor NCOR1[48]. Furthermore, it has also been demonstrated that MSC-derived exosomes could improve anti-cancer therapeutic efficacy through regulating immune response and reversing the chemoresistance[49,50]. The following section describes the effect of MSC-derived exosomes on gastric cancer (GC), hepatoma, pancreatic cancer (PC), and colorectal cancer (CRC) (Table 1).

MSC-DERIVED EXOSOMES FOR GC

GC is the fourth most common malignant neoplasm and the third prominent cause of cancer death globally[51]. Despite routine gastroscopy increasing the rate of early diagnosis, the 5-year survival rate of GC patients is still less than 30%[52]. In the current treatments for GC, perioperative or adjuvant chemotherapy can significantly

Table 1 Effect of mesenchymal stem cells-derived exosomes on gastrointestinal cancer

Tumor type	Exosomes source	Cell lines	Function	Mechanism	Ref.
Gastric cancer	hUCMSCs	HGC-27; MGC-803; SGC-7901	Conferring tumor chemoresistance	(1) Upregulating the expression of multi-drug resistance-associated genes and proteins; (2) Activating calcium/calmodulin-dependent protein kinases (CaMKs) and Raf/MEK/ERK pathway; and (3) Enhancing the functionality of P-gp/MDR	[13]
	hBMSCs	Animal model	Promoting tumor development	(1) Activating ERK1/2 and p38 MAPK pathways; and (2) Enhancing the expression of VEGF	[55]
	hBMSCs	SGC-7901	No effect	NA	[55]
	hUCMSCs	HGC-27	Promoting tumor development	(1) Activating the Akt signal pathway; (2) Inducing the epithelial-mesenchymal transition (EMT); and (3) Enhancing the tumorigenicity and stemness	[61]
	hBMSCs	SGC-7901	Promoting tumor development	Secreting miR-221 to activate Hedgehog signaling pathway	[64]
	GC-MSCs	HGC-27	Promoting tumor development	Increasing the expression of miR-214, miR-221, and miR-222	[66]
	mBMSCs	MFC	Promoting tumor development	Delivering UBR2 to activate Wnt/β-catenin signaling pathway	[69]
Liver cancer	hBMSCs	HepG-2	Inhibiting tumor development	(1) Blocking the cell cycle progression; and (2) Inducing tumor cells apoptosis	[93]
	AMSCs	HepG-2	Inhibiting tumor development and increasing tumor chemosensitivity	Secreting miR-122 to improve chemosensitivity of HepG2 HCC cells and inhibiting tumor development	[83]
	AMSCs	Huh-7; SMMC-7721	Increasing tumor chemosensitivity	Delivering miR-199a-3p to improve liver cancer cell line chemosensitivity	[87]
	mBMSCs	Animal model	Inhibiting tumor development	(1) Promoting tumor cells apoptosis; and (2) Inhibiting angiogenetic activity, metastasis, and invasiveness	[96]
	AMSCs	Animal model	Inhibiting tumor development	Upregulating local and systemic NK cells	[95]
	AMSCs	Huh-7; SMMC-7721	Increasing tumor chemosensitivity	Delivering miR-199a-3p to tumor sites	[87]
Pancreatic cancer	hBMSCs	BxPC-3; PANC-1	Inhibiting tumor development	Secreting miR-1231 to suppress tumor development	[107]
	hBMSCs	PANC-1	Inhibiting tumor development and promoting tumor cells apoptosis	Downregulating the expression of a disintegrin and a metalloproteinase-9 (ADAM9)	[108]
	mBMSCs	AsPC-1; PANC-1	Inhibiting tumor development and promoting tumor cells apoptosis	Delivering miR-124 to regulate the expression of EZH2	[109]
	mBMSCs	CFPAC-1	Inhibiting tumor development	Delivering anticancer agents	[47]
	Normal fibroblast-like MSCs	PANC-1	Inhibiting tumor development	Delivering short interfering RNA or short hairpin RNA to target oncogenic KRAS	[105]
	BMSCs	MiaPaca-2	Inhibiting tumor development	Loading PTX and gemcitabine monophosphate (GEMP) to pancreatic cancer	[100]
	BMSCs	Tumor model	Enhancing tumor immunotherapy	Constructing a dual delivery biosystem to achieve the combined therapy	[106]
Colorectal cancer	hBMSCs	SW-480	Promoting tumor development	Activating ERK1/2, p38, and JNK pathways	[55]
	BMSCs	Caco-2; SW-480; SW-620; LoVo; HT-29	Inhibiting tumor development and promoting tumor cells apoptosis	Upregulating the expression of miR-16-5p to downregulate integrin α2 (ITGA2)	[124]
	BMSCs	DLD-1; HCT-	Inhibiting tumor	Secreting miR-4461 to downregulate the expression of COPB2	[125]

	116; SW-480	development		
BMSCs	SW-1116; Caco-2	Inhibiting tumor development and promoting CSCs phenotype	Secreting miR-142-3p to decrease the expression of Numb. (1) Increasing the expression of Notch target genes; and (2) Secreting miR-142-3p to target CD133 and Lgr5	[126] [127]
hUCMSCs	HT-29; DLD-1	Inhibiting tumor development	(1) Downregulating the expression of Integrin alpha6 (ITGA6); and (2) Inhibiting the activity of transforming growth factor-beta1 (TGF-β1) signaling pathway	[128]
mBMSCs	C-26; MCF-7	Inhibiting tumor development	Loading doxorubicin (DOX) to tumor cells	[132]

MSCs: Mesenchymal stem cells; hBMSCs: Human bone marrow-derived mesenchymal stem cells; AMSC: Adipose-derived mesenchymal stem cells; mBMSCs: Murine bone marrow-derived mesenchymal stem cells; hUCMSCs: Human umbilical cord mesenchymal stem cells; GC-MSCs: Gastric cancer tissue-derived mesenchymal stem cells; MFC: Murine foregastric carcinoma; CSCs: Cancer stem cells; NA: Not available.

improve the therapeutic effect on advanced GC[53]. However, chemoresistance is one of the major obstacles[54]. One recent study has reported that human umbilical cord MSC-derived exosomes could confer chemoresistance to GC cells (HGC-27, MGC-803, and SGC-7901) through upregulating the expression of multi-drug resistance genes and proteins, activating calcium/calmodulin-dependent protein kinases and the Raf/MEK/ERK pathway, and enhancing the functionality of P-gp/MDR. In this way, GC cells are protected from chemotherapy-induced apoptosis[13]. In other words, the efficacy of chemotherapy in GC treatment can be improved by targeting the interaction between MSC-derived exosomes and tumor cells. For example, chemoresistance in GC can be overcome by blocking the CaM-Ks/Raf/MEK/ERK pathway. In conclusion, the therapeutic potential and efficacy of GC treatment can be improved based on the effects of MSC-derived exosomes on drug resistance.

The effect of MSC-derived exosomes on GC development remains controversial. In a mouse model experiment, researchers have observed that human bone barrow-derived MSCs (hBMSCs)-derived exosomes could promote the growth of SGC-7901 gastric tumor cells[55]. Further studies have indicated that MSC-derived exosomes are capable of promoting the incidence and growth of tumors *via* activating angiogenesis and facilitating tumor cell proliferation *in vivo*[56]. After the co-implantation with hBMSC-derived exosomes *in vivo*, MSC-derived exosomes show a tumor-promoting effect in these rat models, and significant up-expression of Bcl-2, phosphorylated ERK1/2, α-smooth muscle actin (a-SMA), CXCR4, VEGF, and MDM2 mRNA, all of which are very essential for tumor growth, metastasis, and angiogenesis, has been detected in the tumor microenvironment[55]. In contrast, it has been discovered that hBMSC-exosomes do not affect SGC-7901 cell proliferation *in vitro*, suggesting that the effect of MSC-derived exosomes on the incidence and growth of tumor is exerted through indirect mechanisms[55]. To further illustrate the mechanism of how hBMSC-exosomes affect tumor growth, researchers have examined the expression levels of VEGF and CXCR4 *in vivo*. They discovered that hBMSC-exosomes could promote tumor growth *via* activating the ERK1/2 and p38 MAPK pathways, and therefore the expression of VEGF is upregulated, which, in turn, activates tumor angiogenesis[55-59]. Previous studies have also shown MSC-derived exosomes could increase the expression of octamer-binding transcription factor 4, ex deter mining region Y-box 2, and Lin28B, and therefore promote the formation of tumor blood vessels and potentiate gastric tumor growth[55,60,61]. Further studies have discovered that hUCMSC-derived exosomes can promote HGC-27 gastric tumor cell invasion and metastasis through increasing the expression of mesenchymal indicators, activating the Akt signaling pathway, and decreasing the expression of epithelial indicators, and therefore the epithelial-mesenchymal transition (EMT) of gastric tumor cells is induced [61]. EMT, an initial stage of tumor metastasis, can stimulate tumor cells to lose epithelial cell polarity, render mesenchymal features, infiltrate into adjacent tissues, and increase self-renewal capacity[62,63]. In addition to contributing to obtaining the EMT, hUCMSC-derived exosomes also contribute to enhancing the tumorigenicity and stemness of HGC-27 cells. After the treatment with hUCMSC-derived exosomes, the expression of Oct4, Sox2, and Lin28B is increased, all of which are stemness-relevant indicators[61].

Other studies have proposed that BMSC-derived exosomes could secret miR-221 as a pro-tumor molecule to activate the Hedgehog signaling pathway, promoting the proliferation and progression of gastric tumors[64,65]. Furthermore, the miR-221 level in the peripheral blood could also be seen as a GC diagnostic marker, and the high expression level of miR-221 is reckoned as an indicator of poor clinical prognosis of

gastric tumors[34]. Another study has found that GC tissue-derived MSCs (GC-MSCs) are capable of increasing the expression of miR-214, miR-221, and miR-222, all of which are positively correlated with the development of GC[66]. For instance, the upregulation of miR-214 can be seen as a sign of venous invasion and unfavorable outcome of GC[67]. The high expression of miR-222 is mainly associated with serosal invasion and lymph node metastasis[66] and miR-221 is mainly involved in advanced stages of node metastasis, local invasion, and lymphatic metastasis of GC[68]. Therefore, it has been reported that the tumor-promoting effects of GC-MSCs could be impaired by using a miRNA inhibitor to downregulate the expression of miR-221[66]. Based on the above discoveries, the exosomal miR-214, miR-221 and miR-222 can be used for the early diagnosis and treatment of gastric tumors in the future.

In a recent preclinical study, Mao *et al*[69] have used the p53 deficient mouse BM-MSC exosomes to deliver UBR2 into murine foregastric carcinoma cells. They found that UBR2 enriched by exosomes could promote the proliferation and migration of these tumor cells through activating the Wnt/β-catenin signaling pathway. Previous studies have demonstrated that the Wnt/β-catenin pathway plays a key role in regulating the growth and metastasis of GC cells, and the maintenance of cancer stem cells (CSCs)[70].

MSC-DERIVED EXOSOMES FOR LIVER CANCER

Liver cancer is the second leading cause of cancer-related death worldwide[71]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for more than 90% of cases[72]. Especially, the prognosis of patients with advanced HCC is poor due to the lack of an effective treatment strategy[73,74]. Currently, although many clinical treatments for HCC such as surgical techniques, conventional chemotherapy, transarterial chemoembolization, radiotherapy, targeted therapy, and liver transplantation have been applied, the 5-year survival rate of liver cancer patients is still not more than 20%[75,76]. In recent years, with more studies focusing on exosomes, it has been proposed that exosomes, especially MSC-derived exosomes, have shown substantial anticancer potential in the clinical application, especially in the treatment of HCC[37].

Chemotherapy has been regarded as the most common curative measure for HCC. However, HCC shows high resistance to conventional chemotherapeutic drugs and agents[77]. Therefore, new therapeutic approaches are needed to enhance HCC chemosensitivity. Previous studies have indicated that miR-122 plays a key role in diagnosing and prognosis of hepatoma[19]. For example, it has been proposed that the loss or downregulation of miR-122 could be reckoned as the sign of poor prognosis and metastasis of HCC[78], and is closely related hepatocarcinogenesis and HCC development[79]. On the other hand, it has been found that miR-122 could increase the sensitivity of liver cancer cells to chemotherapeutic drugs such as 5-fluorouracil (5-FU) and doxorubicin[37,80]. Recent studies have demonstrated that upregulated miR-122 could inhibit the formation and development of HCC, and increase the chemotherapeutic sensitivity of these tumor cells[81,82]. Moreover, another study also reported that adipose MSC (AMSC)-derived exosomes could secret miR-122 to improve chemosensitivity of HepG2 HCC cells[83]. MiR-199a-3p, a highly expressed miRNA in normal liver cells, can increase HCC chemosensitivity by downregulating the gene expression of *YAP1*, *CD151*, and *mTOR*[84-86]. It has been proposed that AMSC-derived exosomes could be used to deliver miR-199a-3p to improve the chemosensitivity of Huh7 and SMMC-7721 liver cancer cell lines[87]. According to the above discoveries, there is no doubt that miR-122-modified exosomes and miR-199a-modified exosomes are two effective liver cancer treatment alternatives. However, finding a safe and effective vehicle for miR-122 and miR-199a-3p delivery is a challenge and researchers have studied how to use exosomes as a biological delivery vehicle for miRNA transfer[88]. Compared to other vehicles, MSC-derived exosomes possess less immunogenicity, higher biocompatibility, and less toxicity[89]. Besides, as the most prolific producers among exosome-producing cells, MSCs are suited for the mass production of exosomes[90]. All in all, MSC-derived exosomes can be used as a new nanocarrier of miRNAs and drugs[37].

Previous studies have indicated that MSCs can both suppress and promote liver cancer progression[6]. The function of MSC-derived exosomes on HCC progression, similar to that of MSCs, is not determined[91-94]. For example, one study has reported that BM-MSC-derived exosomes can inhibit HepG2 cell growth *via* blocking the cell cycle progression and inducing apoptosis *in vitro*[93]. In another study, AMSC-derived

exosomes have been directly injected into nude mice bearing HepG2 cells, and no significant differences compared to the control group have been observed[83]. Moreover, another rat model study has reported that AMSC-derived exosomes could inhibit HCC development through upregulating local and systemic NK cells[95]. The above studies show that mechanisms of the regulation of different MSC-derived exosomes on HCC progression are distinct. Although the experimental results may be interfered by experimental models, tumor types, MSC sources, as well as exosome injection administration, there is no doubt that AMSC-derived exosomes can be effectively used to transfer miR-122 to increase HepG2 liver cancer cell chemosensitivity and inhibit HCC growth and progression, providing a new treatment strategy for HCC[83].

Alzahrani *et al*[96] have conducted a long-term model study and found that BMSC-derived exosomes could inhibit the development of diethylnitrosamine-induced HCC *in vivo*. After BM-MSC-derived exosomes being injected into established HCC, the overexpression of apoptotic genes, *Bax* and *p53*, and the downregulated antiapoptotic gene, *Bcl2*, were observed. In contrast, CSC-derived exosomes are capable of suppressing apoptosis, increasing angiogenetic activity, promoting metastasis and invasiveness, and inducing EMT.

Liver cancer is a highly angiogenic cancer, whose growth requires sufficient blood supply as nourishment. It is acknowledged that VEGF plays an important role during angiogenesis. MSC-derived exosomes can inhibit tumor angiogenesis by downregulating VEGF[45] and suppressing liver cancer cell progression. Some studies have found that MSC-derived exosomes are beneficial for acute liver injury and liver fibrosis *via* activating the proliferative and regenerative responses[97,98]. Moreover, it has been reported that tumor-derived exosomes could work with BM-MSCs to inhibit HCC cell growth through arresting these cells in the G0/G1 phase[99]. All in all, MSC-derived exosomes have shown unlimited potential in liver cancer treatment, but there is still a long way to go before clinical application.

MSC-DERIVED EXOSOMES FOR PANCREATIC CANCER

PC, especially pancreatic ductal adenocarcinoma (PDAC), is a highly fatal malignancy with a 5-year survival rate less than 6%[100]. To date, despite an increasing number of clinical treatments, surgery remains the only curative treatment for PC. However, the surgical resection rate is only approximately 20% as most patients present transforming diseases upon diagnosis, and therefore chemotherapy remains the main strategy for clinical PC treatment[101]. However, traditional chemotherapy is not effective enough due to chemotherapy resistance, abnormally abundant extracellular matrix, and extremely deficient neovascularization in the tumor microenvironment [102,103]. To overcome the pathophysiological barrier of PC, an increasing number of nanotechnology-based drug delivery strategies have been proposed.

Previous studies have shown that MSCs could regulate the tumorous microenvironment and the development of PDAC[104]. With the deepening studies, it has been discovered that MSC-derived exosomes are capable of circumventing the tumor extracellular matrix barrier, overcoming chemoresistance, and efficiently targeting and penetrating tumor cells[100,105]. Therefore, the MSC-derived exosomes can be seen as novel systems to load chemotherapeutics to target PC. For example, one study has used the MSC-derived exosomes to load PTX and gemcitabine monophosphate homing to PC, and these exosomes show more preferable penetration and superior anti-tumor efficacy than the control group both *in vivo* and *in vitro*[100]. In a recent preclinical study, Zhou *et al*[106] have used the BMSC-derived exosomes to construct a dual delivery biosystem, which is capable of carrying both oxaliplatin (OXA) and siRNA for enhancing PDAC immunotherapy. The siRNA-exosomes-OXA nanoparticles can elicit anti-tumor immunity and exert significant therapeutic effects while showing better stability and fewer side effects than traditional synthetic delivery systems. More specifically, the combined therapy of iEXO-OXA could activate innate and adaptive anti-PDAC immunity by inducing the immunogenic cell death of tumor cells, initiating dendritic cell maturation and antigen presentation, and reversing immunosuppression and recruiting antitumoral cytotoxic T lymphocytes. Based on the findings above, it can be concluded that MSC-derived exosomes can serve as a promising nanoscale drug delivery platform for PC over the long run.

In addition to functioning as a carrier for drug delivery, MSC-derived exosomes can also affect PC progression through secreting multiple miRNAs[107]. For example, the expression level of miR-1231 in exosomes derived from the peripheral blood is

correlated with the pathological stage of PC, suggesting that miR-1231 may benefit PC diagnosis. Further studies have proposed that miR-1231 is capable of inhibiting the growth and development of BxPC-3 and PANC-1 pancreatic tumor cells[107]. Based on the above discoveries, it can be concluded that BMSC-derived exosomes with a high expression level of miR-1231 can be efficiently used in anti-cancer medicines, especially medicines for PC. Wu *et al*[108] have transfected miR-126-3p into the exosomes of BMSCs and found that the exosomes could downregulate the expression of a disintegrin and a metalloproteinase-9 and promote the apoptosis while suppressing the proliferation, invasion, and metastasis of PANC-1 pancreatic tumor cells. Therefore, the miR-126-3p can be reckoned as a novel biomarker for PC treatment. In a recent study, Xu *et al*[109] have indicated that miR-124-carried BMSC-derived exosomes could inhibit the proliferation, metastasis, and invasion, and induce apoptosis of PC cells (AsPC-1 and PANC1) by regulating the expression of *EZH2*, which is a target of miR124[110]. Previous studies have also demonstrated that miR-124 serves as a tumor suppressor for many cancers, such as HR-HPV-positive cervical cancer[111], breast cancer[112], and bladder cancer[113]. The above findings suggest that MSC-derived exosomes can be considered as a potential vehicle to transport miR-124 in PC treatment.

MSC-DERIVED EXOSOMES FOR COLORECTAL CANCER

CRC ranks as the third most commonly diagnosed cancer and the most common in GI cancers[71,114]. In recent years, with the improvement of screening tests and therapeutic strategies, the 5-year survival rate of CRC in China has increased to 31% [115]. However, the incidence rates of CRC, especially those in most developing countries, increase sharply due to the lifestyle changes, growing population, and aging of the population[116]. In 2020, the new cases of CRC were more than 1.9 million in 185 countries[71]. Therefore, it is very necessary to explore more effective diagnostic and therapeutic strategies for CRC. Previous studies have pointed out different effects of MSCs on CRC. For example, it has been proposed that hBMSCs could promote the growth of the low-malignancy CRC cell line HT29, but could not affect the progression of the high-malignancy CRC cell line HCT 116[117]. With the discovery of anti-tumor and tumor homing properties, MSCs have been widely used in CRC studies. Despite that MSC therapy in CRC remains controversial due to MSCs can promote immune evasion of tumor cells in the tumor microenvironment, which might be caused by the powerful immunosuppression function of MSCs[118], the application of MSCs is still a promising strategy to ameliorate CRC. First, MSCs are capable of depressing tumor metastasis and complications[119]. For example, one study has reported that MSCs could inhibit CRC metastasis and decrease the formation of malignant ascites by suppressing VEGF expression[120]. It has also been shown that MSCs could inhibit the proliferation of colonic cancer *via* depressing the expression of proinflammatory factors, ERK, STAT3 phosphorylation, and Smad2, and blocking PI3K/AKT signaling pathway[121-123].

On the other hand, MSC-derived exosomes are also involved in CRC proliferation, migration, and invasion. For example, it has been reported that BMSC-derived exosomes are capable of overexpressing miR-16-5p to downregulate integrin α2 (ITGA2), and inhibiting the growth and progression but promoting the apoptosis of CRC cells (Caco-2, SW480, SW620, LoVo, and HT29)[124]. Therefore, miR-16-5p derived from MSC-derived exosomes can be developed as an effective therapy for CRC. Besides, Chen *et al*[125] have transfected BMSC-derived exosomes into CRC cells (DLD1, HCT116, and SW480) and found that the proliferation of these cells is inhibited and the content of miR-4461 increases significantly, indicating that exosomic miR-4461 might inhibit the growth of CRC cells. Further studies have proposed that the expression level of miR-4461 is lower in CRC cells than that in normal cells and miR-4461 is capable of downregulating the expression of coatomer protein complex subunit beta 2 (*COPB2*). Based on these discoveries, it can be concluded that miR-4461 derived from BMSCs exosomes can inhibit CRC tumorigenesis by downregulating the expression of *COPB2*, a target gene of miR-4461. In the future, miR-4461 can be applied for the diagnosis and treatment of CRC.

Li *et al*[126] have treated SW1116 and Caco2 colorectal tumor cells with miR-142-3p and found that miR-142-3p could inhibit the proliferation and invasion of CRC cells but increase the population of CSCs of colon cancer. Further studies have found that miR-142-3p promotes colon CSC-like traits by decreasing the expression of Numb while increasing the expression of Notch target genes, such as Hes1, P21, and Cyclin

D3. On the other hand, the underlying mechanism of inhibited tumor proliferation could be that miR-142-3p can target *CD133* and *Lgr5*[127].

Li *et al*[128] have transfected hUCMSC-derived exosomes containing miR-3940-5p into HT-29 and DLD-1 colorectal tumor cells and found that the exosomes suppress EMT, metastasis, progression, and invasion of these CRC cells by downregulating the expression of Integrin alpha6 (ITGA6) and inhibiting the activity of transforming growth factor-beta1 (TGF-β1) signaling pathway. Previous studies have indicated that overexpression of ITGA6 could trigger CRC progression and migration *via* upregulating transforming growth factor-beta1 (TGF-β1)[129,130].

Significant progress has been made in the development of an efficient vehicle for the delivery of anticancer agents to tumor tissue. Similar to many kinds of natural exosomes, MSC-derived exosomes also possess many distinctive characteristics such as good stability, low toxicity and immunogenicity, good biocompatibility, and long circulation[131]. Therefore, Bagheri *et al*[132] have loaded doxorubicin into MSC-derived exosomes using the electroporation method and found that MSC-derived exosomes inhibit the growth of C26 and MCF7 colon tumor cells more significantly and have proposed that MSC-derived exosomes can be used to construct a novel biomanufacturing drug delivery platform for CRC therapy. One study has proposed that MSC-derived exosomes can also be used in inflammatory bowel disease (IBD) treatment[133] as treatment with MSC-exosomes substantially mitigates IBD through inhibiting inflammatory responses, maintaining intestinal barrier integrity, and polarizing M2b macrophages.

DISCUSSION

It can be concluded from the above findings that MSC-derived exosomes have shown unlimited therapeutic potential for GI cancer treatment. The main methods for developing new treatment strategies are summarized as: (1) To use the nature contents of MSC-derived exosomes to inhibit tumor proliferation and invasion. To date, it has been shown that the main inhibitory factors are some miRNAs and proteins, but the specific mechanisms have not been found out[44,134]. Therefore, in future studies, more efforts are needed to illustrate the possible mechanism; (2) to target the interaction between MSC-derived exosomes and tumor cells. It has been shown that MSC-derived exosomes could promote the growth of some GI cell lines and increase the chemoresistance of these cell lines through upregulating the expression of the factors and proteins or activating some special signal pathways[13]. Therefore, relative receptors can be targeted and the relative pathway can be blocked to improve therapeutic effectiveness; and (3) to modify MSC-derived exosomes as a drug delivery carrier. After being modified with special anti-cancer drugs, these exosomes are capable of homing to tumor sites with less immunogenicity.

Increasing studies have demonstrated that MSC-derived exosomes could exert both anti-tumor and pro-tumor effects on GI malignancies[42-44]. The reasons why MSC-derived exosomes can play different functions in the development of GI cancers are concluded as: (1) The tumor cell lines chosen for the experimental research are different. For example, different types of CRC cells lines, such as Caco-2, SW-480, SW-620, HT-29, HCT-116, and DLD-1, are used in CRCs studies and the experimental results are different, which may be due to that different types of tumor cell lines show different invasion, metastasis, and proliferation capability[135]; (2) the sources of exosomes are different, and the contents, such as factors, signaling lipids, proteins, and miRNAs, of different types of MSC-derived exosomes are distinct. Therefore MSC-derived exosomes can crosstalk with tumor cells through different mechanisms and exert different effects on tumor development; and (3) the experimental methods and models are different. The *in vitro* and *in vivo* studies can show different and even opposite results. Besides, the tumor microenvironment and cell cultivation conditions can both influence the experimental results.

Despite that both MSCs and MSC-derived exosomes can be used in anti-cancer research, MSC-derived exosomes show many potential advantages. First, MSC transplantation may result in the transfer of mutated or damaged DNA into normal cells, and an increasing risk of a new disease[136]. Fortunately, if MSC-derived exosomes are directly transferred into the body, these problems can be effectively avoided. Second, with smaller sizes, MSC-derived exosomes can circulate and pass through various barriers, such as capillary bed and lung barriers easily. Third, with the same infusion dose, the effect of MSCs-derived exosomes can be kept for a longer time than MSCs post-transplant, which can achieve a greater circulation extent[137].

To date, despite that MSC-derived exosomes have showing substantial therapeutic potential in GI treatment, many challenges and obstacles need to be overcome. The most common obstacle is to achieve large-scale production of MSC-derived exosomes. In addition, isolating these exosomes from MSCs without modification of the cargos of these MSCs is also a big challenge. Furthermore, because the sources or donors of MSCs are different, MSC-derived exosomes show heterogeneity and even the exosomes derived from the same type of MSCs can exert opposite effects on tumor development, which might be due to the fact that these exosomes carry di□erent molecules. Therefore, before applying MSC-derived exosomes in clinical trials, researchers need to improve the methods for mass-production, isolation, and homogeneity maintenance of MSC-derived exosomes[138]. The internal living conditions of MSCs can be simulated to achieve a function-specific and large-scale production of MSC-derived exosomes. Besides, more methods for storing and recovering these MSC-derived exosomes and a potency assay for therapeutic efficacy evaluation of exosomes are needed. Based on MSC-based clinical trials, MSC-derived exosome therapies can be developed more rapidly.

CONCLUSION

This review analyzes the effects of MSCs-derived, hBMSC-derived exosomes, mBMSC-derived exosomes, hUCMSC-derived exosomes, and GC-MSC-derived exosomes on GI malignancy development. However, the reasons why different MSC-derived exosomes exert distinct effects on GI malignancies are not determined. In the future, a better understanding of the mechanisms of how MSC-derived exosomes regulate GI cancer development is needed, which will help to develop more promising treatment methods for GI cancer.

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REVIEW

Diabetes mellitus contribution to the remodeling of the tumor microenvironment in gastric cancer

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Abstract

Compelling pieces of evidence derived from both clinical and experimental research has demonstrated the crucial contribution of diabetes mellitus (DM) as a risk factor associated with increased cancer incidence and mortality in many human neoplasms, including gastric cancer (GC). DM is considered a systemic inflammatory disease and therefore, this inflammatory status may have profound effects on the tumor microenvironment (TME), particularly by driving many molecular mechanisms to generate a more aggressive TME. DM is an active driver in the modification of the behavior of many cell components of the TME as well as altering the mechanical properties of the extracellular matrix (ECM), leading to an increased ECM stiffening. Additionally, DM can alter many cellular signaling mechanisms and thus favoring tumor growth, invasion, and metastatic potential, as well as key elements in regulating cellular functions and cross-talks, such as the microRNAs network, the production, and cargo of exosomes, the metabolism of cell stroma and resistance to hypoxia. In the present review, we intend to highlight the mechanistic contributions of DM to the remodeling of TME in GC.

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Core Tip: Compelling shreds of evidence support that diabetes mellitus (DM) is a crucial risk factor in human cancers. Due to its contribution to systemic inflammation, DM can sculpture the gastric tumor microenvironment through different mechanisms, which in turn, may generate highly malignant phenotypes in gastric cancer (GC). We herein discuss the contribution of DM in the remodeling tumor microenvironment in GC, which may then leads to more aggressive tumor phenotypes.

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INTRODUCTION

At present, a compelling body of evidence suggests that diabetes mellitus (DM) patients have not only increased incidence but also worse outcomes when they develop malignant neoplasm, especially those originating from gastrointestinal (GI) organs, such as the pancreas, colon, liver, and stomach[1].

Gastric cancer (GC) is the fifth most common cancer and the fourth most common cause of cancer death globally, with a poor 5-year survival < 20% for advanced stages [2].

Strikingly, data from different epidemiological data suggest that DM and chronic hyperglycemia may even increase the risk and mortality of GC patients[3,4]. Although there is compelling clinical data supporting this association, the molecular mechanisms underlying this association are not fully understood.

DM is considered a systemic inflammatory disorder[5], which triggers a dysregulated metabolism, and is characterized by sustained hyperglycemia[6]. However, the systemic pro-inflammatory effects induced by DM are not only mediated by chronic hyperglycemia but also enhanced by insulin resistance[7]. All these features drive critical modifications in extracellular elements such as ECM and favor the dysregulation of many intracellular signaling pathways[7-9].

Furthermore, DM not only interferes in intercellular communication increasing the biogenesis of exosomes but also altering the delivery of biomolecules to recipient cells and favors a pro-angiogenic and proliferative cross-talk between stromal cells, which could act as a key element in defining the fate of tumor development in these patients [9].

A crucial role in the tumor biology of gastric carcinoma plays the complex network established between cellular and non-cellular elements that composed the tumor microenvironment (TME), which drives the cancer cell fate and plays a critical role in the initiation, progression, and immune evasion[10].

A growing body of evidence suggests that these TME remodeling mechanisms can be crucial in favoring the development of highly malignant phenotypes in DM patients who develop GC[5,11].

In the present review, we intend to highlight the different mechanisms of the contribution of DM to the remodeling of TME in GC, which leads to more aggressive tumors.

EPIDEMIOLOGICAL ASSOCIATION

DM is considered an established risk factor for either higher incidence and increased long-term all-cause mortality rates in many cancer types[12,13], especially for those

originating in the digestive tract[14,15].

Although several observational studies have demonstrated a controversial association between DM and CG[4,16] or restricted only to gender differences[17] a growing body of evidence including both meta-analyses of wide-population cohorts and case-control studies demonstrate an increased risk and mortality of GC in DM patients[3,18-21].

Furthermore, a recent study with a prospective endoscopic follow-up shows that DM is an independent risk factor for GC[3]. Noteworthy, this positive association remains significant even in patients who only present pre-diabetes and hyperglycemic events[22,23].

Additionally, several reports suggest that DM and hyperglycemia are not only associated with a higher incidence of GC but also increased mortality [21,24-26], and may even lead to drug resistance and tumor progression in GC patients[27-29].

At present, *Helicobacter pylori* (*H. pylori*) colonization is a crucial risk factor in the pathogenesis of GC. *H. pylori* infection leads to chronic inflammation of gastric mucosa and then leads to atrophy of the glands, intestinal metaplasia, and GC[30]. In 1994, *H. pylori* was classified as a Group 1 carcinogen by the International Agency for Research on Cancer[31].

Noteworthy, recent reports have not only demonstrated an association between DM and incidence of *H. pylori* infection[32-34] but also, as a higher risk of failure in eradication therapy[35-37]. Furthermore, sustained hyperglycemia influences the expressions of several *H. pylori* virulence factors, leading to promote carcinogenesis [38].

During the last decade, a growing body of evidence has shed light on the mechanisms underlying this epidemiological association (Figure 1).

THE CONTRIBUTION OF DM IN REMODELING THE TME IN GC

Changing stroma cells behavior

The TME is a complex tissue niche with a diverse repertoire of infiltrating host cells, mainly recruited by cancer cells, together with many secreted factors and components of the extracellular matrix, which profoundly influence tumor growth, and dissemination[39].

A convincing body of evidence supports that chronic inflammation caused by *H. pylori* infection is the major risk factor for the development of GC, and thus various types of cells in the gastric mucosa are exposed to an inflammatory environment for long periods. The robust inflammatory response triggered by infection, together with bacterial and host factors determines the transit from the early stages of inflammation through the development of metaplasia, dysplasia, and finally to invasive carcinoma [40].

In the GC microenvironment, the behavior of many cell types of the tumor stroma is influenced by diabetes or hyperglycemia. Noteworthy, all cellular components of tumor stroma express the receptor of advanced glycation end products (RAGE), including tumor cells, and a growing body of pieces of evidence supports the role of the RAGE/advanced glycation end products (AGEs) (RAGE/AGEs) axis on tumor growth. This important modifier of the TME will be covered in the cell signaling disturbances section.

Gastric epithelial homeostasis is maintained by long-lived stem cells surrounded by a supportive niche. Therefore, GC may arise from mutated stem cells that have been accumulating gene mutations during cell half-life, and the subsequent expansion of mutated clones[41]. In this context, the chronic inflammation induced by *H. pylori* infection may then damage gastric epithelial mucosa, followed by the recruitment of bone marrow-derived cells (BMDCs), which may then lead to tissue remodeling, transformation, and potential progression to malignancy[42].

Of note, the diabetic condition has been described to force BMDCs to express tumor necrosis factor-alpha (TNF-α) and thus they become a contributor to fuel inflammation instead of repairing the damaged gastric mucosa[43]. Tumor-associated mast cells are also part of the cell stroma in GC[44]. These infiltrating cells play crucial roles in remodeling the TME[45], by the release of large amounts of preformed and pre-activated inflammatory mediators through degranulation, and supporting tumor progression, immunosuppression, and angiogenesis[46,47].

Hyperglycemia and advanced glycation end-products are known to activate mast cells and increase the expression of proinflammatory cytokines such as TNF-α and favor the degranulation of mast cells[48].

Diabetes contribution to the remodeling of TME in gastric cancer

Changes in stroma cell behavior	ECM remodeling	Cellular signaling disturbance	Disturbance in miRNAs network	Altered exosomes production and cargo	Altered metabolism
Involved mechanisms					
Impaired cytokines production; CAFs formation of fibrotic TME; Alternative M2 polarization of TAMs; TAMCs release of pro-inflammatory mediators	ECM stiffening; Overexpression of LOX; Activation of mechanotransduction-dependent pathways	O-GlcNac modification; Cell cycle dysregulation; Activation of TGF-β pathway; Proangiogenic transcriptional profile; Hyperactivation of RAGE/AGE axis; Hyperactivation of Ins-R/IGF — R system	Overexpression of oncomiRs; Downregulation of tumor supressor miRNAs	Increased releasing rate of exosomes; Proinflammatory and proangiogenic molecular cargo	RAGE upregulation; HIF-1α dependent gene-transcription; Enhanced expression of ChREBP; Increased glycolytic-related gene transcription
Phenotypical changes					
Supporting tumor growth; Reduced anti-tumor immunity; Desmoplastic tumoral stroma	Highly invasive phenotypes; Enhanced cancer cell invasion	Cell death resistance; Supporting tumor angiogenesis; Supporting tumor cell proliferation	Support tumor proliferation; Evasion of growth suppressor mechamisms	Favoring tumor metastasis; Supporting tumor angiogenesis	Proangiogenic cancer cells; Hypoxic resistant phenotype; Tumor growth and progression

Figure 1 Diabetes mellitus can sculpture the tumor microenvironment of gastric cancer through a myriad of different molecular mechanisms ranging from dysregulation of cellular signaling pathways to marked metabolic disturbances. TME: Tumor microenvironment; ECM: Extracellular matrix; miRNAs: MicroRNAs; CAFs: Cancer-associated fibroblasts; TAMCs: Tumor-associated mast cells; LOX: Lysyl oxidase; TGF-β: Transforming growth factor β; RAGE: Receptor of advanced glycation end products; AGE: Advanced glycation end product; ChREBP: Carbohydrate-responsive element-binding protein.

Cancer-associated fibroblasts (CAFs) are prominent components of the TME, and play important roles in GC, such as tumor growth and progression, matrix remodeling, promoting angiogenesis as well as fueling inflammation[49].

CAFs are crucial cells in the production of a desmoplastic stroma, characterized by the formation of dense fibrosis and increased remodeling and deposition of ECM components. CAFs not only produce fibrillar collagens and other interstitial ECM components but also release matrix metalloproteinases[49,50].

Of note, desmoplasia is commonly found in patients with diabetes, where the hyperglycemic condition activates fibrogenic pathways, not only through direct stimulation of the synthesis of ECM components but also by triggering epithelial and endothelial cell conversion to a fibroblast-like phenotype[51].

Cancer cells can recruit and activate fibroblasts in the TME by the induction of their trans-differentiation into CAFs. Recently, the serine/threonine homeodomain-interacting protein kinase 2, has been reported as a crucial regulator of this process, and its downregulation favors tumor progression[52]. Noteworthy, hyperglycemia produces a sustained degradation of this protein[53] and thus favoring the transdifferentiating process.

CAFs play an important role in the progression of GC, by promoting migration and epithelial to mesenchymal transition (EMT) of GC cells, and EMT is fully potentiated by hyperglycemia[54].

Tumor-associated macrophages (TAMs) are crucial cells in sculpturing the TME[55,56]. Furthermore, TAMs have a prognostic significance for GC patients, when combined with the TNM staging system[57].

TAMs are crucial cells in tuning the machinery of inflammatory and host immune responses in TME. Once infiltrated, macrophages undergo a polarization process rendering distinct functional phenotypes, and where classically activated (M1) and alternatively activated (M2) macrophages represent two extreme phenotypes[55,58]. In GC, TAMs are predominantly bearing an M2 phenotype, which is associated with cancer metastasis and a worse prognosis in patients[56-59].

During diabetes, macrophages and other innate immune cells are known to have a pro-inflammatory phenotype, which is believed to contribute to the pathogenesis of various diabetic complications[60]. Noteworthy, hyperglycemia acts in synergy with hypoxia and sensitizes macrophage responses to cytokine stimuli[61], and generates particular M1/M2 cytokine profiles[62].

Recently, hyperglycemia is reported to induce an increased flux through the hexosamine biosynthetic pathway in TAMS resulting in an upregulation of O-GlcNAcylation, which in turn favors the alternative M2 polarization of TAMs and reduced anti-tumor immunity[63].

Tumor-infiltrating neutrophils are very abundant in the GC microenvironment, where they promote GC cell migration and invasion as well as the induction of EMT through the interleukin (IL)-17-mediated JAK2/STAT3 signaling activation, indicating that neutrophils may play an important role in GC metastasis[64,65].

Hyperglycemia is reported to impair granulocyte-colony stimulating factor secretion, thereby hindering the mobilization of antitumor neutrophils, which in turn, leads to increased survival of disseminated tumor cells and consequently increasing the metastatic burden[66].

Obesity is a common comorbidity of diabetes, and some authors have associated obesity with an increased risk of GC[67]. Obesity can impact the TME both locally, and systemically through many signals associated with visceral adipose tissue inflammation, as reported for adipokines, growth factors, and cytokines[68,69]. In this context, the activation of the STAT3 gastric signaling pathway, which is crucial in promoting the malignant transformation of epithelial cells[70], is induced by leptin and IL-6 in obese subjects[71,72].

Modification of extracellular matrix

ECM is known to be a complex non-cellular network composed mainly of glycosaminoglycans and fibrous proteins, such as collagens, fibronectin, elastin, and laminin, which give structural support to tissues and regulate diverse cellular functions such as survival, growth, migration, adhesion, and differentiation[73].

During these cellular-ECM interactions, a complex network of signaling pathways is activated through mechanotransduction receptors, which are capable of sensing changes in the stiffness of the ECM[74,75]. At present, ECM is considered a highly dynamic element that continuously undergoes remodeling induced by several conditions[76].

Compelling evidence support that tumor-associated ECM remodeling and stiffening, are key elements behind the TME of highly invasive phenotypes of several neoplastic cells[77-79]. Strikingly, tumor-associated ECM remodeling and the subsequent stiffening play a critical role in the behavior of cancer cells in the TME[80], and thus, supporting cancer cell survival, progression, and metastatic invasion[81].

Fibronectin and type I collagen are the most common and abundant fibrillar ECM proteins found in cancer-associated ECM[82,83]. Their increase is a result of excessive fibrotic remodeling, also referred to as desmoplasia, which is largely mediated by alpha-smooth muscle actin-expressing myofibroblasts[83,84].

Most ECM fibrous proteins are long-live potential targets for the higher rate of AGEs formation observed in DM and chronic hyperglycemic-state[85]. AGEs cross-links of load-bearing protein lead to ECM stiffening which favors not only tumor cell survival, but also high rates of proliferation, and metastatic cancer cell interaction with the endothelium[86,87].

Furthermore, the chronic hyperglycemia state not only mediates mechanical changes in ECM but also can generate a reservoir of AGEs with the potential to trigger a multitude of RAGE-dependent mechanisms[85].

In addition, *in vivo* studies support that these posttranslational modifications of ECM produced during hyperglycemia also favor cancer cell invasion by activation of mechanotransduction-dependent epidermal growth factor receptor (EGFR) signaling pathway[88,89]. The interplay between highly glycated-ECM and increased EGFR activity could be a key element in the enhanced cancer cell invasion in DM patients.

In this context, lysyl oxidase (LOX) plays a central role in modulating the formation of molecular cross-linkages of ECM components[90]. LOX is known to play a significant role in the GC microenvironment[91]. Interestingly, the DM milieu favors the overexpression of LOX[92], which is associated with increased ECM modifications and high invasion activity of GC in DM patients[93,94].

Cellular signaling disturbances

One of the earliest pieces of evidence supporting that DM represents an active landscape for cellular signaling disturbances, coming either from the complex network of mechanisms underlying diabetes complications and the altered insulin sensitivity observed in obesity and type 2 diabetes[95,96].

At present, a growing body of evidence supports that the hyperglycemic condition can activate different signaling mechanisms, such as the polyol pathway, the advanced-glycation end-product formation, and the subsequent activation of the

RAGE/AGEs axis, as well as the activation of Protein Kinase C and hexosamine pathway. All these activated pathways may then lead to the over-expression of reactive oxygen species (ROS), activation of the transcriptional factors nuclear factor-κappa beta, and consequently the increased production of proinflammatory mediators, the activation of leukocytes, as well as increased apoptosis, and a desmoplastic reaction[97,98].

These pro-inflammatory signaling pathways have supported the new concept called meta-inflammation, which is characterized by a low-grade systemic and chronic inflammation and is associated with the pathogenesis of diabetic complications[99,100].

Hyperglycemia results in calpain-1 upregulation in the mitochondria, which in turn leads to a reduction in ATP synthase activity and increased mitochondrial ROS formation[101]. Mitochondrial ROS is crucial for the stabilization of hypoxia-inducible transcription and thus leading to an activation of a transcriptional profile supporting tumor angiogenesis[102]. Additionally, ROS is a well-known driver of myofibroblast differentiation, through the activation of the TGF-B pathway[103], and myofibroblasts are recognized as a major source of the CAFs[104].

Chronic hyperglycemia is the hallmark of DM. This condition leads to an accelerated formation of AGEs, a heterogeneous group of compounds resulting from the non-enzymatic reaction of reducing sugars with the free amino group of proteins lipids and nucleic acids[105].

The high rate of AGEs formation in DM patients favors the overexpression of RAGE, and the hyperactivation of the RAGE/AGE axis[98,106,107]. This receptor is involved not only in the adhesion of H. pylori to gastric epithelial cells but also in the inflammatory response to infection[108].

The activation of this signaling pathway is an important contributor to inflammation-related tumorigenesis through different signaling mechanisms, including the resistance to apoptotic insults and hypoxia, interfering with antitumor immunity, stimulating angiogenesis, and supporting invasiveness[109].

Interestingly, hyperglycemia can disturb cell cycle regulation not only in normal cells[110] but also in cancer cells[111]. High levels of glucose and insulin are reported to enhance cyclin D, cyclin-dependent kinase 4 (Cdk4), and Cdk2 expression and suppress cyclin-dependent kinase inhibitors p21 and p15/16[112].

On the other hand, the protein O-linked β-N-acetylglucosamine (O-GlcNAc) modification is a dynamic post-translational modification affecting a wide variety of proteins involved in cell cycle regulation, and this modification is considered as a major contributor to the deleterious effects of hyperglycemia[113]. This post-translational modification relies on the addition of a single N-acetyl-glucosamine molecule to the OH residues of serine or threonine by the action of the O-GlcNAc-transferase, and where some oncogenic factors, such as p53, Myc, and β-catenin, as well as other cell cycle regulators are O-GlcNAcylated[114,115] and is increased in many human neoplasias, including GC[116,117].

A growing body of evidence supports the role of the Wnt/β-catenin pathway in the development, progression, and metastasis of GC[118,119]. High glucose levels can produce profound effects on Wnt/β-catenin signaling in cancer cells, leading to an increased expression of WNT target genes[120] by either a sustained increment of the p300 acetyltransferase activity or decreased sirtuin 1 deacetylase activity. In this way, hyperglycemia renders high levels of β-catenin acetylation, which in turn, allows nuclear accumulation and transcriptional activation of Wnt-target genes[121].

An overactive TGF-β1 signaling pathway has been reported in diabetes patients [122] and its role as a critical profibrotic factor in the progression of chronic kidney disease in diabetes is widely documented[27,123]. The role of TGF-β in the biology of GI cancers has been extensively studied showing crucial roles in regulating processes such as tumor progression, evasion of growth suppressors, and resistance to cell death, angiogenesis, invasion, and metastasis[124].

TGF-β1 is overexpressed in GCs and the stromal tissues surrounding the cancer cells [125,126]. The main source of TGF-β1 is stromal cells, such as fibroblasts, lymphocytes, and macrophages[127], and therefore, the coexistence of the burden due to diabetes reinforces its impact on the TME.

Another key element in the signaling network in the gastric TME is the EGFR family, which consists of four related receptor tyrosine kinases (ErbB1 to ErbB4)[128], and all members of the family are expressed in gastric tumors[129].

On the other hand, diabetic kidney disease is a common microvascular complication of DM and the leading cause of end-stage renal disease[130]. Activation of the EGFR signaling pathway is linked to the onset and progression of renal damage in DM, by promoting cell proliferation, inflammatory processes, and ECM modification[131].

Interestingly, these signaling pathways can be activated by several ligands, but also by other biological mediators such as ROS, TGF-β, and PKC, all of which are upregulated in DM[122,132,133].

The COX-2/PGE2 signaling pathway plays a critical role in the inflammatory nature of gastric tumors. COX-2 is upregulated in GC and its precursor lesions, and it provides valuable clinical information as a prognostic factor[134]. Of note, the high levels of COX-2 expression are an earlier event reported during the *H. pylori* infection of gastric mucosa[135].

Many PGE2-mediated mechanisms supporting tumor growth, new vessel formation, and enhancing metastasis have been described[136]. Additionally, COX-2/PGE2-mediated signaling pathways are key contributors to many diabetes complications[137,138].

Noteworthy, the activation of the RAGE/AGEs axis, which is highly expressed in diabetic tissues and cells, can significantly increase both COX-2 messenger RNA and protein expression, together with a rise in PGE2 Levels[139].

Hyperinsulinemia is strongly associated with type 2 diabetes and it has been recently postulated to be a risk factor for GC[140] Emerging data suggest that insulin may be a crucial regulator in some human neoplasias, including GC[141-145].

In addition, hyperinsulinemia also increases the hepatic production and systemic bioavailability of IGF1 but also reduces the hepatic protein production of the insulin-like growth factor binding proteins 1 (IGFBP-1) and 2 (IGFBP-2). These two coordinated actions may, in turn, hyperactivate the Ins-R/IGF1-R system, and thus triggering their proliferative and anti-apoptotic programs in cancer cells[142]. Additionally, the activation of the RAGE/AGEs axis also upregulates the expression of both IGF1 mRNA and protein levels[143].

Disturbances in microRNAs network

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which act as posttranscriptional regulators of gene expression[146], and are involved in several cellular activities such as cell growth, differentiation, development, and apoptosis in many cancer types[147]. Notably, recent research has demonstrated that dysregulation in the miRNAs network has crucial consequences in the cellular behavior of neoplastic cells [147], by modulating multiple signaling pathways, especially within the gastric TME [148].

Noteworthy, hyperglycemia, and hyperinsulinemia in DM patients are two conditions that induce major changes in miRNA expression profile, especially those that are involved in gastric carcinogenesis[149].

DM patients have a particularly pro-tumoral miRNA profile, characterized by downregulation of tumor suppressor miRNAs such miR-497, miR-495p, and miR-203, which can inhibit tumor cell proliferation and migration[150,151], and its decreased expression has been associated with poor prognosis in GC patients[152,153].

In addition, the expression of some members of the family of Let-7 miRNAs, which are known by their roles in regulating oncogenes and controlling cellular differentiation and apoptosis[154], is often downregulated in several cancers, and thus derepressing some relevant oncogenic targets in GC, such as K-ras, and c-Myc[155]. Noteworthy, Let-7 miRNAs are downregulated under DM conditions not only by hyperglycemia but also by insulin resistance[151].

Conversely, a diabetogenic milieu and chronic hyperglycemia favor the overexpression of some oncomiRs, such as miR-17-5p[150,156]. Furthermore, recent research support that increased stiffness of ECM significantly induces the expression of miR-17-5p and thus rendering a loop towards the support of tumor growth and invasion[157].

Altered exosomes production and cargo

Exosomes are submicron-sized extracellular vesicles that are involved in cell-to-cell and organ-to-organ communication[158]. Recently, exosomes are involved in the pathogenesis of various disorders, including inflammatory diseases and cancer[159].

In addition, these small vesicles are now emerging as important modulators of the interchange of bioactive molecules within the TME[148].

At present, a growing body of evidence supports that either chronic hyperglycemic or hyperinsulinemic state alters not only the molecular cargo of exosomes but also their production in DM patients. These changes consequently induce critical changes in cellular function that enhance the cross-talk communication between neoplastic and non-neoplastic stromal cells[9,160].

Noteworthy, recent studies suggest that exosome release from individuals with diabetes expresses a skewed profile of pro-inflammatory molecular cargo[160,161], which influences the neoplastic transformation and favors cancer cell dissemination,

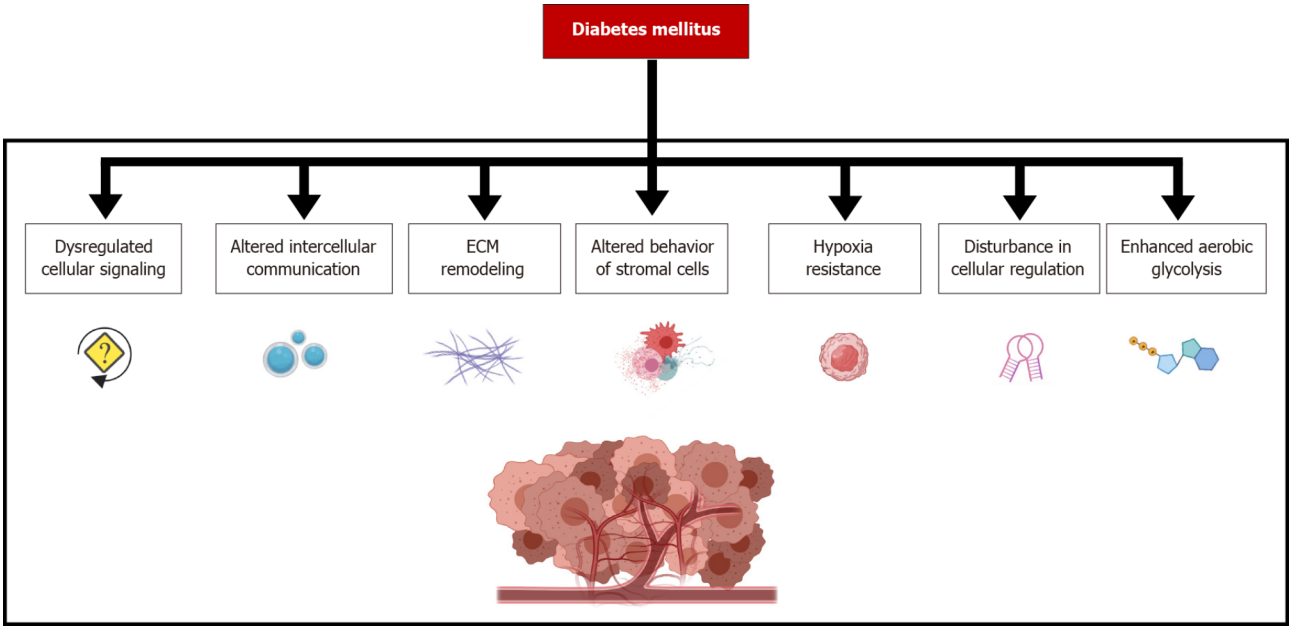

Figure 2 The molecular mechanisms involved in the contribution of diabetes mellitus to the remodeling of gastric cancer microenvironment determine crucial phenotypical changes not only on tumor cells but also in many other infiltrating cells. All changes may then result in a supporting tumor-growth niche that favors angiogenesis, invasion, and metastasis, as well as interference with anti-tumor immunity and thus generating more aggressive tumor phenotypes. ECM: Extracellular matrix.

especially in gastric tumors[162].

In addition, the altered exosomes-profile in a hyperglycemic milieu drives an increased expression of pro-angiogenic factors such as VEGF and HIF-1[160,161], which give rise to an increased intercellular cross-talk between endothelial cells, and subsequently leading to a highly angiogenic gastric microenvironment phenotype, thus promoting cancer growth and progression[163].

Besides the alterations in the molecular cargo of exosomes in the DM milieu, the increased release rate of exosomes due to hyperinsulinemia could enhance the exosome-dependent molecular transfer associated with the peritoneal dissemination of GC[160,161,164], and thus affecting the prognosis of DM patients who develop GC [165].

Altered metabolism

The Warburg effect refers to the enhanced glucose uptake and lactate production observed in cancer cells, even in the presence of oxygen and fully functioning mitochondria, also known as aerobic glycolysis[166]. Aerobic glycolysis provides glycolytic intermediates, which function as important precursors required for the synthesis of carbohydrates, fats, and proteins by cancer cells[167].

In the context of hyperglycemia, all these requirements are covered; however, hyperglycemia also promotes glycolysis by inducing the expression of glycolytic-related genes[168-170]. However, the metabolic effects of hyperglycemia on cancer cells can go further than the Warburg effect, considering that the activation of some oncogenes can subsequently proceed to an increase in ATP production[171].

Hyperglycemia also enhances the expression of the carbohydrate-responsive element-binding protein (ChREBP) in cancer cells, a well-known promoter of lipogenesis[172]. This is particularly interesting considering many tumor cells produce *de novo* almost the total of the monounsaturated and saturated fatty acids required, which are used in many cellular events crucial for tumor growth and progression[173].

In tumor cells, high glucose levels can promote HIF-1α expression under both normoxic and hypoxic conditions[168,174]. In the GC microenvironment, the HIF-1 complex activates the transcription of crucial target genes in conferring the adaptation to the hypoxic milieu and its expression correlates with an aggressive tumor phenotype and a poor prognosis[175].

In summary, DM contributes through a myriad of molecular mechanisms to the remodeling of the GC microenvironment and thus renders crucial phenotypical changes, which in turn generates tumors that are more aggressive (Figure 2).

CONCLUSION

At present, a compelling body of evidence supports the contribution of DM not only to higher cancer incidence but also to an increased mortality rate of DM patients who develops GC.

In recent years, considerable efforts in experimental research have allowed elucidating the molecular mechanisms underlying this association. In this regard, growing data suggest that the mechanical alterations induced in ECM by AGEs-mediated cross-linking and the profound changes in stromal cell behavior influenced by diabetes are pivotal elements in supporting tumor growth and progression.

Furthermore, the underlying pro-inflammatory signaling supporting the meta-inflammation in DM patients, favors several disturbances in intra- and intercellular signaling pathways, which ultimately converge in favor of the development, progression, and dissemination of GC. In addition, the chronic metabolic dysregulation observed in DM patients favors crucial changes in tumor cell metabolism that will ultimately contribute to highly hypoxic-resistant neoplastic cells as well as to a more aggressive tumoral phenotype.

Although in recent years crucial advances have been made in the knowledge of the mechanisms induced by DM in generating a TME that is supportive of tumor growth and spread, the strengthening of clinical research is essential to achieving a better understanding of the mechanisms underlying this epidemiological association.

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REVIEW

Macrophages play a role in inflammatory transformation of colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most common and fatal cancers worldwide, and it is also a typical inflammatory cancer. The function of macrophages is very important in the tissue immune microenvironment during inflammatory and carcinogenic transformation. Here, we evaluated the function and mechanism of macrophages in intestinal physiology and in different pathological stages. Furthermore, the role of macrophages in the immune microenvironment of CRC and the influence of the intestinal population and hypoxic environment on macrophage function are summarized. In addition, in the era of tumor immunotherapy, CRC currently has a limited response rate to immune checkpoint inhibitors, and we summarize potential therapeutic strategies for targeting tumor-associated macrophages.

Key Words: Colorectal cancer; Macrophages; Inflammatory transformation; Tumor microenvironment

Core Tip: In this review, we provide a comprehensive review of the research progress of macrophages in intestinal inflammation and colorectal cancer. It is of great significance to discuss the intestinal macrophages under steady-state and inflammatory conditions and tumor-associated macrophages in the immune microenvironment. With the research on macrophages in intestinal inflammation and tumor diseases, targeted macrophage therapy will benefit patients with intestinal inflammation or colorectal

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor. Changes in bowel habits and stool characteristics, abdominal discomfort, thigh lumps, intestinal obstruction, anemia, and other systemic symptoms can be related to disease progression, but no obvious clinical manifestations are present in the early stage[1]. According to the latest statistics by the American Cancer Society, the incidence and mortality of CRC rank third among all malignant tumors[2]. The latest statistics on cancer from China show that CRC has become the third most common cancer in terms of incidence, with the fifth highest mortality rate[1]. Most patients are already in a moderate or advanced stage when they are diagnosed, which imposes a great burden on their family and society. Therefore, early detection and screening, correct diagnosis of CRC, and early intervention and treatment to slow down the progression of the disease are particularly important. In recent years, an increasing number of studies have shown that macrophages play an important role in the occurrence and development of CRC. This article will review the research status of intestinal macrophages, the role and regulatory factors of tumor-associated macrophages (TAMs), and the research progress related to targeted TAM therapy to provide new ideas for the clinical diagnosis and treatment of inflammatory bowel disease and CRC.

INTESTINAL MACROPHAGES

Macrophages play an important role in intestinal inflammatory immunity, injury repair, epithelial-mesenchymal transition, and tumor development. Traditionally, macrophages differentiate from monocytes and play an immunomodulatory role[3]. Further study found that there are two main sources of intestinal macrophages: Gut-resident macrophages (gMacs) and monocytes (monocyte-derived macrophages). Resident tissue macrophages (RTMs) are derived from embryonic precursors, which accumulate in tissues before birth and are maintained by renewal in adulthood[4]. In contrast to the self-renewal and self-maintenance of Kupffer cells and microglia, whether the gMac population is maintained by contributions from mononuclear macrophages is not clear. Although traditional studies have concluded that embryonic macrophages in the intestinal tract are replaced by bone marrow-derived Ly6Chi monocytes in a microorganism-dependent manner, an experiment evaluating intestinal macrophage heterogeneity determined that the self-sustaining population of macrophages is produced by embryonic precursors and adult bone marrow-derived monocytes, which persist throughout adulthood, and that these cells settle in specific niches, including the vascular system, submucosa, muscular plexus, sites of Pan's cells, and Peyer's patches. Single-cell analysis has shown that gMacs have a unique transcriptional profile, which supports the vascular structure and permeability in the lamina propria (LP) and also regulates neuronal function and intestinal peristalsis in the LP and muscularis externa[5].

Origin and differentiation of intestinal macrophages

The gene expression profiles of macrophages in tissues and sites vary[6]. Although no study has shown that the origin changes the macrophage life span or biological functions[7], recent studies have shown that macrophage origin influences the gene expression profile[8,9]. After treatment with chlorophosphate liposomes, mice with a monocyte-derived Kupffer cell population reacted more acutely to excessive paracetamol than mice with an intact embryonic Kupffer cell population. However, this functional difference might also be attributed to tissues because the difference

disappeared after monocyte-derived Kupffer cells were placed in the liver for 60 d without an overdose of acetaminophen[10]. Epigenetic analyses show that macrophages of different cell origins are relatively similar and are mainly influenced by living tissues. There are some epigenetic differences among macrophages derived from different precursors, which may be related to the changes in the local tissue environment caused by whole body irradiation[8]. To date, it is necessary to explore the differences in epigenetics and function, not only origin, among different macrophages in detail.

Surface markers of intestinal macrophages

The unique transcriptome of tissue macrophages endows different functions to these cells and allows them to play specific biological functions in the microenvironment[11-13].

Some of the main challenges in this field are to identify intestinal macrophages and their subgroup markers and determine how to regulate these cells to meet the biological functional requirements of their living environment. In mice, F4/80 is the best and most commonly used marker to identify macrophages[14]. However, conventional dendritic cells (cDCs) and eosinophils can also express F4/80[15,16]. Intestinal macrophages highly express CD11C and MHCII, which can identify cDCs and are related to the polarization of M1 macrophages[17]. However, intestinal macrophages also express CD206 and CD163 but do not express arginine[18]. Therefore, intestinal macrophages are not suitable for M1 and M2 typing. The identification of intestinal macrophages requires a multiparameter method.

gMacs and cDCs can be distinguished by CX3CR1 and CD64 in combination with CD11C and MHCII. Compared with cDCs, gMacs highly express the chemokine receptor CX3CR1[18,19], which is mainly located in the LP of the intestine, connective tissue under the skin, intestinal wall, submucosa, and muscle[19-22]. CX3CR1 is a key regulator of macrophage function in the inflammatory state[23,24], while the CX3CR1^{+} myeloid cell-Treg axis plays a central role in maintaining intestinal homeostasis[25]. CX3CR1^{+} macrophages resident in the mucosa can recruit and activate antigen-presenting cells displaying epitopes to CD4^{+} T cells and B cells at an invasion site[26], effectively inhibiting the production of IL-17 by CD4^{+} T cells by promoting Treg activity dependent on IFN-β[27]. Although there are reports that IFN-β can inhibit the production of IL-17 in mouse and human CD4^{+} T cells, the mechanism is not clear[28,29]. The expression of CD11c differs among gMacs at different sites; CD11c^{+} gMacs are enriched in the LP, while CD11$^{c-/lo}$CX3CR1^{hi} gMacs are enriched in the muscle[29,30]. LP gMacs actively participate in host defense, maintain the integrity of the barrier, have high phagocytic activity, promote the constitutive secretion of interleukin-10 (IL-10), maintain FoxP3^{+} T cells, and protect mucous membranes[31]. The development and survival of CD64^{+} mononuclear phagocytes are highly dependent on colony-stimulating factor 1 (CSF1), while CD64-CD11c^{+} MHC II^{+} mononuclear phagocytes, which are highly dependent on the CDC-specific growth factor FLT3[32], migrate to the mesenteric lymph nodes (MLNs) and participate in the initiation of T cell responses in a CCR7-dependent manner[33,34]. An experiment evaluating Tim-4- and CD4-labeled gMacs also provided evidence for the development and heterogeneity of intestinal macrophages[35]. However, the function of these cells is not clear. Tracking CD64^{+} gMacs with YFP in hybrid offspring from Cx3cr1^{CreERT2} mice and Rosa26-LSL-YFP mice successfully identified self-sustaining gMac subsets[5].

Intestinal macrophages under steady-state and inflammatory conditions

Intestinal macrophages are the main participants in establishing and maintaining intestinal homeostasis. gMacs produce a variety of cytokines and mediators (PGE2, BMP2, WNT ligand, *etc.*) to maintain the proliferation of intestinal epithelial cells and the physiology of intestinal neurons and endothelial cells[36]. gMacs also promote the expansion of antigen-specific CD4^{+} CD25^{+} regulatory T cells by producing IL-10, prevent inflammatory reactions in the microbial environment, and support intestinal tolerance[37]. Intrinsic receptors (including LPS (CD14), fcα (CD89), fcγ (CD64, CD32, and CD16), Cr3 (CD11b/CD18), and Cr4 (CD11c/CD18)) are not expressed in gMacs [38]. gMacs also lack trigger receptors expressed on myeloid cells 1 (TREM-1)[39], which is a cell-surface molecule expressed on neutrophils and monocytes/macrophages in the peripheral blood. The activation reaction mediated by TREM-1 can increase the expression of proinflammatory mediators (such as TNF, IL-1β, and IL-6) and upregulate the levels of cell-surface molecules (CD40, CD86, and CD32)[40], leading to oxidative stress. Therefore, when intestinal macrophages play an effective scavenging role, they usually do not induce inflammation or damage intestinal homeostasis.

Monocytes and macrophages can induce cytotoxicity and proinflammatory mediators, eliminate apoptotic and damaged cells, and promote tumor progression when tissue is damaged[41,42]. The CCL2-CCR2 axis plays an important role in the migration of monocytes from the bone marrow to the peripheral blood. CCR2-deficient and CCR2-positive mice have been widely used in the study of monocytes and monocyte-derived cells in the development of tissue damage and elimination of pathogens[43,44]. During inflammation, the transportation of $CCR2^{-/-}$ monocytes to the small intestine is obviously decreased, but interestingly, the recruitment of circulating monocytes to other tissues, such as the liver and spleen, is not affected by CCR2 deficiency[45]. Silencing CCR2 also significantly reduces repaglinide tolerance, which may be related to the stability of β-catenin regulated by AKT/GSK3[46]. Recent studies have shown that the exogenous antiaging factor Klotho can inhibit the progression of CRC by inhibiting the expression of CCL2[47]. The chemokines CCL2 and CXCL12 synergistically induce M2 macrophage polarization[19]. Targeting CCL2/CCR2 without affecting transport to other tissues provides new hope for the treatment of CRC.

Under steady-state conditions, monocytes gradually differentiate into $CX3CR1^{hi}$ macrophages that express genes related to the function of tolerant macrophages. According to the expression of Ly6C and MHCII, monocytes and macrophages in the small intestine can be divided into three subgroups: $Ly6C^{+}$ $MHCII^{-}$, $Ly6C^{+}$ $MHCII^{+}$, and $Ly6C^{-}MHCII^{+}$. Based on the expression of CX3CR1, $Ly6C^{-}MHCII^{+}$ cells can be divided into $CX3CR1^{int}$ and $CX3CR1^{hi}$ cells, which can reflect the different stages of monocyte differentiation in the small intestine and colon[18,48,49]. Transcriptomic analysis also shows significant differences in gene expression among different stages. In addition to CX3CR1, the expression of CD64, CD11c, and CD206 increases with the development of $Ly6C^{+}$ MHCII monocytes into small intestinal $Ly6C^{-}MHCII^{+}$ $CX3CR1^{hi}$ macrophages. In contrast, monocytes immediately adapt to different expression patterns in a TREM-1-dependent manner after they enter the intestine in an inflammatory state. Inflammation fundamentally changes the kinetics and mode of monocyte differentiation in tissues[45]. In contrast to intestinal homeostasis, inflammatory injury results in the accumulation of $Ly6C^{+}$ monocytes in large numbers. In a study, the expression of CD64 was high, while that of CX3CR1 was always low. On the third day of inflammation, CD64+ Ly6C−$MHCII^{int}$ monocytes were divided into two subsets: $MHCII^{hi}CX3CR1^{int}$ (seen in the inflamed colon)[50]) and MHCII. In the $Ly6C^{-}MHCII^{int}$ population, the CX3CR1 expression level was slightly higher than that in the $Ly6C^{+}$ $MHCII^{-}$ and $Ly6C^{+}MHCII^{int}$ populations but lower than that in $Ly6C^{-}MHCII^{hi}$ macrophages. These cells may represent the intermediate stage of monocyte differentiation in intestinal inflammation. However, there was no differential expression of genes with enhanced expression during homeostasis in the inflammatory intestinal environment. The levels of some inflammation-related genes gradually decreased, while that of CD169 increased significantly.

Studies have shown that macrophages play a key role in the pathogenesis of IBD and that these cells are present throughout the occurrence, progression, and recovery of intestinal inflammation in both humans[18,51] and mice[52,53]. Macrophages regulate the progression of colitis by producing proinflammatory factors, such as TNF, IL-1β, IL-23, IL-6, reactive oxygen species (ROS), and NO[50]. Intestinal macrophages release IL-1β, IL-6, IL-23, and TGF-β and mediate the Th17 immune response, which plays an important role in the pathogenesis of IBD[54].

Intestinal flora and intestinal macrophages

The intestinal flora maintains the integrity of the epithelial barrier, shapes the mucosal system, and balances host defense through metabolites, its own components, and adhesion to host cells. The metabolites and bacterial components of intestinal microorganisms can send signals to immune cells and regulate intestinal immunity.

Dietary fiber can directly enter the cecum and colon, where it can be fermented and metabolized by microorganisms to produce short-chain fatty acids (SCFAs)[55]. SCFAs are the energy source of colon cells and regulate the physiological functions of intestinal epithelial cells and intestinal immune cells. SCFA-mediated histone deacetylase (HDAC) inhibition has anti-inflammatory effects. Butyrate inhibits the differentiation of dendritic cells and proinflammatory macrophage effectors from bone marrow stem cells in the LP through HDACs and reduces the immune system response to beneficial symbionts[56]. In addition, macrophages and dendritic cells develop anti-inflammatory properties under the stimulation of butyrate-mediated GPR109A signaling. $Foxp3^{+}$ Tregs and $CD4^{+}$ T cells accumulate in the colon, activating immunosuppressive mechanisms and maintaining intestinal homeostasis[57].

CX3CR1^{hi} mononuclear phagocytes do not migrate during intestinal homeostasis [58]. Symbiotic bacteria and pathogenic bacteria can regulate the host immune response by activating TLR pathways in the intestine. TLR/MyD88 signal transduction limits the transport of CX3CR1^{hi} monocytic phagocytes from the LP to the MLNs[59]. MyD88 deficiency and malnutrition lead to the migration of CX3CR1^{hi} mononuclear phagocytes to the MLNs, enhance the Th1 response to noninvasive pathogens in the MLNs, and increase IgA. TLR signaling mediated by the intestinal microbiota can regulate IL-10 production by intestinal macrophages[60]. The probiotic *Clostridium butyricum* promotes the accumulation of F4/80$^+$CD11b$^+$ CD11c macrophages in the inflamed intestinal mucosa through the TLR2/MyD88 signaling pathway and the production of IL-10 and prevents colitis in mice[52]. It has also been shown that the LPS/TLR4 pathway can trigger CCL2 and promote the accumulation of monocyte-like macrophages (MLMs)[61], which can produce IL-1β, promote Th17 cell expansion, aggravate malnutrition and inflammation, and lead to tumor progression tumor formation[62].

TUMOR-ASSOCIATED MACROPHAGES

Peripheral mononuclear cells or RTMs infiltrate near tumor masses or into tumor tissue to form TAMs, which are the main inflammatory cells in the tumor matrix[63].

Recent studies have shown that TAMs originate from RTMs and newly recruited monocytes[64]. The evolution of cells was inferred by the RNA velocity of single cells, and it was confirmed that FCN1$^+$ monocyte-like cells with tumor enrichment may be the precursors of TAMs and have a tumor-promoting transcriptional program. Transcriptional tracking of macrophages[65,66] indicated that FCN1$^+$ monocyte-like cells produce C1QC$^+$ TAMs and SPP1$^+$ TAMs from different RTMs. C1QC$^+$ TAMs may develop through IL1B$^+$ RTMs and express genes involved in phagocytosis and antigen presentation. SPP1$^+$ TAMs are linked to NLRP3$^+$ RTMs, which are rich in angiogenesis-regulating factors and have specific enrichment of rectal adenocarcinoma and metastatic liver cancer pathways, suggesting that SPP1$^+$ TAMs can promote tumor development and metastasis[67]. However, these subsets do not conform to the M1 and M2 classification of TAMs[68].

Dual role of tumor-associated macrophages

The plasticity of macrophages determines the polarization state, and the function of macrophages varies with the macrophage phenotype and tumor type[69,70]. The phenotype of polarized TAMs depends on the stage of tumor progression: In the early stage of cancer, that is, the stage of tumor elimination with local chronic inflammation in the tumor, cytokines and chemokines induce TAM polarization to the M1 type[71], which can induce an inflammatory response and phagocytosis[72]. Subsequently, M2 polarization occurs, and these cells secrete cytokines or chemokines and inhibit the antitumor immune response with changes in the tumor microenvironment (TME) and external stimuli as the tumor progresses[73].

In most human cancers, a large number of TAMs are significantly related to a poor disease prognosis, and basic research also shows that macrophages have a tumor-promoting function[74,75]. A study of 120 CRC patients with liver metastasis showed that M1 macrophages were negatively correlated with tumor metastasis, while M2 macrophages were positively correlated with lymph node and liver metastasis and the degree of tumor differentiation. M2 macrophages and the M2/M1 ratio can be used as accurate predictors of liver metastasis in CRC patients[76]. Based on an analysis of peripheral blood mononuclear cell samples from 360 CRC patients at the European Oncology Center, polarized circulating mononuclear cells can be used as biomarkers for CRC diagnosis and may be useful for follow-up and treatment evaluation[77].

M1 macrophages have high expression of major histocompatibility complex-II (MHC-II), exhibiting an effective antigen-presenting ability, and secrete proinflammatory factors and immunostimulatory cytokines, such as IL-12, IL-23, CXCL9, and CXCL10; thus, these cells function to kill bacteria and viruses, promote TH1 cell polarization and recruitment, and enhance the type 1 immune response[78]. M2 macrophages express a large number of anti-inflammatory cytokines (IL-10), immune mediators (TGF-β), prostaglandins, indoleamines, growth factors (VEGF), chemokines (CCL2, CCL17, and CCL22), and matrix metallopeptidases; thus, M2 macrophages participate in anti-inflammatory activity, tissue remodeling, wound healing, angiogenesis, and tumor development[79]. Prior research and a meta-analysis showed that M1 macrophages prevent the occurrence and development of tumors, while M2

macrophages promote tumor cell proliferation and invasion, enhance angiogenesis, and accelerate tumor growth and metastasis[80,81]. However, CRC exhibits a paradox in the function of specific groups of immune cells. A study of 205 CRC patients showed that there were a large number of infiltrating $CD163^+$ macrophages in the CRC patients with less lymphatic metastasis and a lower tumor grade, and the patients with more $CD163^+$ macrophages exhibited a survival benefit. Unexpectedly, $iNOS^+$ macrophages did not show any advantage[82]. In CRC stage III patients, high TAM levels are related to a better prognosis in patients who receive chemotherapy but not to the prognosis of patients who do not receive chemotherapy[83].

The type 1 immune response can inhibit the progression of CRC[84]. However, the molecular mechanism regulating antitumor activity and promoting tumor inflammation in CRC is still unclear. NF-κB is a key regulator of inflammation, and its activation and inhibition are controlled at a variety of regulatory levels, which can regulate the function of macrophages[85]. NF-κB p50 promotes the transcriptional program of M2 macrophages[86]. In a model of colitis-associated cancer (CAC) induced by AOM combined with DSS[87], the number of tumor lesions was significantly decreased in p50-/- mice, accompanied by increases in Th1/M1 inflammatory genes (*Il12b, Il27, Ebi3, Cxcl9, Cxcl10, Nos2*, and *Ifng*) and gene products (TNF-α, IL12, and iNOS). An analysis of CRC stage II/III patients showed that nuclear accumulation of p50 in TAMs inhibited Th1 cell/M1 macrophage-dependent antitumor reactions, which was related to the expression of M2 macrophage-related genes (*IL10, TGF-β, Ccl17,* and *Ccl22*) and increases in tumor-promoting genes (*TNF-α* and *IL23*). The expression of NF-κB p50 plays important roles in the development of colitis and CAC, but negative regulators (including p50) that only block inflammatory reactions also cause adverse reactions[88]. Type 1 proinflammatory factors (IL-12 and CXCL-10) can offset adverse reactions and restore antitumor immunity, which still needs to be evaluated in large-scale clinical studies.

Tumor microenvironment and tumor-related macrophages

The TME is composed of cellular components and noncellular components. The cellular components include cancer cells, mesenchymal cells, infiltrating immune cells, and tumor-related fibroblasts, while the noncellular components are composed of cytokines and chemokines[89]. The TME can regulate the infiltration of macrophages and promote the development of CRC through the synergistic effects of cytokines and cells.

Chemotactic factors

The chemokine family includes important signaling molecules in the TME. CCL3, CCL4, CCL5, CCL8, and CCL22 are highly expressed in various tumors and participate in the action of TAMs[90]. Recent studies have shown that CCL5 plays an important role in the development of CRC and that $CD8^+$ T cell infiltration is significantly increased in the primary colorectal tumor site of $CCL5^{-/-}$ mice[91]. *In vivo* and *in vitro* experiments show that CCL5 secreted by macrophages mediates the formation of the p65/STAT3 complex, induces upregulation of PD-L1, inhibits the $CD8^+$ T cell response, and promotes immune escape and CRC development in cancer cells. Macrophage infiltration decreases significantly after anti-CCL5 and C-15 treatment [92]. Inhibition of the CCL5-CCR5 axis is expected to be a new cancer treatment strategy[93].

CCL2 plays an important role in regulating the TME[94]. CCL22 secreted by tumor cells plays a pivotal role in immunosuppression in the tumor microenvironment by binding with $Foxp3^+$ Tregs, which highly express CCR4[95]. CCL22 was recently identified to have potential as a molecular biomarker for evaluating chemotherapy and tumor progression. Moreover, M2 macrophages transfer CCL22 to cancer cells and contribute to the development of 5-FU resistance and the epithelial-mesenchymal transition (EMT) program in CRC cells[96]. CCL22 and its receptor CCR4 can also promote the migration and invasion of gastric cancer cells[97], and M2 macrophage-derived CCL22 can enhance the migration of tumor cells in patients with liver cancer [98].

Hypoxia

Hypoxia in the TME can lead to angiogenesis, EMT, TGF-β signal transduction, and increases in tumor cell migration and metastasis[99,100]. Tissue hypoxia affects TAMs in two ways: Hypoxia can induce tumor cells and the stroma to produce monocyte-recruiting factors (CCL2, CCL5, CXCL12, CSF1, and VEGF). After monocytes are recruited into hypoxic areas, the expression of cytokine receptors is downregulated, and TAMs are trapped in the hypoxic microenvironment[101]. Furthermore, macro-

phages capture oxygen through hypoxia inducible factors (HIFs), and decreased expression of ARG1 and immunosuppressive activity occur *in vitro* in the absence of HIF1α[102]. HIF2α deficiency weakens macrophage infiltration and cytokine production[103]. TAMs secrete "vascular factors" (VEGF, Sema3A, MMP2, and MMP9) [104]. TAMs in $Nrp^{L/L}$ mice fail to enter the hypoxic tumor area, resulting in decreased angiogenesis and a weakened immunosuppressive ability, which leads to decreased vascular branches and a Th1 cell/CTL-mediated antitumor immune response[105]. Th1 cells release TAMs recruited by IFN-γ and other cytokines, initiating feed-forward circulation and enhancing antitumor immunity[106]. Reduced angiogenesis and tumor perfusion also trigger feed-forward circulation, resulting in hypoxia and recruitment of more TAMs[107]. However, when Nrp1 is absent, these TAMs will not enter the hypoxic area and thus maintain the antitumor phenotype, which may explain observations made with clinical tumor biopsies: A higher number of TAMs are not necessarily related to a poor prognosis, and the clinical correlations between TAMs in different locations and the prognosis and survival of tumor patients are different[108].

Cluster analysis showed that the degree of M2 macrophage infiltration increased obviously under hypoxia but that the degree of M1 macrophage infiltration did not increase. The levels of $CD163^+$ and $CD206^+$ macrophages in the hypoxic subgroup were much higher than those in the normoxic subgroup. Hypoxia activates the RAS signaling pathway independently of KRAS mutation and activates the IL-6/JAK/STAT3 signaling pathway by increasing the infiltration of M2 macrophages, thus regulating the progression of CRC[109]. The effect of lactic acid on macrophages under normoxic conditions is weak, but the combination of hypoxia and lactic acid can significantly promote the M2 polarization of macrophages through HIF-1, Hedgehog, and mTOR pathways[110].

Metabolism of tumor-related macrophages

Tumor metabolism plays important roles in promoting tumor growth and metastasis [111,112]. Amino acids and fatty acids provide substrates for tumor cells to produce metabolites and energy to meet the metabolic needs for proliferation and TME development. M1 macrophages mainly produce ATP through glycolysis, while M2 macrophages preferentially obtain energy through the oxidative TCA cycle coupled with oxidative phosphorylation. Compared with M1 macrophages, M2 macrophages have opposing arginine metabolism[113]. Increasing evidence shows that the lipid metabolism of immune cells, especially that of TAMs, plays important roles in the occurrence and development of tumors. In recent years, research on the process of lipid metabolism in TAMs has focused on the regulatory mechanisms of lipid metabolism-related enzymes.

In vitro and *in vivo* mouse experiments have shown that[114] the level of the lipolytic coactivator ABHD5 in CRC-associated macrophages is increased significantly, while that of monoacylcerolipase (MGLL) is decreased. ABHD5 can promote the growth of CRC by inhibiting the production of spermidine, which depends on SRM in TAMs. MGLL deficiency may lead to an increase in fatty acid glycerides[115]. The upregulation of ABHD5 may lead to a decrease in triglycerides and an increase in diglyceride[116]. A transplanted tumor model including mouse myeloid cells overexpressing ABHD5 showed that TAM ABHD5 could inhibit peritoneal and pulmonary metastasis of tumor cells (MC-38 and B-16 cells) and that macrophage ABHD5 regulated the migration and metastasis of tumor cells through the IL-1β/NF-κB/MMP pathway. The MMTV-PyMT mouse model of spontaneous breast cancer also verified that macrophage ABHD5 could inhibit lung metastasis of spontaneous breast cancer [114].

Phospholipid metabolism can affect the TME by regulating tumor-related immune cells[117-119]. The lysophosphatidic acid acyltransferase β-AGPT4 is highly expressed in CRC patients, and the survival rate of CRC patients is reduced with high expression of AGPT4. *Agpat4* knockdown can increase the expression of the proinflammatory factors IL-1β, IL-6, and TNF-α by increasing the LPA content, inducing polarization of M1 macrophages and enhancing antitumor effects[124]. An animal experiment performed with mice treated with ethoxymethane and sodium dextran sulfate showed that[120] Lipin-1, a phospholipid acid phosphatase, could promote the infiltration of $F4/80^+$ macrophages by participating in the production of CXCL1/2 (the infiltration of other immune cells, such as T cells, was not changed), upregulating the level of Nos2/iNOS and promoting dysplasia-cancer metastasis in colorectal tumors.

Targeting tumor-related macrophages

A large number of studies have proven the role of the CSF1-CSF1R axis in TAM recruitment, and inhibition of CSF1-CSF1R signaling leads to apoptosis and death in

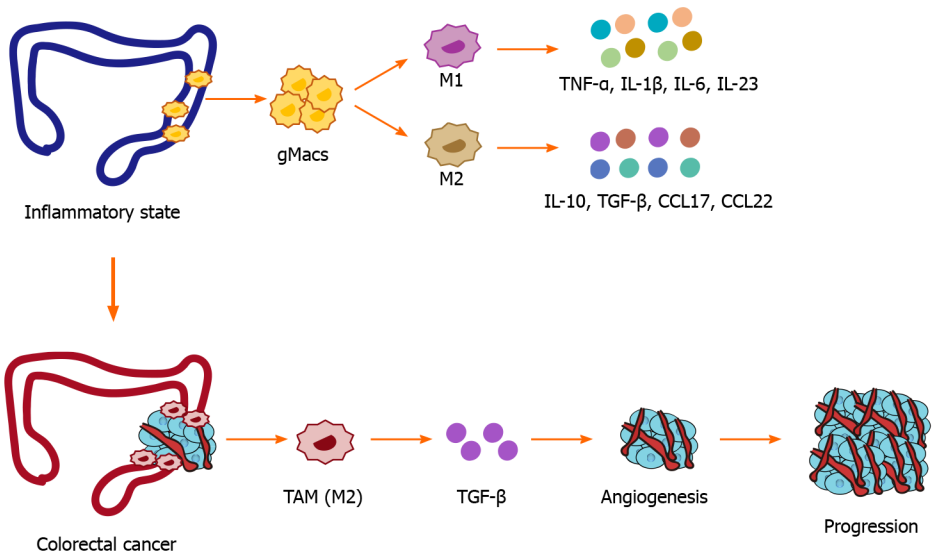

Figure 1 Etiology of macrophages in inflammatory bowel disease and colorectal cancer. gMacs: Gut-resident macrophages; TNF-α: Tumor necrosis factor; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; IL-23: Interleukin-23; IL-10: Interleukin-10; TGF-β: Transforming growth factor-beta; CCL17: C-C motif chemokine 22; CCL22: C-C motif chemokine 22.

most TAMs[121]. CSF1-CSF1R blockers can improve the efficacy of various immunotherapeutic methods, including administration of CD40 agonists or PD1 or cytotoxic T lymphocyte antigen 4 (CTLA4) antagonists and adoptive T cell therapy[122-124]. Anti-CSF1R treatment can specifically deplete C1QC$^+$ TAMs but cannot deplete the entire SPP1$^+$ macrophage population, which can promote tumor growth. This finding may explain why anti-CSFR1 antibodies are not effective as monotherapies in tumor patients[67]. CSF1R inhibition combined with radiotherapy or chemotherapy can improve the T cell response and enhance the therapeutic effect in a large number of animal models[125-127].

CXCR4-CXCL12 is an important signal transduction axis involved in TAM recruitment, which can promote tumor invasion and regeneration[128]. Monocytes secrete the chemokine CXCL12 and express the receptors CXCR4 and CXCR7, which lead to autocrine/paracrine loops; promote the differentiation of different types of macrophages; enhance the expression of CD4, CD14, and CD163; and decrease the ability to stimulate antigen-specific T lymphocyte responses[129]. The CXCR4 antagonist peptide R (PEP R) can reduce the growth of HCT116 cells and improve the therapeutic effect of conventional chemotherapy (CT) or chemoradiotherapy (RT-CT). This effect depends on the decreases in cell growth and mesenchymal stem cell transformation induced by CT/RT-CT[130]. PEPR can also target CXCR4$^+$ stromal cells and further decrease EMT and chemoresistance[131]. Combined administration of PEP R and the CXCL12 antagonist noxa-012 can improve the function of anti-PD1 antibodies in mice with CRC[132].

Macrophages in different functional states maintain cell activity through different metabolic pathways and metabolites[133]. Mammalian target of rapamycin (mTOR) signaling *via* mTORC1 and mTORC2 plays a central role in tracking nutrition, oxygen, and metabolites to guide the metabolic processes of macrophages[134]. Rapamycin (an mTORC1 inhibitor) can stimulate M1 macrophages and cause them to have an antitumor effect[135]. mTORC1 inhibitors can reduce immunosuppressive inflammation and tumor occurrence. Rad001 (a rapamycin derivative) ameliorates CRC induced by AOM/DSS in mice by limiting inflammation[136]. Signaling molecules (such as PI3Kγ, Akt, and PTEN) upstream of mTOR also participate in the polarization and remodeling of TAMs, making the mTOR pathway a potential anticancer target [137]. The expression of a PI3Kγ inhibitor (PTEN) or silencing of AKT1 can also promote the polarization of antitumor M1 macrophages[138].

Iron participates in the interaction between tumor cells and their environment[139]. Unlike M1 macrophages, M2 macrophages express iron transporters and downregulate ferritin and heme oxygenase, all of which promote iron release[140]. In addition, conditioned medium from M2 macrophages can promote the proliferation of tumor cells, while iron chelation can inhibit the proliferation of tumor cells[141]. Recent studies have shown that iron chelation can reverse the iron-processing function of M2 macrophages, switching from iron release to chelation, and block the tumor-

promoting effect of M2 macrophages[142].

CONCLUSION

Macrophages play a crucial role in the occurrence and development of CRC. As the disease progresses, macrophages tend to differentiate into different subsets that play different biological functions. The dual functions of TAMs and the regulatory effects of the TME on TAMs are worthy of further study. Subsets of macrophages cannot be simply classified according to the traditional M1 and M2 phenotypes. Single-cell technology will benefit the phenotypic classification of macrophages and provide further insights into their function (Figure 1).

The complexity of tumors highlights the advantages of combined therapeutic approaches. The clinical application of immune checkpoint inhibitors such as PD-1 and PD-L1 monoclonal antibodies provide additional evidence for tumor immunotherapy, and studies have shown that targeting tumor-associated macrophages can significantly improve the efficacy of existing immunotherapy. Future research needs to have a clear understanding of drug mechanisms of action and drug resistance mechanisms to design effective combined therapies. In addition, more clinical data are needed to clarify the relationships between macrophage infiltration or phenotype and the prognosis of patients and to guide whether TAM antagonists can be used in patients to overcome immunotherapy resistance. Despite these challenges, the use of macrophages to improve the prognosis of cancer patients still has great potential.

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MINIREVIEWS

Advancement of chimeric antigen receptor-natural killer cells targeting hepatocellular carcinoma

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Abstract

With the advance of genome engineering technology, chimeric antigen receptors (CARs)-based immunotherapy has become an emerging therapeutic strategy for tumors. Although initially designed for T cells in tumor immunotherapy, CARs have been exploited to modify the function of natural killer (NK) cells against a variety of tumors, including hepatocellular carcinoma (HCC). CAR-NK cells have the potential to sufficiently kill tumor antigen-expressing HCC cells, independent of major histocompatibility complex matching or prior priming. In this review, we summarize the recent advances in genetic engineering of CAR-NK cells against HCC and discuss the current challenges and prospects of CAR-NK cells as a revolutionary cellular immunotherapy against HCC.

Key Words: Chimeric antigen receptors; Natural killer cells; Hepatocellular carcinoma; Immunotherapy; Genome engineering

Core Tip: Chimeric antigen receptors (CARs)-based immunotherapy is an emerging therapeutic strategy for tumors. This review summarizes the recent advances in genetic engineering of CAR-natural killer (NK) cells against hepatocellular carcinoma and discuss the current challenges and prospects of CAR-NK cells as a revolutionary cellular immunotherapy against hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer, the sixth most commonly diagnosed cancer, and the third leading cause of cancer death worldwide[1,2]. The key risk factors of HCC include infection with hepatitis B virus or hepatitis C virus, alcohol consumption, non-alcoholic fatty liver disease, and aflatoxin exposure[3]. Surgery is the prevalent treatment for HCC. However, recurrence of HCC severely decreases the survival rate.

Locoregional therapies, including percutaneous ablation and intra-arterial chemotherapy, are alternative treatment approaches and rely on the grade of HCC. Unfortunately, more than 70% of patients with advanced HCC have little chance for transplant, surgery, or locoregional therapy. In the recent decade, cancer immunotherapy has become a novel emerging therapeutic strategy, improving treatment effect *via* mobilizing patients' immune systems to launch efficient anti-tumor reactions against cancer progression. Adoptive transfer of immune cells, as an immunotherapeutic approach, is expected to improve HCC prognosis by passively infusing autologous or allogenic leukocytes after *ex vivo* expansion and activation. Natural killer (NK) cells are a persistent research focus in the field of immunotherapy, due to their significant anti-tumor activity.

NK CELL BIOLOGY

NK cells, which make up 5%-15% of human blood leukocytes and 50% of hepatic innate immune cells, are the major innate lymphocytes executing anti-tumor and anti-viral immunity[4]. Based on the expression of cluster of differentiation (CD)16 and CD56, human NK cells can be divided into two subpopulations. The majority (85%-95%) of the peripheral blood NK cells are $CD56^{dim}CD16^{high}$, showing a developmentally mature phenotype, and mediate high cytotoxicity upon encountering target cells. The remaining minority of NK cells are the $CD56^{bright}CD16^{low/-}$ subset, which have an immature phenotype and lower cytotoxicity upon activation[5].

Unlike T cells, the cytotoxicity of NK cells does not rely on T cell receptor (TCR)-mediated specific antigen recognition and is thus independent of major histocompatibility complex (MHC) expression. Instead, NK cell activity is regulated by a wide spectrum of activating and inhibitory receptors (Table 1) that induce positive and negative signals to comprehensively control NK cell behavior upon contacting target cells[6]. In this regard, tumor cells that downregulate MHC expression are sensitive to NK cell cytotoxicity, owing to weaker killer cell immunoglobulin-like receptor (commonly known as KIR)-mediated inhibitory signals. Similar to cytotoxic T cells, activated NK cells express and release lytic molecules, such as perforin and granzyme B, to kill tumor cells. Additionally, NK cells secrete an array of cytokines, including interferon-g, tumor necrosis factor (TNF), and granulocyte-macrophage colony-stimulating factor, to induce the activation of T cells and innate immune cells (dendritic cells, macrophages, and neutrophils), and result in the promotion of immunity against malignant cells and the tumorigenic microenvironment[4].

CARS FOR TUMOR TREATMENT

Thanks to genome editing technology, CARs were initially designed for T cell-mediated immunotherapy against relapsed or refractory B-cell malignancies[7]. A CAR is an antibody-derived antigen-recognizing domain, linked with T cell signaling domains. When expressed by a T cell, the CAR targets a specific antigen and transduces activating signals to that T cell. A CAR consists of three domains, namely an ectodomain, a transmembrane domain, and an intracellular signaling domain[8]. The ectodomain is a single-chain variable fragment (scFv) comprising the variable regions of heavy and light chains of an antibody connected with a short linker peptide of 10-25 amino acids. The intracellular domain is conventionally derived from the TCR CD3ζ chain and/or other key accessory proteins for relaying activating signals. Three generations of CARs have been developed to date. The first-generation CARs contain CD3ζ alone. The second-generation CARs integrate an extra costimulatory domain from CD28 or 4-1BB. The third-generation CARs contain more than one costimulatory domain[9]. The latest ¾ fourth-generation ¾ CARs possess supplementary exogenous proteins, such as cytokines, to enhance the lifespan and effector function of NK cells [10].

Table 1 Activating and inhibitory receptors on human natural killer cells

	Receptor	Ligand
Activating receptors	CD16	Fc region of IgG
	NKG2D	MICA/B, ULBP1-6
	NKp30 (CD337)	B7-H6, BAT3, HCMV pp65
	NKp44 (CD336)	Viral HA and HN, PCNA, MLL5, PDGF-DD
	NKp46 (CD335)	Viral HA and HN, CFP
	NKp65	KACL
	NKp80	AICL1
	CD94-NKG2C/E/H	HLA-E (for CD94-NKG2C)
	2B4 (CD244)	CD48
	DNAM-1 (CD226)	PVR (Necl5, CD155), nectin 2 (CD112)
	Activating KIRs	HLA-A11, -Bw4
	CRTAM (CD355)	Necl-2
	Tactile (CD96)	CD155
Inhibitory receptors	Inhibitory KIRs	HLA class I
	CD94-NKG2A/B	HLA-E
	NKR-P1A	LLT1
	TIGIT	PVR (Necl5, CD155), nectin 2 (CD112)
	LIR-1 (ILT-2/CD85j/LILRB1)	HLA (α3), HCMV UL18
	IRp60 (CD300a)	PS, PE
	CEACAM1 (CD66)	CEACAM1 (CD66), TIM-3 (HAVCR2)

AICL: Activation-induced C-type lectin; BAT3: Human leukocyte antigen-B-associated transcript 3; CEACAM: Carcinoembryonic antigen-related cell adhesion molecules; CFP: Complement factor P; CRTAM: Class I-restricted T cell-associated molecule; DNAM-1: DNAX accessory molecule 1; H60: Minor histocompatibility protein 60; HA: Hemagglutinin; HAVCR2: Hepatitis A virus cellular receptor 2; HCMV: Human cytomegalovirus; HN: Hemagglutinin-neuraminidase; IgG: Immunoglobulin G; ILT-2: Immunoglobulin-like transcript 2; IRp60: Inhibitory receptor protein 60; KACL: Keratinocyte-associated C-type lectin; KIR: Killer-cell immunoglobulin-like receptor; LILRB1: Leukocyte immunoglobulin-like receptor, subfamily B member 1; LIR-1: Leukocyte immunoglobulin-like receptor 1; LLT1: Lectin-like transcript 1; MHC: Major histocompatibility complex; MIC: MHC class I chain-related protein; MLL5: Mixed lineage leukemia 5; MULT-1: Mouse UL-16- binding protein-like transcript 1; Necl: Nectin-like molecules; PCNA: Proliferating cell nuclear antigen; PDGF: Platelet-derived growth factor; PE: Phosphatidylethanolamine; PILR: Paired immunoglobulin-like type 2 receptor; PS: Phosphatidylserine; PVR: Poliovirus receptor; Rae-1: Retinoic acid early inducible 1; TIGIT: T cell immunoglobulin and immunoreceptor tyrosine-based activation motif domain; TIM-3: T cell immunoglobulin domain and mucin domain 3; ULBP: UL16 binding proteins.

Although T cells armed with CARs (hereinafter referred to as "CAR-T cells") have demonstrated unprecedented efficacy in boosting anti-tumor immunity, but they have intrinsic shortcomings[11,12]. First, CAR-T cells require prior antigen priming, which depends on the expression of MHC molecules on target cells. However, various types of tumor cells downregulate MHC expression and therefore escape from the attack of CAR-T cells. Second, before infusion of CAR-T cells, patients have to receive lymphodepleting treatment to facilitate the *in vivo* persistence of the CAR-T cells. However, a large number of CAR-T cells in the recipient increases the risk of graft *vs* host disease (commonly known as GVHD). Third, activated CAR-T cells produce enormous amounts of cytokines, including interleukin (IL)-1a, IL-2, IL-6, TNF-α, MCP-1, IL-8, IL-10 and IL-15, rendering the cytokine release syndrome (otherwise known as CRS) and neurotoxicity. Compared to T cells, NK cells overcome these disadvantages because they function in an MHC-independent manner, without triggering GVHD, and produce a distinct spectrum of cytokines.

CAR-NK CELLS

CAR constructs for NK cells

For NK cells, the CAR structure is quite similar to that of T cells. The extracellular scFv is also derived from an antibody against a tumor antigen. So far, the tumor antigens targeted by engineered scFv involve CD19 and CD20 (B-cell acute and chronic leukemia antigen)[13], human epidermal growth factor receptor 2 (commonly known as HER2; breast cancer)[14], GD2 (neuroblastoma and melanoma)[15], CD138 (multiple myeloma)[16], CD4 (T cell lymphoma)[17], and epidermal growth factor receptor (EGFR) which is overexpressed in multiple tumors[18]. Some researchers have designed CAR-NK cells targeting certain immunosuppressive cells, such as alternatively-activated macrophages and myeloid-derived suppressor cells, to enhance antitumor immunity[19,20]. Moreover, a recent endeavor has been made to create a bispecific CAR featuring an scFv that targets two antigens simultaneously. NK cells equipped with this bispecific CAR are supposed to execute cytotoxicity more efficiently, in case one antigen is weakly expressed or absent on certain tumor cells[21].

The transmembrane domain of a CAR belongs to CD8a, CD28, CD3, or ICOS, with some exceptions derived from NKG2D[22]. Recent findings suggest that the sequences and locations of the transmembrane domain should be delicately designed to achieve the best function[23]. The intracellular domain is key to the signal transduction activity and consequent NK cell activation upon the engagement of the scFv with tumor antigens. Most CAR-NK cells exploit CD3ζ as a signaling domain to transduce activating signals because the immunoreceptor tyrosine-based activation motif (also known as ITAM) of CD3ζ initiates NK cell killing[24-26]. Additional costimulatory domains derived from CD28, CD137 (4-1BB), or CD244 (2B4) are usually required for efficient killing[22,27]. In recent research, replacing CD3ζ with the intracellular region of DAP12 has resulted in a better effect on glioblastoma[28]. The most common CAR constructs used in CAR-NK cells have been summarized elsewhere[8].

Sources of CAR-NK cells

Currently, clinical-grade NK cells are manufactured on a large scale from the following sources.

NK92 cell line: The NK-92 cell line was established from a patient with large granular lymphoma in 1994. It does not attack recipients' allogeneic cells but kills a wide range of tumor cells, such as leukemia and melanoma. The cell line is easy to grow *in vitro*, making it an attractive agent for adoptive cancer immunotherapy. However, its tumorigenicity potential, deficiency in CD16 and NKp44 expression, and the requirement for lethal irradiation before adoptive transfer pose concerns about clinical safety and therapeutic efficiency[29].

Peripheral blood mononuclear cells (PBMCs): Sufficient allogeneic NK cells can be enriched from PBMCs of a patient or healthy donor, followed by *ex vivo* stimulation, expansion, and genetic engineering. PBMC-derived CAR-NK cells are predominantly $CD56^{dim}CD16^{+}$ mature NK cells with high cytotoxicity, making them especially suitable for either autologous or allogeneic transfer[8].

Umbilical cord blood (UBC): As an alternative NK cell source, UBC with identified MHC types can be obtained from UBC banks. Nonetheless, the NK cell abundance in UBC is relatively low, so it takes a long *ex vivo* expansion and activation procedure before sufficient NK cells are generated. Another issue involves UBC-derived NK cells exhibiting an immature phenotype and being less cytotoxic to target cells[30].

Hematopoietic progenitor cells (commonly known as HPCs): $CD34^{+}$ HPCs are sorted from bone marrow, embryonic stem cells, mobilized peripheral blood, or UBC. They are expanded and differentiated into NK cells *ex vivo*, under the effect of a set of cytokines. HPC-derived NK cells are mature and significantly cytotoxic to leukemia cells[31].

Induced pluripotent stem cells (iPSCs): iPSCs are derived from mature somatic cells that have been reprogrammed back into a pluripotent stem cell state. They enable the development of an unlimited source of multiple cell types needed for therapeutic purposes. Theoretically, one iPSC is competent to generate a large quantity of homogeneous CAR-NK cells. However, iPSC-derived NK cells are relatively immature and less cytotoxic, posing the challenge of inducing both phenotypically and functionally mature NK cells[8]. A recent study developed a robust and efficient manufacturing system for the differentiation and expansion of high-quality iPSC-

derived NK cells that produced inflammatory cytokines and exerted strong cytotoxicity against an array of hematologic and solid tumors[32].

CAR-NK CELLS IN HCC THERAPY

Both academia and industry have made rapid progress in developing CAR-engineered immune cells for HCC[33,34]. One of the major challenges for CAR-based immunotherapy for HCC is to target a specific, safe, and effective tumor antigen. Currently, the following tumor antigens are considered candidate targets of CAR-based immunotherapy for HCC: glypican-3 (GPC3), α-fetoprotein, epithelial cell adhesion molecule (commonly known as EpCAM), and mucin-1 (commonly known as MUC1)[35,36]. Of note, glypican-3 is a glycoprotein overexpressed on the cell surface of HCC tissues but not in healthy liver[37]. A bispecific CAR targeting GPC3 and the asialoglycoprotein receptor 1 (commonly known as ASGR1) and featuring CD3ζ and 28BB (containing CD28 and 4-1BB signaling domains) has been tested in an HCC xenograft mouse model[38].

Although CAR-NK cells have been tested in a few clinical trials against leukemia, lymphoma, and several solid tumors, to our knowledge, no clinical trials have been performed to evaluate the efficacy of CAR-NK cells in HCC treatment. Several laboratory studies of CAR-NK cells against HCC have been conducted and showed the bright prospect of CAR-NK-based HCC immunotherapy.

The first report of CAR-NK cells against HCC was published in 2018. Yu *et al*[39] developed GPC3-CAR-NK-92 cells and demonstrated the potent anti-tumor properties of these cells. The CAR construct comprised a CD8α signal peptide, a humanized GPC3-specific scFv (known as "hu9F2"), a CD8α hinge region, and a CD28 transmembrane region followed by the intracellular domains of CD28 and CD3ζ. The GPC3-CAR-NK-92 cells showed potent cytotoxicity and cytokine production upon encountering HCC cells with either high or low GPC3 expression, both *in vitro* and *in vivo*. Notably, the GPC3-CAR-NK-92 cells were cytotoxic to GFP3-negative HCC cells. Huang *et al*[40] made a further modification to GPC3-CAR-NK-92 cells by replacing the intracellular domains of CD28 and CD3ζ with the costimulatory domains of DNAM1 and 2B4. DNAM1, also known as CD226, is a costimulatory receptor in cytotoxic T cells, NK cells, and monocytes[41]. DNAM1 cross-linking results in phosphorylation of its cytoplasmic tyrosine residues and drives NK cell cytotoxicity [42]. 2B4, as above-mentioned, is known as CD244 and harbors a cytoplasmic domain containing immunoreceptor tyrosine-based switch motifs (commonly known as ITSMs) that are responsible for interacting with multiple signaling adaptors and transmitting activating signals[43]. This modification endowed GPC3-CAR-NK-92 cells with faster expansion, lower apoptosis and higher cytotoxic abilities than their original counterparts[40].

Tseng *et al*[44] reported that NK cells transduced with a CAR that targets CD147, a cell surface marker that is significantly upregulated in HCC, can effectively kill various malignant HCC cell lines *in vitro*, as well as HCC tumors in xenograft and patient-derived xenograft mouse models. In this study, the CD147-CAR contained the scFv of the anti-CD147 antibody derived from clone 5F6 with optimization, a human IgG1-CH2CH3 spacer, a transmembrane domain of CD28, the intracellular domain of CD28-4-1BB, and intracellular signaling domains of the TCR-ζ chain. The CAR-encoding retrovirus was transduced into the NK-92MI cell line, which is an IL-2-dependent NK cell line derived from PBMCs obtained from a patient with rapidly progressive non-Hodgkin's lymphoma. These CD147-CAR-NK-92MI cells were effectively activated by CD147+ HCC cell lines and demonstrated impressive cytotoxicity against the target HCC cell lines. When using primary PBMC-derived NK cells to prepare CD147-CAR-NK cells, the researchers observed efficient killing effects on susceptible target cells, including SKHep1, Huh7, and HepG2, *etc*. Furthermore, primary NK cells isolated from different zones of HCC liver tissue and engineered to express CD147-CAR can kill CD147+ HCC cell lines selectively and specifically. This study offers a valuable insight into manufacturing CD147-CAR-NK cells as either an autologous or an allogeneic off-the-shelf cell-based product.

Bouattour *et al*[45] developed c-MET-CAR-NK cells and tested the efficacy against HCC cell lines. c-MET is the product of the proto-oncogene *MET* and acts as a tumorigen in HCC, since enhanced c-MET activity initiates and contributes to the progression of HCC. The CAR construct contained an scFv of an anti-c-MET antibody fused with truncated human EGFR (referred to as huEGFRt). The intracellular domain of the CAR comprised the 4-1BB and the DAP12 cytoplasmic domain. c-MET-CAR-

encoding lentivirus was then prepared and transduced into human PBMC-derived NK cells. The resultant c-MET-CAR-NK cells remarkably killed HepG2 cells *in vitro*; however, no data were generated to demonstrate the effect of c-MET-CAR-NK cells *in vivo*. Of note, c-MET-CAR-NK cells could be potentially used to treat other solid tumors, because the upregulation of c-MET has been found in breast cancer, lung cancer, and colorectal cancer[46-48].

CHALLENGES TO BE ADDRESSED

Although CAR-NK cells have demonstrated their potency and advantages in tumor killing, technical or clinical issues remain to be addressed that will optimize CAR-NK-based immunotherapy against HCC.

First and foremost, stringent screening of HCC-specific antigens is necessary to minimize or avoid severe adverse effects due to on-target/off-tumor toxicity. On-target/off-tumor toxicity arises from the simultaneous expression of a target antigen on both tumor and healthy tissues[49]. This toxicity, sometimes lethal, can be reduced by careful design of CAR constructs that improve the recognition of tumor cells. One such approach is to affinity-tune CARs so that they detect tumor cells with a high density of surface antigens and do not react against normal cells that have low antigen densities[50]. Altering the scFv binding domain *via* mutagenesis or recombination of heavy and light chains can genetically tune the affinity of a CAR to fulfill this purpose [51,52]. The intracellular signaling domain of a CAR can also be tuned to induce a moderate activating signal to weaken CAR-mediated direct cytotoxicity.

Another issue is the promotion of CAR-NK cell expansion and persistence *in vivo*. Insufficient *in vivo* expansion of CAR-NK cells after adoptive transfer owing to unidentified factors is a particularly serious challenge. A recent clinical trial has demonstrated that incorporating IL-15 into CAR-NK cells remarkably augmented *in vivo* expansion[53].

The third issue involves the effect of CAR-NK cells on solid tumors like HCC, which remains unsatisfactory. The heterogeneity of solid tumors, the harsh tumor microenvironment that induces NK cell exhaustion, the limited infiltration from the bloodstream into tumor sites, and the immunosuppression caused by immunosuppressive cells and metabolic disturbances comprehensively inhibit CAR-NK cell function[54]. More efforts have to be made to deal with these challenges, one-by-one, to build more efficient CAR-NK cells against HCC and other solid tumors. With the advance of genetic engineering science, CAR-NK cells equipped with genetically-incorporated cytokines, antibodies, survival factors, constitutively-active signaling molecules, *etc*. will greatly promote NK cell proliferation, persistence, migration, and penetration into HCC.

CONCLUSION

CAR-NK cell therapy is an emerging immunotherapy against tumors, including HCC. Its advantage in manufacturing off-the-shelf cellular therapy products with high clinical availability and safety makes it a promising anti-HCC approach. Recent breakthroughs in genome editing techniques have potentiated the production of novel CAR-NK cells with high anti-HCC specificity and activity and low on-target/off-tumor toxicity. Innovative engineering tools including CRISPR-Cas9, zinc finger nucleases (referred to as ZFNs), transcription activator-like effector nucleases (referred to as TALENs), and meganucleases are expected to revolutionize the design and creation of CAR constructs for NK cells in the future. With the great efforts being made to enhance safety and activity through laboratory studies and clinical trials, we are confident in the eventual overcoming of the remaining challenges to CAR-NK cell therapy, bringing hope to HCC patients and their families.

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MINIREVIEWS

Current status of first-line therapy, anti-angiogenic therapy and its combinations of other agents for unresectable hepatocellular carcinoma

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Abstract

Globally, hepatocellular carcinoma (HCC) is a frequently diagnosed malignancy with rapidly increasing incidence and mortality rates. Unfortunately, many of these patients are diagnosed in the advanced stages when locoregional treatments are not appropriate. Before 2008, no effective drug treatments existed to prolong survival, until the breakthrough multi-tyrosine kinase inhibitor (TKI) sorafenib was developed. It remained the standard treatment option for advanced HCC for 10 years, with a battery of other candidate drugs in clinical trials failing to produce similar efficacy results. In 2018, the REFLECT trial introduced another multi-TKI, lenvatinib, which has non-inferior overall survival compared with sorafenib. Thus, offering patients and their treating physicians two effective treatment options. Recently, immunotherapy-based drugs, such as atezolizumab and bevacizumab, have shown promising results in patients with unresectable HCC. This review summarizes clinical trial and real-world data studies of sorafenib and lenvatinib in patients with unresectable HCC. We offer guidance on the optimal choice between the two treatments and discuss the potential of immunotherapy-based combination; when more data become available, this will likely make the choice between sorafenib and lenvatinib somewhat obsolete.

Key Words: Hepatocellular carcinoma; Immune checkpoint inhibitor; Lenvatinib; Multi-tyrosine kinase inhibitor; Sorafenib

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Core Tip: Recently, an immunotherapy-based combination of atezolizumab and bevacizumab was shown to prolong survival compared to sorafenib in unresectable hepatocellular carcinoma patients who did not receive prior therapy. In addition, the combination of lenvatinib and pembrolizumab has yielded promising results in the same patient setting. This review article summarizes the results obtained with sorafenib and lenvatinib in patients with unresectable hepatocellular carcinoma in pivotal clinical trials and real-world studies. We offer guidance on the optimal choice between sorafenib or lenvatinib in an individual patient and discuss the immunotherapy-based combination, which will likely make the choice between sorafenib and lenvatinib somewhat obsolete.

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INTRODUCTION

Currently, liver cancer ranks as the seventh most common cancer type in the world, being the fourth most common cause of cancer-related death[1]. The vast majority of these liver cancers (approximately 80%) arise from hepatocytes and are referred to as hepatocellular carcinoma (HCC)[2]. Over the last decades, the global incidence of HCC has been increasing, with a 75% incidence increase of newly diagnosed HCC cases from 1990 to 2015[3]. Risk factors for developing HCC consist of viral hepatitis, extreme alcohol intake, and non-alcoholic fatty liver disease (NAFLD)[2]. Furthermore, obesity increases the risk of developing NAFLD, and given the ever-increasing obesity epidemic in many parts of the world, the significance of NAFLD-related HCC is predicted to have profound effects in the coming years[4].

For patients with initial-stage HCC, curative therapy strategies, such as liver resection, liver transplantation, or radiofrequency ablation, can still provide long-term survival[2,5]. However, many HCC patients receive their diagnosis at progressive stages of the disease when locoregional treatment is no longer an option. When not treated, patients with advanced-stage HCC have a very poor prognosis, with a median overall survival (OS) of 9 mo and a 6-mo OS of 56.6%[6]. Until 2008, not a single effective systemic treatment option was available for these patients, an unparalleled situation in oncology. In fact, advanced HCC proved to be notoriously difficult to treat. HCC is not only a very chemo-resistant tumor type, but the constant threat of declining liver function often compromises an effective treatment. Over the last decades, improved insights into the molecular processes that initiate and promote the tumor progression in HCC have facilitated the development of novel molecular treatment modalities that specifically target these disrupted molecular pathways.

In 2008, the multi-tyrosine kinase inhibitor (TKI) sorafenib became the first effective systemic therapy for patients with advanced HCC with a preserved liver function[7]. Following its successful introduction into the treatment paradigm for advanced HCC, several other targeted drugs were tested in this setting. Unfortunately, this resulted in a decade of disappointing phase III randomized controlled trials (RCTs). Until 2017, the oral multi-TKI regorafenib reported increased survival rates in patients who received sorafenib[8]. Shortly thereafter, the multi-TKIs cabozantinib and ramucirumab were also shown to delay the disease progression and prolong the OS of HCC patients progressing on sorafenib [for ramucirumab, this benefit was limited to patients with an increased alpha-fetoprotein (AFP) concentration][9,10]. However, until very recently, not a single clinical trial was able to demonstrate a survival benefit compared to sorafenib in the first-line treatment for patients with progressive HCC. Finally, in 2018, the multi-TKI lenvatinib emerged as a feasible first-line alternative for sorafenib in these patients. In fact, results of the phase III REFLECT trial established that the multi-TKI lenvatinib was non-inferior to sorafenib as a first-line treatment for patients with advanced HCC[11]. Thus, physicians now have a choice between two equally effective multi-TKIs in the first-line treatment of these patients. In this article, we review the clinical trial data and real-world data generated with sorafenib and

lenvatinib, with a particular focus on the differences between both agents that can be used to steer the treatment choice in an individual patient.

In recent years, the growing interest in immune checkpoint inhibition as a new pillar of the cancer treatment paradigm has also spurred the evaluation of these drugs in patients with unresectable HCC. Clinical trials using these immune checkpoint inhibitors (ICIs) in monotherapy demonstrate only a moderate clinical benefit[12,13]. In contrast, RCTs evaluating combinations of a TKI and an ICI have generated more convincing results. In fact, results of the phase III IMbrave150 trial recently highlighted that atezolizumab plus bevacizumab had a significantly prolonged OS compared to sorafenib in the first-line treatment of patients with advanced HCC[14]. Furthermore, new data are emerging for other first-line ICI-TKI combinations (*e.g.*, pembrolizumab-lenvatinib). This review summarizes the results obtained from clinical trials and real-world studies of sorafenib and lenvatinib in patients with unresectable HCC. We offer guidance on the optimal choice between sorafenib or lenvatinib in an individual patient and discuss the potential of immunotherapy-based combinations, which, with more data, will likely make a choice between sorafenib and lenvatinib somewhat obsolete.

SORAFENIB: THE LONG-STANDING STANDARD

Clinical trial data

Sorafenib is an oral multi-TKI that checks and arrests several tyrosine kinases involved in tumor angiogenesis, progression, and apoptosis. It inhibits both vascular endothelial growth factor receptor and platelet-derived growth factor receptor and also targets fms-like tyrosine kinase 3, c-Kit, and several kinases involved in the mitogen activated protein kinase signaling pathway[15]. The application of sorafenib as the standard treatment choice in the first-line therapeutic management of patients with advanced HCC was based on the results of two pivotal phase III RCTs.

In the phase III SHARP trial, 602 (mainly European) patients with advanced HCC, who did not receive prior systemic treatment, were randomly assigned to receive either sorafenib (400 mg twice daily) or placebo. In order to be eligible for the trial, patients had to have an Eastern Cooperative Oncology Group performance status of ≥ 2 and have a preserved liver function (Child-Pugh class A)[7]. The median age of patients in the trial was 65 years, more than 80% had Barcelona Clinic Liver Cancer (BCLC) stage C disease, and the vast majority (97%) were rated as Child-Pugh A at baseline. The study reached its primary endpoint by proving a significant OS benefit for sorafenib compared to placebo, with a median OS of 10.7 and 7.9 mo, respectively [hazard ratio (HR) 0.69; 95% confidence interval (CI): 0.55-0.87; $P < 0.001$]. Also, in terms of the time to radiological progression, sorafenib outperformed the placebo (median: 5.5 mo *vs* 2.8 mo; HR 0.58; 95%CI: 0.45-0.74; $P < 0.001$). Objective response rates (ORR) were rare in both arms of the study, with only 2% partial responses with sorafenib compared to 1% with placebo (no complete responses were reported). However, looking at the disease control rate (DCR), a significant benefit was seen for sorafenib compared to placebo (43% *vs* 32%; $P = 0.002$)[7]. In the SHARP trial, the incidence of treatment-related adverse events (TRAEs) was reported at 80% with sorafenib compared to 52% with placebo. The most common grade 3/4 TRAEs of sorafenib were diarrhea (8% with sorafenib *vs* 2% with placebo; $P < 0.001$) and hand-foot skin reactions (8% *vs* 1%; $P < 0.001$). Despite the relatively low rate of high-grade TRAEs, the rate of therapy discontinuations due to adverse events (AEs) was high at 38%.

The second pivotal trial with sorafenib in patients with progressive HCC was conducted in the Asia-Pacific region and yielded fairly similar results[16]. In that trial, a total of 226 advanced HCC patients with a Child-Pugh A liver score were randomly assigned (2:1) to receive either sorafenib (400 mg twice daily) or placebo. Sorafenib also showed a significantly prolonged OS compared to placebo, with a median OS of 6.5 and 4.2 mo for sorafenib and placebo, respectively (HR 0.68; 95%CI: 0.50-0.93; $P = 0.014$). In addition to this, patients who received sorafenib had a significantly longer time to progression (TTP) compared to patients treated with placebo (median 2.8 mo *vs* 1.4 mo; HR 0.57; 95%CI: 0.42-0.79; $P = 0.0005$)[16].

Real-world experience

In the years following the registration of sorafenib for patients with advanced HCC, several studies were set up to evaluate the performance of sorafenib in a real-world setting. In the Italian SOFIA study, 269 advanced HCC patients were treated with

sorafenib (400 mg twice daily), resulting in a median OS of 10.5 mo (8.4 mo for patients with BCLC-C disease and 20.6 mo for BCLC-B patients)[17]. The most common grade 3/4 AEs reported in SOFIA were fatigue (25%), hand-foot skin reactions (9%), hypertension (7%), and diarrhea (6%). Similar to what was reported in SHARP, 40% of patients treated with sorafenib in the SOFIA study had to discontinue therapy due to an AE[17].

The prospective, non-interventional INSIGHT trial also evaluated the safety and efficacy of sorafenib in real-world clinical practice. In this study, including a safety set of 788 HCC patients, the rate of TRAE discontinuations was much lower than in SHARP and SOFIA, as only 15.5% of patients discontinued their therapy because of unacceptable toxicity[18]. A possible explanation for this lower rate could be the fact that this study was reported a decade after the introduction of sorafenib. Thus, it is likely that the increased experience of physicians with this agent and the increased knowledge on how to deal with its toxicity profile ultimately resulted in this lower rate of TRAE discontinuation.

Similarly, a large retrospective study from the United States reported lower rates of TRAE discontinuations with sorafenib than those reported in SHARP. In that study, published in 2017, a total of 3094 advanced HCC patients received sorafenib at the normal dose of 800 mg *per* day. Of them, only 22.4% had to discontinue their therapy for reasons of toxicity[19]. In an attempt to reduce further the TRAE drop-out, the possibility of introducing sorafenib at a lower dose (< 800 mg/d) was explored, resulting in a decreased pill burden that was less expensive. In addition to this, there were fewer treatment discontinuations due to safety/toxicity concerns (19.6%). With the standard dosing, the median OS reported in this cohort was 233 d (approximately 7-8 mo), which is in line with the SHARP trial[7,19]. Among patients who received a reduced dose of sorafenib, the median OS was shorter at 200 d. However, given the fact that patients who received the reduced dose were generally sicker than patients who were deemed to be eligible for the full dose, this comes as no surprise. When compensating for the differences in patient and disease characteristics between patients in the full and reduced dose cohort (propensity score-matched analysis), no difference in OS was found (HR 0.92; 95%CI: 0.83-1.01)[19].

Among the patients enrolled in these RCTs, many of them had a stable hepatic function with reference to Child-Pugh A disease (SHARP: 95% and Asia-Pacific study: 97%). However, in real-world settings, there are patients with hepatic dysfunction (Child-Pugh B or C), and for these, the RCTs do not provide a clear answer on the potential benefit of sorafenib. In this respect, real-world data can provide guidance to physicians. Not surprisingly, results of a French case-control study (n = 120) indicate that advanced HCC patients with a Child-Pugh A status have a better OS when they are treated with sorafenib than patients who have more advanced liver damage (Child-Pugh B), with a median OS of 13 and 4.5 mo, respectively (P = 0.0008)[20]. A similar observation was seen in the INSIGHT trial, where the median OS with sorafenib was reported as 17.6 mo for patients with a Child-Pugh A status, decreasing to 8.1 and 5.6 mo for patients with Child-Pugh B or C disease, respectively[18]. Finally, the observational GIDEON registry showed that Child-Pugh A patients have a longer OS when treated with sorafenib than patients with Child-Pugh B disease (median OS: 13.6 and 5.2 mo, respectively)[21]. However, the fact that Child-Pugh B patients do worse on sorafenib than patients with a preserved liver function should not be a reason to reserve sorafenib for patients with Child-Pugh A disease alone. In fact, GIDEON also shows that the overall safety profile and dosing strategy of sorafenib are similar across the different Child-Pugh subgroups[21]. In another prospective study by Leal *et al*[22], in a separate prospective score, specifically focusing on the use of sorafenib in Child-Pugh B patients, a median OS of 6.5 mo was reported, which was longer than historical controls for this population. In this study, sorafenib also proved to be tolerable, with a relatively low rate of TRAE discontinuations (27.7%)[22]. As such, these results highlight that selected Child-Pugh B patients may also derive benefit from treatment with sorafenib, with a manageable toxicity profile.

Overall, a large body of real-world data convincingly validate sorafenib as a safe and effective therapy option for patients with advanced HCC and confirm the results obtained in the pivotal RCTs. Furthermore, real-world data have also indicated that given adequate patient selection, sorafenib can also be safe and effective in patients who do not meet the strict inclusion criteria of the SHARP and Asia-Pacific trial.

LENVATINIB: AT LEAST AS GOOD AS SORAFENIB

Since its introduction as a treatment option for patients with advanced HCC, sorafenib has been evaluated against several other targeted agents. However, sunitinib did not prove to be better than sorafenib, and two non-inferiority studies testing brivanib and linifanib against sorafenib turned out to be negative[23]. In addition to this, the phase III SEARCH trial, assessing the potential benefit of adding erlotinib to sorafenib in the first-line treatment of patients with advanced HCC, also failed to show a benefit[24]. In the background of these numerous negative studies, the positive outcome of the phase III REFLECT trial in 2018, showing non-inferiority of lenvatinib to sorafenib as a first-line treatment for patients with unresectable HCC, came somewhat as a surprise[11].

Clinical trial data

Similar to sorafenib, lenvatinib is a multi-TKI. It primarily inhibits the vascular endothelial growth factor receptor 1–3, fibroblast growth factor receptor 1–4, KIT, and RET (Figure 1)[25]. In a single-arm phase II trial, including 46 patients with advanced HCC, lenvatinib at a fixed dose of 12 mg/d was found to have substantial clinical activity. With this regimen, a median OS of 18.7 mo was reported, with 37% of patients obtaining a partial response. However, this came at the cost of considerable toxicity, necessitating a dose reduction and treatment discontinuation in 74% and 22% of patients, respectively[26]. Further in-depth analyses of this trial revealed a close correlation between lenvatinib treatment discontinuation and body weight. Based on this finding, the investigators opted to use a weight-adapted lenvatinib dosing in the subsequent phase III trial.

In the randomized phase III REFLECT trial, a total of 954 patients who did not receive treatment for unresectable HCC were randomly assigned (1:1) to receive either lenvatinib (12 mg/d for patients weighing ≥ 60 kg; 8 mg/d for patients weighing < 60 kg) or sorafenib (400 mg twice daily). In order to be eligible for the study, patients had to have a Child-Pugh A liver status and were not allowed to have portal vein invasion at the main portal branch. In addition, patients with a platelet count below 75000 cells/μL were excluded. The study's primary outcome was to demonstrate non-inferiority for lenvatinib compared to sorafenib regarding the OS, with a non-inferiority margin of 1.08. The median age of the patients enrolled in REFLECT was 62 years; 69% had a body weight of ≥ 60 kg, and two-thirds came from Asia-Pacific regions. Extrahepatic spread at baseline was seen in 61% of the patients, while 21% exhibited macroscopic portal vein invasion (macroscopic portal vein invasion and/or extrahepatic spread in 70%). The majority of patients (79%) were classified as having BCLC stage C disease, and 57% had more than one involved disease site. Most of the patient and disease characteristics were well-proportioned between both arms, with some important exceptions. In fact, the number of patients with a hepatitis C etiology was higher in the sorafenib arm than in patients treated with lenvatinib (19% *vs* 26% for lenvatinib and sorafenib, respectively), while the opposite was true for the proportion of hepatitis B-related HCC (53% *vs* 48%). Finally, a marked imbalance was seen in the number of patients with an AFP level of ≥ 200 ng/mL (46% *vs* 39%)[11].

The median OS for patients treated with lenvatinib in the REFLECT trial was reported at 13.6 mo, which was shown to be non-inferior to the 12.3 median OS seen in patients who received sorafenib (HR 0.92; 95%CI: 0.79-1.06). Besides, lenvatinib induced a significant progression-free survival (PFS) than sorafenib. In fact, compared to sorafenib, the median PFS for patients treated with lenvatinib was more than twice as long as the median PFS obtained with sorafenib (7.4 *vs*. 3.7 mo; HR 0.66; 95%CI: 0.57-0.77; $P < 0.0001$). Importantly, lenvatinib was also shown to be associated with a significantly increased rate of ORR compared to sorafenib. With lenvatinib, an ORR of 24.1% was reported, while only 9.2% of sorafenib-treated patients obtained a partial or complete response (odds ratio; OR 3.13; 95%CI: 2.15-4.56; $P < 0.0001$) (Table 1)[11].

With respect to safety, lenvatinib was found to be associated with a slightly increased rate of grade ≥ 3 treatment-emergent AEs compared to sorafenib (57% *vs* 49%). This difference was mainly fueled by a higher rate of grade ≥ 3 hypertension (23% *vs* 14%) and an increased rate of grade ≥ 3 decreased appetite (5% *vs* 1%) and grade ≥ 3 weight loss (8% *vs* 3%) among patients treated with lenvatinib. In contrast, lenvatinib was associated with a substantially lower incidence of hand-foot skin reactions (all grade: 27% *vs* 52%; grade ≥ 3: 3% *vs* 11%)[11]. The proportion of patients requiring a dose interruption (40% *vs* 32%), dose reduction (37% *vs* 38%), and treatment discontinuation (9% *vs* 7%) was similar in the lenvatinib and sorafenib arms.

Table 1 Efficacy outcomes in the phase III REFLECT trial[11]

	Lenvatinib (*n* = 478)	Sorafenib (*n* = 476)	HR (95%CI)	*P* value
Median OS	13.6 mo	12.3 mo	0.92 (0.79-1.06)	
Median PFS	7.4 mo	3.7 mo	0.66 (0.57-0.77)	*P* < 0.0001
Median TTP	8.9 mo	3.7 mo	0.63 (0.53-0.73)	*P* < 0.0001
ORR	24.1%	9.2%	OR (95%CI): 3.13 (2.15-4.56)	*P* < 0.0001
DCR	75.5%	60.5%	-	

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; ORR: Overall response rate; DCR: Disease control rate; HR: Hazard ratio; OR: Odds ratio; CI: Confidence interval.

Real-world experience

Obviously, as lenvatinib only entered the HCC arena in 2018, the real-world evidence with this agent is more limited compared with sorafenib data. To date, the available real-world data with lenvatinib almost universally originate from Japan.

The largest real-world dataset for lenvatinib reported to date includes data from 105 unresectable HCC patients treated with lenvatinib across 48 clinics in Japan[27]. After excluding patients who started lenvatinib at a reduced dose and with a short observation time, 77 patients were eligible for a response (33 TKI-naïve, 44 TKI-exposed). Both the ORR and the DCR at 4 wk did not differ significantly between TKI-naïve and TKI-pretreated patients (38.5% *vs* 32.4% and 80.8% *vs* 70.8%, respectively). The AE profile of lenvatinib seen in this study was largely in line with what was reported in REFLECT[27].

In a second study, a total of 41 patients with unresectable HCC were treated with lenvatinib. Interestingly, of these patients, 23 (56%) would not have been eligible for the REFLECT trial, mainly because of a prior history of TKI use (*n* = 16), a Child-Pugh B score (*n* = 5), and the presence of bile duct invasion (*n* = 4). In this cohort, lenvatinib was associated with an ORR of 61.0% and a DCR of 90.2%. Overall, 5 patients (12.2%) experienced a complete response to lenvatinib. Interestingly, both the ORR and the DCR did not differ between patients who met the REFLECT criteria or not (*P* = 0.83 and 0.79, respectively). In patients with a Child-Pugh B score, the ORR was 60% (3/5), while this was 100% in the 4 patients with bile duct invasion. With respect to safety, no major differences were seen between REFLECT eligible and ineligible patients, with a similar rate of grade ≥ 3 AEs. Lenvatinib in this cohort caused the following AEs most commonly: Hypertension (68.3%, grade ≥ 3 12.2%), appetite loss (68.3%; 2.4%), fatigue (58.5%; 0%), and hand-foot skin reactions (56.1%, 14.6%). As such, these real-world data demonstrate that lenvatinib induces a high early response rate with good tolerability in advanced HCC patients who did and did not meet the REFLECT trial inclusion criteria[28].

A third Japanese real-world study yielded fairly similar results, in which 57 unresectable HCC patients were treated with lenvatinib, of whom 53 were eligible for response (34 TKI-naïve, 19 TKI-exposed). In this cohort, lenvatinib therapy resulted in an ORR of 49.1% (26/53) and a DCR of 96.2% (51/53). Of note, the ORR was higher in patients receiving lenvatinib in first-line (61.8%) compared to patients receiving a second- (33.3%) or third-line (20.0%) treatment. The median TTP in the entire cohort was reported at 8.5 mo, and also for this endpoint, the outcome was better when lenvatinib was used as a first-line treatment. In addition, this real-world study revealed that patients with a better liver functional reserve had a higher response rate to lenvatinib and a longer TTP. Similar to REFLECT, the most common AEs with lenvatinib were hypertension (54.7%, grade ≥ 3: 15.1%), fatigue (49.1%, 7.5%), and a decreased appetite (37.7%, 0%). Hand-foot skin reactions were reported in 26.4% of the patients (all grade 1/2)[29].

In a multi-center retrospective study, including 77 patients with advanced HCC, lenvatinib was associated with an ORR of 29.9% (similar to REFLECT) and a DCR of 77.9%. Interestingly, thyroid dysfunction and appetite loss were found to be associated with a worse and shorter PFS[30]. In this respect, Hiraoka *et al*[31] also identified appetite loss as a dismal prognostic factor for advanced HCC patients treated with lenvatinib[31]. As such, these AEs should be managed with care in patients treated with lenvatinib.

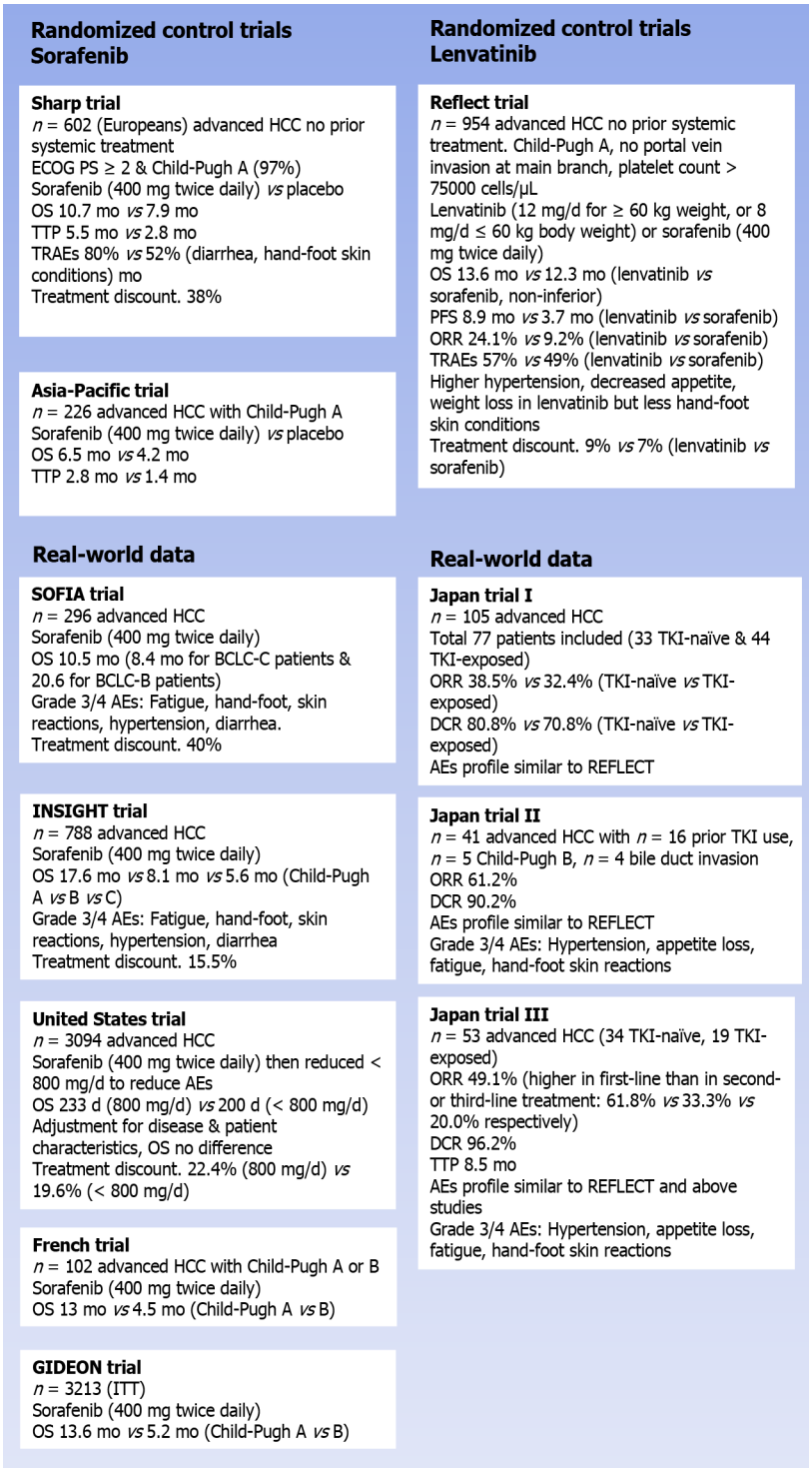

Figure 1 Evolution of clinical trials and real-world data for sorafenib and lenvatinib. AEs: Adverse events; BCLC: Barcelona Clinic Liver Cancer; DCR: Disease control rate; ECOG: Eastern Cooperative Oncology Group; HCC: Hepatocellular carcinoma; ITT: Intent-to-treat population; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival; TKI: Tyrosine-kinase inhibitor; TRAE: Treatment-related adverse event; TTP: Time to progression.

LENVATINIB OR SORAFENIB: HOW TO CHOOSE?

As illustrated above, robust RCT data and convincing real-world results have identified both sorafenib and lenvatinib as effective and safe first-line treatment options for patients with unresectable HCC. This brings us to a logical next question: How should physicians choose between both agents? When making such a decision, several patient and disease characteristics, including liver function and concomitant medication, need to be taken into account. In addition, financial implications need to be considered, especially given the ever-increasing pressure on healthcare budgets.

The phase III REFLECT trial showed non-inferiority of lenvatinib compared to sorafenib and even demonstrated a numerically longer median OS in lenvatinib-treated patients compared to patients treated with sorafenib (13.6 mo *vs* 12.3 mo). This difference did not meet the statistical threshold to demonstrate superiority of lenvatinib compared to sorafenib. However, in a critical appraisal of REFLECT, as acknowledged by the authors, this lack of superiority might have been influenced by elements in the study design[32]. First of all, both the baseline AFP level and the presence of macrovascular invasion were not used as a stratification factor for the randomization in REFLECT. As a result, a higher proportion of patients in the lenvatinib arm had macrovascular invasion (23% *vs* 19%) or an elevated AFP level (≥ 200 ng/mL: 46% *vs* 39%)[11]. It is likely that this higher incidence of poor prognostic factors in the lenvatinib cohort had an influence on the survival outcome of these patients. Another demographic imbalance that might have influenced the trial outcome relates to the HCC etiology. In fact, a higher proportion of patients in the sorafenib arm had HCC with a hepatitis C etiology compared to the lenvatinib arm (19% *vs* 26%)[11]. This difference is of clinical importance given the fact that the treatment effect of sorafenib depends on the hepatitis status of patients, with the best OS prospects for patients with hepatitis C virus-positive HCC. A third and final element from REFLECT that might have diluted the OS benefit of lenvatinib is that patients with invasion of the main portal vein and patients with a disease bulk of more than 50% of the liver were excluded from the study. As a result, the trial selected patients who were more likely to be eligible for subsequent therapy after disease progression on the study drug. This hypothesis is confirmed by the high proportion of patients in both the lenvatinib arm (33%) and sorafenib arm (39%) who received some form of post-study anticancer therapy in REFLECT[11]. These subsequent therapies have likely prolonged the post-progression survival of patients in both treatment arms, diluting the potential OS benefit obtained with one of the two agents in the first-line setting. The fact that the median OS obtained with sorafenib in REFLECT was the longest ever reported with sorafenib in a large RCT further supports the idea that post-study therapies had an important influence on the OS analysis of this trial[7,11,16,23,24]. As such, several elements of the REFLECT trial design might have mitigated the true OS benefit of patients treated with lenvatinib *vs* sorafenib. However, it is important to underscore that these statistical speculations should only be seen as hypothesis-generating. This should not be used as an argument to claim a survival superiority of lenvatinib over sorafenib in the first-line treatment of advanced HCC patients.

As such, a critical evaluation of the OS analysis of REFLECT does not help physicians to make a choice between both TKIs in their clinical practice. Perhaps, more practical advice can be derived from the detailed subgroup analysis performed in the trial. In general, the effect of lenvatinib and sorafenib on OS was consistent across all the investigated subgroups. Nevertheless, some subgroups seemed to have a slightly better OS when treated with lenvatinib instead of sorafenib. With respect to HCC etiology, particularly patients with a hepatitis B virus infection seemed to derive a more pronounced OS benefit from lenvatinib compared to sorafenib (median OS: 13.4 mo *vs* 10.2 mo; HR 0.83; 95%CI: 0.68-1.02). Regional differences were also seen: While patients with a Western origin had a fairly similar median OS with lenvatinib and sorafenib (13.6 mo *vs* 14.2 mo), patients from Asia-Pacific displayed a numerically longer median OS with lenvatinib (13.5 mo *vs* 11.0 mo; HR 0.86; 95%CI: 0.72-1.02). Finally, the presence of macroscopic portal vein invasion and/or extrahepatic spread (median OS: 11.5 mo *vs* 9.8 mo; HR 0.87; 95%CI: 0.73-1.04) and a baseline AFP level ≥ 200 ng/mL (median OS: 10.4 mo *vs* 8.2 mo; HR 0.78; 95%CI: 0.63-0.98) seemed to be associated with a more pronounced treatment effect with lenvatinib[11].

As indicated earlier, lenvatinib was found to be superior to sorafenib in terms of response rate[11]. This finding can be used to make treatment decisions in clinical practice. It is well established that sorafenib mainly induces its survival benefit in patients with advanced HCC by stabilizing the disease. However, in some patients (*e.g.*, patients with bulky disease), a tumor response may be warranted to alleviate symptoms. When faced with such a patient, lenvatinib is probably the better choice.

Both sorafenib and lenvatinib come with a specific toxicity profile, and these differences should be taken into account when opting for one of the two agents. For example, given the high incidence of hypertension reported with lenvatinib, it seems wise to avoid this agent in patients with baseline hypertension or other cardiovascular risk factors.

Finally, the treatment cost should be considered, especially in the context of the ever-increasing pressure on healthcare budgets. In this respect, two independent cost-effectiveness analyses demonstrate that lenvatinib is more cost-effective than sorafenib

in the first-line treatment of patients with unresectable HCC[33,34].

As of now, no standard therapies are available for patients who encountered lenvatinib failure. Sorafenib can be considered in such cases, given that about one-fourth of patients in the REFLECT trial received sorafenib while taking lenvatinib as the first-line medication[35]. Recently, a Japanese pilot study has suggested the potential therapeutic benefit from ramucirumab after lenvatinib failure in HCC patients; nevertheless, another study with more patients could not confirm such benefits in the post-progression treatment[36,37].

IS A CHOICE FOR TKI MONOTHERAPY STILL RELEVANT? IMMUNOTHERAPY-BASED COMBINATION THERAPIES AS A NEW STANDARD IN THE FIRST-LINE TREATMENT OF ADVANCED HCC

Since 2008, sorafenib has been the long-standing standard of care in the first-line treatment for patients with unresectable HCC. It took until 2018, with the publication of the REFLECT trial, before an alternative for sorafenib became available. Recently, results presenting the potential benefits of immunotherapy-based combinations may bring in a crucial change in therapeutic strategies for patients with advanced HCC. In the phase III IMbrave 150 trial, atezolizumab anti-PD-L1) plus bevacizumab (anti-vascular endothelial growth factor) was compared to sorafenib as a first-line treatment of patients with advanced HCC. The atezolizumab-bevacizumab combination showed a significant and clinically meaningful OS improvement compared to sorafenib (median OS not reached *vs* 13.2 mo; HR 0.58; 95%CI: 0.42–0.79; $P = 0.006$). At the 12-mo landmark, 67.2% of patients in the combination arm were still alive, 12% more than the 54.6% OS rate seen with sorafenib at 12 mo. Besides, the median PFS was 6.8 mo *vs* 4.3 mo (atezolizumab-bevacizumab *vs* sorafenib; HR 0.59; 95%CI: 0.47–0.76; $P < 0.0001$). This combination regimen had an expected drug safety profile, with a late deterioration in patients' quality of life[14]. Based on these findings, in May 2020, the United States Food and Drug Administration approved this combination for treating patients with unresectable HCC who had not previously received systemic treatment. In addition, the European Society for Medical Oncology updated its HCC guidelines and endorsed the atezolizumab-bevacizumab combination as a regimen that can be considered a first-line treatment option for advanced HCC patients[35]. While the groundbreaking outcome of the trial led to the approval of the new treatment in 16 countries, the same has been questioned for its generalizability, owing to the short duration of the trial follow-up and the lack of both safety and efficacy data in Western patients, in whom liver cancer has a different molecular profile than in Asian patients and in patients with metabolic tumors, autoimmune disorders, and transplanted organs. Interesting results were also obtained with a second immunotherapy-based combination consisting of pembrolizumab (anti- programmed death-ligand 1) and lenvatinib. In an open-label phase Ib trial, 104 patients with advanced HCC (BCLC stage B or C, Child-Pugh A, and an Eastern Cooperative Oncology Group performance score of 0-1) were treated with lenvatinib (12 mg/d in patients weighing ≥ 60 kg; 8 mg/d in < 60 kg) in combination with pembrolizumab (200 mg intravenously every 3 wk). Among the 100 eligible patients, an impressive ORR of 46% was obtained, which is markedly higher than 24% and 17% ORR obtained with lenvatinib or pembrolizumab monotherapy in the REFLECT and Keynote-224 trials, respectively[11,38,39]. The responses to the lenvatinib-pembrolizumab combination also proved to be durable, with a median response duration of 8.6 mo. The median OS reported with this combination was unprecedented at 22 mo, while patients had a median PFS of 9.3 mo[36]. Based on these findings, the United States Food and Drug Administration granted a breakthrough designation for the use of this combination in the first-line treatment of patients with advanced HCC. However, after the publication of the IMbrave 150 results, this breakthrough designation was put on hold (at that point, pembrolizumab-lenvatinib no longer showed evidence of meaningful improvement over available therapies and, as a result, no longer met the criteria for accelerated approval). Currently, the lenvatinib-pembrolizumab combination is being compared to lenvatinib alone in the randomized phase III LEAP-002 trial, which includes 750 unresectable HCC patients who have not received previous treatment for HCC (NCT03713593). The results of this study are eagerly awaited.

CONCLUSION

Since 2008, sorafenib has been the undisputed standard of care for patients with unresectable HCC who have not received previous treatment for their advanced disease. It took until 2018 for an alternative drug to emerge. In fact, the publication of the pivotal REFLECT trial demonstrated that lenvatinib is non-inferior to sorafenib in terms of OS in the first-line treatment of patients with unresectable HCC. In addition, lenvatinib was shown to be associated with a higher ORR and significantly longer PFS than sorafenib. This leaves patients and clinicians with two equally effective first-line treatment options for patients with unresectable HCC. For physicians to choose which TKI is best to use, they need to consider the individual patient and disease characteristics and consider the specific toxicity profile of both agents. The recent publication of the IMbrave 150 trial demonstrating the superiority of atezolizumab-bevacizumab combination over sorafenib in this setting will radically change the way we treat this disease type. Additionally, the results with the pembrolizumab-lenvatinib combination are very promising but require further validation in larger, randomized trials. Overall, the results obtained with these immunotherapy-based combination regimens are very convincing and will likely make a choice between sorafenib and lenvatinib in this patient group somewhat obsolete.

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MINIREVIEWS

Endoscopic or percutaneous biliary drainage in hilar cholangiocarcinoma: When and how?

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Abstract

Hilar cholangiocarcinoma (hCCA) is a primary liver tumor associated with a dim prognosis. The role of preoperative and palliative biliary drainage has long been debated. The most common techniques are endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic biliary drainage (PTBD); however, recently developed endoscopic ultrasound-assisted methods are gaining more atention. Selecting the best available method in any specific scenario is crucial, yet sometimes challenging. Thus, this review aimed to discuss the available techniques, indications, perks, pitfalls, and timing-related issues in the management of hCCA. In a preoperative setting, PTBD appears to have some advantages: low risk of postprocedural complications (namely cholangitis) and better priming for surgery. For palliative purposes, we propose ERCP/PTBD depending on the experience of the operators, but also on other factors: the level of bilirubin (if very high, rather PTBD), length of the stenosis and the presence of cholangitis (PTBD), ERCP failure, or altered biliary anatomy.

Key Words: Hilar cholangiocarcinoma; Endoscopic biliary drainage; Percutaneous biliary drainage; Endoscopic ultrasound biliary drainage; Surgical oncology

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Core Tip: Hilar cholangiocarcinoma (hCCA) is a primary tumor of the liver with dim prognosis. The role of biliary drainage in curative and palliative setting has long been debated. Endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic biliary drainage (PTBD) as the most commonly used techniques. This review will highlight the available techniques, their indication, advantages or drawbacks, and also timing in the management of hCCA. In a preoperative setting, PTBD appears to win the argument as there is a lower risk of postprocedural complications and better priming for surgery. For palliative purposes, we propose ERCP/PTBD depending on the experience of the operators, biological and anatomy factors, and the presence of cholangitis.

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INTRODUCTION

Cholangiocarcinoma (CCA) is, by definition, a primary tumor of the biliary duct system. Although it is relatively rare compared to the other hepatobiliary tumors, its burden is disproportionately high due to its typically dim prognosis. Per available reports, CCA amounts for approximately 20% of hepatobiliary tumors[1] and is the second most common primary hepatobiliary tumor, accounting for up to 25% of cases in some geographical areas[2,3]. The current gold standard for treatment is surgical resection, yet only a small portion of the patients are optimal surgery candidates. Moreover, the current standard of care is less than ideal, since the five-year recurrence-free survival for radical resection barely exceeds 33% according to the most optimistic reports[4-6].

Based on its anatomic location, CCA is classified as intra- and extrahepatic, the latter accounting for up to 90% of cases. Extrahepatic CCA is further classified as either hilar CCA (hCCA), accounting for approximately two-thirds of cases and, distal CCA amassing up to 30%[6]. As the focus of our current work, hCCA is located between the emergence of the left and right hepatic ducts and the junction between the common hepatic and the cystic ducts[7].

Due to its origin and characteristics, hCCA obstructs the hepatic bile flow, leading to painless jaundice as the main clinical staple, as it occurs in 90% of cases at diagnosis.

Furthermore, accompanying systemic manifestations such as anorexia, weight loss, and fatigue are commonplace at diagnosis, affecting more than half of the patients and rendering a poor outcome due to their association with advanced or metastatic disease [7].

Depending on initial staging, patients with hCCA are typically dichotomized into the following therapeutic pathways: curative-intent surgery or palliative care.

Either path, however, must cross the same common roadblock — addressing obstructive jaundice and reestablishing adequate bile flow. At this point, the clinician might face multiple dilemmas with regards to the benefit, timing, and method of biliary drainage. The main approaches to biliary decompression are endoscopic and percutaneous. The endoscopic approach most commonly consists of bile duct stenting *via* endoscopic retrograde cholangiopancreatography (ERCP) and, to a smaller extent, endoscopic ultrasound (EUS)-assisted methods. The percutaneous approach typically consists of ultrasound or radiological-guided transhepatic tube drainage.

In the curative-intent setting, the jury is out on whether pre-operative drainage adds a practical benefit concerning major outcomes. However, the empirical argument appears to be straightforward, as the biliary obstruction is associated with an increased risk of liver failure and cholangitis.

In the palliative setting, the role of biliary drainage can range from allowing a patient to benefit from systemic therapy with an impact on survival, to treating and preventing cholangitis, alleviating symptoms, and, not least, reducing social stigma by resolving jaundice.

The current review aims to chart a course in the field of biliary drainage of hCCA, based on the most recently available data. In the following parts, the discussion will focus on the available techniques, their indications, advantages, potential drawbacks, and timing, to further clarify the role of endoscopic and percutaneous drainage in the therapeutic arsenal of hCCA.

ENDOSCOPIC DRAINAGE

Most patients with obstructive jaundice due to hCCA can be managed non-surgically using ERCP. Although it is less invasive compared to the percutaneous approach, selective endoscopic stenting is technically difficult and can cause severe infectious complications, such as cholangitis. Current guidelines recommend palliative drainage of malignant hilar strictures through ERCP for Bismuth types I and II, and percutaneous transhepatic biliary drainage (PTBD) or a combination of PTBD and ERCP for Bismuth types III and IV[8].

Plastic stents vs self-expanding metal stents

Plastic biliary stents come in various shapes and sizes. They are made either of polyethylene, polyurethane, or Teflon. Their diameter can range from 5F to 12F, while their length ranges between 1 and 18 cm. Furthermore, there are numerous configurations available. Pigtail stents are coiled at one (single pigtail) or both ends (double pigtail), with side holes placed along the curved ends. Flanged stents may be straight, angled, or curved. They have a single flap proximally and distally with a side hole or 4 flaps without side holes[9].

Self-expanding metal stents (SEMS) are made of different metal alloys, such as nickel and titanium. They range from 4 cm to 12 cm in length and 6 mm to 10 mm in diameter when fully expanded. Biliary metallic stents can be fully covered, partially covered, or uncovered, depending on the presence or absence of a polyurethane or silicone layer.

All plastic and metal stents are radiopaque. Most SEMS models have additional proximal and distal markers, with flared ends to prevent migration[9]. Biliary stent placement is performed under radiologic guidance. Regardless of stent type, the first step is to endoscopically locate the papilla, followed by the selective catheterization of the biliary tree. Biliary sphincterotomy is not always mandatory, as stenting without prior sphincterotomy doesn't appear to increase the incidence of post-ERCP pancreatitis[10]. Subsequently, the bile ducts are visualized using a contrast agent, which allows the characterization of the location and extent of the stenosis. The length of the stent must be carefully selected to exceed the proximal end of the stenosis.

For plastic stents, a radiopaque guidewire is placed into the intrahepatic bile ducts. The stent is then advanced over a catheter (which acts as a pusher), which is itself placed over the guidewire. Once the stent has been adequately positioned, the catheter and guidewire are withdrawn, and the stent remains in place.

Metal stents are compressed by an outer, introducer sheath. After the desired position is achieved *via* the guidewire, the outer sheath is withdrawn, allowing the stent to expand. The guidewire is subsequently removed.

The main goal of endoscopic stenting is to drain at least 50% of the liver, which would reduce bilirubin levels by at least 50% in patients with normal liver function [11]. Secondly, cost-effectiveness must be taken into account, as well as ensuring the stent patency for as long as possible. Given these considerations, plastic stents and SEMS have been compared in several studies and meta-analyses. SEMS are associated with longer patency [odds ratio (OR) 0.16; 95% confidence interval (CI): 0.04-0.62], lower therapeutic failure (OR 0.43; 95%CI: 0.27-0.67) occlusion (OR 0.28; 95%CI: 0.19-0.39) and re-intervention rates (mean difference, -0.49; 95%CI: -0.8 to -0.19)[12-16].

SEMS are more expensive than plastic stents. However, given the higher occlusion rates in plastic stents which impose hospitalization and performing ERCP, SEMS seem to be more cost-effective in the long run[17].

Even if the diameter of fully-expanded SEMS is larger than that of plastic stents, ensuring longer patency, they are thinner when preloaded in their delivery system (5.4-8.5 Fr)[18]. Therefore, they are easier to maneuver and pass through tight strictures than their plastic counterpart. Also, SEMS' delivery system has a sharp tip that acts as a dilatator, facilitating passage through the strictures. Uncovered SEMS also allow the drainage of the biliary tree, side branches, as opposed to plastic stents. This is especially important when performing unilateral drainage in the case of Bismuth type III-IV hilar strictures.

Taking these into account, plastic stents are no longer considered standard of care in Bismuth III-IV type CCA, but they can still be used in Bismuth type I-II[8].

Unilateral vs bilateral stenting

Endoscopic drainage in the case of Bismuth type I-II hilar strictures is in many ways similar to distal biliary strictures, namely patients can be fully drained with a single stent, either plastic or SEMS. However, in the case of Bismuth type III-IV malignant hilar strictures, unilateral and bilateral drainage using SEMS have been compared in several randomized control trials (RCT) and meta-analyses. In one meta-analysis that included 683 patients, side by side metal stenting (n = 317) yielded better clinical success rates (CSRs) (OR: 3.56; 95%CI: 1.62-7.82, P = 0.002) and a reduced incidence of stent dysfunction (OR: 1.74; 95%CI: 1.16-2.61, P = 0.007) compared to unilateral stenting (n = 366). Complication rates seemed to be lower in the unilateral group, although they did not reach statistical significance (OR: 0.51; 95%CI: 0.30-1.00, P = 0.05)[19].

In contrast, another recent meta-analysis which included a total of 21 studies with 1292 patients demonstrated that unilateral and bilateral stenting are comparable in terms of efficacy and safety, although technical success was significantly higher in the unilateral group (P = 0.003). One of the limitations of this meta-analysis was that the authors were unable to perform subgroup analysis based on etiology or Bismuth classification[20].

Similarly, a multicenter international study of 187 patients showed that unilateral and bilateral drainage had comparable CSR irrespective of the Bismuth classification, but with a higher incidence of complications and deaths in the bilateral group (11.7% *vs* 0%, P = 0.007)[21].

Bilateral drainage with SEMS is technically difficult and should be reserved for patients where placing a unilateral stent does not ensure drainage of at least 50% of the liver. Injection of a contrast agent into a liver segment that cannot be subsequently drained can lead to infectious complications such as cholangitis and the formation of liver abscesses, which negatively affect patient survival rates. For this reason, pre-interventional hepatobiliary imaging using computed tomography (CT) or magnetic resonance imaging (MRI) with the calculation of liver volume is paramount.

Stent-in-stent vs side-by-side stent placement

If bilateral stenting is chosen, either stent-in-stent (SIS) or stent-by-stent (SBS) drainage can be used, depending on the endoscopist's experience and preference. However, the left lobe, right anterior, or right posterior biliary tree should be selected based on pre-interventional CT or MRI imaging to ensure the best drainage. Atrophied liver secondary to longstanding biliary obstruction or portal vein thrombosis should be avoided[22].

In the case of SBS stent placement, after catheterization of the common bile duct (CBD), two guidewires are advanced in the left and right hepatic ducts. The first stent is then advanced on the corresponding guidewire, with the recommendation that it should be placed in the hepatic duct where access is more difficult. The second stent is then advanced parallel to the first on the second guidewire as quickly as possible. The stents must be placed so that their distal ends are at the same level in the CBD or should cross the papilla, which will facilitate subsequent access if revision is necessary.

For SIS deployment, similarly to SBS deployment, two guidewires are inserted into both intrahepatic ducts bilaterally. The first stent is then inserted and deployed into the left or right intrahepatic duct. Subsequently, the guidewire used to deploy the first stent is retracted and passed through the central part of the deployed stent into the contralateral bile duct. The second stent is then advanced and deployed through the central portion of the wire mesh of the first stent. As is the case with SBS stenting, the branch that is more difficult for guidewire insertion should be selected as the first stent placement target.

When performing SIS placement, one thing to consider is that not all SEMS have the same structure. Wire mesh can be either small closed-cell or large open-cell, the latter being easier to dilate as it is weaker in its central part[18]. Hence, using an open-cell SEMS might allow an easier SIS placement.

Regarding their efficacy, one prospective (n = 69) and one retrospective (n = 64) study showed that SIS and SBS deployment seem to be similar in terms of clinical success, stent patency and adverse events[22,23] . In addition, a meta-analysis of four studies which included 158 patients in total revealed no significant differences with respect to the rates of successful placement (P = 0.799), successful drainage (P = 0.617), early complications (P = 0.738), late complications (P = 0.744) and stent occlusions (P = 0.606)[24].

Complications of endoscopic stenting

A common complication of stent placement is duodenal biliary reflux, with secondary bacterial colonization of the biliary tract and sludge/stone formation. Another complication is related to stent deployment too far inside the duodenum, or its migration, with subsequent impaction of the stent flanges in the duodenal wall and perforation. If stents migrate in the bowel they can also become stuck, mostly in the ileocecal valve, leading to bowel obstruction.

Plastic stent dysfunction is managed by stent removal and replacement either with another plastic stent or a SEMS. In the case of SEMS dysfunction, if the occlusion is due to debris, this can be removed using balloon catheters. If the occlusion is caused by tissue ingrowth or overgrowth, a secondary plastic stent or SEMS can be inserted inside the existing stent.

IS THERE ANY ROOM FOR EUS-GUIDED BILIARY DRAINAGE IN THE MANAGEMENT OF HCCA?

Although ERCP and PTBD are the two established biliary decompression techniques in the management of hCCA, EUS-guided biliary drainage (EUS-BD) has gained more and more interest in the gastroenterology community[25]. In theory, EUD-BD seems to provide multiple advantages to the management of hCCA, since it does not require the passage of the biliary stricture. However, in clinical practice, it appears to only be used when ERCP has failed, in surgically altered anatomy, or failed re-interventions for blockage of transpapillary placed stents[26]. Although scarce, the most common adverse effects associated with EUS-BD are bleeding, peritonitis, pneumoperitoneum, cholangitis, bile leak, and stent migration[27]. Moreover, its limitations also reside in the insufficient number of expert endosonographers, typically found only in tertiary referral centers.

The types of EUS-BD performed in malignant hilar obstruction are: (1) EUS-guided hepato-gastrostomy (EUS-HGS); (2) Bridging therapies; and (3) EUS-guided hepatico-duodenostomy (EUS-HDS).

EUS-HGS is one of the most commonly used EUS-BD procedures *via* an intrahepatic approach[27]. Technically, the EUS-HGS procedure drains only the left hepatic lobe, leaving the right biliary system undrained and thus increases the risk of potential life-threatening cholangitis.

To address this caveat, Ogura *et al*[28] developed a novel technique of EUS-BD for right intrahepatic biliary obstruction by adding an uncovered metal stent to the EUS-HGS to bridge the obstruction. In brief, after catheterizing the biliary tract *via* the stomach and reaching the left liver lobe using a 19-gauge fine-needle aspiration and a guidewire, the needle is replaced by a standard catheter, a guidewire is passed through the hilar stricture and into the right hepatic biliary system. Functional success was reported in all patients and no severe adverse events were noted. Dismally, the bridging method is technically very challenging when passing the guidewire to the right intrahepatic biliary system and requires trained experts.

Last but not least, the right intrahepatic biliary channels could be accessed by EUS-HDS in a similar manner to EUS-HGS, the only variation being the approach *via* the duodenum. However, since the technique is performed on the lateral side of the duodenal bulb or proximal second duodenum, a long endoscope position might be unstable and risky. For this reason, its use is very limited in the management of hCCA [29,30].

To date, there are several studies carried out to evaluate the use of EUS-BD in clinical practice. Unfortunately, the majority used EUS-BD as a salvation technique (leading to an important selection bias) and the number of patients involved is small [28,31-33]. Nevertheless, there are some advantages of EUS-BD that could make it a more beneficial procedure in the future. The combination of ERCP and EUS-BD (CERES) appears to be more appealing than PTBD in the treatment of Bismuth III-IV CCA. In 2021, Kongkam *et al*[34] reported a similar technical success rate (TSR), CSR and complications rate (CR) of CERES *vs* PTBD as follows: TSR = 84.2% (16/19) *vs* 100% (17/17) (P = 0.23), CSR = 78.9% (15/19) *vs* 76.5% (13/17) (P = 1), and CR = 26.3 (5/19) *vs* 35.3 (6/17) (P = 0.56), respectively. Moreover, regarding recurrent biliary obstruction within 3 and 6 mo, authors reported improved results of the CERES procedure[34]. Several retrospective and prospective studies comparing EUS-BD *vs* PTBD in malignant distal obstruction favor EUS-BD as a better tool for biliary drainage [35,36]. Moreover, a multicenter survey evaluating patient preference for either EUS-BD or PTBD has shown that more than 80% of patients preferred EUS-BD, citing an

increased quality of life without the discomfort of an external drain tube (78.1%), a higher success rate with relatively lower morbidity (43.8%) and the opportunity to be performed at the same time as ERCP (28.3%)[37]. However, no study compared EUS drainage with PTBD in patients with hCCA. While certainly promising, limited experience and low availability diminish the use of EUS-BD. To this point, there is insufficient data to suggest that EUS-BD can replace PTBD as a more efficient biliary drainage tool, with current applicability in large centers with vast EUS experience.

PTBD AND PERCUTANEOUS BILIARY STENTING

PTBD can be performed in two clinical scenarios: (1) Before surgery to relieve biliary obstruction and subsequent cholestasis since an improved survival was documented [38,39]; and (2) As a palliative technique with the ultimate goal to decrease the bilirubin levels to a level that can allow chemotherapy.

Compared to endoscopic drainage, the use of the PTBD method has the advantage that a specific duct can be easily targeted to maximize the drainage of functional parenchyma[40-42]. In high bile duct obstruction, right and left hepatic ducts are typically isolated, with no distal communication. There are three types of isolation: (1) Complete: Cholangiography doesn't result in any opacification; (2) Effective: Isolated ducts are opacified but they do not drain; and (3) Impending isolation: The biliary duct is opacified and drains, but has a central narrowing that is likely to progress to complete isolation. This is important as the latter two increase the risk of subsequent cholangitis.

The first (and probably the most important) steps when assessing a patient for PTBD are: (1) To evaluate the viability of liver parenchyma by high-quality CT or MRI (*e.g.*, drainage of a portion of the liver without an intact portal venous blood supply with ipsilateral duct obstruction will not result in the improvement of liver function; and (2) Pre-procedural antibiotic prophylaxis as cholangitis could result in serious complications that could delay or complicate further management.

Types of PTBD

Currently, there are three modalities of PTBD: (1) External biliary drainage; (2) Internal-external biliary drainage; and (3) Percutaneous self-expanding metallic stent placement.

The point of access is typically chosen depending on the location of the stenosis and the type of intervention. Typically, the right-sided access may be preferred for stent placement in high obstruction, as it offers anatomical continuity between the right hepatic duct and the CBD. When the obstruction is below the duct bifurcation, a left approach is advisable due to a lower risk of catheter displacement. When ascites or segmental isolation of the right duct are present, a left-sided approach may provide more benefit. Peripheral access is preferred because of the lower risk of bleeding and inadequate drainage.

There are two types of approaches: Fluoroscopy-guided PTBD (F-PTBD) and a combined ultrasound-guided approach (US-PTBD). The US-PTBD has more advantages: reduction of fluoroscopy time, faster access to the bile ducts, reduced number of punctures, and, consequently, significantly lowers rates of complications. Moreover, a meta-analysis showed the superiority of US-PTBD *vs* F-PTBD, as US-PTBD was associated with fewer severe early complications and procedure-related deaths (overall complication rates range from 5%-100% for F-PTBD (median, 21%) and from 0%-22% for US-PTBD (median, 5%)[43,44]. After localizing the best access pathway, the real-time US-guided puncture of the liver parenchyma is performed with a 21-gauge Chiba needle. In the case of F-PTBD, after injecting the contrast agent, the targeted bile duct is accessed *via* fluoroscopy. Afterward, the inner stylet is withdrawn, and a guidewire is inserted through the needle into the collecting system.

Subsequently, progressive 6, 8, and 10 Fr coaxial sheaths are advanced over the guidewire for tract dilation, and ultimately an 8 Fr or 10 Fr biliary drainage catheter is placed. If the obstruction can be passed, the directional catheter is advanced into the small bowel. The catheter can be then exchanged over a stiffer wire for a multisidehole drainage catheter. This allows the bile to drain both externally (into a bag), and internally (into the duodenum) to preserve the normal enterohepatic circulation of bile.

Finally, a percutaneous SEMS is a third option for drainage. Stent placement can be performed as either a one-step (primary stenting technique) or a two-step (secondary stenting technique) procedure; the latter will give the clinician more time to plan and

is particularly useful in case of intraprocedural bleeding. A randomized controlled trial that compared the clinical effectiveness of percutaneous covered Viabil stents *vs* uncovered metallic Wallstents demonstrated improved survival in patients with hCCA who received a covered (median survival, 243 d) *vs* uncovered stent (median survival, 180 d) (P = 0.039). The incidence of stent dysfunction was significantly lower in the covered stent group[45].

Post transhepatic percutaneous biliary drainage complications

Virtually, PTBD complications are overlapped to those of endoscopic biliary drainage, hence we won't reiterate the matter. However, in the group of patients with resectable hCCA, a long-term complication that can be a game-changer is represented by tumor seeding. Takahashi and colleagues reported an alarming 5.2 percent catheter tract recurrence after PTBD in patients with hCCA which is much higher than previously reported. However, the duration of PTBD (over 60 d) was an important independent risk factor for tract metastasis and shortened postoperative survival. In the curative surgery setting, this points out the importance of a short delay until surgery to prevent this troublesome complication[46,47].

Combined techniques — "Rendez-vous": how and when?

Rendez-vous (RV) procedure is an appealing option for treating obstructive jaundice in the case of an unsuccessful ERCP[48-50]. Apart from ERCP failure, RV is a rescue therapy for in the case of complex biliary interventions that require combined access routes: patients with surgically altered enteric anatomy, tight hilar biliary stricture passable only by the guidewire, and in patients with a preexistent PTBD that can be easily used as an anterograde route for percutaneous RV[50,51].

Clinical scenarios: Single or dual drainage?

In the case of distal biliary obstruction, a straightforward approach is sufficient. However, in the case of Bismuth type II, III, IV hCCA controversy exists as to whether partial or total biliary drainage is more suitable in a palliative setting. There are two majors advocates for complete[52] or incomplete[53] drainage.

Schima *et al*[54] studied a group of 41 patients with hilar obstruction and compared long-term outcomes. Single stents were placed for unilateral drainage in 27 patients, while 14 patients had bilateral stents. They found no significant difference regarding mean stent patency.

Kaiho *et al*[55] performed either complete (n = 12) or partial (n = 9) drainage in a group of 21 patients with hilar obstruction. There were three, seven, and eleven patients with Bismuth types II, III, and IV obstructions, respectively. They found no difference in stent patency between complete and partial drainage.

Inal *et al*[56] evaluated the necessity of draining more than one hepatic duct in 138 patients with malignant hilar obstruction. Single-duct drainage was achieved in 74 patients (54%) by placing one stent (n = 59), two stents (n = 41) or a single transhepatic tract in a "T" configuration (n = 23). There were no differences between single and dual stenting in Bismuth type I, II, and III hCCA. However, in Bismuth type IV, the deployment of two parallel stents resulted in significantly higher patency rates.

Lee *et al*[57] suggest that when a repeat procedure in proximal hCCA is necessary, placement of internal/external drainage catheters provides better palliation than putting in new metal stents, as life expectancy is limited in this patient group.

ENDOSCOPIC *VS* PERCUTANEOUS BILIARY DRAINAGE — PRO AND CONS

After decades of clinical research, it is still unclear whether endoscopic (ERCP) or percutaneous drainage is the preferred method of biliary drainage in patients with hCCA. Both can be performed before surgery (in patients suited for curative treatment) or as a palliative treatment. Choosing one technique over the other is not an easy clinical decision. A straightforward selection can only be made in patients with modified anatomy[58].

However, for the majority of patients with hCCA choosing the best technique depends on several factors.

Biliary drainage before surgery

In one retrospective study, technical success in the ERCP group (n = 87) was 78%

compared to 98% in the PTBD group (n = 42; P = 0.04). The therapeutic success rate was also higher in the PTBD group (79% *vs* 49%; P = 0.02)[59]. Another retrospective study showed higher technical success for PTBD *vs* ERCP (100 % *vs* 81%; P = 0.203)[60]. However, neither technical nor therapeutic success should be the sole primary outcomes when comparing the two methods. There is only one multicentric RCT comparing endoscopic and percutaneous biliary drainage. The primary outcome was the number of severe complications in the timespan between randomization and surgery. In total, 54 patients were randomly assigned to benefit from either PTBD or ERCP. The study was prematurely interrupted due to significantly higher mortality in the PTBD group [11 (41%) of 27 patients] *vs* the ERCP group [three (11%) of 27 patients; relative risk 3.67, 95%CI 1.15-11.69; P = 0.03][61]. Indeed this study provides the highest level of evidence we have to this point for decision making in clinical practice. However, these data should be interpreted with caution for several reasons: (1) Only one patient with Bismuth type 1 was included in the PTBD group; (2) Although not statistically significant, both technical and therapeutic success were higher in the PTBD group; (3) 55% of the patients in the ERCP group had subsequent PTBD; and (4) Only 54 patients were randomized, making the study prone to a type-I error. Nevertheless, the expertise of centers performing PTBD is highly relevant and could explain these results. In one study[62], low-volume centers showed a higher occurrence of serious complications related to PTBD, whereas high-volume centers showed a similar proportion of complications between endoscopic and percutaneous drainage. In terms of procedure-related complications (*e.g.*, cholangitis and pancreatitis) two other studies found PTBD to be superior to ERCP[63,64]. Another critical aspect is the cost associated with each method. One recent study found ERCP to be more expensive than PTBD (P = 0.005)[63]. Some patients with hCCA are better suited for ERCP drainage, while others might be more appropriate for PTBD. Discriminating between these two categories is crucial. One study showed that patients with Bismuth 3a or 4 hCCA and a total bilirubin level above 8.8 mg/dL should be considered for initial PTBD rather than ERCP[65].

ERCP appears to perform better in Bismuth II hCCA, as it is associated with fewer postprocedural complications, namely cholangitis[66].

Until now, four meta-analyses comparing the two techniques have been published [67-70]. All of them found PTBD to be superior to some extent over ERCP. More data about the meta-analyses is provided in Table 1.

Biliary drainage in palliation

A single meta-analysis comparing ERCP and PTBD in palliation of advanced malignant hilar obstruction has been published to this point. It included a total of nine studies and 546 patients, yet not all of them had hCCA. The results showed that palliation with PTBD was associated with higher rates of successful biliary drainage and lower rates of cholangitis while palliative ERCP had lower bleeding complications [71].

A key aspect in a palliative setting is the patient's quality of life. In this light, the presence of the external drainage tube for the remainder of a patient's life (in the case of PTBD) might generate a significant alteration, especially compared to the placement of an internal stent. Surprisingly, based on a controlled study by Saluja *et al*[72], quality of life after PTBD was rated higher than ERCP according to the World Health Organization Quality of Life physical and psychological scores at one and three months. A potential cause might be the relatively high incidence rate of fever in the endoscopic biliary stent implementation group. Moreover, percutaneous stenting after PTBD is also possible, eliminating the burden of caring for an external drainage tube for the entire life. One study which included 85 patients with advanced Bismuth type III and IV hCCA showed that percutaneous SEMS was superior to endoscopic stenting regarding successful biliary decompression (92.7% *vs* 77.3%; P = 0.49)[73]. There is not enough evidence to suggest one technique over the other. Moreover, the implementation of RCTs is problematic. The results of an unsuccessful RCT were recently published. Lack of funding, provider/institutional bias in favor of one procedure, and logistical challenges were cited as possible responsible factors of failure[74]. Therefore, until high-quality observational data or RCTs become available, one must rely on personal judgment, according to expertise and specific conditions. Based on the aforementioned discussion, we propose an algorithm on when and how to use ERCP and PTBD in patients with hCCA, depicted in Figure 1.

Table 1 Meta-analysis comparing endoscopic *vs* biliary drainage before surgery in patients with hilar cholangiocarcinoma

Ref.	No studies	No. patients	Main findings
Liu *et al*[68]	6	359-EBD; 286-PTBD	Similar technical success rate, R0 resection, incidence of total complications after resection, post-operative hospitalization time, resection time and recurrence; The incidence of total complications were higher in the EBD group ($P < 0.05$)
Hameed *et al*[69]	15	398-EBD; 1036-PTBD	There was a trend towards higher procedure conversion (RR 7.36, $P = 0.07$) and cholangitis (RR 3.36, $P = 0.15$) in the EBD group
Al Mahjoub *et al*[70]	4	275-EBD; 158-PTBD	Overall procedure related mortality was higher in EBD group ($P = 0.0009$); Similar initial technical failure; Conversion rate was higher in EBD group ($P < 0.001$); Risk of pancreatitis was higher in EBD group ($P < 0.001$); Risk of cholangitis was higher in EBD group ($P < 0.001$); Similar postoperative morbidity and mortality
Tang *et al*[67]	9	498-EBD; 414-PTBD	PTBD was associated with a lower risk of cholangitis ($P < 0.001$); PTBD was associated with a lower risk of pancreatitis ($P = 0.003$); A higher successful rate of palliative relief of cholestasis in PTBD group ($P < 0.001$); The incidence of hemorrhage was similar ($P = 0.59$)

EBD: Endoscopic biliary drainage; PTBD: Percutaneous biliary drainage; hCCA: Hilar cholangiocarcinoma; RR: Relative risk.

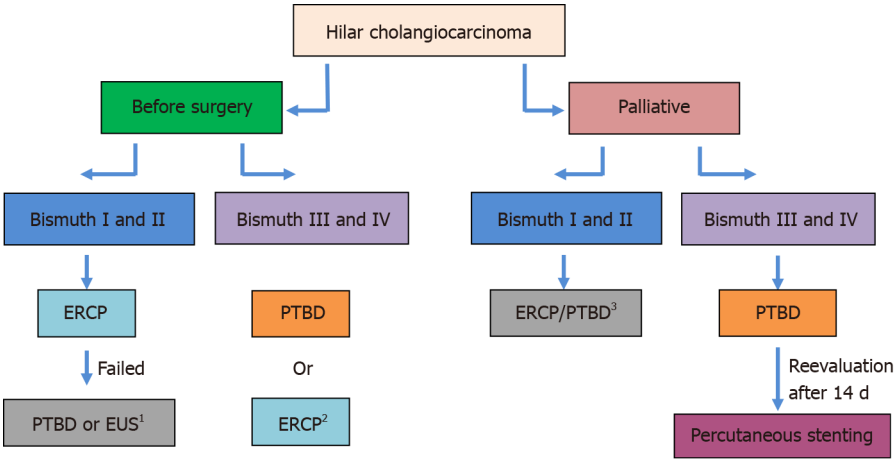

Figure 1 Management algorithm in hilar cholangiocarcinoma. [1]In high-volume centers with expertise in EUS. [2]In centers specialized in ERCP and little experience in PTBD. [3]Depending on the experience and preference of the patient. ERCP: Endoscopic retrograde cholangio-pancreatography; EUS: Endoscopic ultrasound; PTBD: Percutaneous transhepatic biliary drainage.

THE SURGICAL POINT OF VIEW

Biliary drainage is an established safe hCCA treatment strategy as a bridge therapy before surgery. To date, the surgical standard of treatment in hCCA is complete resection combined surgery. Although there is debate about the effect of biliary drainage on surgical outcomes in patients with hCCA, it has been demonstrated that liver failure caused by obstructive jaundice can be a significant risk factor in major liver resection. This is especially relevant in the case of hCCA, for which extended hepatectomy might be needed to provide the best chance for a cure. Therefore, it is preferable to perform the biliary decompression of the future remnant liver to preserve postoperative liver function[75].

There are two preferred methods for biliary drainage in hCCA: Endoscopic biliary drainage (ERCP) and PTBD. ERCP might be a less invasive technique, but it may come with a price: it carries an increased risk of ascending cholangitis and procedure-related complications, such as duodenal perforation and pancreatitis[59-60,64,67,76]. On the other hand, PTBD could lead to several complications such as bleeding, portal vein thrombosis, tumor seeding, patient discomfort, and has been widely reported to be associated with malpractice[64,77-80].

Several meta-analyses have compared the two methods in hCCA patients[67-70]. All of them showed some superiority of PTBD over ERCP when performed before surgery.

In our center, as surgeons, we always prefer PTBD drainage over endoscopic drainage. From our own experience several other factors must be taken into account: (1) First, the cornerstone of surgical treatment is to obtain an R0 resection, which

translates in performing an accurate dissection of the hepatic pedicle and lymph node dissection. This is significantly easier in the absence of inflammation surrounding the main biliary duct (MBD), which might be caused by a prior ERCP[81], resulting in greater intraoperative blood loss and prolonged operative time; (2) In the case of PTBD, inflammation is absent or minimal, which leads to an easier dissection and an accurate lymph node dissection[82]; and (3) The inflammation determined by the stent could give a false appreciation of inoperability[83]. The inflammation surrounding the MBD can be a blunder in mimicking a direct invasion of the important vascular structures, such as the portal vein or the hepatic artery, which would falsely classify the patient as inoperable. Consequently, with ERCP, we consider that the best timing for surgery is within the first seven days, to avoid MBD inflammation. A concern is that the seven-day timeline could not suffice to obtain a normal liver function, a problem that doesn't exist for PTBD.

CONCLUSION

Endoscopic or percutaneous biliary drainage? Decades of experience, a lot of research, new stents, new techniques but the same disease: hCCA remains one of the most challenging cancers. Biliary drainage, then chemotherapy, stent occlusion/external tube removal then stop chemotherapy and re-drain, and the story goes on. Treating patients according to the proposed algorithm (Figure 1), although based on low-quality data and personal experience and educated guesses might at least help the decision-making process. In the palliative setting, we would choose between ERCP and PTBD generally based on operator experience, as well as other relevant factors: stenosis length, bilirubin level (if very high, rather PTBD), cholangitis (PTBD), ERCP failure, or altered biliary anatomy. Not least, one should always consider patient preference It is not hard to understand that (from the patient perspective) there is only one answer to the question: endoscopic or percutaneous biliary drainage? EUS biliary drainage is a relatively new technique with only a few hCCA patients treated. Yet it is likely to gain more interest in the years to come, hoping to improve the current management of hCCA.

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MINIREVIEWS

Current status of non-surgical treatment of locally advanced pancreatic cancer

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Abstract

Pancreatic cancer is the 7th leading cause of death due to cancer in industrialized countries and the 11th most common cancer globally, with 458918 new cases (2.5% of all cancers) and 432242 deaths (4.5% of all cancer deaths) in 2018. Unfortunately, 80% to 90% of the patients present with unresectable disease, and the reported 5-year survival rate range between 10% and 25%, even after successful resection with tumor-free margins. Systemic chemotherapy, radiotherapy, and minimally invasive image-guided procedures that have emerged over the past years, are used for the management of non-operable PC. This review focuses on currently available non-surgical options of locally advanced pancreatic cancer.

Key Words: Interventional radiology; Oncology; Radiotherapy; Pancreatic cancer; Ablation

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Core Tip: Currently available non-surgical treatment options for locally advanced pancreatic cancer include systemic chemotherapy, radiotherapy, and minimally invasive image-guided procedures. The latter have emerged over the past years and include radiofrequency, microwave ablation, laser ablation, cryoablation, irreversible electroporation, high intensity focused ultrasound, and trans-arterial embolization procedures. Although initial results were not deemed satisfactory, mainly due to unacceptable complication rates, cumulative experience and technological advances have led to the improvement of outcomes of image-guided procedures and their incorporation in the treatment algorithm.

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INTRODUCTION

Pancreatic cancer (PC) is the 7th leading cause of death due to cancer in industrialized countries and the 11th most common cancer globally with 458918 new cases (2.5% of all cancers) and 432242 deaths (4.5% of all cancer deaths) in 2018[1]. Its incidence is variable among regions (age-standardized incidence highest in Europe: 7.7 per 100000 people and lowest in Africa: 2.2 per 100000 people) and is expected to increase[1,2]. Both incidence and mortality of the disease correlate with increasing age and male gender[2].

Modifiable risk factors include alcohol (increased risk high alcohol consumption > three drinks per day, but no association with low-to-moderate alcohol intake), smoking (relative risk 1.74 for current and 1.2 for former smokers; risk persists for at least 10 years after cessation), obesity [body mass index (BMI): 25.0-29.9 kg/m^2 or BMI ≥ 30 kg/m^2 in early adulthood], dietary factors (red meats, processed meats, cholesterol, foods containing nitrosamines) and exposure to metalworking, pesticides, cadmium, and arsenic[2,3]. Non-modifiable risk factors are gender (global incidence 5.5/100000 for men and 4.0/100000 for women), age (> 50 years), ethnicity (significantly higher in black people than any other racial group), diabetes mellitus (1.8-fold risk increase), family history (double risk when at least two first-degree relatives have PC and 32-fold higher in kindreds with three or more first-degree relatives) genetic factors (10% of patients have some kind of germ-line mutation), chronic infections (*Helicobacter pylori*), non-O blood group and chronic pancreatitis (1.8% patients longstanding pre-existing chronic pancreatitis will develop PC within 10 years from diagnosis and 4% after 20 years)[2,4].

PC symptomatology is frequently non-specific (abdominal pain, jaundice, pruritus, dark urine, and acholic stools, anorexia, early satiety, dyspepsia, nausea, and eventually weight loss), while the early-stage disease remains frequently asymptomatic and most patients present at an advanced stage with poor prognosis[5,6]. Early detection could reduce mortality and screening of selected sub-groups such as those with a family history), using blood markers could be deemed beneficial in the future. Nevertheless, screening of the general population is not currently recommended[7,8]. Diagnosis and staging include imaging modalities such as contrast-enhanced, triphasic pancreatic computed tomography (CT) protocol, magnetic resonance imaging. Endoscopic ultrasound-guided fine-needle aspiration is indicated for cytological confirmation with a sensitivity of approximately 80%), while cancer antigen 19-9 assists in the confirmation of diagnosis, prediction of prognosis, and recurrence following surgery[7].

Surgical resection remains the only curative therapy for PC. However, 80% to 90% of the patients present with unresectable tumors and the reported 5-year survival rates are low, ranging between 10% and 25%, even after successful resection with tumor-free margins[2,9]. Over the past years, alongside with systemic chemotherapy and radiotherapy (RT), minimally invasive image-guided procedures have emerged for the

management of non-operable or recurrent locally advanced PC. These include ablative modalities such as radiofrequency ablation (RFA), microwave ablation (MWA), laser ablation, cryoablation (CA), reversible electrochemotherapy (ECT) and irreversible electroporation (IRE), and high intensity focused ultrasound, while trans-arterial embolization procedures have also been suggested and investigated[10-12]. Following the initially non-satisfactory results, which were correlated with unacceptable complication rates, outcomes of minimally-invasive image-guided procedures were significantly improved, mainly due to the cumulative experience, and technological advances of the devices used, and their incorporation in the treatment algorithm for PC has been gradually accepted in specialized centers[10].

ABLATIVE TECHNIQUE

Patients with stable or partial response RECIST (response evaluation criteria in solid tumors) disease following neoadjuvant therapy, and not eligible for surgery should be considered as potential candidates for ablative therapies[13-16]. Ablative treatment can be divided into thermal (RFA, CA, MWA) and non-thermal (RE, IRE).

Thermal ablation

RFA uses needle electrodes to apply high-frequency alternating current to solid tumors. This process generates high temperatures, resulting in thermal coagulation, necrosis, and protein denaturation within the tumor[15]. It can be performed during laparotomy, percutaneously, or using endoscopic ultrasound. Usually, RFA is adopted for tumor debulking rather than complete ablation, as a safety margin of at least 5 mm from the ablation zone is necessary to avoid thermal damage to vital structures. This treatment is also contraindicated in small tumors with a perivascular growth pattern [17]. Early studies investigating the efficacy and safety of RFA in the treatment of LAPC reported high rates of morbidity (4%-37%) and mortality (0%-25%) due to thermal injury to bile ducts, pancreatic duct, duodenum, vital vessels, and heat-sink effect, resulting in incomplete tumor ablation. Recent literature shows an improved overall survival (OS) ranging between 19.0 and 25.6 mo when combining RFA with chemotherapy[17] by optimizing ideal parameters for temperature range (< 90 degrees), treatment time, and probe placement[15].

CA induces rapid argon-gas-based freezing and thawing of target lesions. The freeze-thaw cycles, based on the Joule-Thompson effect, cause cellular destruction by vascular-mediated cytotoxicity, endothelial damage, and cell death. CA with and without immunotherapy for LAPC treatment in term of OS was studied in a retrospective study: Median OS was higher in the cryoimmunotherapy (13 mo) and cryotherapy groups (7 mo) than in the chemotherapy group (3.5 mo; both $P < 0.001$) and was higher in the cryoimmunotherapy group than in the cryotherapy ($P < 0.05$) and immunotherapy groups (5 mo; $P < 0.001$)[15].

MWA promotes tissue coagulation by the oscillation of water molecules, ultimately generating tissue necrosis. This technique has advantages over RFA in the ease of setup and larger ablation zones in a shorter period but recent literature on the use of MW ablation in PC with survival data is very limited[15].

Non-thermal ablation

IRE uses ultrashort high voltage direct current pulses to create an electric field across the cell membrane. This process disrupts membrane homeostasis and irreversibly alters transmembrane potential, which activates the apoptotic pathway and leads to cell death (Figure 1). IRE has the unique ability to preserve the extracellular matrix, critical vessel structures, bile ducts, intestines and minimize heat-sink effects resulting in potentially incomplete ablation. The technology is commercially available (NanoKnife; Angiodynamics Latham, NY, United States) with 510(k) clearance by the Food and Drug Administration (FDA) for ablation of soft tissue tumors[15,18]. In addition to its cytoreductive abilities, the evidence is emerging on IRE's capability to induce systemic immunomodulation through active *in vivo* vaccination against PC cells. IRE induces a systemic immune response following apoptosis and necrosis of tumor cells with the release of antigens and damage-associated molecular pattern molecules (DAMPs). These DAMPs promote the maturation of dendritic cells and other antigen-presenting cells that can subsequently take up the activation of lymph nodal T-cells potentially inducing a durable antitumor T-cell response. This response could then lead to regression in distant metastases, a process known as the "abscopal effect". These effects in combination with immunotherapy may offer a new treatment

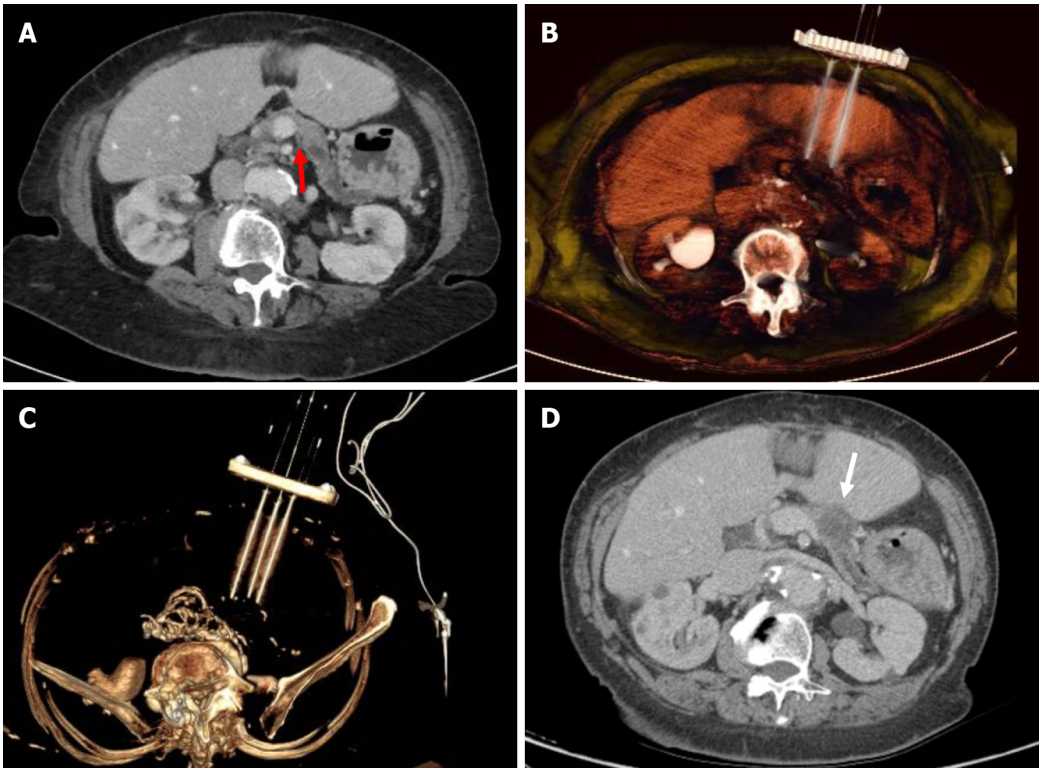

Figure 1 Pancreatic cancer percutaneous irreversible electroporation. A: Axial computed tomography (CT) showing 2 cm lesion (red arrow) in the body of the pancreas in keeping with ductal adenocarcinoma; B and C: 3D volume rendering CT with parallel needles positioning within the lesion; D: 1-mo CT follow up showing complete ablation of the tumor (white arrow).

paradigm for tumors with low immunogenic potential, like pancreatic ductal adenocarcinoma[16]. IRE can be performed during open or laparoscopic surgical exploration or percutaneous procedure with CT guidance. For IRE, 2-6 electrodes are typically placed around the tumor, with a maximum spacing of 2.0-2.5 cm, using image guidance and an upper limit of 5 cm in tumor diameter is highly recommended [17]. Given the high-risk profile of pancreatic IRE procedures, it is important to consider absolute and relative contraindications and to carefully assessed patients for treatment: it is advised to exclude patients with irreversible bleeding disorders, epilepsy, or any other unstable condition that precludes general anesthesia like patients with a past medical history of cardiac disease (*i.e.*, cardiac arrhythmia, implantable cardioverter defibrillator, pacemaker) given the risk of inducing cardiac arrhythmias when applying the electrical pulses[18]; pervasive involvement of the duodenum is considered an absolute contra-indication; if the patient is affected by biliary obstruction, adequate biliary drainage must be guaranteed prior to treatment and if the tumor is closed to the common bile duct it is highly recommended to ensure biliary protection prior to treatment, as post-IRE swelling can impede passage through the central bile ducts; if a patient suffers from a partially occluded portal vein prior to IRE, portal vein stenting should be performed to prevent acute complete occlusion due to postprocedural swelling. Most frequent adverse events comprise GI-related symptoms including pain, diarrhea, nausea, vomiting, loss of appetite, and delayed gastric emptying[16]. Previous cohort studies report heterogeneous outcomes, with median OS rates varying between 15-32 mo when combining IRE with systemic treatment. A recent systematic review by Moris *et al*[16] shows an overview of all studies that have utilized IRE for LAPC, with a median OS following IRE between 7 and 27 mo. This variance may be due to selection bias, the utilized IRE approach, the diverse LAPC tumor biology, personalized (neo-) adjuvant chemo- and/or RT protocols, performance status including comorbidities, and other interpersonal patient differences. In general, major complications (*e.g.*, portal vein thrombosis, bleeding, duodenal perforation) are reported in 0%-30% of patients, with mortality rates ranging between 0%-11%[17]. The average cumulative morbidity for surgical and percutaneous IRE was 36% *vs* 24%, with an average periprocedural mortality rate of 2% *vs* 0%, respectively[16].

Reversible ECT is a new non-thermal ablation technique that avoids possible thermal injury to the peripancreatic vessels like portal mesenteric vein combining the use of chemotherapeutic drugs (bleomycin) with electric pulses for cell membrane electroporation. A transient cell membrane improve permeability is determined by electric pulses, permitting the exposure of the cell to chemotherapeutic drugs[19]. The procedure is divided into four steps: Laparotomy or laparoscopic or percutaneous approach and intraoperative ultrasound to confirm that the pancreatic tumor was unresectable and to exclude distant metastases, needle insertion, bleomycin infusion and electroporation. Eight minutes after the bleomycin infusion, electric pulses were applied and delivered using four single long needle electrodes having 1.2 mm in diameter, and 3 or 4 cm active part. ECT was performed mostly in young patients (mean age, 63 years), with a good performance status and normal BMI. ECT was safe, according to the absence of acute intraoperative adverse effects related to electroporation and effects related to the bleomycin[19]. Nevertheless there is few studies regarding ECT in literatures[20,21] and additional studies should be carried out.

INTRA-ARTERIAL THERAPIES

Since the 1950s, regional intra-arterial chemotherapy (RIAC) was introduced in an attempt to increase cancer survival rates. Intra-arterial chemotherapy generates high drug concentrations in the target areas while maintaining low systemic drug levels. Clinical trials demonstrated that regional intraarterial infusion with Gemcitabine (GEM) improved the response and resectability rates for advanced PC and was well tolerated by patients[22,23]. In 2012, a systematic review and meta-analysis of six randomized controlled trials, comparing systemic chemotherapy, with RIAC, reported that the latter resulted in higher partial remission, clinical benefits and response rates with fewer complications including myelosuppression[24].

With regards to efficacy, it is known that the effect of chemotherapy is concentration-dependent, therefore intra-arterial local infusion which generates higher drug concentrations within targeted regions, could be proven more efficient. In fact, according to the results of two RCTs, RIAC improved the 1-year OS (41.2%-28.6%) compared with systemic chemotherapy[25,26].

Other Authors considered the use of RIAC as neoadjuvant regional chemotherapy with continuous infusion of GEM intending to improve resectability rates in case of locally advanced PC[27]. A study was carried out to investigate the prognostic factors in patients who received GEM-based intra-arterial infusion for advanced PC; young age, pretreatment CA19-9 value < 1000 U/mL, and tumor located at the head of the pancreas indicated better response to RIAC and improved survival[28].

A recent retrospective cohort study of 454 patients with advanced PC compared RIAC *via* angiographically placed celiac axis catheters *vs* isolated upper abdominal perfusion (upper abdominal perfusion with stop flow balloon catheters in the aorta and vena cava)[29]. The isolated perfusion group demonstrated superior survival compared to intra- arterial infusion (median survival rates: 12 and 8 mo in stage III; 8.5 and 7 mo in stage IV; respectively)[29].

Future perspectives in local chemotherapy include novel infusion techniques such as Trans-Arterial Micro-Perfusion (TAMP) using the RenovoCath catheter (RenovoRx, Inc.) which was recently approved by the FDA. The TAMP procedure involves arterial segment isolation using proximal and distal occlusion balloons generating increased intra-arterial luminal pressure above the interstitial pressure and forcing the drug across the arterial wall within the tumoral tissue. The specific catheter has the potential to deliver higher drug concentrations within the tumor, while limiting systemic exposure. The TIGeR-PaC Phase III randomized clinical trial is currently enrolling patients with unresectable LAPC to investigate TAMP *vs* systemic chemotherapy. The goal of the trial is to prove extended median survival and improved quality of life through targeted delivery of therapy. In Phase, I/II studies, TAMP resulted in over two years survival in more than half the patients. The TIGeR-PaC trial, currently includes approximately 30 active clinical sites, and is expected to involve 200 participants in 40 centers in the United States and Europe (clinicaltrials.gov NCT03257033).

ONCOLOGY

LAPC is in general considered incurable and the management remains unclear and controversial. Treatment includes chemotherapy, which can have a potential role as

neoadjuvant treatment or in combination with RT on a case-based approach. The aim is to control disease progression, palliation, and improvement of OS. Patients with LAPC have a poor prognosis, with a median OS of 12-14 mo following systemic therapies[14]. The first step before choosing the appropriate treatment plan is the assessment of the patient's performance status. Patients with locally advanced disease received therapies based on their performance. Patients with a poor performance status are candidates to single-agent chemotherapy or palliative radiation therapy or best supportive care; patients with a good performance status can be considered for a more intensive oncological strategy as chemotherapy or chemoradiation[30].

Formerly, standard treatment included GEM for six months. However, in 2011, the Eastern cooperative Oncology group reported improved OS with the addition of radiation therapy (for a total of 50.4 Gy) to GEM[31].

In 2016, Suker *et al*[32] conducted a patient-level meta-analysis of 11 studies (315 patients) indicating that LAPC patients treated with FOLFIRINOX demonstrated a median OS of 24.2 mo, which was significantly higher compared to that achieved by GEM therapy (6-13 mo).

While systematic chemotherapy has become the standard for patients with LAPC, surgery remains the only potentially curative therapy. Therefore, the development of a new neoadjuvant treatment that improves survival rate is of the utmost importance. Neoadjuvant chemotherapy should aim in a possible resection, or radiation therapy or IRE. Clinicians and researchers should investigate cancer biology and invest to define predictive and prognostic factors, patients-related features, and biological criteria to identify patients that would benefit from aggressive surgery or chemotherapy and chemoradiation[33].

When surgery is not possible after induction chemotherapy, chemoradiotherapy (intensity modulated radiation therapy or stereotactic body radiation therapy), is a possible option to incite tumor shrinkage and enable secondary resection in a small percentage of patients, while palliation of cancer-related pain is also a significant goal in unresectable tumors. The LAP07 phase III randomized controlled trial was designed to investigate the effect of chemoradiotherapy and erlotinib in the OS of patients with LAPC controlled after 4 mo of GEM-based induction chemotherapy. Unfortunately, both chemoradiotherapy and erlotinib did not provide any benefit in patients with LAPC[34].

On the other hand, in an updated 2018 review and meta-analysis of 41 studies (1018 patients receiving consolidation chemoradiation after induction chemotherapy and 954 patients receiving chemotherapy alone) the authors noted a significant survival benefit for chemoradiation after induction chemotherapy in cases in which chemotherapy lasted for a period of at least 3 mo[35].

Currently, according to the results of the SCALOP multicenter, open-label, randomized, two-arm, phase 2 trial, a capecitabine-based regimen should be preferred over a GEM-based regimen in the context of consolidation chemoradiotherapy after a course of induction chemotherapy for locally advanced PC[36].

Only recently, novel minimally invasive approaches, such as IRE, have been evaluated for treatment of LAPC after induction chemotherapy. The PANFIRE-2 multicenter, prospective, single-arm study investigated the safety and efficacy of percutaneous CT-guided IRE alone or after induction chemotherapy with GEM alone or FOLFIRINOX. In total 50 patients were enrolled (40 with LAPC and 10 with local recurrence) and target median OS was exceeded in patients with LAPC (17 mo) and those with local recurrence (16 mo). Notably, 14 minor and 21 major complications occurred, while one probable IRE-related death was reported[14]. Some data suggest the potential role of IRE after FOLFIRINOX chemotherapy as better results have been noted in specific subgroups of patients. IRE could be considered as a less invasive potentially curative approach for LAPC[37]. Nevertheless, randomized clinical trials are necessary to provide comparative data regarding the efficacy of these three methods.

RT

About 80%-90% of the patients with PC, present with locally advanced disease at diagnosis and consequently a very poor prognosis of less than 5% OS at 5-years[38, 39]. In this group of patients, RT associated with systemic therapy plays a crucial role in achieving satisfactory local control (LC). As noted above, the LAP 07 phase III study failed to demonstrate any significant advantage on OS, but a substantially decreased local progression rate (32% *vs* 46%, P = 0.03) for chemoradiotherapy was noted,

without increase in grade 3 to 4 toxicity (except for nausea)[34]. In this context, for patients demonstrating good performance status, the aim of combining RT with systemic therapy is to provide satisfactory LC and avoid or delay local disease progression. However, it is crucial that during RT a higher radiation dose should be delivered to the tumor, limiting doses to healthy tissue.

Conventionally, the standard RT dose has been set around of 50-54 Gy administered over 6 wk using the 3D conformal radiation technique (3D-CRT), a dose initially established on the tolerance of large-field radiation to organ at risks. However, these doses showed a limited LC of the disease, while higher doses often induce treatment-related toxicity that can significantly affect the patient's quality of life[40].

Over the past decade, modern technological advances such as intensity-modulated RT (IMRT), the introduction of respiratory management methods and improved imaging guidance during therapy, have enabled dose-escalation therapy and facilitated the possibility to reduce treatment volumes, to improve ablative doses to the tumor and consequently clinical outcomes, concurrently reducing treatment toxicity[41]. In fact, like other mobile tumors, pancreatic tumors require assessment of respiratory movements, while RT dosimetry is additionally complicated by the presence of nearby hollow digestive organs.

In a 2015 systematic review, 13 IMRT studies were analyzed and compared with 7 3D-CRT series, in order to compare toxicity and tumor outcomes between these two different RT-techniques for the management of LAPC. Even though no differences in terms of OS and PFS were obtained, IMRT was correlated with reduced risk of side effects[42]. Surely, the absence of OS advantage could be explained by the conventionally delivered dose, which is not sufficient to efficiently control the disease[43].

In a study published by Krishnan *et al*[44], patients who received an intensified dose with a biological effective dose (BED) > 70 Gy demonstrated higher OS compared to those who received a standard dose BED ≤ 70 Gy. In this scenario, in which the main limitation remains the risk of severe intestinal toxicity, stereotactic body RT (SBRT) has also been largely explored as a potentially effective treatment for patients with LAPC (Figure 2)[45-61]. Of note, poor prognosis related to these tumors has motivated physicians to limit the number of RT sessions and concentrate treatment within one to two weeks, thereby reducing the period of chemotherapy interruption and increasing patient's compliance.

According to recent data on SBRT treatment for LAPC, a large heterogeneity in total dose prescription and fractionations was reported. Generally, the total doses used ranged from 30 Gy to 45 Gy in 3-5 fractions resulting in effective biological dose (BED) superior to 60 Gy (assuming an $\alpha/\beta = 10$) in most of the cases[46-60]. This higher dose/fraction can theoretically produce enhanced cell destruction than conventional fractionation[62]. Due to the aggressiveness of the disease which causes a rapid evolution of the metastatic sites, OS is not greatly decreased in SBRT series.

Despite satisfactory LC and median freedom from local progression rates of approx. Eighty percent at 1 year, and very low acute toxicity obtained by SBRT single fraction treatments (25 Gy/1 fr)[45,47,48,51,52], high rates of late toxicity have limited the use of this approach. Therefore, multi-fraction treatment schemes are preferably used. In a retrospective cohort of 167 LAPC patients, 45% of whom were treated in single fraction SBRT and 54% in five-fraction SBRT, five-fraction protocol showed significantly lower gastrointestinal toxicity compared to the single fraction treatment. However, good LC rate was noted in both groups[54].

Similar conclusions were drawn following several retrospective studies that acknowledged the benefit in terms of reduced toxicity when multi-fractionated (3-5 fractions) RT regimens were applied compared to single fraction treatment, while obtaining the same efficacy in terms of LC[49,50,52,53,55-57]. All patients investigated in these studies, received systemic therapy (mainly GEM) during different time points of SBRT treatment (before, after or both before and after SBRT) and therapeutic schemes were variable based on the patients' clinical status and the grade of the disease. Notably, the indication for intensified local treatment using SBRT in LAPC patients may be abruptly compromised due to the high metastatic potential of the disease.

Only few, non-randomized, retrospective studies compared SBRT and conventional fractionated RT[63] in the local advanced PC curative or neo-adjuvant setting. SBRT was associated with significantly improved OS compared to conventional fractionated RT. Additionally, SBRT was associated with significantly increased rates of pathological complete response and margin-negative resection in neo-adjuvant setting. These are promising results and provide the basis for consideration for prospective validation.

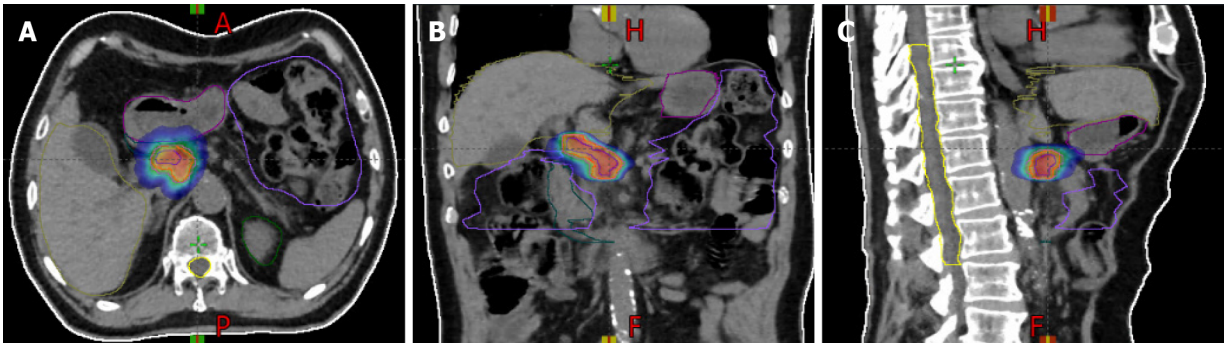

Figure 2 Radiation treatment plan for a patient treated with stereotactic body radiotherapy for local advanced pancreatic cancer. A-C: The plans show isodose levels in the axial plane (A), coronal plane (B), and sagittal plane (C).

However, the indication for SBRT in order to intensify local treatment may be affected by the high metastatic potential of the disease.

CONCLUSION

The prognosis of LAPC remains poor although recent advancements in multimodality treatment seem to provide some improvement in clinical outcomes. Combined modality treatment using systemic chemotherapy, minimally invasive image-guided procedures and RT are currently under investigation. Neoadjuvant chemotherapy could be used to enable curative surgical resection, radiation therapy, or ablation. SBRT may be employed to obtain satisfactory LC and reduce side effects. Mounting experience with percutaneous thermal ablation and IRE provides superior outcomes with less complications. Considering the currently unsatisfactory results in terms of OS improvement, different treatment options should be investigated to optimize therapy. As the pathogenesis of LAPD is multifactorial and has been associated with genetic factors (mainly germ-line BRCA2 gene mutations, but also various syndromes such as the Lynch syndrome, hereditary breast and ovarian cancer syndrome, familial adenomatous polyposis and Li-Fraumeni syndrome) environmental factors (obesity, smoking habit, diabetes, alcohol consumption, dietary factors such as red meat consumption, and occupational exposure to nickel cadmium and arsenic, and the human microbiome), future treatment directions should focus on the investigation of these factors to provide personalized therapeutic schemes and improve survival[2,64,65].

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MINIREVIEWS

Prospect of lenvatinib for unresectable hepatocellular carcinoma in the new era of systemic chemotherapy

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Abstract

The phase III clinical trial of the novel molecular targeted agent (MTA) lenvatinib for patients with advanced hepatocellular carcinoma (HCC) (REFLECT trial) found that lenvatinib was non-inferior to sorafenib in overall survival. Recently, the efficacy of multiple MTAs, including lenvatinib, in practice has been reported, and therapeutic strategies for Barcelona Clinic Liver Cancer (BCLC) intermediate stage HCC are undergoing major changes. Based on these results, lenvatinib could be recommended for patients with transcatheter arterial chemoembolization (TACE)-refractory, ALBI grade 1, within the up-to-seven criteria in the BCLC intermediate stage. Lenvatinib provides a more favorable outcome than TACE, even in cases with large or multinodular HCC beyond the up-to-seven criteria with Child-Pugh grade A. When patients meet the definitions of TACE-refractory or TACE-unsuitable, switching to systemic chemotherapy, including lenvatinib, is for favorable for preserving liver function. If initial treatment, including MTA, has a significant therapeutic effect and downstaging of HCC is obtained, additional TACE or surgical resection should be considered. Lenvatinib also has a therapeutic effect for poorly differentiated type and non-simple nodular type HCC thanks to the survival-prolonging effect of this drug. Furthermore, a significant therapeutic effect is expected in tumors with more than 50% liver involvement or main portal vein invasion, which have traditionally been considered to have a poor prognosis in patients. This suggests that at the start of lenvatinib treatment, HCC patients with ALBI grade 1 may be able to maintain liver functional reserve.

Key Words: Hepatocellular carcinoma; Lenvatinib; Molecular targeted agent; TACE-

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refractory; TACE-unsuitable; Barcelona Clinic Liver Cancer intermediate stage

Core Tip: For about 10 years, first-line systemic chemotherapy for patients with advanced hepatocellular carcinoma (HCC) had been limited to sorafenib. The Phase III clinical trial of lenvatinib for patients with advanced HCC showed lenvatinib to be non-inferior to sorafenib with respect to overall survival (OS). The OS of patients is still far from satisfactory, and there is a great unmet medical need for more effective therapies. This review focuses on the current understanding of the therapeutic efficacy and safety of lenvatinib in the world and outlines the role of lenvatinib in the new era of chemotherapy for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid cancers and a major cause of cancer-related deaths globally[1]. According to the Global Cancer Observatory in 2020, HCC is ranked third in mortality, causing over 830000 deaths per year. Despite increasing global incidence as a major cause of cancer death, the development of new anticancer drugs for HCC has been inadequate. Traditionally, HCC has a poor prognosis. However, this might be partly due to the confined treatment options for patients with advanced HCC[2,3].

Since the publication of the practice guidelines of the American Association for the Study of Liver Diseases (AASLD) on the management of HCC in 2005, the Barcelona Clinic Liver Cancer (BCLC) staging system has been widely accepted and is also being used in many clinical trials of new drugs to treat HCC. These take into account factors including tumor burden, liver function, and general health conditions to determine prognosis and the best treatment. Accordingly, patients at an early stage are those with HCC ≤ 5 cm or up to three nodules < 3 cm each (BCLC stage A). Patients exceeding these limits, without vascular invasion or extrahepatic spread, fit into the intermediate stage (BCLC stage B). Patients with evidence of a performance status ≤ 2 or an aggressive tumor pattern (vascular invasion or extrahepatic spread) correspond to the advanced stage (BCLC stage C)[4].

Systemic chemotherapy is the only therapeutic option for patients with Child-Pugh grade A at BCLC stage B with unresectable HCC and stage C. Prior to the development of the molecular targeted agent (MTA), patients in the advanced stage had a survival time of approximately 6 mo. Systemic chemotherapy for HCC has changed since the introduction of MTA sorafenib in 2007. The SHARP trial demonstrated that sorafenib prolonged median overall survival (OS) compared to placebo in patients who had not received systemic chemotherapy [10.7 mo *vs* 7.9 mo, hazard ratio (HR) = 0.69, 95%CI: 0.55-0.87, $P < 0.001$][5]. The subsequent Asia-Pacific trial confirmed these results in Asian patients[6]. Therapeutic options for extrahepatic metastases (*e.g.*, lung, lymph node, or bone) and vascular invasion (*e.g.*, portal vein tumor thrombus) have been demonstrated, and relatively long survival has been achieved for patients with BCLC stage C.

However, sorafenib does not shrink or induce necrosis in tumors and has relatively severe adverse events (AEs), including hand-foot-skin reactions. Therefore, the development of a novel MTA that can substitute for sorafenib is much anticipated.

For the past 10 years, sorafenib has been the only available first-line systemic chemotherapy for patients with advanced HCC. Many clinical trials of candidate systemic chemotherapeutic agents for advanced HCC have failed to demonstrate superiority or non-inferiority to sorafenib[7-9]. The phase III clinical trial of the

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lenvatinib for patients with advanced HCC (REFLECT trial)[10] showed lenvatinib to be non-inferior to sorafenib with respect to OS (13.6 mo *vs* 12.3 mo, HR = 0.92, 95%CI: 0.79-1.06). Furthermore, the secondary efficacy endpoints [progression-free survival (PFS) and objective response rate (ORR)] in the lenvatinib group showed a significant improvement compared with sorafenib. Based on the REFLECT trial, lenvatinib has been approved in the United States, European Union, and other countries as a first-line treatment option alongside sorafenib for advanced HCC, making it the first such drug to be used in Japan. A recent Phase III trial (IMbrave150) showed that combination immunotherapy with atezolizumab plus bevacizumab improved outcomes, including OS, PFS, ORR, and disease control rate, compared with sorafenib monotherapy[11]. Based on these results, both the 2020 AASLD and the 2021 European Society for Medical Oncology liver treatment options depends on BCLC staging and treatment guidelines which recommended atezolizumab+bevacizumab as first-line systemic therapy in stage B with transcatheter arterial chemoembolization (TACE)-unsuitable HCC and stage C. In addition, even with the latest version of the treatment algorithm in the Clinical Practice Guidelines for HCC 2020 in Japan, the first-line drug therapy for unresectable HCC is atezolizumab+bevacizumab combination therapy. Sorafenib and lenvatinib, which were previously the first-line treatments, are now second-line treatments. Regorafenib, ramucirumab, and cabozantinib can be used as third-line treatments (Figure 1).

Although various systemic chemotherapies are available for HCC, the OS of patients is still far from satisfactory, and there is a great unmet medical need for more effective therapies. Furthermore, in the future, issues are likely to arise regarding the order and combination of treatment options for HCC.

This review focuses on the current understanding of the therapeutic efficacy and safety of lenvatinib and outlines the role of lenvatinib in the new era of chemotherapy for HCC.

MOLECULAR MECHANISMS OF LENVATINIB

Lenvatinib is an MTA that suppresses vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), proto-oncogene tyrosine-protein kinase receptor RET, platelet-derived growth factor receptor α, and stem cell factor receptor[12]. Because these targets act as drivers in cancer, lenvatinib has been reported to exhibit antitumor and immunomodulatory activities in a variety of preclinical cancer models[13].

Inhibition of the VEGF/VEGFR signaling pathway

Angiogenesis, mostly regulated by the VEGF pathway, is an essential event in tumor growth and metastasis[14]. In particular, VEGFR-2 is a high-affinity VEGF receptor in vascular endothelial cells[15,16]. Ligand binding activates certain signaling pathways, such as the phospholipase-Cγ, phosphoinositide 3-kinase (PI3K)/V-akt murine thymoma viral oncogene homolog (AKT) pathways, and rat sarcoma (Ras)/mitogen-activated protein kinase (MAPK)[16]. These signaling pathways have been implicated in endothelial cells and vascular permeability during tumor enlargement[17]. Moreover, VEGFR is highly expressed in HCC cells. Lenvatinib has been shown to inhibit tumor angiogenesis in various preclinical models. In patient-derived and PLC/PRF/5 cell-transplanted tumor models, Lenvatinib administration resulted in a reduction in microvessel density of tumor[18]. In addition, lenvatinib had been found to suppress various types of cancers[19-23], by blocking the VEGFR pathway. These data indicate that lenvatinib exhibits potent anti-angiogenic activity and may have a stronger effect than sorafenib in preclinical models.

Inhibition of the FGF/FGFR signaling pathway

Activated fibroblast growth factor (FGF) signaling can directly facilitate cell proliferation and survival, as well as promote tumor angiogenesis and progression[24]. The binding of FGF to FGFR leads to the activation of the RAS/MAPK and PI3K/AKT signaling pathways[25,26]. FGF and FGFR are typically overexpressed in HCC, and the expression of FGF19/FGFR4 contributes to HCC progression[27]. Analysis of the effects of selective FGFR inhibitors and FGFR small interfering RNAs on cancer stem-like cells (CSCs) in HCC showed that lenvatinib diminished CSCs in HCC by inhibiting FGFR1-3 signaling; however, FGFR4 signaling was not affected. FGF2 and FGF19 are involved in maintaining CD44High/CD133High CSCs in HCC, potentially *via* FGFR1-3[28]. Preclinical studies have shown that lenvatinib inhibits the prolif-

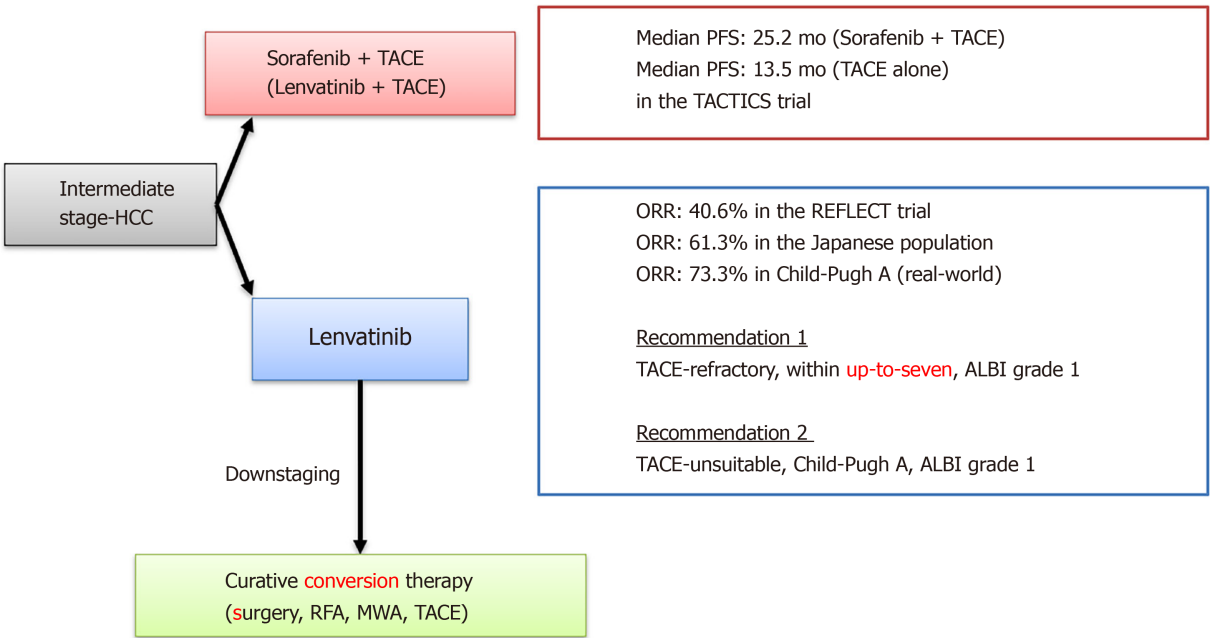

Figure 1 Treatment strategy for intermediate stage hepatocellular carcinoma. HCC: Epatocellular carcinoma; MWA: Microwave ablation; ORR: Objective response rate; PFS: Progression-free survival; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

eration of FGF19 and FGFR-overexpressing cell lines[18,28,29].

Inhibition of the RET signaling pathway

RET activates downstream signaling pathways through mutations or chromosome rearrangements, promoting tumor cell growth[30]. The autophosphorylation of specific tyrosine residues of RET allows for the recruitment adaptor proteins that connect the RET receptor to RAS/MAPK and PI3K/AKT signaling pathways, thereby promoting cell growth, proliferation, survival, and differentiation[31]. Lenvatinib can inhibit cell proliferation by blocking RET autophosphorylation[12].

Immunomodulatory activity

The immune escape of tumor cells is the main mechanism of tumorigenesis[32]. It has been suggested that VEGF-A has immunosuppressive properties[33]. VEGF-A produced by tumor cells enhances the expression of immunosuppressive receptors in CD8+ T cells and promotes immune escape[34]. Immune inhibitory receptors cause CD8+ T cell exhaustion by recognizing tumor antigens[32]. Lenvatinib reduced the infiltration of tumor-associated macrophages and increased the percentage of activated CD8+ T cells in HCC[13].

LENVATINIB THERAPY IN INTERMEDIATE STAGE HCC

Efficacy of Lenvatinib in intermediate stage HCC

In recent years, the efficacy of multiple MTAs, including lenvatinib, has been reported. As a result, therapeutic strategies for BCLC intermediate-stage HCC are undergoing major changes. In the REFLECT trial, the ORR of lenvatinib was 40.6% in the mRECIST evaluation, and in the sub-analysis, the ORR of BCLC intermediate stage HCC in the Japanese population was 61.3%[35]. Furthermore, Kudo *et al*[36] reported that a very high response rate (RR) of 73.3% was obtained for Child-Pugh A in BCLC intermediate stage HCC. Tomonari *et al*[37] reported from their real-world data that BCLC intermediate stage HCC cases had fewer AEs, could maintain the dosage amount of lenvatinib, and had a good therapeutic effect. Many reports have demonstrated the high therapeutic effect of lenvatinib in BCLC intermediate stage HCC.

Efficacy of TACE in intermediate stage HCC

TACE is the guideline-recommended standard of care for intermediate stage HCC. The AASLD Consensus Conference showed that locoregional TACE may still be the best approach if the patient's tumor volume is small and nodules can be accessed

superselectively[38]. According to a systematic review of patients treated with lipiodol-based TACE, the survival rates at one, three, and five years were reported to be 70.3%, 40.4%, and 32.4%, respectively, with a median OS of 19.4 mo[39]. But, the efficacy of TACE was not observed equally in all cases. Intermediate-stage HCC varies widely in terms of tumor burden and liver function. The heterogeneity of tumors in the BCLC intermediate stage has led to the development of several prognostic scores, including the Kinki criteria, which are based on liver function and tumor burden that attempt to determine who may obtain the maximum benefit from TACE[40]. The up-to-seven criteria, defined as the sum of the maximum tumor diameter in the liver (cm) and the number of tumors[41], has prognostic value and can predict the recurrence and maintenance of Child-Pugh grade in patients who undergo initial conventional TACE[42]. In intermediate stage HCC, preserving liver function is as important as achieving a high objective response because the treatment goal is to prolong OS. Additional TACE has a lower RR than the initial TACE and increases the risk of liver function loss[43]. Hence, repeated TACE is not recommended, as it leads to decreased liver function and reduced therapeutic efficacy.

Recently, the characteristics of TACE-resistant tumors, which are prone to being TACE-refractory and to exacerbate to Child-Pugh grade B by TACE, have been clarified[36,44]. In cases in which the tumor is a non-simple nodular type, occupying multiple lobes, a large tumor mass, such as beyond the up-to-seven criteria, and of a histopathologically poorly differentiated type, TACE has little effect. As a result, these cases may become TACE-refractory at an early stage[45].

EFFICACY OF LENVATINIB IN TACE-REFRACTORY CASES

Therapeutic alternatives are needed for patients who are TACE-refractory. Repeated TACE could worsen liver function, thereby narrowing the time window for a switch to MTAs, which is recommended for patients with Child-Pugh grade A. In the era of sorafenib, TACE failure/refractoriness was proposed for switching to systemic chemotherapy[46]. The OPTIMIS trial showed that the survival time in TACE-refractory patients was longer in patients who received sorafenib than in those who continued TACE.

A recent study showed that the median PFS times in TACE-refractory patients treated with lenvatinib, sorafenib, and TACE was 5.8, 3.2, and 2.4 mo, respectively [47]. In a Cox regression analysis, lenvatinib treatment and being within the up-to-seven criteria were identified as independent factors for PFS (lenvatinib, $P < 0.0001$; within the up-to-seven criteria, $P = 0.001$). Similarly, decision-tree analysis showed that patients treated with ALBI grade 1 beyond the up-to-seven criteria had longer PFS than patients treated with ALBI grade 2 beyond the up-to-seven criteria. Therefore, lenvatinib could be recommended to patients with TACE-refractory, ALBI grade 1, and within the up-to-seven criteria in the BCLC intermediate stage. Thus, treatment with lenvatinib could give rise to good outcomes in TACE-refractory intermediate-stage HCC patients.

EFFICACY OF LENVATINIB IN TACE-UNSUITABLE CASES

In patients beyond the up-to-seven criteria, TACE treatment was reported to be likely to worsen liver function, potentially resulting in losing the opportunity to be treated with MTA[42,48]. Recently, the Asia-Pacific Primary Liver Cancer Expert (APPLE) consensus statement proposed the criteria for TACE unsuitability[49].

A proof-of-concept study demonstrated that, in patients with intermediate stage HCC who exceeded the up-to-seven criteria, the lenvatinib group showed a significantly higher ORR (73.3% *vs* 33.3%; $P < 0.001$) and a significantly longer median PFS than the conventional TACE group (16.0 mo *vs* 3.0 mo; $P < 0.001$)[50]. The ALBI score was maintained in the lenvatinib group during treatment, whereas it worsened in the TACE group. Therefore, in the case of large or multinodular intermediate stage HCC with Child-Pugh grade A, lenvatinib provides a better outcome than TACE.

LENVATINIB AS AN UPFRONT SYSTEMIC CHEMOTHERAPY

When patients meet the definitions of TACE-refractory or TACE-unsuitable, switching

to systemic chemotherapy including lenvatinib may be advisable in order to preserve liver function. Upfront lenvatinib therapy may also be suitable for patients with a high tumor burden or those who are considered TACE-refractory. If the tumor responds well and downstaging is possible with lenvatinib, additional TACE (or surgical resection or ablation) could be considered. However, the efficacy and safety of this strategy have not yet been validated.

Recently, a randomized, controlled trial comparing the efficacy and safety of TACE plus sorafenib to TACE alone (TACTICS trial) found that median PFS was significantly longer in the TACE plus sorafenib group than in the TACE alone group (25.2 mo vs 13.5 mo; $P = 0.006$)[51]. MTA treatment is thought to improve the clinical outcome of TACE by promoting the normalization of tumor vessels and contributing to a higher density of lipiodol deposition[49]. Lenvatinib has a higher RR than sorafenib, suggesting that Len-TACE sequential therapy may be a promising treatment for patients with moderately differentiated HCC.

EFFICACY OF LENVATINIB FOR POORLY DIFFERENTIATED HCC

In the REFLECT study, poorly differentiated HCC was diagnosed in 42 patients (4.4%), with 21 in the lenvatinib group and 21 in the sorafenib group. The ORR was 47.6% in the lenvatinib group and 14.3% in the sorafenib group. Thus, lenvatinib is expected to have an effect on poorly differentiated HCC[49].

HCC shows great diversity of tumor differentiation even in the same nodule, and it is difficult to examine the heterogeneity of all tumors by liver tumor biopsy. Therefore, it is extremely important to evaluate the degree of tumor differentiation using a non-invasive method. Poorly differentiated HCC is characterized by tumors that show a heterogeneous enhancement pattern with irregularly shaped ring structures in the arterial phase of dynamic computed tomography (CT)[52] and tumors in which lesions are detected on FDG-positron emission tomography[53]. Based on the prediction of tumor differentiation by the enhancement pattern of dynamic CT[54], the therapeutic effect of lenvatinib is also recognized for poorly differentiated type and non-simple nodular type, and it has been reported that it has a survival-prolonging effect for all of them[54]. Tumors with a heterogeneous enhancement pattern and irregularly shaped ring structures had an RR of 84%, which was significantly better than the RR of tumors with a homogeneous enhancement pattern, which was 53%. Furthermore, there was no significant difference in the PFS between the two groups. The therapeutic effect of lenvatinib, regardless of the degree of tumor differentiation, will have a significant impact on future HCC treatment[54].

Traditionally, non-simple nodular, poorly differentiated HCC has a poor prognosis with existing treatments, including hepatectomy. In particular, it was difficult to control the progression of poorly differentiated HCC using TACE. Therefore, these factors are considered to be among those that are TACE-unsuitable. It is important to identify TACE-unsuitable cases even during the course of TACE treatment and to identify Len-suitable cases while maintaining hepatic reserve.

IMPACT OF LENVATINIB ON PRESERVING LIVER FUNCTION

A sub-analysis of the REFLECT trial examined the time to progression of Child-Pugh score 6 before lenvatinib treatment to a Child-Pugh score of 7. The median time to progression to Child-Pugh score 7 was 23.7 mo in the sorafenib group, 23.9 mo in the lenvatinib 8 mg group, and 15.9 mo in the lenvatinib 12 mg group, respectively. There was no significant difference between the sorafenib and lenvatinib groups. Both sorafenib and lenvatinib affected liver functional reserve, but the difference was not significant[35].

Terashima *et al*[55] reported that patients treated with lenvatinib had a Child-Pugh score that was maintained or improved after 4 and 12 wk compared with those treated with sorafenib ($P = 0.048$ and $P = 0.036$, respectively) in clinical settings. Lenvatinib was identified as one of the factors associated with maintaining Child-Pugh scores. On multivariate analysis, a worse Child-Pugh score after 4 wk was an independent predictor of poor OS. Patients treated with lenvatinib for advanced HCC maintained their liver functional reserves better than those treated with sorafenib.

Uchikawa *et al*[56] assessed the ALBI score as an index of liver function during sorafenib and lenvatinib treatment. The median ALBI score was -2.53 before MTA treatment and -2.45, -2.44, and -2.36 post-2, -4, and -6 mo, respectively. The ALBI

scores tended to increase during MTA treatment. When examined separately in the sorafenib and lenvatinib groups, no significant difference was observed between the two groups. However, the ALBI scores of the sorafenib group increased 2 mo after treatment initiation, and at 4 and 6 mo, significant differences were observed (P < 0.01). Based on the above, although the ALBI score may gradually decrease with the course of MTA treatment, lenvatinib may have a lower effect on the deterioration of the ALBI score than sorafenib.

Hiraoka *et al*[57] compared the ALBI score at the start of lenvatinib with scores after 2 and 4 wk; decreased liver function was common in the early stages after starting lenvatinib (within 4 wk, especially within 2 wk). It is important to introduce MTA to patients with as good liver function as possible, taking into account the early decrease in liver function due to lenvatinib. These results suggest that ALBI grade 1 and lenvatinib at the start of MTA treatment may be related to the maintenance of liver functional reserve.

TREATMENT OUTCOME FOR THOSE WHO DO NOT MEET THE REFLECT TRIAL ELIGIBILITY CRITERIA

In the REFLECT trial, patients were excluded if they had a treatment history of MTA, large HCC with more than 50% liver occupation, HCC with main portal vein invasion, Child–Pugh grade B, platelet count < 75 × 10^9/L, or apparent bile duct invasion.

We reported the efficacy and safety of lenvatinib in patients with unresectable HCC who did not meet the REFLECT eligibility criteria in clinical settings[58,59]. The ORR and the median PFS was similar between patients who met the REFLECT inclusion criteria and those who did not. Thus, the study results support the use of lenvatinib for patients with unresectable HCC who do not meet the REFLECT inclusion criteria.

But, the efficacy and tolerability of lenvatinib treatment differed according to the eligibility criteria of the REFLECT trial.

Chuma *et al*[60] reported that lenvatinib treatment offers benefits in highly advanced HCC (tumors with more than 50% liver occupation or main portal vein invasion) patients with good liver function or nodular-type tumors. Maruta *et al*[61] also reported that lenvatinib had potential profits for patients with advanced HCC with second- or later-line therapies and a high burden of intrahepatic lesions. The various characteristics identified in these studies may be useful as indicators for lenvatinib treatment in highly advanced HCC cases, which are considered treatment-resistant cancers.

POST-PD TREATMENT AFTER LENVATINIB

In the REFLECT trial, among the 954 patients randomized to receive first-line lenvatinib (n = 478) or sorafenib (n = 476), 340 patients received subsequent anticancer medication during the survival follow-up period: 156 patients (32.6%) had received first-line lenvatinib, and 184 patients (38.7%) had received first-line sorafenib[62]. Of the patients who were treated with first-line lenvatinib, the most common subsequent carcinostatic substance was sorafenib (25.3%), and the most common subsequent non-anticancer medication treatment was TACE (56.6%). The OS of patients who were initially randomized to first-line lenvatinib (versus first-line sorafenib) and who received any subsequent anticancer medication was 20.8 mo *vs* 17.0 mo (HR = 0.87; 95%CI: 0.67-1.14). The OS of patients who initially received first-line lenvatinib (versus first-line sorafenib) and who did not receive any subsequent carcinostatic substance was 11.5 mo *vs* 9.1 mo (HR = 0.90, 95%CI: 0.75-1.09). In the -hoc analysis of all patients in the REFLECT study, the OS of those who received subsequent anticancer medication was prolonged compared with patients who did not receive any subsequent anticancer medication. In the REFLECT trial, the lenvatinib group had significantly longer PFS than the sorafenib group, but superiority in OS could not be demonstrated because of the effect of post-treatment on post-progression survival prolongation.

Lenvatinib is the preferred agent for TACE-unsuitable patients in the intermediate stage based on the high RR, survival benefit over TACE, and the possibility of conversion to resection or ablation therapy[49]. Sorafenib combined with surgical resection is a feasible option in advanced HCC patients; if sorafenib is effective, long-term survival may be achieved[63]. Additional surgery was the most significant factor

predicting survival exceeding 3 years ($P < 0.0001$) and represents an independent prognostic factor (HR = 0.07, 95%CI: 0.003-0.40, $P = 0.01$)[64]. Long-term survival may be obtained for select patients with HCC receiving adequate additional surgical treatment, even after sorafenib induction. Therefore, lenvatinib treatment, which has a higher RR than sorafenib, is expected to increase the number of cases that can be switched to conversion therapy.

CONCLUSION

Many MTAs and immunotherapies have become available as treatment options for advanced HCC. Lenvatinib has been shown to have a good therapeutic effect, even in TACE-refractory and TACE-unsuitable cases. Intermediate-stage HCC beyond the up-to-seven criteria with Child-Pugh grade A/ALBI grade 1 usually does not benefit from TACE, whereas lenvatinib provides a better outcome than TACE.

Lenvatinib also has good therapeutic performance even in cases of non-simple nodular, poorly differentiated, tumor masses with more than 50% involvement of the liver and main portal vein invasion, which are generally recognized as having a poor prognosis with existing treatments. To maximize the therapeutic effect of lenvatinib in such cases, it is necessary to preserve liver function in patients with Child-Pugh grade A and ALBI grade 1 at the start of treatment.

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ORIGINAL ARTICLE

Basic Study

Dysbiosis of the duodenal microbiota as a diagnostic marker for pancreaticobiliary cancer

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Abstract

BACKGROUND

Pancreaticobiliary cancer (PB Ca) is a lethal disease, and a useful diagnostic marker is urgently needed. A correlation between the human microbiota and malignant gastrointestinal diseases was recently reported.

AIM

To investigate the efficacy of the duodenal microbiota for diagnosing PB Ca.

METHODS

We recruited 22 patients with benign pancreaticobiliary diseases (benign group) and 12 patients with PB Ca (malignant group). The duodenal microbiota of each patient was analyzed by the 16S rDNA terminal restriction fragment length polymorphism method. Patient characteristics, tumor markers, and relative abundances of the duodenal microbiota were compared between the benign and malignant groups.

RESULTS

Cancer antigen 19-9 (CA19-9), *Bifidobacterium*, *Clostridium* cluster XVIII, and *Prevotella* levels differed significantly between the benign and malignant groups. *Clostridium* cluster XVIII had the greatest area under the receiver operating characteristic curve (AUC) among the four factors with respect to diagnosing PB

statement: This study was approved by the Institutional Review Board of Fukushima Medical University (Approval No. 2451).

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Ca (cutoff value: 3.038%; sensitivity: 58.3%; specificity: 95.2%; AUC: 0.81). The combination of *Clostridium* cluster XVIII (cutoff value: 3.038%) and CA19-9 Levels (cutoff value: 18.8 U/mL) showed 91.7% sensitivity and 71.4% specificity for diagnosing PB Ca.

CONCLUSION
The duodenal microbiota may be useful for PB Ca screening.

Key Words: Pancreaticobiliary cancer; Diagnostic marker; Duodenal microbiota; *Clostridium* cluster XVIII; Cancer antigen 19-9

Core Tip: Recently, a correlation between the human microbiota and malignant gastrointestinal diseases was reported. In this report, the efficacy of the duodenal microbiota for diagnosing pancreaticobiliary cancer (PB Ca) was investigated. The combination of *Clostridium* cluster XVIII (cutoff value: 3.038%) and cancer antigen 19-9 Levels (cutoff value: 18.8 U/mL) showed 91.7% sensitivity and 71.4% specificity for diagnosing PB Ca. In conclusion, the duodenal microbiota may be useful for PB Ca screening.

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INTRODUCTION

Pancreaticobiliary cancer (PB Ca) is a lethal disease[1]. Surgery is the only radical treatment for pancreatic cancer, but unfortunately, many pancreatic cancer patients have advanced-stage lesions or other organ metastases, and they thus are not candidates for surgery[2]. For those who can undergo surgical treatment, the 5-year survival rate is reported to be 20%-30%[3,4].

In general, biliary tract cancer is difficult to diagnose. Conventional diagnostic methods include evaluating tumor markers [cancer antigen 19-9 (CA19-9) or carcinoembryonic antigen (CEA)], biliary biopsy, biliary juice cytology, and brush cytology, but the diagnostic power of these methods is not sufficient[5-19]. It has been reported that serum CA19-9 elevation is observed in 85% of patients with cholangiocarcinoma, though elevation of this marker can also be found in benign obstructive jaundice. Similarly, elevated serum CEA, which is not seen in obstructive jaundice, occurs in only 30% of patients with cholangiocarcinoma[20]. Therefore, effective diagnostic methods for the early diagnosis of PB Ca are urgently needed. Recently, a correlation between the human microbiota and malignant gastrointestinal diseases was reported[21-26]. In addition, oral and salivary microbiota communities have been reported to be effective in diagnosing pancreatic cancer or predicting the onset of pancreatic cancer[27-29], and the risk of pancreatic cancer is reportedly increased in patients with a history of periodontal disease[30]. Furthermore, serum antibodies against oral microbiota are reported to be a risk factor for the onset of pancreatic cancer[31]. However, the mechanism by which this dysbiosis leads to pancreatic cancer is unknown, especially as the pancreas is relatively distant from the mouth.

Thus, we hypothesized that the duodenal microbiota would be more efficient than the oral microbiota for diagnosing PB Ca because the duodenum is closer to the bile duct and pancreas than the oral cavity. The aim of this study was to determine the efficacy of the duodenal microbiota for diagnosing PB Ca.

L-Editor: A
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MATERIALS AND METHODS

Ethical approval

This study was approved by the Institutional Review Board of Fukushima Medical University.

Patients

We assessed 34 patients with pancreaticobiliary disease who visited our hospital over two years. Twenty-two patients were diagnosed with benign pancreaticobiliary diseases (benign group) [chronic pancreatitis: 6; intraductal papillary mucinous neoplasm (IPMN): 5; gallbladder adenomyomatosis: 3; autoimmune pancreatitis: 3; benign common bile duct (CBD) stricture of unknown origin: 2; serous cystic neoplasm: 2; and CBD stone: 1] (Table 1). The other 12 patients were diagnosed with PB Ca (malignant group) (pancreatic cancer: 9; bile duct cancer: 3). The patients provided written informed consent to participate in this study. For all pancreatic cancer cases, the lesion was located in the head. Eight pancreatic cancer patients were diagnosed by endoscopic ultrasound-guided fine needle aspiration. One pancreatic cancer patient was diagnosed with intraductal papillary mucinous carcinoma with evident worsening of the lesion by imaging. Benign diseases were diagnosed by no histological malignancy or unchanging lesions after a clinical course of at least six months. Furthermore, the IPMN patients in the benign group did not have high-risk stigmata or worrisome features[32]. The cases of bile duct cancer were diagnosed by biliary biopsy or surgery. According to the cytology grade, classes IV and V were diagnosed as malignancies. The stage of PB Ca was determined based on the UICC classification, ver. 8.

The patients did not receive antibiotic agents for at least a week prior to duodenal juice collection, and they did not receive steroids at all.

Sample collection and DNA extraction

An endoscope was used under sedation with midazolam. The endoscope was advanced to the duodenum, and 0.5-1.0 mL of duodenal juice was collected through a catheter and stored at -20°C. The endoscope used was Q260 and Q260H, and the catheter was a PR-109Q-1 or PR-104Q-1 (Olympus, Tokyo, Japan).

Bacterial DNA was extracted from duodenal juice samples in accordance with a previous report by Takahashi *et al*[33].

Terminal restriction fragment length polymorphism

Terminal restriction fragment (T-RF) length polymorphism (T-RFLP) was performed by TechnoSuruga Laboratory (Shizuoka, Japan) according to Nagashima's methods[34, 35]. The 16S rRNA gene was amplified from the extracted DNA using the primers 5′ FAM-labeled 516F (5′-TGCCAGCAGCCGCGGTA-3′) and 1510R (5′- GGTTACCTTGT-TACGACTT-3′) and HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) with a Thermal Cycler Dice (Takara, Shiga, Japan). The amplification program used was as follows: preheating at 94°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min; and a terminal extension at 72°C for 10 min. DNA amplification was verified by electrophoresis of the polymerase chain reaction (PCR) products (2 μL) through a 1.0% agarose gel with Tris-acetate-EDTA buffer. The amplified DNA was purified by a MultiScreen PCR96 Filter Plate (Millipore, Billerica, MA, United States).

The purified PCR product (3 μL) was digested with 10 U of Fast Digest *BseL*I (*Bsl*I) (Thermo Fisher Scientific) in a total volume of 15 μL at 37°C for 10 min. The restriction digestion products (0.5 μL) were mixed with 0.1 μL of a DNA fragment-length standard size marker and 10 μL of deionized formamide. The standard size marker was MapMarker X-Rhodamine Labeled 50-1000 bp (Bio Ventures, Murfreesboro, TN, United States). The samples were denatured at 95°C for 2 min and then placed immediately on ice. The T-RF length was established using an ABI PRISM 3130xl genetic analyzer (Thermo Fisher Scientific), and the length and peak area were determined using the genotyping software GeneMapper (Thermo Fisher Scientific). The fragment sizes were estimated using the Local Southern method in GeneMapper software (Thermo Fisher Scientific). If the peak height was less than 50 fluorescence units, the T-RF was excluded from the analysis. The fragments were resolved to one base pair by manual alignment of the size standard peaks from different electropherograms, and the predicted T-RFLP patterns of the 16S rDNA of known bacterial species were obtained using publicly available sequences. T-RFs were divided by operational taxonomic units (OTUs), and bacterial classification was performed

Table 1 Final patient diagnoses

Benign group (*n* = 22)		Malignant group (*n* = 12)	
Chronic pancreatitis	6	Pancreatic cancer, stage (I/II/III/IV)	9 (2/5/1/1/)
IPMN	5	Biliary ductal cancer, stage (I/II/III/IV)	3 (1/2/0/0)
GB ADM	3		
Autoimmune pancreatitis	3		
CBD stricture of unknown origin	2		
Serous cystic neoplasm	2		
CBD stone	1		

IPMN: Intraductal papillary mucinous neoplasm; GB ADM: Gallbladder adenomyomatosis; CBD: Common bile duct.

according to the ratio of each OTU per total OTU area. The OTUs were identified by correspondence to a database of human intestinal flora (https://www.tecsrg.co.jp/t-rflp/index.html).

Analyzed traits

Patient characteristics and tumor markers (age, sex, reduction in body weight ≥ 5 kg within 6 mo prior to duodenal juice sampling, intake of proton pump inhibitors, CA19-9) were compared between the two groups. The body weight marker was selected for the following reasons. The composition ratio of the microbiota has been reported to be different between subjects with obesity and those with a normal body mass index[36]. Because the intake of high-fat foods influences the quantity and composition of bile acid, the intestinal bacterial flora might change[37]. The relative abundances of duodenal microbiota members (*Bacteroides*, *Bifidobacterium*, Lactobacillales, *Prevotella*, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, *Clostridium* cluster IX, *Clostridium* cluster XI, *Clostridium* cluster XVIII, and others) were compared between the benign and malignant groups.

Statistical analysis

Normally distributed continuous variables were compared using Student's *t* test and nonnormally distributed continuous variables using the Mann-Whitney *U* test. Nominal variables were compared with Fisher's exact test. A receiver operating characteristic (ROC) curve was employed to compare the accuracy of the biomarkers. The *P* value < 0.05 was considered statistically significant.

These statistical analyses were performed using the EZR platform (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R-commander that was designed to perform functions that are frequently used in biostatistics[38].

RESULTS

Among the patient characteristics and tumor markers, age and CA19-9 Levels were significantly different between the benign and malignant groups (mean ± SD, age: 63.3 ± 12.2 *vs* 73.0 ± 8.3 years, *P* value = 0.016; median (range), CA19-9: 5.4 (2.0-54.8) *vs* 22.8 (2.0-9893.2), *P* value = 0.03) (Table 2).

Comparison of microbiome components revealed *Bifidobacterium*, *Clostridium* cluster XVIII, and *Prevotella* to be significantly different between the benign and malignant groups (median (range), *Bifidobacterium*: 0 (0-0.5)% *vs* 0.3 (0-4.5)%, *P* value < 0.05; *Clostridium* cluster XVIII: 1.3 (0-3.9)% *vs* 3.6 (0.8-14.9)%, *P* value = 0.006; *Prevotella*: 2.2 (0-25.9)% *vs* 0.1 (0-10.9)%, *P* value = 0.04) (Figure 1 and Table 3).

To determine the influence of age on CA19-9 Levels and microbiome composition, these factors were compared between the subgroup of patients < 69 years and the subgroup of those ≥ 69 years (the median age of all patients was 69 years). According to the results, CA19-9 Levels, *Bifidobacterium*, *Clostridium* cluster XVIII, and *Prevotella* were not influenced by age (Table 4).

Table 2 Comparison of patient characteristics, tumor markers, and microbiomes

	Benign group (*n* = 22)	Malignant group (*n* = 12)	*P* value
Age, yr (mean ± SD)	63.0 ± 12.2	73.0 ± 8.3	0.016
Sex (male/female)	8/14	4/8	1.0
Reduction in body weight ≥ 5 kg within 6 mo before duodenal juice sampling, *n* (%)	1 (4.5)	2 (16.7)	0.28
Intake of proton pump inhibitors, *n* (%)	4 (18.2)	2 (16.7)	1.0
CA19-9, U/mL, median (range)	5.4 (2.0-54.8)	22.8 (2.0-9893.2)	0.03

CA19-9: Cancer antigen 19-9.

Table 3 Microbiome comparison

	Benign group (*n* = 22)	Malignant group (*n* = 12)	*P* value
Bacteroides, %, median (range)	4.3 (0-26.1)	5.6 (0-46.4)	0.55
Bifidobacterium, %, median (range)	0 (0-0.5)	0.3 (0-4.5)	< 0.05
Clostridium cluster IV, %, median (range)	2.9 (0-10.8)	3.4 (0-8.8)	0.80
Clostridium cluster IX, %, median (range)	4.7 (0.6-19.5)	4.9 (0-17.8)	0.68
Clostridium cluster XI, %, median (range)	0 (0-0)	0 (0-0)	
Clostridium cluster XVIII, %, median (range)	1.3 (0-3.9)	3.6 (0.8-14.9)	0.006
Clostridium subcluster XIVa, %, median (range)	5.1 (0-23.1)	6.4 (2.9-13.7)	0.38
Lactobacillales, %, mean ± SD	63.0 ± 19.7	62.6 ± 18.3	0.95
Prevotella, %, median (range)	2.2 (0-25.9)	0.1 (0-10.9)	0.04
Others, %, median (range)	4.9 (2.5-20.4)	4.1 (1.5-6.3)	0.14

Table 4 Effects of age on cancer antigen 19-9 levels and the human microbiome

	Age < 69 yr (*n* = 17)	Age ≥ 69 yr (*n* = 17)	*P* value
CA19-9, U/mL, median (range)	7.1 (2-129.3)	4.9 (2-9893.2)	0.77
Bifidobacterium, %, median (range)	0.3 (0-0.5)	0 (0-4.5)	0.3
Clostridium cluster XVIII, %, median (range)	2.6 (0-5.7)	1.8 (0-14.9)	0.82
Prevotella, %, median (range)	1.3 (0-18.2)	1.9 (0-25.9)	0.56

CA19-9: Cancer antigen 19-9.

We assessed the ability of the microbiota to diagnose PB Ca by calculating the area under the ROC curve (AUC) and found that *Clostridium* cluster XVIII had the highest AUC (cutoff value: 3.038%, sensitivity: 58.3%, specificity: 95.2%, AUC: 0.81) among the three microbiome components (*Bifidobacterium*, *Clostridium* cluster XVIII, *Prevotella*) and CA19-9 Levels (Figure 2).

The combination of *Clostridium* cluster XVIII (cutoff value: 3.038%) and CA19-9 Levels (cutoff value: 18.8 U/mL) was also examined as a marker to diagnose PB Ca; the sensitivity of this combination was 91.7% (11/12), and the specificity was 71.4% (15/21) (Table 5). CA19-9 data were missing for one patient in the benign group.

DISCUSSION

In this study, we investigated which members of the duodenal microbiota could aid in diagnosing PB Ca and found *Clostridium* cluster XVIII to be more useful than CA19-9 Levels and other bacteria for diagnosing PB Ca. Notably, the combination of

Table 5 Diagnosis of pancreaticobiliary cancer by the combination of *Clostridium* cluster XVIII and cancer antigen 19-9 levels

	Cutoff value	Sensitivity	Specificity
CA19-9	18.8 U/mL	66.7% (8/12)	76.2% (16/21[1])
Clostridium cluster XVIII	3.038%	58.3% (7/12)	95.2% (20/21[1])
Combination of *Clostridium* cluster XVIII and CA19-9		91.7% (11/12)	71.4% (15/21[1])

[1]CA19-9 data were missing for a patient in the benign group.
CA19-9: Cancer antigen 19-9.

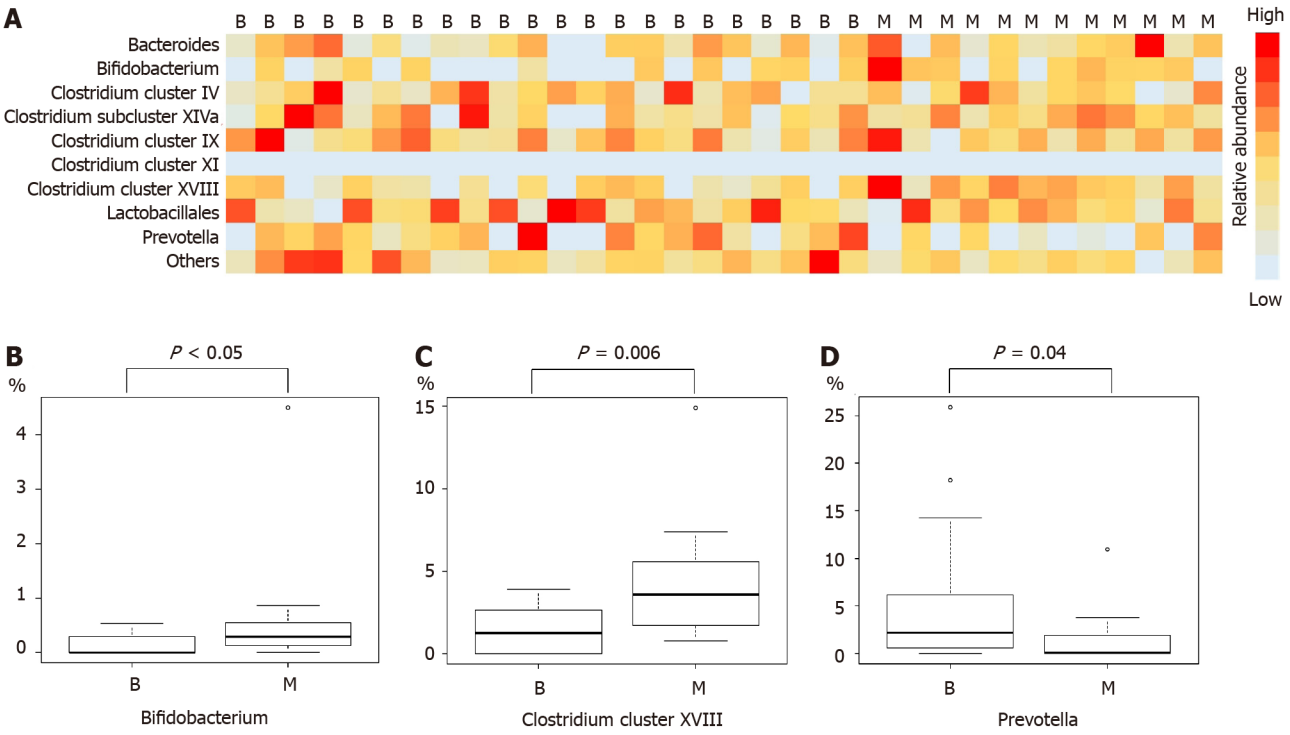

Figure 1 Analysis of the duodenal microbiota. A, B: *Bifidobacterium* levels were significantly higher in the malignant group than in the benign group; A, C: *Clostridium* cluster XVIII levels were significantly higher in the malignant group than in the benign group; A, D: *Prevotella* levels were significantly higher in the benign group than in the malignant group. B: Benign group; M: Malignant group.

Clostridium cluster XVIII and CA19-9 Levels showed high sensitivity, indicating that this combination is valuable for screening patients for PB Ca.

As mentioned above, the oral microbiota has been considered to be a biomarker in pancreatic cancer. First, Michaud *et al*[30] reported that a history of periodontal disease was a risk factor for pancreatic cancer. After that, several oral microbes were reported to be more abundant and possible predictors and risk factors for survival in pancreatic cancer (Table 6)[27-29,39,40]. In addition, antibodies against *Porphyromonas gingivalis* can serve as a risk factor for pancreatic cancer onset[27].

Although the salivary microbiome may be useful for the medical care of patients with pancreatic cancer, it is influenced by differences in oral hygiene, mastication, and swallowing among individuals[27,41,42]. In contrast, the duodenal microbiota is more relevant to the pancreas and bile duct than is the salivary microbiota. Therefore, the duodenal microbiota was hypothesized to directly reflect the dysbiosis associated with PB Ca, and in fact, the results of this study reveal that the duodenal microbiota might be beneficial for screening PB Ca. The microbiota around the pancreas and biliary duct have been reported. Microbes of the duodenal mucosa, bile juice, cancer tissue, and cyst fluid of IPMN in pancreaticobiliary tumor patients have been investigated (Table 6)[30,43-47]. However, the methods used to analyze the microbiota around the pancreas and bile duct in these reports were more invasive than duodenal juice sampling.

Table 6 Past reports on microbes and pancreaticobiliary cancer

Disease	Ref.	Microbes	Sample	Role
Pancreatic cancer	Michaud *et al* [30]	A history of periodontal diseases		Risk factor
	Farrell *et al* [28]	A combination of *Neisseria elongate* and *Streptococcus mitis*	Oral	Distinguishing from healthy controls
	Torres *et al* [29]	Ratio of *Leptotrichia* to *Porphyromonas*	Saliva	Higher in pancreatic cancer patients
	Fan *et al*[27]	*Porphyromonas gingivalis*	Oral, antibody	Risk factor
	Olson *et al*[39]	Firmicutes	Oral	More abundant
	Lu *et al*[40]	*Leptotrichia, Fusobacterium, Rothia, Actinomyces, Corynebacterium, Atopobium, Peptostreptococcus, Catonella, Oribacterium, Filifactor, Campylobacter, Moraxella, Tannerella*	Tongue coating	More prevalent
	Mei *et al*[46]	*Acinetobactor, Aquabacterium, Oceanobacillus, Rahnella, Massilia, Delftia, Deinococcus, Sphingobium*	Duodenal mucosa	More abundant
	Mitsuhashi *et al*[43]	*Fusobacterium species*	Cancer tissue	Poor prognosis
	Riquelme *et al* [44]	*Pseudoxanthomonas, Streptomyces, Saccharopolyspora, Bacillus clausii*	Cancer tissue	Long-term survival
Pancreatic and ampullary cancer	Di Calro *et al* [45]	*Escherichia coli, Klebsiella pneumoniae*	Bile juice	Predictor for survival
IPMN with high-grade dysplasia	Gaiser *et al* [47]	*Granulicatella adiacens, Fusobacterium nucleatum*	Cyst fluid	More abundant

IPMN: Intraductal papillary mucinous neoplasm.

The mechanism by which microbes lead to PB Ca remains unknown. The etiology with respect to the salivary microbiota has been considered in past reports. *P. gingivalis* can interrupt signaling pathways by modulating receptor expression and cytokine secretion to evade the host's immune system[48-52]. Moreover, *P. gingivalis* activates the Toll-like receptor signaling pathway[53,54], which has been reported to be related to pancreatic carcinogenesis[55,56]. In other reports, oral bacteria were found outside of the oral cavity in the gastrointestinal tract. Immune responses against these bacteria can cause inflammation and carcinogenesis in the pancreas. Lipopolysaccharide has also been reported to drive pancreatic carcinogenesis by blocking the MyD88-dependent, Toll-like receptor 4 and MyD88-independent pathways[57]. In another report, the mechanism was described as follows. Bacterial ligands detected by Toll-like receptors cause a Th1/Th2/Th17 imbalance in the tumor microenvironment, promoting tumorigenesis in combination with *Kras* mutation. In the duodenum, microbes may reach the pancreatic duct or biliary duct through the Vater papilla. In the pancreas or bile duct, pattern recognition receptors (such as Toll-like receptors) are stimulated by the pathogenic molecular patterns of bacterial ligands and induce lower levels of immune suppression, leading to the development of PB Ca[58]. These results from past reports suggest that some type of immune system response is the link between the duodenal microbiota and PB Ca.

On the one hand, the relationship between *Clostridium* cluster XVIII and carcinogenesis has not been reported, even though *Clostridium* cluster XVIII is reported to have the potential to enhance regulatory T (Treg) cells[59]. Many Treg cells exist in tumor tissue and prevent the immune response to tumors. Therefore, Tregs contribute to tumor progression and poor prognosis[60-64]. Thus, *Clostridium* cluster XVIII may increase in response to cancer and activate Tregs. Alternatively, *Clostridium* cluster XVIII may activate Tregs, with oncogenesis advancing.

This report has some limitations. First, this study was small and performed at a single institution. However, based on the data from *Clostridium* cluster XVIII, the average value of the malignant group was 4.5%, and that of the benign group was 1.5%. Total thirty patients were needed to achieve an α error of 5% and a β value of 0.2. When *Clostridium* cluster XVIII was the main outcome, the minimum necessary sample size was secured. Although this is the first report to describe the relationship between the duodenal juice microbiota and PB Ca, the diseases in the malignant group were not

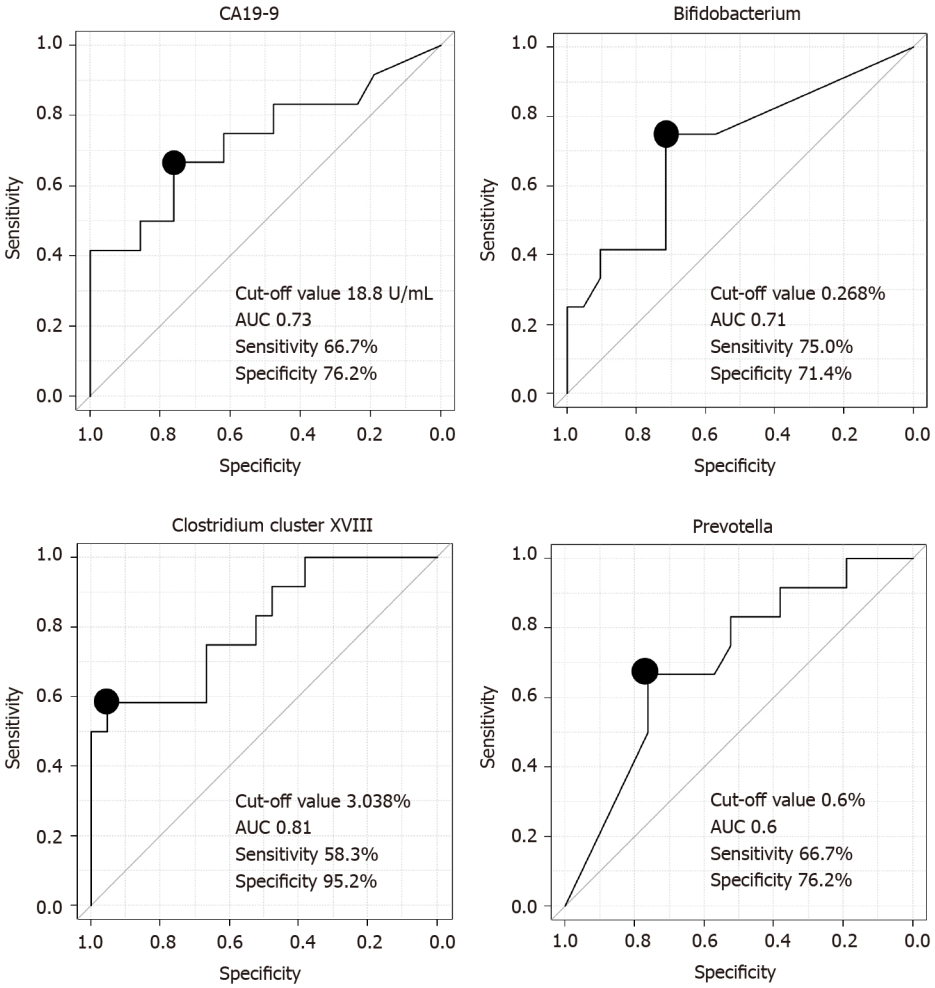

Figure 2 Comparison of the ability of microbiome components and cancer antigen 19-9 Levels to diagnose pancreaticobiliary cancer. The area under the receiver operating characteristic curve of *Clostridium* cluster XVIII was the highest among the three microbes and cancer antigen 19-9 Levels. CA19-9: Cancer antigen 19-9; AUC: The area under the curve.

uniform. If subgroup analyses of pancreatic diseases were performed, the abundance of some duodenal microbes would be significantly different between the benign and malignant groups (Table 7). We hope that a future study with a larger number of patients will confirm our results for both pancreatic cancer and biliary cancer. Second, healthy control subjects were not enrolled in this study. However, as esophagogastroduodenoscopy under sedation is rarely performed in healthy patients, this limitation was unavoidable in the study design. Third, T-RFLP was applied. Investigations into the duodenal microbiota have been limited because duodenal juice cannot be collected in large volumes (less than 0.5 mL is typically collected). However, the measurement of the duodenal microbiota was demonstrated to be possible. Follow-up studies using next-generation sequencing are warranted[65]. Fourth, examining the duodenal microbiota requires a somewhat invasive technique. In the future, the development of serum antibody testing for the duodenal microbiota should be pursued.

CONCLUSION

In conclusion, the duodenal microbiota may contribute to PB Ca screening.

Table 7 Microbiome comparison in patients with pancreatic disease

	Benign pancreatic diseases (*n* = 16)	Pancreatic cancer (*n* = 9)	*P* value
Bacteroides, %, median (range)	2.1 (0-26.1)	5.8 (0-46.4)	0.17
Bifidobacterium, %, median (range)	0 (0-0.5)	0.48 (0-4.5)	0.03
Clostridium cluster IV, %, mean ± SD	4.0 ± 3.3	3.6 ± 2.9	0.76
Clostridium cluster IX, %, mean ± SD	5.3 ± 3.6	6.4 ± 5.2	0.57
Clostridium cluster XI, %, median (range)	0 (0-0)	0 (0-0)	
Clostridium cluster XVIII, %, median (range)	1.4 (0-3.9)	3.0 (0.8-14.9)	0.04
Clostridium subcluster XIVa, %, median (range)	3.8 (0-21.4)	6.0 (2.9-13.7)	0.32
Lactobacillales, %, mean ± SD	68.4 ± 19.3	59.7 ± 20.4	0.3
Prevotella, %, median (range)	4.2 (2.5-20.4)	4.0 (1.5-6.3)	0.3
Others, %, median (range)	2.0 (0-18.3)	0.3 (0-11.0)	0.3

ARTICLE HIGHLIGHTS

Research background

Pancreaticobiliary cancer (PB Ca) is a lethal disease; however, there are currently no appropriate diagnostic and prognostic markers. Recently, the human microbiota was reported to be a causative factor, diagnostic marker, and prognostic marker for gastrointestinal malignant diseases.

Research motivation

The oral and fecal microbiota have been reported to be useful diagnostic markers for gastrointestinal cancer. The duodenum is located closer to the pancreas and bile duct than the oral cavity and colon. Therefore, we hypothesized that assessment of the duodenal microbiota might improve the diagnostic accuracy for PB Ca.

Research objectives

To investigate the diagnostic accuracy of duodenal microbiota evaluation for PB Ca.

Research methods

Thirty-four PB Ca and benign pancreaticobiliary disease patients were recruited for this study, and their duodenal juice was aseptically collected by endoscopy. The duodenal microbiota was analyzed, and the relative abundances of species in the duodenal microbiota were compared between PB Ca patients and benign pancreaticobiliary disease patients. The PB Ca diagnosability was compared between a conventional tumor marker and species in the duodenal microbiota with significantly different abundances in PB Ca patients *vs* benign pancreaticobiliary disease patients.

Research results

The abundances of cancer antigen 19-9 (CA19-9), *Bifidobacterium*, *Clostridium* cluster XVIII, and *Prevotella* were significantly different between PB Ca patients and benign pancreaticobiliary disease patients. The diagnostic capacity of *Clostridium* cluster XVIII was the highest among the four markers (CA19-9, *Bifidobacterium*, *Clostridium* cluster XVIII, and *Prevotella*). The combined assessment of *Clostridium* cluster XVIII and CA19-9 Levels was useful for PB Ca diagnosis.

Research conclusions

It was possible to investigate the microbiota of duodenal juice. Duodenal microbiota evaluation may contribute to the diagnosis of PB Ca.

Research perspectives

In the future, novel diagnostic and prognostic markers and treatments could be developed by investigating the relationship between the duodenal microbiota and PB Ca.

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ORIGINAL ARTICLE

Basic Study

MutL homolog 1 methylation and microsatellite instability in sporadic colorectal tumors among Filipinos

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Author contributions: Enriquez MLD conceptualized the project; Cabral LKD designed and optimized the experiments; Mapua CA screened data of the colorectal cancer databank and performed statistical analysis; Cabral LKD and Sukowati CHC analyzed data and wrote the manuscript; Cortez ER and Natividad FF coordinated specimen collection and funds allocation as study group head and division head respectively; all authors read and approved the manuscript.

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Abstract

BACKGROUND

Colorectal cancer (CRC) ranks third in terms of incidence and second in mortality worldwide. In CRC, the silencing of mismatch repair genes, including the mutL homolog 1 (*hMLH1*) has been linked to microsatellite instability (MSI), the lengthening or shortening of microsatellite repeats. Very limited data have been presented so far on the link of *hMLH1* methylation and MSI in Southeast Asia populations with sporadic CRC, and on its clinical significance.

AIM

To investigate the significance of the MSI status and *hMLH1* methylation in CRC Filipino patients.

METHODS

Fifty-four sporadic CRC patients with complete clinical data were included in this study. Genomic DNA from CRC tumor biopsies and their normal tissue counterparts were profiled for MSI by high resolution melting (HRM) analysis using the Bethesda Panel of Markers (BAT25, BAT26, D2S123, D5S346, and D17S250). *hMLH1* methylation screening was performed using bisulfite conversion and methylation specific polymerase chain reaction. Statistical analysis was conducted to calculate their associations to clinicopathological characteristics

Data sharing statement: The authors may share the data for justified reason.

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and survival relevance (Kaplan-Meier curves and the log-rank test).

RESULTS
hMLH1 methylation was observed in 9% and 35% of CRC and normal samples, respectively. Higher incidence of consistently methylated *hMLH1* found in both normal and CRC was noticed for relation to location of tumor ($P < 0.05$). As for MSI status, D2S123 the most common unstable microsatellite and MSI-high (MSI-H) was the most common MSI profile, counted for 46% and 50% of normal and CRC tissues, respectively. The presence of MSI-low (MSI-L) and microsatellite stable (MSS) was 43% and 11% for normal, and 31% and 19% for CRC samples. The mean month of patients' survival was shorter in patients whose normal and tumor tissues had methylated compared to those with unmethylated *hMLH1* and with MSI-H compared to those with MSI-L/MSS ($P < 0.05$). This was supported by significant difference in Kaplan-Meier with log-rank analysis. This data indicated that *hMLH1* methylation and high MSI status have prognostic value.

CONCLUSION
This study showed the clinical significance of *hMLH1* methylation and MSI status in sporadic CRC Filipino patients, especially in the normal part of the tumor.

Key Words: Sporadic colorectal cancer; DNA methylation; Microsatellite instability; Population genetic; Colorectal cancer

Core Tip: Colorectal cancer (CRC) ranks third in terms of incidence and second in mortality worldwide. In CRC, the silencing of mismatch repair genes, including the mutL homolog 1 (*hMLH1)* has been linked to microsatellite instability (MSI). This study investigated the status of *hMLH1* methylation and MSI in normal and tumor tissues of Filipinos sporadic CRC patients and their clinical significances.

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INTRODUCTION

Colorectal cancer (CRC) occurs when malignant tumors form in the lining of the large intestine, which includes the ascending, transverse, and descending colon, and the rectum. CRC ranks third in terms of incidence and second in mortality worldwide, for both sexes. Over 1.9 million new CRC cases and 935000 deaths were estimated to occur in 2020, accounting for about 1/10 cancer cases and deaths[1]. In the Philippines, CRC is currently the third leading site of malignancy. The incidence of CRC had almost doubled from 2010 to 2015 with survival rates under 50%[2,3]. In terms of mortality rate, Filipinos (and Chinese) ethnicity had significantly decreased risk of death compared with Caucasians[4].

CRC, like many other cancers, is a malignancy caused by DNA changes causing abnormal behavior of cells. DNA deletions, duplications, substitutions, mutations, and rearrangements can either activate or inactivate a gene or several genes and consequently affect cellular function[5]. Depending on the origin of the mutation, CRC can be classified as sporadic (70%), inherited (5%), and familial (25%)[6]. Sporadic CRC is characterized by the carcinogenesis pathway, derived from point mutations which can occur in different genes[7].

In an inherited CRC, the hereditary non-polyposis CRC (HNPCC), germline mutation is involved in one or two of the mutator or mismatch repair (MMR) genes, including the mutL homolog 1 (*hMLH1*). Mutations in *hMLH1* have been linked to microsatellite instability (MSI), the lengthening or shortening of microsatellite repeats

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(sequences with 1-6 repeating nucleotides). When instability is left unrepaired, it may accumulate as mutations and can affect other genes that have microsatellite repeats in their coding regions[8,9]. MSI is found in 85%-90% of HNPCC patients[10].

Recent studies demonstrated that in a subset of sporadic CRC, DNA methylation, the transfer of a covalent methyl group to the C5 position of the cytosine to form 5-methylcytosine by DNA methyltransferases, may cause the loss of function of a MMR gene. Likewise, it leads to the accumulation of MSI[11,12]. Tumors with methylated *hMLH1* and high levels of MSI present with a distinct characteristic from other CRC tumors. CRC with methylated *hMLH1* had a delayed onset and was associated with female gender[13]. This linked *hMLH1* and MSI in the development of sporadic CRC, suggesting that the two molecular profiles are closely related[14].

So far, very limited data have been presented on the link of *hMLH1* methylation and MSI in Southeast Asia populations with sporadic CRC. This study presents the detection and characterization of MSI status and *hMLH1* methylation in CRC and its paired non-tumoral adjacent tissues in Filipino patients.

MATERIALS AND METHODS

Patients and samples

Fifty-four sporadic CRC patients with complete clinical data were included in this study. Diagnosis of CRC was based on the presence of malignancy in the initial biopsy. Patients should be Filipino by descent with no family history of cancer. From each patient, paired tumor and its corresponding normal tissue were obtained from surgical resection at the St. Luke's Medical Center, Quezon City, Philippines. Normal tissues were collected approximately 6 inches away from the margin of the tumor. Upon pathological confirmation, fresh frozen sections were stored in -80°C. The project of the Colorectal Cancer Study Group was approved by the Institutional Ethics Review Board of St. Luke's Medical Center (Project Code No. 06-015). All patients enrolled in the study signed an Informed Consent Form allowing the use of their tissues and clinical data in the Colorectal Cancer (CRC) Databank of St. Luke's Medical Center.

CRC cell lines SW480 (ATCC® CCL-228) and SW48 (ATCC® CCL-23) were purchased from American Type Culture Collection (ATCC) as controls for MSI and *hMLH1* methylation. SW480 is a CRC cell line that has stable microsatellite and unmethylated *hMLH1*, while SW48 is high MSI and methylated *hMLH1*. Lymphocytes from patients who underwent colonoscopy and who were found to be free of cancer and polyp were used as additional controls for stable microsatellite.

Isolation of genomic DNA

Genomic DNA (gDNA) extraction was performed using the QIAamp® DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Briefly, tissues were finely minced and put in lysis solution and proteinase K until completely lysed. After DNA precipitation with ethanol, DNA extract was washed twice and eluted with appropriate buffer. DNA quality and quantity were assessed using Nanodrop® v1000 spectrophotometer (Thermo Fisher Scientific). Final working concentration of 50 ng of gDNA was used for each sample in the succeeding analysis.

Bisulfite conversion and methylation specific polymerase chain reaction for hMLH1

gDNA from normal and tumor specimens was subjected to bisulfite treatment to differentiate methylated cytosines from unmethylated ones. Bisulfite chemically modifies non-methylated cytosines into uracil, which is then converted to thymidine in polymerase chain reaction (PCR) cycles.

Bisulfite treatment of the DNA sample was performed using EZ DNA Methylation Lightning Kit (Zymo Research) according to the manufacturer's suggestion. Briefly, 200-500 ng gDNA was incubated in the conversion reagent and then treated with binding buffer in a spin column. Converted DNA was then subjected to desulphonation and clean-up using washing buffer. DNA (approximately 10 μL) was eluted and collected for methylation specific PCR (MS-PCR)[15]. Reaction was carried out using Qiagen Taq Core Kit in a reaction volume of 25 μL with 10x PCR Buffer, 2.5 mmol/L of $MgCl_2$, 50 pmol of primer, 1.25 mmol/L of dNTPs and 1.25 units of Taq DNA polymerase added to 1.5-2 μL of converted DNA. Primer sets used for *hMLH1* MS-PCR are taken from published work of Fox *et al*[15].

MSI test by high resolution melting analysis

MSI test was carried out using PCR for five markers from the Bethesda panel that included BAT25, BAT26, D2S123, D5S346, and D17S250, as in a previous study[16]. PCR conditions of each marker were optimized and validated in our earlier study on HNPCC (Evangelista & Enriquez, unpublished work).

In brief, PCR was performed using Qiagen Taq PCR core kit with EvaGreen dye (Biotium) in a 25 μL reaction volume containing 50 ng of gDNA. All reactions were done in triplicates. PCR and high resolution melting (HRM) analysis were carried out using Rotor-Gene™ 6000 (Qiagen) system with data collected over the range from 55 °C to 95 °C with ramp rising at 0.1 °C/s[17]. Melting curve was analyzed using Rotor-Gene Q (RGQ) Scanning Software version 2.0.2 (Qiagen). Raw melting-curve data were normalized by manual adjustment of linear regions before (pre-; 100% fluorescence) and after (post-; 0% fluorescence) the melting transition. The melting curve of DNA from SW480 cells which exhibits microsatellite stable (MSS) phenotype [18] was used as the normal/stable control. The RGQ software assigned the profile of each sample in reference to the stable control. The confidence percentage (cut-off), was optimized by analyzing non-cancer patients' DNA, with values not lower than 60%. Therefore, the confidence value of ≥ 60% was regarded as MSS, while any confidence value of < 60% was regarded as unstable.

MSI score was defined as MSI-high (MSI-H) where HRM instability was observed in ≥ 2 markers; MSI-low (MSI-L) where instability was only in one marker; and MSS if no instability was observed in any of the markers. For validation, amplified PCR products of the stable and unstable control were subjected to Sanger sequencing to verify their microsatellite repeats.

Statistical analysis

Statistical analysis was constructed using software GraphPrism version 5.01 (GraphPad Software, Inc., La Jolla, CA, United States). Associations between clinicopathological characteristics (age, sex, tumor location, grade and stage), MSI status and methylation status were determined using Pearson's chi-square test or Fisher's exact test. Survival relevance was analyzed using Kaplan-Meier curves and the log-rank test based on the *hMLH1* methylation and MSI status grouping. All tests were two-tailed and values were considered significant at P value of less than 0.05.

RESULTS

Patients' demographic and tumor features

This study analyzed 54 Filipino CRC patients with comparable male to female ratio (30M:24F). The age mean was 56.9 ± 11.8 years old, with 5 patients under 40 years old and 49 patients above 40 years old. Regarding tumor parameters, CRC was mostly found in the distal part of the colon (38; 70%), followed by the rectum (10; 19%) and proximal part of the colon (6; 11%). By histology, moderately-differentiated grade CRC was noticed in 44 (81%), poorly-differentiated in 8 (15%), and well-differentiated in 2 (4%) patients. By disease stage, stage 1 CRC was observed in 6 (11%), stage 2 in 14 (26%), stage 3 in 30 (56%), and stage in 4 (7%) of patients. None of the patients had family history of CRC.

hMLH1 methylation status in Filipino CRC patients

We analyzed the *hMLH1* DNA methylation by MS-PCR. From 54 paired CRC and its normal adjacent tissue, most of the samples were unmethylated (91% and 65%, respectively). Representative MS-PCR gel electrophoresis is shown in Figure 1. *hMLH1* methylation was noticed only in 5 (9%) and 19 (35%) of CRC and normal samples, respectively. It is interesting to note that the methylated *hMLH1* in tumor tissues was accompanied by methylation in its paired normal tissues (4/5, 80%). Only 1 sample showed tumoral *hMLH1* methylation without methylation in its normal tissue (Figure 2).

The distribution of *hMLH1* methylation status in patients' demography and tumor parameters is shown in Table 1 (left panel). In normal tissues, the incidence of *hMLH1* methylation was slightly higher in female compared to male (42% *vs* 30%), and in tumors located in the proximal colon compared to distal/rectum location (67% *vs* 31%). The same pattern was found for *hMLH1* methylation in tumor tissues. Methylation was higher in females compared to males (13% *vs* 7%), and in tumors located in the proximal colon compared to those in distal colon or rectum (33% *vs* 6%, P = 0.031).

Table 1 *hMLH1* methylation status in the Filipinos colorectal cancer patients

		Normal tissue (%)			Tumor tissue(%)			Normal/tumor (%)			
		M	U		M	U		M/M	U/U	M/U or U/M	
	Freq	19	35	*P* value	5	49	*P* value	4	34	16	*P* value
Gender											
Male	30	9 (30)	21 (70)	NS	2 (7)	28 (93)	NS[1]	1 (3)	20 (67)	9 (30)	NS
Female	24	10 (42)	14 (58)		3 (12)	21 (88)		3 (13)	14 (58)	7 (29)	
Age											
≤ 40	5	3 (60)	2 (40)	NS[1]	0 (0)	5 (100)	NS[1]	0 (0)	2 (40)	3 (60)	NS
> 40	49	16 (33)	33 (67)		5 (10)	44 (90)		4 (8)	32 (65)	13 (27)	
Location of tumor											
Proximal	6	4 (67)	2 (33)	NS[1]	2 (33)	4 (67)	0.031	2 (33)	2 (33)	2 (33)	0.029
Distal/rectum	48	15 (31)	33 (69)		3 (6)	45 (94)		2 (4)	32 (67)	14 (29)	
Tumor grade											
Poor	8	5 (62)	3 (38)	NS	1 (12)	7 (88)	NS	1 (12)	3 (38)	4 (50)	NS
Moderate	44	13 (30)	31 (70)		4 (9)	40 (91)		3 (7)	30 (68)	11 (25)	
Well	2	1 (50)	1 (50)		0 (0)	2 (100)		0 (0)	1 (50)	1 (50)	
Tumor stage											
I-II	20	6 (30)	14 (70)	NS	2 (10)	18 (90)	NS[1]	2 (10)	14 (70)	4 (20)	NS
III-IV	34	13 (38)	21 (62)		3 (9)	31 (91)		2 (6)	20 (59)	12 (35)	

[1]Fisher's exact test.

NS: Not significant; M: Methylated; U: Unmethylated; In paired normal/tumor tissues: M/M: Methylated/methylated; U/U: Unmethylated/unmethylated; M/U: Methylated/unmethylated; U/M: Unmethylated/methylated.

The signature of *hMLH1* status in paired normal and tumor tissue is presented in Table 1 (right panel). Although the incidence was not statistically significant, a higher incidence of methylated *hMLH1* in both normal and tumor tissues (M/M) was noticed in females, in > 40 years old patients, and in poorly differentiated cancer. A significant difference was found for the tumor location (P = 0.029), where M/M signature was noticed in 33% of proximal tumors, while U/U was in 67% of distally located or rectal tumors.

MSI status in Filipino sporadic CRC patients

MSI status was assessed using HRM of the Bethesda panel that included BAT25, BAT26, D2S123, D5S346, and D17S250. As controls, cell lines SW480 and SW48 were used to represent a MSS and MSI-H, respectively. MSS feature was also checked in lymphocytes DNA from a normal individual. A representative HRM analysis of BAT26 gene and its direct Sanger sequencing are shown in Figure 1.

From the Bethesda panel markers, the microsatellite with the highest instability was D2S123 and this was found in 32 (59%) and 33 (61%) samples of normal and tumor, respectively. On the other hand, BAT26 instability was observed only in 1 (2%) sample of each normal and tumor tissues. In normal tissues, the instability of BAT25, D17S250, and D5S346, were 2 (4%), 14 (26%), and 29 (54%), respectively. Among the CRC tissues, instability was found in BAT25, BAT26, D17S250, and D5S346 accounting for 5 (20%), 12 (22%), and 25 (46%), respectively. There was no statistical difference in the instability of these markers between normal and tumor tissues.

The distribution of MSI status in patients' demography and tumor parameter is shown in Table 2 (left panel). In normal tissue samples, 25 (46%), 23 (43%), and 6 (11%) samples were MSI-H, MSI-L, and MSS, respectively. In female, the highest percentage was for MSI-H (54%), followed by MSI-L (33%), and MSS (13%). Similar pattern was observed in tumoral tissues with the following rates: MSI-H with 27 (50%), MSI-L with 17 (31%), and MSS with 10 (19%). Instability rates of microsatellites in tumors tissues in the female group were the exactly the same as in the normal tissues.

Table 2 Microsatellite instability status in the Filipinos colorectal cancer patients

		Normal tissue (%)				Tumor tissue (%)				Normal/tumor (%)					
		MSI-H	MSI-L	MSS	*P* value	MSI-H	MSI-L	MSS	*P* value	H/H	L/L	S/S	L/S or S/L	L/H or H/L	*P* value
	Freq	25	23	6		27	17	10		19	8	3	10	14	
Gender															
Male	30	12 (40)	15 (50)	3 (10)	NS	14 (47)	9 (30)	7 (23)	NS	9 (30)	4 (13)	1 (3)	8 (27)	8 (27)	NS
Female	24	13 (54)	8 (33)	3 (13)		13 (54)	8 (33)	3 (13)		10 (42)	4 (17)	2 (8)	2 (8)	6 (25)	
Age															
≤ 40	5	3 (60)	2 (40)	0 (0)	NS	2 (40)	1 (20)	2 (40)	NS	2 (40)	0 (0)	0 (0)	2 (40)	1 (20)	NS
> 40	49	22 (45)	21 (43)	6 (12)		25 (51)	16 (33)	8 (16)		17 (35)	8 (16)	3 (6)	8 (16)	13 (27)	
Location of tumor															
Proximal	6	4 (67)	2 (33)	0 (0)	NS	4 (67)	2 (33)	0 (0)	NS	4 (67)	2 (33)	0 (0)	0 (0)	0 (0)	NS
Distal/rectum	48	21 (44)	21 (44)	6 (12)		23 (48)	15 (31)	10 (21)		15 (31)	6 (13)	3 (6)	10 (21)	14 (29)	
Tumor grade															
Poor	8	5 (62)	3 (38)	0 (0)	NS	5 (63)	2 (25)	1 (12)	NS	4 (50)	1 (13)	0 (0)	1 (13)	2 (25)	NS
Moderate	44	19 (43)	19 (43)	6 (14)		21 (48)	15 (34)	8 (18)		14 (32)	7 (16)	3 (7)	8 (18)	12 (27)	
Well	2	1 (50)	1 (50)	0 (0)		1 (50)	0 (0)	1 (50)		1 (50)	0 (0)	0 (0)	1 (50)	0 (0)	NS
Tumor stage															
I-II	20	9 (45)	9 (45)	2 (10)	NS	8 (40)	10 (50)	2 (10)	NS	7 (35)	6 (30)	0 (0)	4 (20)	3 (15)	NS
III-IV	34	16 (47)	14 (41)	4 (12)		19 (56)	7 (21)	8 (23)		12 (35)	2 (6)	3 (9)	6 (18)	11(32)	

MSI-H: Microsatellite instability-high; MSI-L: Microsatellite instability-low; MSS: Microsatellite stable; NS: Not significant; In paired normal/tumor tissues: H/H: Microsatellite instability-high/microsatellite instability-high; L/L: Microsatellite instability-low/microsatellite instability-low; S/S: Microsatellite stable/microsatellite stable; L/S: Microsatellite instability-low/microsatellite stable; S/L: Microsatellite stable/microsatellite instability-low; L/H: Microsatellite instability-low/microsatellite instability-high; H/L: Microsatellite instability-high/microsatellite instability-low.

The signature of MSI status in paired normal and tumor tissue is presented in Table 2 (right panel). Analysis of data using showed that the MSI status between normal and tumor tissues was not statistically significant. Nonetheless, a slightly higher incidence of MSI-H in *hMLH1* was observed in both normal and tumor tissues (H/H) among females, similar to methylated *hMLH1*.

Correlation of MSI and hMLH1 methylation status

Table 3 shows the MSI and methylation status of each sample according to the paired groupings described previously. There was no statistically significant correlation between MSI and *hMLH1* methylation status of all the sample pairs. Thirteen paired samples (24%) were unmethylated with MSI-H (H/H and U/U). The clinicopathological profile of this cancer group showed that 11 samples came from the distal colon/rectum (85%), 12 (92%) were > 40 years old, 10 (77%) were moderately differentiated, and 8 (62%) were stage III/IV cancers.

Correlation of MSI and hMLH1 methylation status with prognosis

To determine the prognostic values of both *hMLH1* methylation and MSI status, we performed Kaplan-Meier analysis on patient survival for independent groups. The survival was defined as death event (in months) post-surgery. Patients were followed up until 60 mo after surgery.

Table 3 Correlation of *hMLH1* methylation and microsatellite instability status of paired samples

		hMLH1 methylation (%)					
		M/M	M/U	U/M	U/U	Total	*P* value
MSI status	H/H	1 (5)	5 (26)	0 (0)	13 (68)	19	NS
	L/L	1 (13)	4 (50)	0 (0)	3 (37)	8	
	S/S	0 (0)	1 (33)	0 (0)	2 (67)	3	
	L/S or S/L	1 (10)	3 (30)	0 (0)	6 (60)	10	
	L/H or H/L	1 (7)	2 (14)	1 (7)	10 (71)	14	
	Total	4 (7)	15 (28)	1 (2)	34 (63)	54	

MSI: Microsatellite instability; M: Methylated; U: Unmethylated; In paired normal/tumor tissues: M/M: Methylated/methylated; U/U: Unmethylated/unmethylated; M/U: Methylated/unmethylated; U/M: Unmethylated/methylated; In paired normal/tumor tissues: H/H: Microsatellite instability-high/microsatellite instability-high; L/L: Microsatellite instability-low/microsatellite instability-low; S/S: Microsatellite stable/microsatellite stable; L/S: Microsatellite instability-low/microsatellite stable; S/L: Microsatellite stable/microsatellite instability-low; L/H: Microsatellite instability-low/microsatellite instability-high; H/L: Microsatellite instability-high/microsatellite instability-low; NS: Not significant.

Our results show that the mean survival was relatively shorter in patients with methylated compared to those with unmethylated *hMLH1*, both in tumor [18 (6-29) mo *vs* 44 (34-54) mo] and in normal tissues [34 (21-46) mo *vs* 44 (33-54) mo], expressed as mean (95% confidence interval). A significant difference was also observed in tumor tissues with MSI-H compared to those with MSI-L/MSS [27 (19-36) mo *vs* 56 (44-67) mo]. For the MSI status in normal tissues, again a shorter survival was found for MSI-H compared to MSI-L/MSS [27 (18-36) mo *vs* 54 (39-68) mo].

Kaplan-Meier analysis was performed to associate the *hMLH1* methylation and MSI status with patients' survival in both cohorts. In concordance with the mean survival above, we found the association of MSI-H status in both normal and tumor tissues with shorter probability of survival ($P < 0.05$), compared to that of MSI-L/MSS (Figure 3). Our data indicated that *hMLH1* methylation and high MSI status might have a prognostic value.

DISCUSSION

CRC is a classic example of disease progression accompanied by molecular changes. Its carcinogenesis is composed of stage-specific molecular changes in gene expression and the accumulation of mutations. A mutation in the MMR genes may lead to accumulation of DNA damage. Consequently, the tumor suppressor genes or oncogenes are affected, leading to the development of the cancer[8].

DNA methylation has been linked to tumor suppressor gene silencing[19], including the silencing of *hMLH1* MMR gene[20]. In a subset of sporadic CRC, the loss of function of *hMLH1* gene led to the accumulation of instability in microsatellite regions of the DNA[11,12].

In this study, there was a notable higher incidence of methylation of *hMLH1* in normal tissue as compared to its paired tumor tissue (35% *vs* 9%). This is in agreement with other studies showing elevated *hMLH1* methylation in the normal gastric epithelia in patients with stomach cancer as compared to those of non-cancer patients [21]. Methylation in the normal mucosa was proposed as a marker of risk for the development of CRC[22]. As demonstrated recently, the acceleration of ageing-related DNA methylation drift in normal mucosa was correlated with an earlier age of diagnosis[23].

hMLH1 methylation in non-neoplastic epithelia was shown also to exhibit MSI[24]. It is interesting to note that the methylated *hMLH1* in tumor was accompanied by methylation in its paired normal tissue. This finding warrants further investigation on the clinical utility of *hMLH1* methylation in normal tissue as a useful marker not only in CRC but in also in other gastrointestinal cancers.

However, in this study, a high incidence of non-methylated *hMLH1* in MSI-H samples (both normal and tumor) suggests that *hMLH1*methylation did not bring about MSI. We assumed, at least in our cohort, that unstable microsatellites can be attributed to methylation in other MMR genes, such as hSMH2[25,26] and mutation in

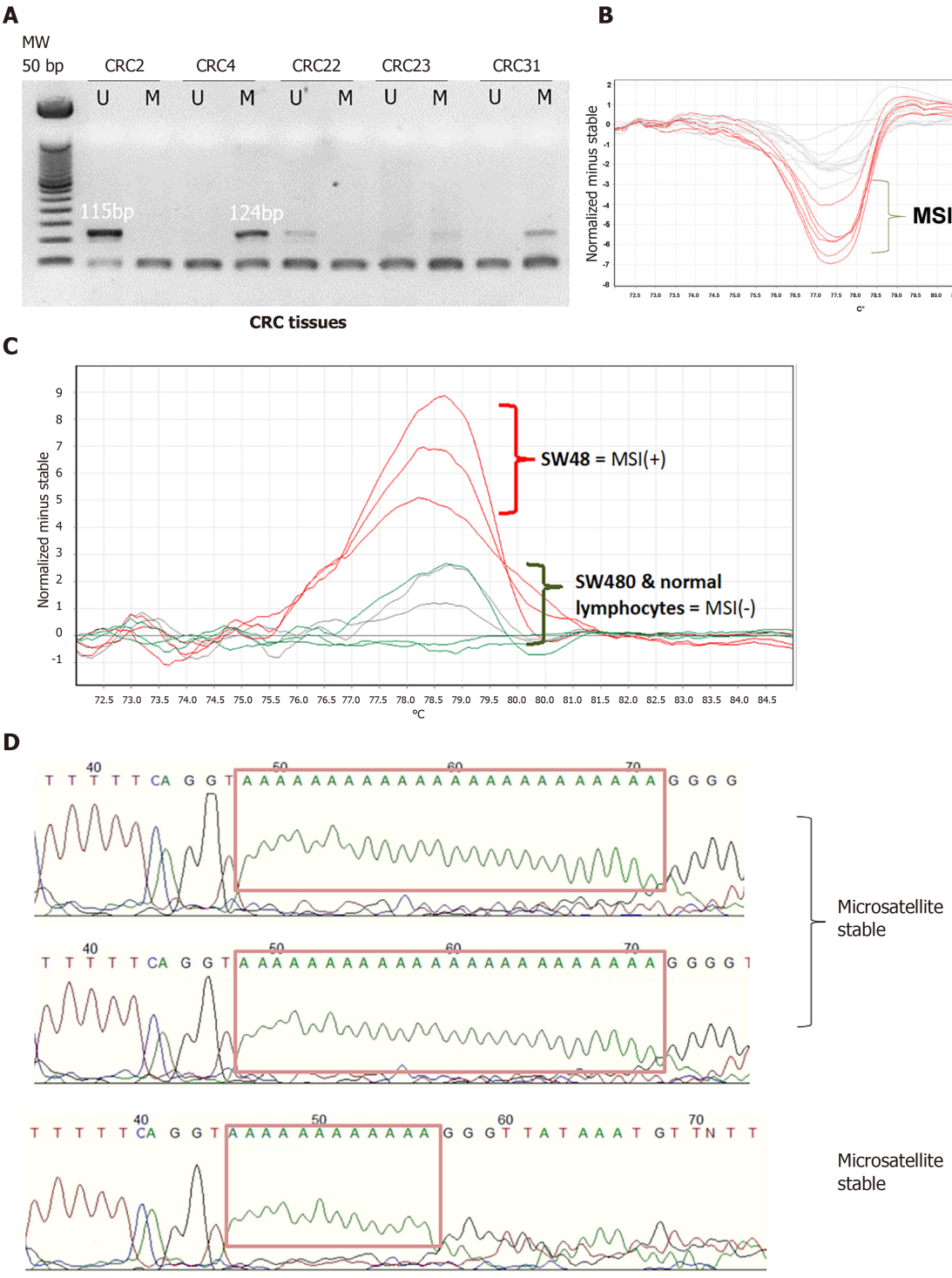

Figure 1 Determination of *hMLH1* methylation by methylation-specific polymerase chain reaction and microsatellite instability status by high resolution melting analysis. A: Gel images showing presence of 124 bp product indicating an unmethylated *hMLH1* and the 115 bp product for the methylated *hMLH1* in clinical samples; B: Normalized difference curves of clinical samples showing microsatellite instability-high profiles in red; C: Difference curve of the high resolution melting analysis; D: Electropherograms showing deletion of 12 adenine repeats in microsatellite instable control SW48 using BAT26 compared to the 24 adenine repeats in stable control SW480 and lymphocytes from a normal individual. MS-PCR: Methylation specific polymerase chain reaction; HRM: High resolution melting; MSI: Microsatellite instability; MSI-H: Microsatellite instability-high; CRC: Colorectal cancer.

hSMH6[27]. It was also noted that MSI may also result from other defects in the component of base excision repair of the proteins (DNA glycosylase, AP endonuclease, DNA polymerase, *etc.*)[28]. MSI tumors were also grouped to Type A (≤ 6 bp change) and Type B (≥ 8 bp change). In a report on MMR gene knock-out animals, no Type B MSI was observed, suggesting that a kind of MSI group may involve malignancies apart from MMR deficiency[29].

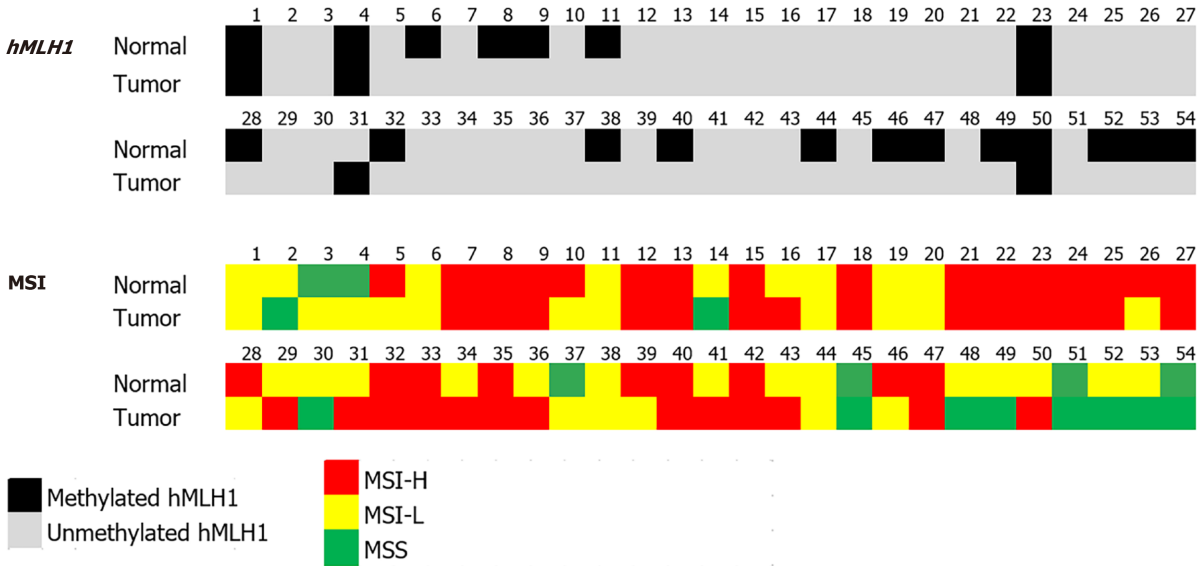

Figure 2 *hMLH1* methylation and microsatellite instability analysis results of paired normal and tumor tissues. *hMLH1* methylation analysis was determined using bisulfite converted genomic DNA and methylated and unmethylated primer sets for methylation specific polymerase chain reaction. Microsatellite instability profiling was done by high-resolution melting analysis using the five Bethesda panel of markers. MSI: Microsatellite instability; MSI-H: Microsatellite instability-high; MSI-L: Microsatellite instability-low; MSS: Microsatellite stable.

Significant differences for *hMLH1* methylation were found for the location of the tumor. Colon is divided into two anatomical regions: the proximal/right colon and the distal/left colon and each with very unique features[30-32]. This may be partially attributed to their difference in embryological development and physiological circumstances. The proximal colon develops from the hindgut while the distal colon originates from the midgut[33]. Proximal and distal colon are also different in the genetic patterns[34].

Since methylation is affected by environmental exposure, the difference might also be related to function. The proximal colon is the site where breakdown of complex carbohydrates occurs while the undigested dietary proteins are broken down in the distal colon. This process of digestion in the distal colon generates by-products such as ammonia, phenols, indoles and sulfurs, that may inhibit DNA methyltransferase activity[35]. Among African-American race, higher vegetable intake diet was shown being associated with greater odds for high *hMLH1* methylation[36]. Because methylation is less inhibited in proximal region, this may explain the higher incidence of methylated normal and tumor tissue (M/M), in contrast to high percentage of unmethylation in distal region.

In this study, we observed that from both the average of survival data and log-rank test of Kaplan-Meier analysis, *hMLH1* methylation in normal tissues and MSI status in both normal and CRC tissues had prognostic value. Unmethylated *hMLH1* and MSI-L/MSS status were positively associated with better patients' survival. Our data may support earlier findings where expression of *hMLH1* is considered an independent prognostic and predictive factor stage II-III CRC in Chinese population[37]. Data from this study, however, in contrast with previous studies showing the association between MSI-H with improved overall and disease-free survival[38,39], showed that population genetics and the disease stages might influence the prognosis of CRC patients.

Although there was no statistical difference, the incidence of MSI and *hMLH1* methylation showed a similar trend in association with sex and grade. MSI and *hMLH1* methylation were more common in females and in poorly differentiated tumors. This non-significant correlation may be attributed to the small sample size, thus studies using larger number of samples that are well distributed in terms of tumor clinicopathological parameters will be needed. Moreover, significance of both markers in relation to patients' prognosis, especially for Southeast Asian population, needs to be validated. Investigation of the methylation status of two or more MMR genes and their correlation with MSI status is also recommended, since this study only looked into one MMR gene out of the six. A recent data from metastatic CRC showed that a novel epigenetic signature of eight hypermethylated genes was able to identify CRC with poor prognosis. These genes were characterized with CpG-island high

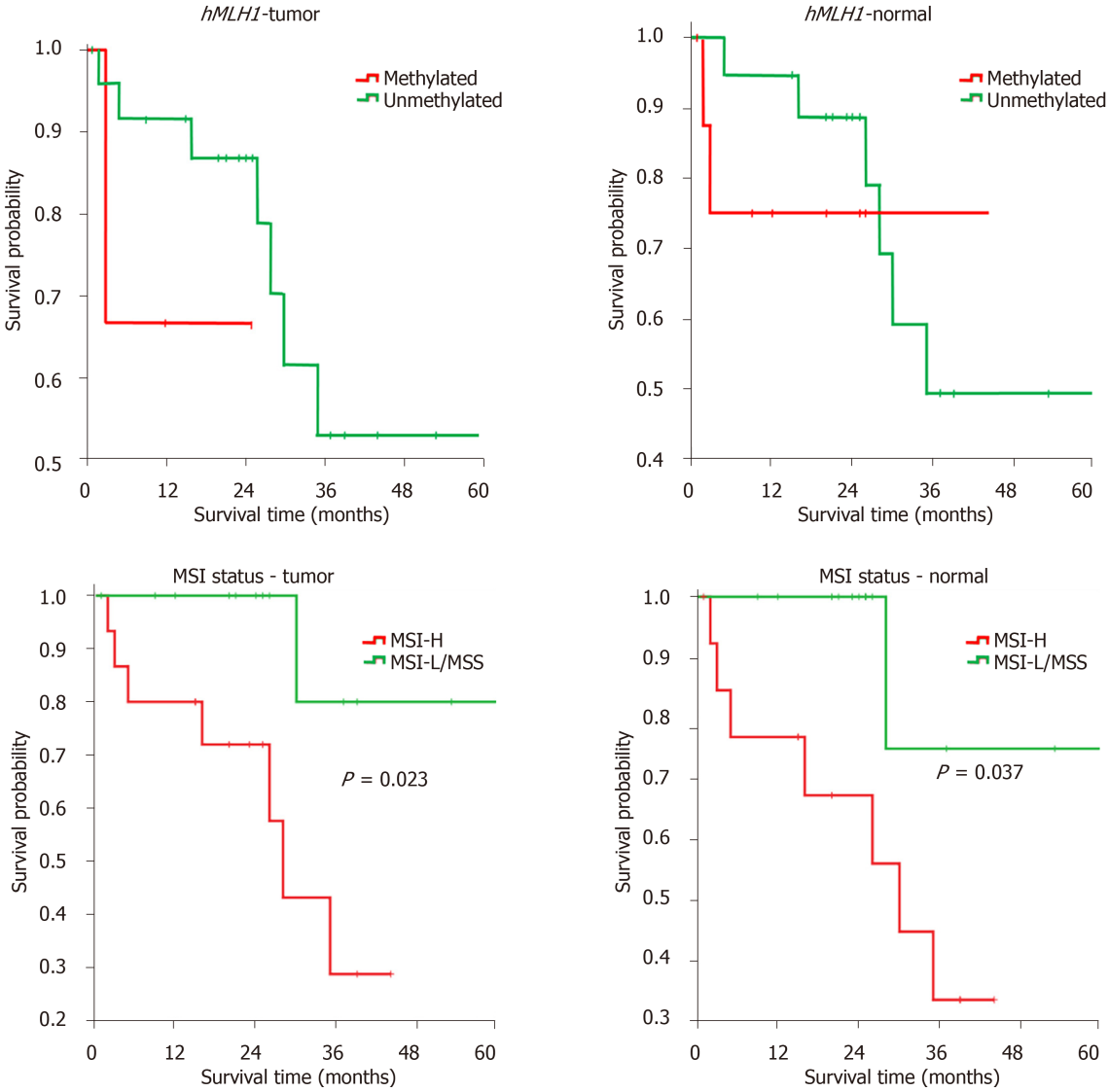

Figure 3 *hMLH1* methylation and microsatellite instability analysis for patients' prognosis. Kaplan-Meier analysis of colorectal cancer patients' overall survival in 60-mo of patients' follow-up after surgery. MSI: Microsatellite instability.

methylator and MSI-like phenotype[40].

The establishment of molecular biomarkers in diseases is essential for an effective management and treatment protocol for cancer patients. For the precision treatment in the future, especially for immunotherapy, it was shown that patients with MSI tumors exhibited significant response to anti-PD-1 inhibitors after failure of conventional therapy[41,42]. The role of MSI and *hMLH1* methylation is shedding light in the management of sporadic CRC in clinical practice in other countries[43,44] but not yet in the Philippines.

CONCLUSION

To conclude, we showed the clinical significance of *hMLH1* methylation and MSI status in sporadic CRC Filipino patients, especially in the normal part of the organ. This study is one of the few attempts to establish the molecular profile of Filipino CRC patients in the local setting, to highlight the importance of epigenetic modification. Understanding the diverse molecular and genetic key players involved in cancer development will ultimately translate to improvement in patient care.

ARTICLE HIGHLIGHTS

Research background

A distinct molecular signature marks a particular subset of sporadic colorectal cancer (CRC). It involves the mismatch repair (MMR) genes silencing due to DNA methylation, leading to microsatellite instability (MSI).

Research motivation

To improve the management of the CRC patients based on their distinct molecular subtypes.

Research objectives

To examine the association of mutL homolog 1 (*hMLH1*) methylation (MMR gene) and the MSI phenotype in relation to cancer characteristics and patient survival among Filipino sporadic CRC patients.

Research methods

Paired tissues (normal and tumor) from sporadic CRC patients was screened for *hMLH1* methylation using methylation specific polymerase chain reaction. Subsequent MSI typing was done by high resolution melting analysis.

Research results

The results of this study showed that *hMLH1* methylation was mostly noticed in proximal tumors. Low overall survival was observed in methylated *hMLH1* and MSI tumors.

Research conclusions

The epigenetic silencing of *hMLH1* as well as MSI may present a distinct pattern of CRC in Filipino patients.

Research perspectives

This is an initial attempt to characterize sporadic CRC in Filipino population. It may shed light in understanding molecular epigenetic modification in CRC as well as its role in tumor development and management.

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ORIGINAL ARTICLE

Basic Study

Propofol induces ferroptosis and inhibits malignant phenotypes of gastric cancer cells by regulating miR-125b-5p/STAT3 axis

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Abstract

BACKGROUND

Gastric cancer is a common malignancy with poor prognosis, in which ferroptosis plays a crucial function in its development. Propofol is a widely used anesthetic and has antitumor potential in gastric cancer. However, the effect of propofol on ferroptosis during gastric cancer progression remains unreported.

AIM

To explore the function of propofol in the regulation of ferroptosis and malignant phenotypes of gastric cancer cells.

METHODS

MTT assays, colony formation assays, Transwell assays, wound healing assay, analysis of apoptosis, ferroptosis measurement, luciferase reporter gene assay, and quantitative reverse transcription polymerase chain reaction were used in this study.

RESULTS

Our data showed that propofol was able to inhibit proliferation and induce apoptosis of gastric cancer cells. Meanwhile, propofol markedly repressed the invasion and migration of gastric cancer cells. Importantly, propofol enhanced the erastin-induced inhibition of growth of gastric cancer cells. Consistently, propofol increased the levels of reactive oxygen species, iron, and Fe^{2+} in gastric cancer cells. Moreover, propofol suppressed signal transducer and activator of transcription (STAT)3 expression by upregulating miR-125b-5p and propofol induced ferroptosis by targeting STAT3 in gastric cancer cells. The miR-125b-5p inhibitor or STAT3 overexpression reversed propofol-attenuated malignant phenotypes of gastric cancer cells.

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CONCLUSION
Propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis. Propofol may serve as a potential therapeutic candidate for gastric cancer.

Key Words: Gastric cancer; Progression; Ferroptosis; Propofol; miR-125b-5p; STAT3

Core Tip: In this study, we discovered that propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis. Propofol may serve as a potential therapeutic candidate for gastric cancer.

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INTRODUCTION

Gastric cancer is a severe, lethal type of cancer worldwide[1]. Despite improvement in treatment, gastric cancer remains the leading cause of cancer-associated death[2]. Adjuvant therapy, radical surgery, and early diagnosis enhance survival rates and prognosis for gastric cancer, but mortality is still unsatisfactory[3,4]. Ferroptosis is a type of regulated cell death that differs from apoptosis and is repressed by erastin, especially in RAS-mutated cancer cells[5]. It has been identified that the suppression of ferroptosis contributes to cancer progression, and ferroptosis has a crucial role in the development of gastric cancer[6-8]. However, the exploration of a treatment that can target ferroptosis is still limited.

Propofol is a broadly applied anesthetic because of rapid recovery and has some nonanesthetic functions in cancer development[9]. It has been identified that propofol inhibits cell invasion and growth and induces apoptosis in pancreatic cancer[10]. Meanwhile, propofol decreases cell proliferation and increases apoptosis of lung cancer cells by regulating caspases-3, Bim, forkhead box (FOX)O1, and FOXO3, in which miR-486 inhibitor can reverse this effect[11]. Propofol also represses the malignant progression of promyelocytic leukemia cells[12]. Moreover, it has been reported that propofol induces an inhibitory effect on gastric cancer cell invasion and migration in patients with gastric cancer[13,14]. However, the function of propofol in the modulation of ferroptosis during gastric cancer progression remains elusive. miRNAs can modulate gene expression in different cellular processes[15]. Previous studies have revealed that miRNAs participate in the development of gastric cancer [16,17]. Meanwhile, a recent study has shown that miR-125b-5p is a tumor suppressor and involved in the modulation of gastric cancer[18]. In addition, signal transducer and activator of transcription (STAT)3 has been identified as an oncogene in gastric cancer[19-21]. However, the effect of propofol on miR-125b-5p and STAT3 during the development of gastric cancer remains obscure.

In this study, we focused on the investigation of the function of propofol in the development of gastric cancer. We found that propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis.

MATERIALS AND METHODS

Cell culture

The SGC7901 and BGC823 cells were maintained in the laboratory. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Solarbio, China) with 0.1 mg/mL streptomycin (Solarbio), 100 U/mL penicillin (Solarbio), and 10% fetal bovine

serum (Solarbio), under the conditions of 37 °C with 5% CO_2. The pcDNA3.1-STAT3 overexpression vector, miR-125b-5p mimic, miR-125b-5p inhibitor, and corresponding control were purchased from Genscript Biotech Corporation (Nanjing, China) and GenePharma Co. Ltd. (Shanghai, China). The transfection in the cells was performed by Liposome 3000 (Invitrogen, Carlsbad, CA, United States). Propofol was purchased from Sigma (St. Louis, MO, United States).

MTT assays

SGC7901 and BGC823 cells were treated with propofol for 48 h. MTT assays analyzed the proliferation of SGC7901 and BGC823 cells. About 2 × 10^5 cells were plated in 96-well plates and incubated for 24 h. To assess cell viability, the cells were cultured with MTT solution (5 mg/mL) and incubated for 4 h, and 150 μL dimethyl sulfoxide (DMSO) was applied to treat the cells. The cell viability was measured at 570 nm absorbance by applying an ELISA browser (Bio-Tek EL 800, Winooski, VT, United States).

Colony formation assays

The SGC7901 and BGC823 cells were treated with propofol for 48 h. Colony formation assays measured proliferation. About 10^4 SGC7901 and BGC823 cells were placed in six-well plates and cultured in DMEM at 37 C. The cells were cleaned with phosphate-buffered saline (PBS) after 2 wk, washed in methanol for ~30 min, and stained with 1% crystal violet dye, after which, the number of colonies was calculated.

Transwell assays

To analyze cell migration, the cells were cultured for 24 h and resuspended in serum-free culture medium, then plated into the apical chamber of a Transwell chamber at 5 × 10^3 cells/well. The culture medium was made up to 150 μL and 600 μL complete culture medium was added to the basolateral chamber. After 24 h culture at 37°C and 5% CO_2, the cells were fixed with 4% paraformaldehyde for 10 min, stained by crystal violet dye for 20 min, followed by analysis using the intelligent biological navigator (Olympus, Tokyo, Japan). The migrated cells were recorded and calculated by using the ImageJ software.

To analyze cell invasion, Matrigel was melted overnight at 4 °C and diluted by presold serum-free culture medium (ratio 8:1). The medium (50 μL) was plated into the Transwell polycarbonate membrane with a pore diameter of 8 μm, covering all the wells with Matrigel at 37°C for 2 h. The cells were cultured for 24 h and resuspended in serum-free culture medium, plated into the Transwell apical chamber at 10^5 cells/well, and the medium was made up to 150 μL. Complete medium with 50% FBS (600 μL) was added to the basolateral chamber. After 24 h, the cells were fixed using 4% paraformaldehyde for 15 min and stained with crystal violet dye for 10 min. The invaded cells were analyzed and calculated using the ImageJ software.

Wound healing assay

SGC7901 and BGC823 cells were treated with propofol for 48 h. Approximately 3 × 10^5 SGC7901 and BGC823 cells were plated into the 24-well plates and incubated overnight to reach a fully confluent monolayer. A 20-μL pipette tip was applied to slowly cut a straight line across the well. The well was washed by PBS three times and the medium was changed to serum-free medium and culture was continued. The wound healing percentage was calculated.

Analysis of apoptosis

he SGC7901 and BGC823 cells were treated with propofol for 48 h. Approximately 2 × 10^5 SGC7901 and BGC823 cells were plated in six-well dishes. Apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit (Cell Signaling Technology, Danvers, MA, United States). About 2 × 10^5 washed cells were collected by binding buffer and stained at 25 C, followed by flow cytometry analysis.

Ferroptosis measurement

SGC7901 and BGC823 cells were cotreated with erastin (5 mmol/L) or ferrostatin (1 mmol/L). After 48 h, the cell viability was analyzed by MTT assay. Elevated iron level and accumulated lipid reactive oxygen species (ROS) were representative characteristics of ferroptosis. We used an iron assay kit (Beyotime, China) to examine the level of intracellular Fe^{2+}. The cells were homogenized to collect the supernatant, incubated with iron reducer, followed by labeling with iron probe. OD 590 nm was detected in a microplate reader (PerkinElmer, Waltham, MA, United States). For detection of lipid

ROS, cells were stained with BODIPY C-11 dye (Beyotime) for 30 min, and subsequently detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, United States). The levels of malondialdehyde (MDA) and glutathione peroxidase (GSH) was measured by the MDA detection kit (Beyotime) and GSH assay kit (Cayman, Ann Arbor, MI, United States), respectively.

Luciferase reporter gene assay

The luciferase reporter gene assays were performed using the Dual-luciferase Reporter Assay System (Promega, Madison, WI, United States). SGC7901 and BGC823 cells were treated with miR-125b-5p mimic, pmirGLO-STAT3 (contained STAT3 3′UTR), and pmirGLO-STAT3 mutant transfected into the cells using Lipofectamine 3000 (Invitrogen), followed by analysis of luciferase activities, in which Renilla was applied as a normalized control.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted by TRIZOL (Invitrogen). The first-strand cDNA was manufactured according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, United States). qRT-PCR was carried out by SYBR Real-time PCR I kit (Takara, Japan). The standard control for miRNA and mRNA/circRNA was U6 and GAPDH, respectively. Quantitative determination of the RNA levels was conducted in triplicate independent experiments. The primer sequences were as follows: miR-125b-5p forward: 5′-TCCCTGAGACCCTAACTTGTGA-3′; reverse: 5′-AGTCTCAGGGTC CGAGGTATTC-3′; STAT3 forward: 5′-GGCCATCTTGAGCACTAAGC-3′, reverse: 5′-CGGACTGGATCTGGGTCTTA-3′; GAPDH forward: 5′-TATGATGATATCAAGA GGGTAGT-3′, reverse: 5′-TATGATGATATCAAGAGGGTAGT-3′; U6 forward: 5′-CTCGCTTCGGCAGCACA-3′, U6 reverse: 5′-AACGCTTCACGAATT TGCGT-3′.

Western blot analysis

Total proteins were isolated from the cells with RIPA buffer (Cell Signaling Technology) and analyzed by BCA Protein Quantification Kit (Abbkine, United States). The protein was separated by 12% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States). The membranes were treated with 5% milk and incubated overnight at 4°C with the primary antibodies for STAT3 (Rabbit monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology), GPX4 (Rabbit monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology) and SLC7A11 (Rabbit monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology); and β-actin (Mouse monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology), E-cadherin (Rabbit monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology) and vimentin (Rabbit monoclonal, 1:1000, diluted by 5% milk; Cell Signaling Technology), in which β-actin served as the control. The corresponding secondary antibodies (Abcam, Cambridge, MA, United States) were incubated with the membranes for 1 h at room temperature, followed by visualization using an Odyssey CLx Infrared Imaging System.

Xenograft assays

All animal experiments were performed under the approval of Animal Ethics Committee of The First Affiliated Hospital of Harbin Medical University. Specific-pathogen-free male nude mice aged 5-6 wk and weighted around 20 g were purchased from Vitalriver (China). All mice were maintained in a 12-h circadian rhythm, and had free access to water and food. SGC7901 and BGC823 cells were subcutaneously injected into the right flank of mice. Propofol (50 mg/kg/d) was administrated intraperitoneally after tumor volume approached 100 mm^3 for 20 d[22]. For the control group, the mice were treated with an equal volume of DMSO. Tumor volume and body weight were monitored every 5 d. The tumor size was calculated using the formula: length × $width^2/2$.

Statistical analysis

Data were expressed as mean ± SD, and the statistical analysis was conducted using GraphPad Prism 7. The unpaired Student's *t* test was used to compare two groups, and one-way analysis of variance was used to compare among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

Propofol decreases proliferation and induces apoptosis of gastric cancer cells

We evaluated the effect of propofol on proliferation and apoptosis of gastric cancer cells. Propofol repressed viability of SGC7901 and BGC823 cells in a dose-dependent manner and 10 μmol/L propofol had a greater effect, which was selected in the subsequent analysis (Supplementary Figure 1). Propofol was able to inhibit viability of SGC7901 and BGC823 cells (Figure 1A and B). Similarly, propofol markedly reduced proliferation of SGC7901 and BGC823 cells (Figure 1C and D). Apoptosis of SGC7901 and BGC823 cells was enhanced by propofol (Figure 1E and F), suggesting that propofol decreases proliferation and induces apoptosis of gastric cancer cells.

Propofol reduces invasion and migration of gastric cancer cells

We further measured the effect of propofol on the migration and invasion of gastric cancer cells. Transwell assays indicated that the migration and invasion of SGC7901 and BGC823 cells were markedly decreased by propofol (Figure 2A and B). Consistently, the treatment of propofol significantly repressed wound healing in SGC7901 and BGC823 cells (Figure 2C and D), indicating that propofol is able to attenuate the migration and invasion of gastric cancer cells. Consistently, propofol enhanced E-cadherin expression and reduced vimentin expression in SGC7901 and BGC823 cells (Figure 2E).

Propofol enhances ferroptosis in gastric cancer cells

To analyze the impact of propofol on ferroptosis, we assessed the role of propofol in the erastin-induced inhibition of cell growth and the intracellular levels of ROS, iron and Fe^{2+}, and expression of GPX4 and SLC7A11, which are considered to be ferroptosis markers. Propofol enhanced the erastin-induced inhibitory effect on SGC7901 and BGC823 cell growth, in which erastin served as an activator of ferroptosis (Figure 3A and B). Iron levels were induced by propofol in SGC7901 and BGC823 cells (Figure 3C). Propofol significantly promoted the levels of ROS in SGC7901 and BGC823 cells (Figure 3D). Propofol increased accumulation of Fe^{2+} in SGC7901 and BGC823 cells (Figure 3E). Consistently, the expression of GPX4 and SLC7A11 was inhibited by propofol in SGC7901 and BGC823 cells (Figure 3F). GSH levels were reduced and MDA levels were enhanced in SGC7901 and BGC823 cells by treatment with propofol (Figure 3G and H), suggesting that propofol enhances ferroptosis in gastric cancer cells.

Propofol represses STAT3 expression by upregulating miR-125b-5p in gastric cancer cells

We explored the potential mechanisms underlying propofol-mediated gastric cancer progression. Given that propofol can regulate colon cancer metastasis by regulating STAT3 signaling[23], we assessed the correlation of propofol with STAT3 in gastric cancer. Significantly, we identified that propofol was able to upregulate expression of miR-125b-5p in SGC7901 and BGC823 cells (Figure 4A). We identified the binding site between miR-125b-5p and STAT3 mRNA 3′ UTR in a bioinformatic analysis using Targetscan (http://www.targetscan.org/vert_72/) (Figure 4B). Treatment with miR-125b-5p mimic reduced luciferase activities of wild-type STAT3, but not STAT3 with miR-125b-5p-binding site mutant in SGC7901 and BGC823 cells (Figure 4C and D). mRNA and protein expression of STAT3 was significantly suppressed by miR-125b-5p mimic in SGC7901 and BGC823 cells (Figure 4E). Propofol inhibited expression of STAT3, which was reversed by miR-125b-5p inhibitor (Figure 4F), indicating that propofol represses STAT3 expression by upregulating miR-125b-5p in gastric cancer cells.

Propofol enhances ferroptosis by targeting STAT3 in gastric cancer cells

We confirmed whether propofol modulated ferroptosis by targeting STAT3 in gastric cancer cells. As expected, overexpression of STAT3 was able to rescue propofol-inhibited cell growth in the erastin-treated SGC7901 and BGC823 cells (Figure 5A). Similarly, STAT3 overexpression reversed propofol-induced levels of Fe^{2+}, iron and ROS (Figure 5B-D), suggesting that propofol induces ferroptosis by inhibiting STAT3 in gastric cancer cells.

Propofol attenuates gastric cancer progression by miR-125b-5p /STAT3 axis

We further investigated the role of the propofol/miR-125b-5p/STAT3 axis in regulating gastric cancer malignant phenotypes. Overexpression of STAT3 or miR-

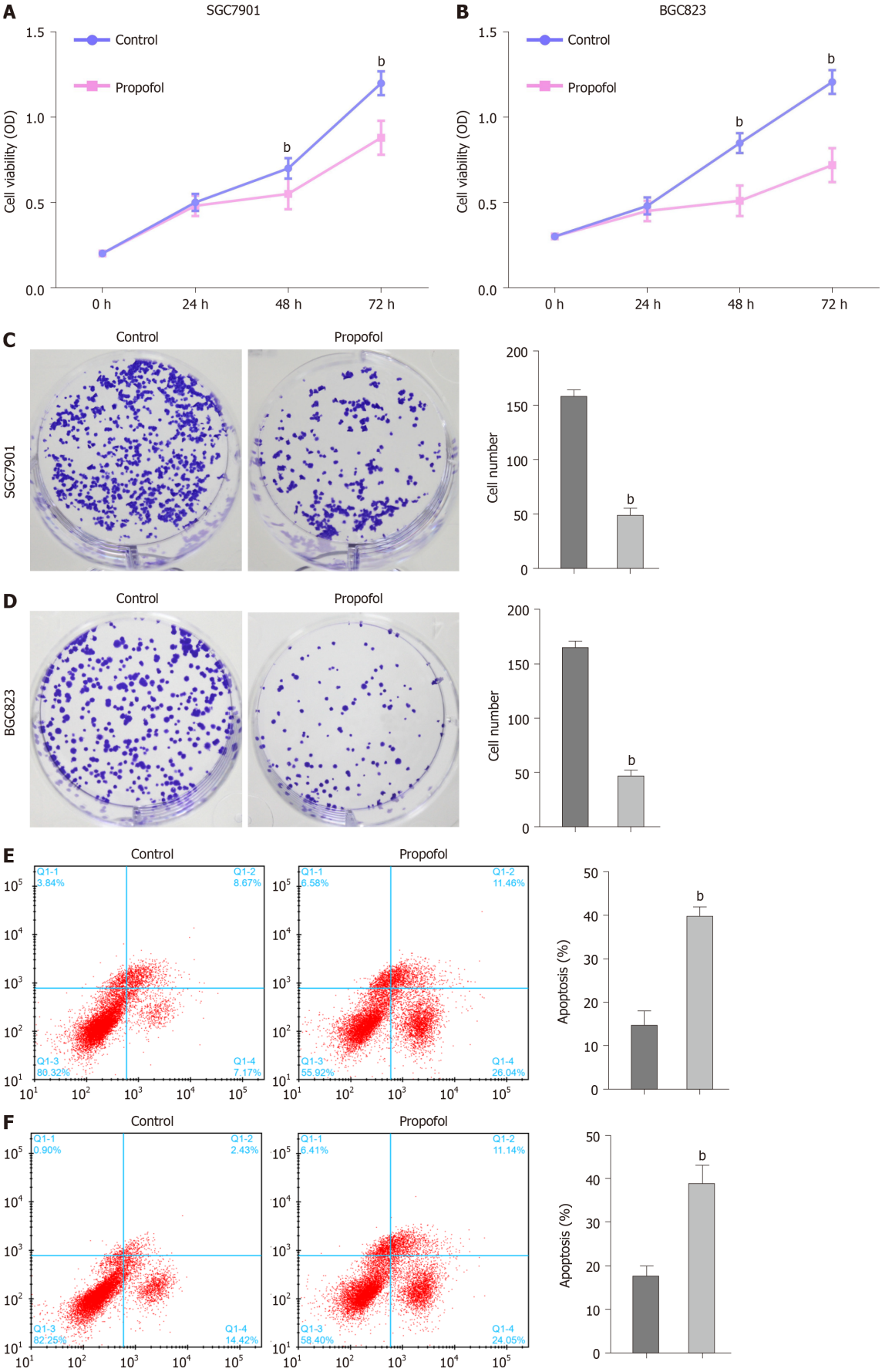
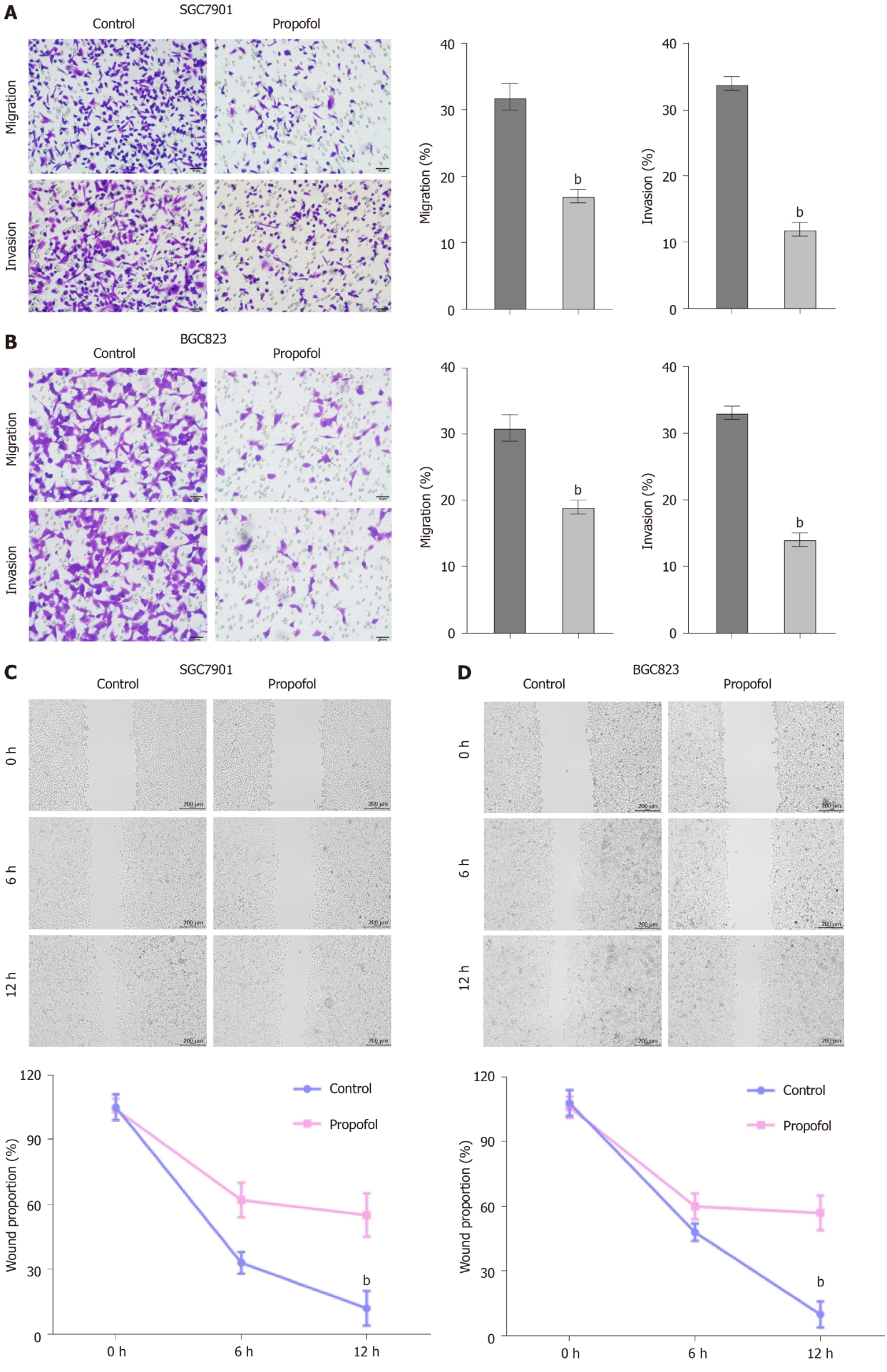


Figure 1 Propofol decreases proliferation and induces apoptosis of gastric cancer cells. A–E: SGC7901 and BGC823 cells were treated with propofol (10 μmol/L). A and B: MTT assays analyzed cell viability; C and D: Colony formation assays measured cell proliferation; E and F: Flow cytometry analysis tested cell apoptosis. n = 3, mean ± SD, $^{b}P < 0.01$.


A
SGC7901
Control
Propofol
Migration
Invasion
Migration (%)
Invasion (%)
b
B
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C
SGC7901
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BGC823
0 h
6 h
12 h
Wound proportion (%)
Control
Propofol

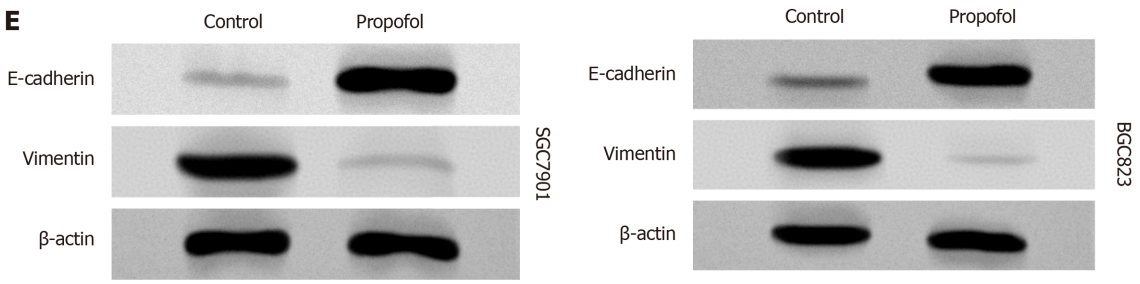

Figure 2 Propofol reduces invasion and migration of gastric cancer cells. A-D: SGC7901 and BGC823 cells were treated with propofol (10 μmol/L). A and B: Transwell assays analyzed cell migration and invasion; C and D: Wound healing assays examined migration and invasion. The wound healing proportion is shown. E: Protein levels of E-cadherin and vimentin were measured by western blotting. n = 3, mean ± SD, $^{b}P < 0.01$.

125b-5p inhibitor promoted propofol-inhibited viability in SGC7901 and BGC823 cells (Figure 6A and B). Consistently, apoptosis of SGC7901 and BGC823 cells was induced by propofol, and overexpression of STAT3 or miR-125b-5p inhibitor reversed this effect in SGC7901 and BGC823 cells (Figure 6C), implying that propofol has an inhibitor effect on gastric cancer malignant phenotypes *via* the miR-125b-5p/STAT3 axis. We confirmed that propofol-induced levels of Fe^{2+}, iron and ROS were reversed by inhibition of miR-125b-5p in SGC7901 and BGC823 cells (Supplementary Figure 2).

Propofol attenuates growth of gastric cancer cells in vivo

We evaluated the effect of propofol on gastric cancer cell growth in a tumorigenicity analysis in nude mice. Tumor growth of SGC7901 or BGC823 cells was attenuated by propofol in nude mice (Figure 7A-C and Supplementary Figure 3A-C), as demonstrated by the reduced tumor size, weight and volume. As expected, expression of miR-125b-5p was enhanced and STAT3 expression was reduced in the tumor tissues of propofol-treated mice compared with the control group (Figure 7D and E and Supplementary Figure 3D and E). Expression of GPX4 and SLC7A11 was also downregulated by propofol in the tumor tissues of mice (Figure 7F and Supplementary Figure 3F).

DISCUSSION

Gastric cancer is a prevalent malignancy with high mortality[1], in which ferroptosis plays a critical role in its development[6-8]. Propofol is a widely used anesthetic and has inhibitory effects on cancer progression. Nevertheless, the effect of propofol on ferroptosis during the development of gastric cancer is still unreported. In this study, we showed that propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis.

Propofol has presented significant anticancer functions in several models. It has been reported that propofol inhibits the development of nonsmall cell lung cancer by downregulating the miR215p/MAPK10 axis[24]. Propofol represses malignant progression of pancreatic cancer cells by reducing NMDA receptor[25]. Propofol induces apoptosis of cervical cancer cells through inhibition of the HOTAIR/mTOR pathway[26]. Moreover, it has been found that propofol represses proliferation, migration and invasion by enhancing miR-195 in gastric cancer cells[14]. Propofol improves cisplatin sensitivity in gastric cancer by MALAT1/miR-30e/ATG5 signaling *via* inhibiting autophagy[27]. Propofol suppresses the survival and growth of gastric cancer by inducing expression of ING3[28]. We found that propofol decreased proliferation, migration and invasion and induced apoptosis of gastric cancer cells. Importantly, propofol enhanced ferroptosis in gastric cancer cells. Our data indicate an unreported function of propofol in the modulation of ferroptosis during gastric cancer progression, elucidating the novel role of the anesthetic in the ferroptosis of cancer development. Ferroptosis is one of the critical malignant phenotypes mediated by propofol in gastric cancer progression. The importance of ferroptosis and apoptosis should be compared by more complex investigations. Meanwhile, it has been reported that the regulation of ferroptosis may benefit the inhibition of gastric cancer development, and targeting ferroptosis may be a promising strategy for gastric cancer therapy.

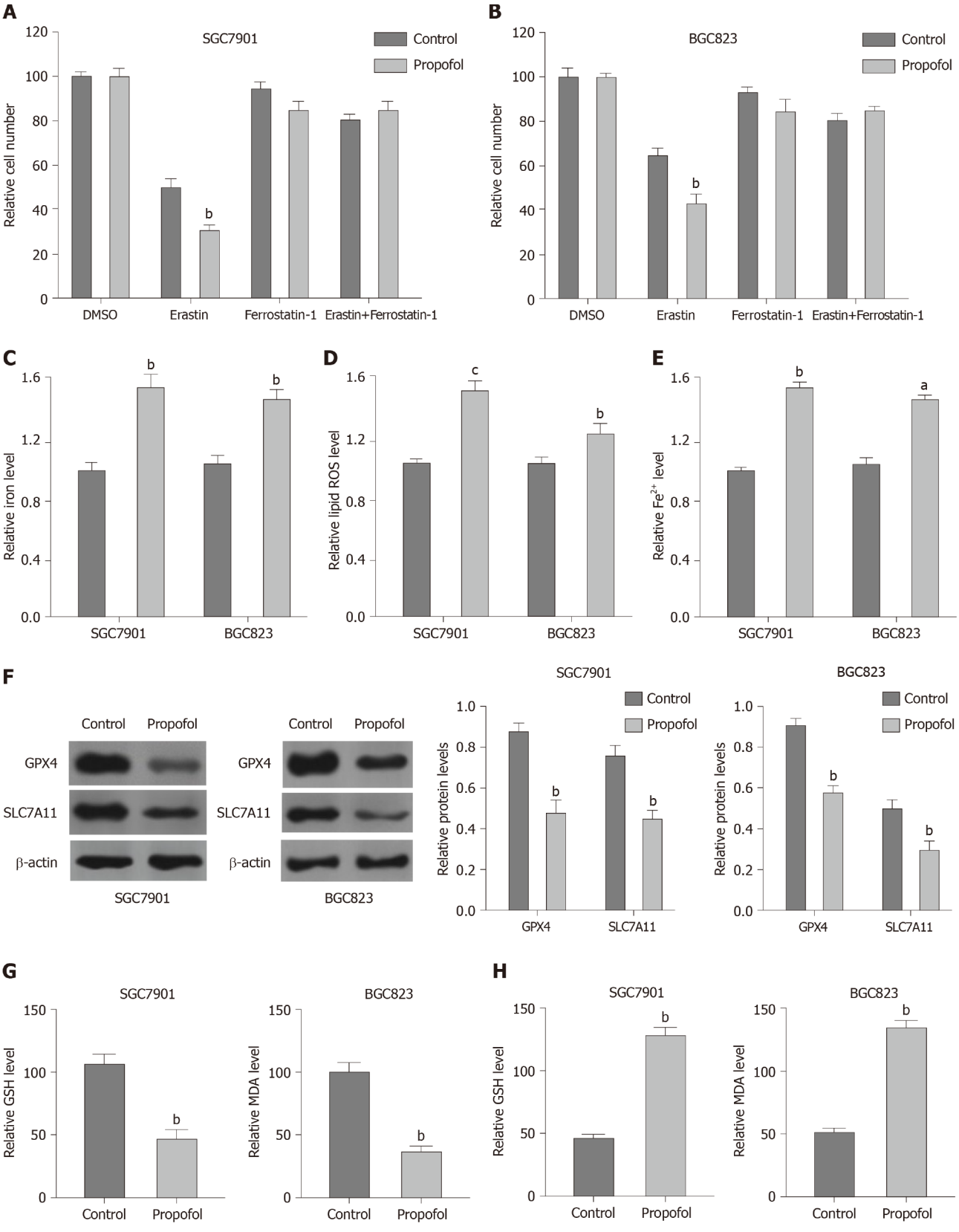

Figure 3 Propofol enhances ferroptosis in gastric cancer cells. A and B: SGC7901 and BGC823 were cotreated with 5 mmol/L erastin or ferrostatin (1 mmol/L) and propofol (10 μmol/L). Cell growth was analyzed by MTT assays. C–F: SGC7901 and BGC823 cells were treated with propofol (10 μmol/L). C: Flow cytometry measured the levels of ROS. D and E: Iron Assay Kit analyzed the levels of iron and Fe^{2+}. F: Western blotting analysis tested the expression of GPX4, SLC7A11 and β-actin. G and H: Levels of GSH and MDA were analyzed by the detection kit. n = 3, mean ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

miRNAs function as crucial regulators and are involved in gastric cancer progression. It has been reported that miR-96-5p enhances gastric cancer cell proliferation *via* inhibiting FOXO3[29]. miR-27b inhibits gastric cancer metastasis by

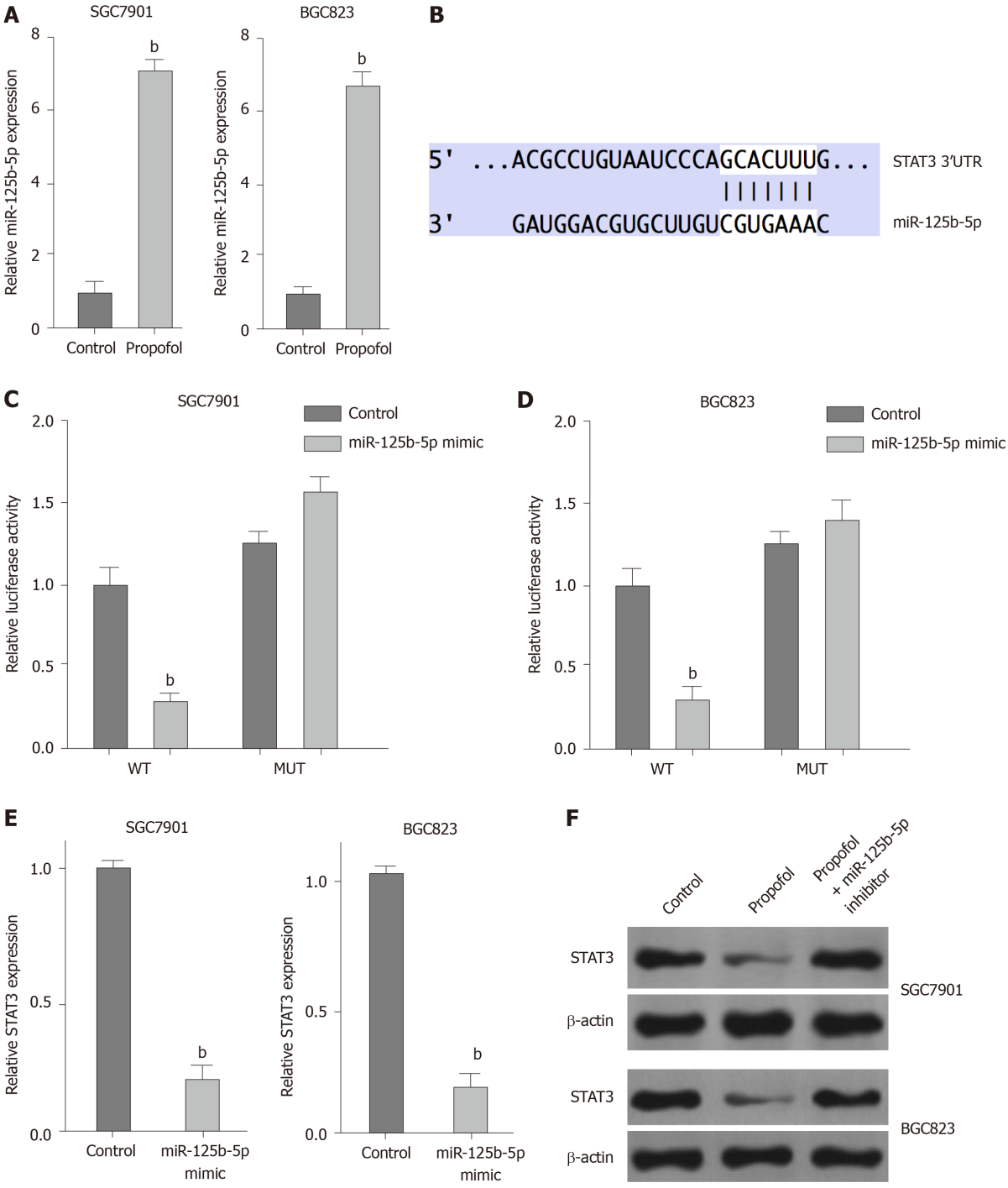

Figure 4 Propofol represses signal transducer and activator of transcription (STAT)3 expression by upregulating miR-125b-5p in gastric cancer cells. A: SGC7901 and BGC823 cells were treated with propofol (10 μmol/L). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) measured expression of miR-125b-5p. B: The binding site of miR-125b-5p and STAT3 3' UTR was identified by bioinformatic analysis using Targetscan (http://www.targetscan.org/vert_72/). C–E: SGC7901 and BGC823 cells were treated with the miR-125b-5p mimic or control mimic. C and D: Luciferase reporter gene assays determined the luciferase activities. E: qRT-PCR analyzed mRNA expression of STAT3. F: SGC7901 and BGC823 cells were treated with propofol, or cotreated with propofol and miR-125b-5p inhibitor. Western blotting assessed protein expression of STAT3 and β-actin. n = 3, mean ± SD, $^{b}P < 0.01$.

downregulating NR2F2 (nuclear receptor subfamily 2 group F member 2)[30]. miR-558 contributes to the progression of gastric cancer by repressing Smad4-regulated heparanase expression[31]. Moreover, it has been reported that miR-125b-5p represses invasion, migration and proliferation of breast cancer cells by inhibiting KIAA1522 [32]. miR-125b-5p suppresses invasion, migration and proliferation of hepatocellular carcinoma cell by downregulating thioredoxin reductase 1[33]. miR-125b-5p inhibits the progression of bladder cancer by attenuating PI3K/AKT signaling and targeting hexokinase 2[34]. In the present study, we found that propofol inhibited STAT3 expression by upregulating miR-125b-5p in gastric cancer cells. Propofol induced

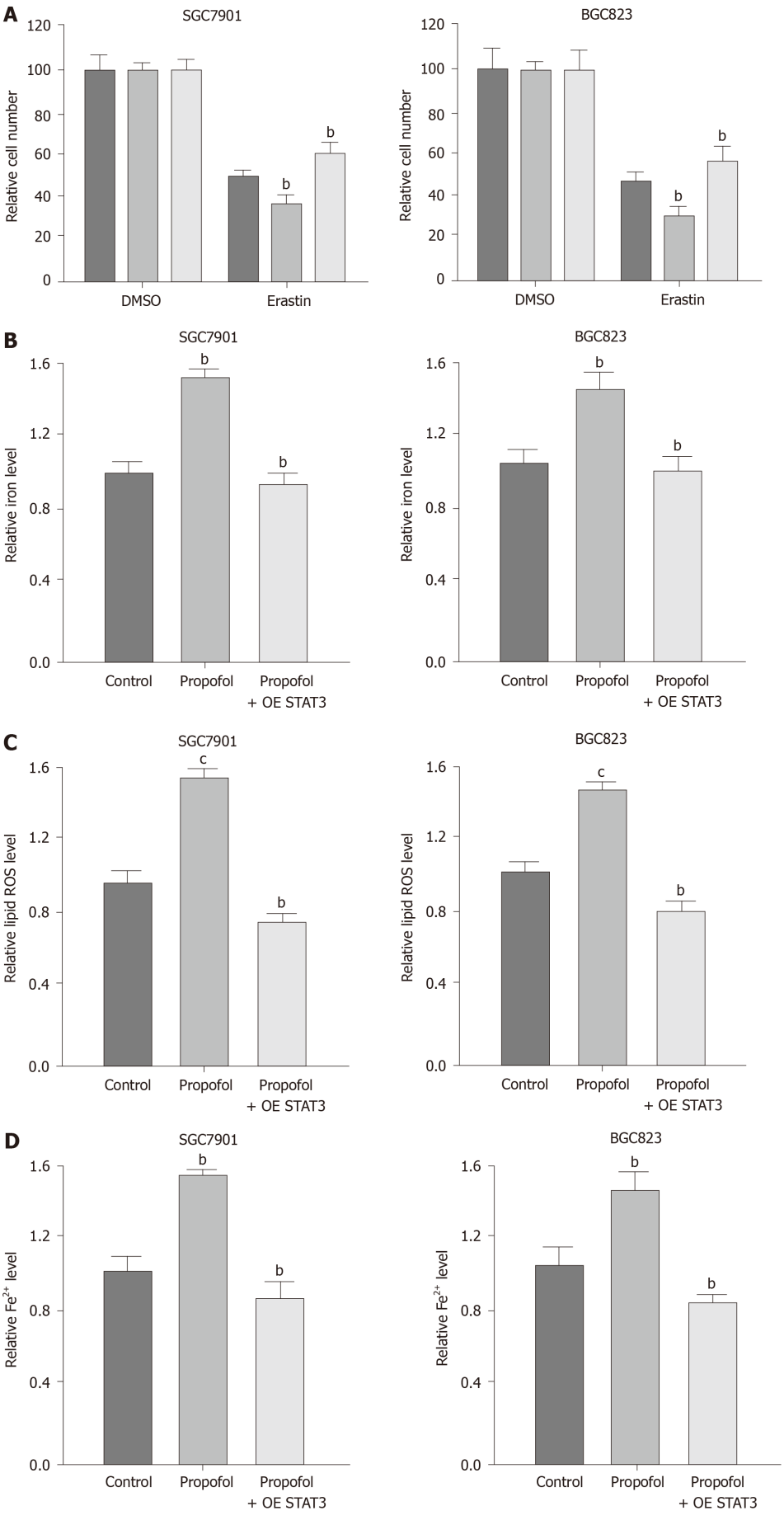

Figure 5 Propofol enhances ferroptosis by targeting signal transducer and activator of transcription (STAT)3 in gastric cancer cells. A:

SGC7901 and BGC823 cells were treated with 5 mmol/L erastin, cotreated with 5 mmol/L erastin and propofol, or cotreated with 5 mmol/L erastin, propofol, and pcDNA.1-STAT3. MTT assays measured cell growth. B–D: SGC7901 and BGC823 cells were treated with propofol, or cotreated with propofol and pcDNA.1-STAT3. B: Iron Assay Kit analyzed the levels of iron; C: Flow cytometry analysis tested the levels of ROS; and D: Iron Assay Kit analyzed the levels of Fe^{2+}. n = 3, mean ± SD, $^{b}P < 0.01$, $^{c}P < 0.001$.

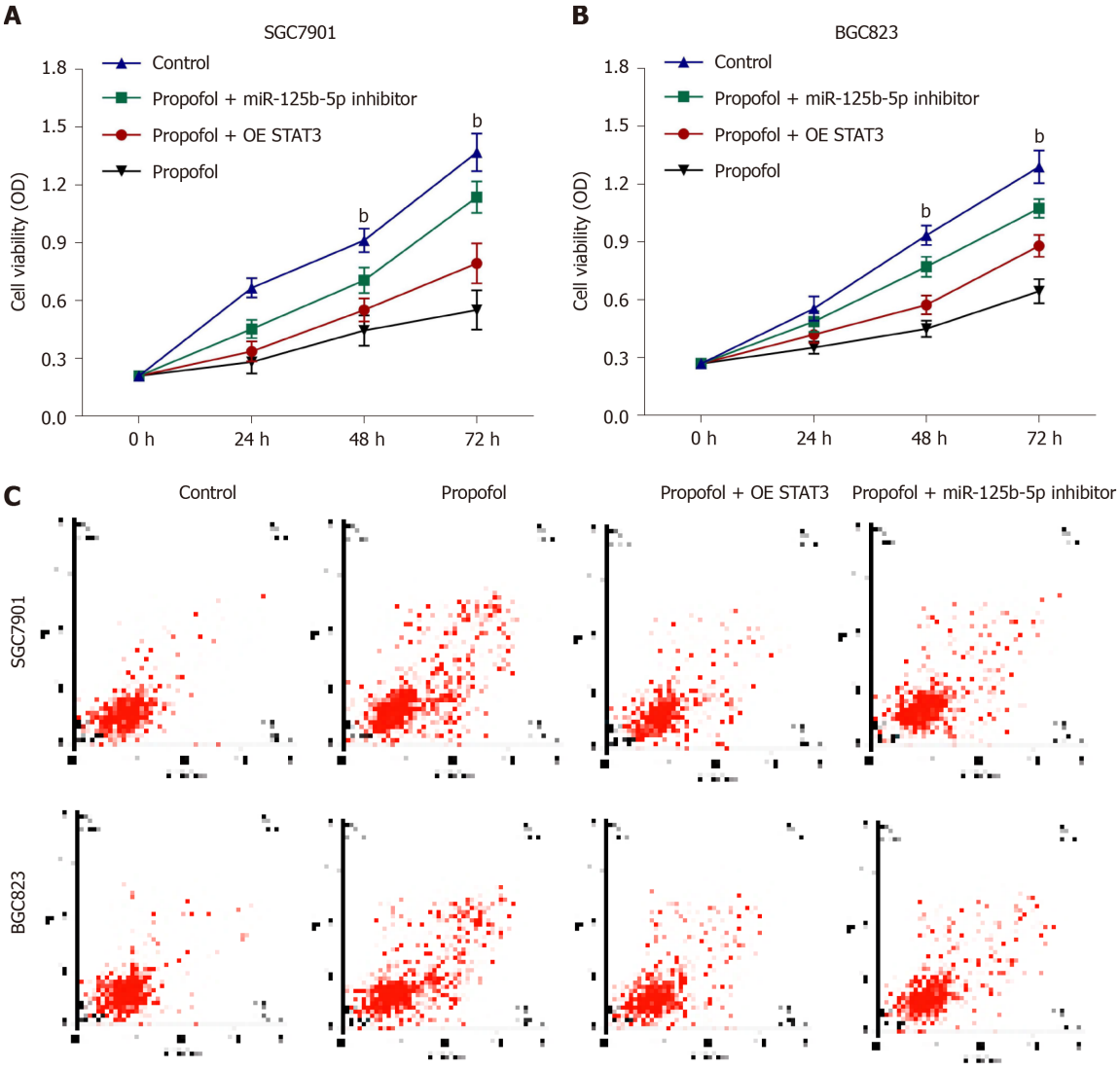

Figure 6 Propofol attenuates gastric cancer progression by miR-125b-5p/STAT3 axis. A–C: SGC7901 and BGC823 cells were treated propofol, or cotreated with propofol and miR-125b-5p inhibitor or pcDNA.1-STAT3. A and B: MTT assays analyzed the cell viability; C: Flow cytometry measured apoptosis. n = 3, mean ± SD, $^{b}P < 0.01$.

ferroptosis by targeting STAT3 in gastric cancer cells. The overexpression of STAT3 and miR-125b-5p inhibitor could reverse propofol-attenuated malignant phenotypes of gastric cancer cells. It uncovers a novel mechanism involving propofol, miR-125b-5p and STAT3 in the regulation of gastric cancer, enriching the understanding of the anticancer effect of propofol.

CONCLUSION

We discovered that propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis. Propofol may serve as a potential therapeutic candidate for gastric cancer therapy.

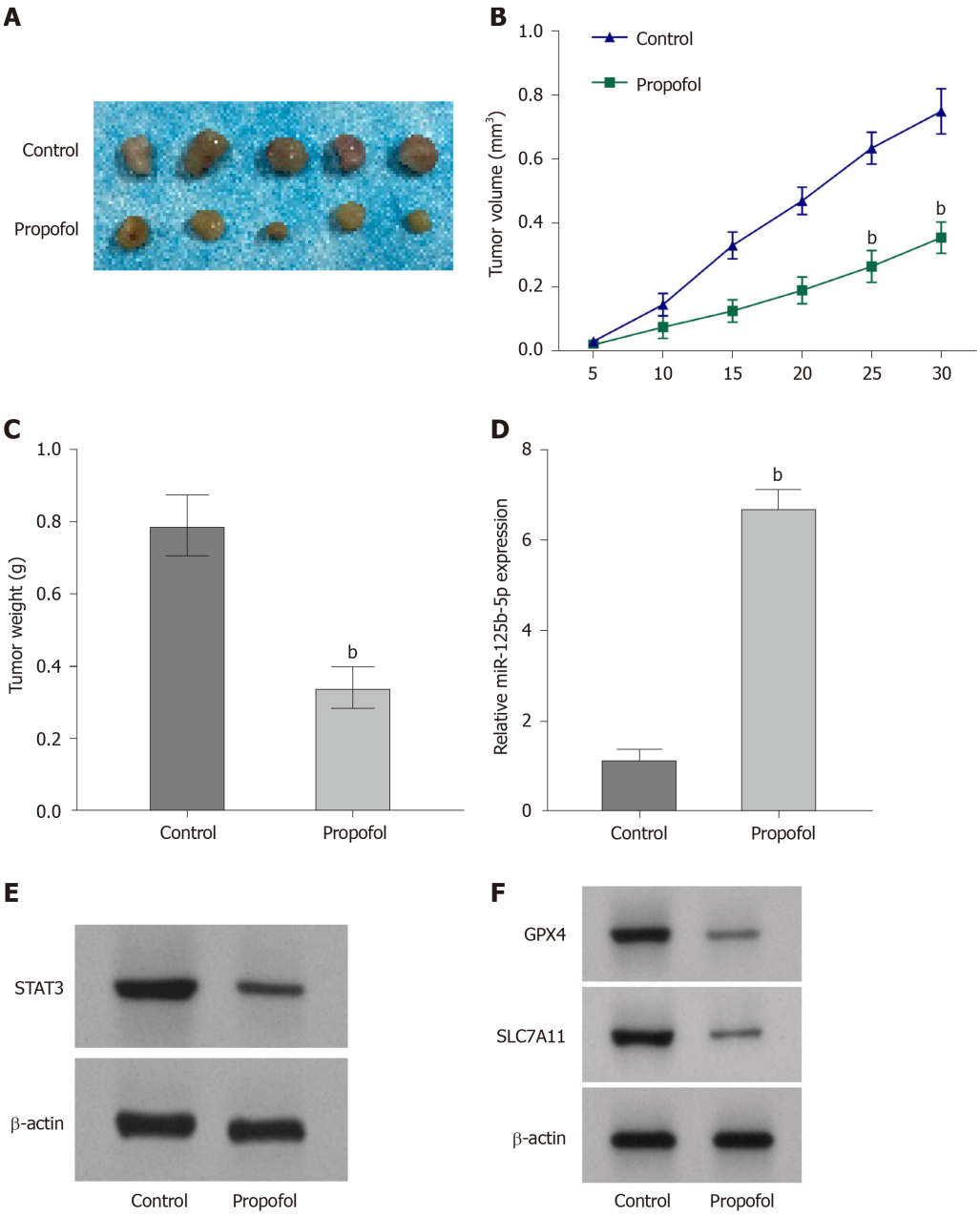

Figure 7 Propofol attenuates growth of gastric cancer cells *in vivo*. The nude mice were injected with SGC7901 cells and intraperitoneally treated with propofol (50 mg/kg). A: Tumor tissues; B: Tumor volume; and C: Tumor weight; D: Expression of miR-125b-5p was analyzed by quantitative reverse transcription polymerase chain reaction. E: Protein expression of STAT3 was detected by western blotting. F: Protein expression of GPX4 and SLC7A11 was measured by western blotting. $n = 5$, mean ± SD, $^{b}P < 0.01$.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer is a common malignancy with poor prognosis, in which ferroptosis plays a crucial role in its development. Propofol is a widely used anesthetic and has shown antitumor potential in gastric cancer. However, the effect of propofol on ferroptosis during gastric cancer progression remains unreported.

Research motivation

This study aims to identify the function of propofol in the regulation of ferroptosis and malignant phenotypes of gastric cancer cells.

Research objectives

To explore the role of propofol in the regulation of ferroptosis and malignant phenotypes of gastric cancer cells.

Research methods

MTT assays, colony formation assays, Transwell assays, wound healing assay, analysis of cell apoptosis, ferroptosis measurement, luciferase reporter gene assay and quantitative reverse transcription-PCR were used in this study.

Research results

Propofol was able to inhibit proliferation and induce apoptosis of gastric cancer cells. Propofol markedly repressed the invasion and migration of gastric cancer cells. Importantly, propofol enhanced the erastin-induced inhibitory effect on the growth of gastric cancer cells. Consistently, propofol increased the levels of ROS, iron and Fe^{2+} in gastric cancer cells. Propofol suppressed STAT3 expression by upregulating miR-125b-5p and propofol induced ferroptosis by targeting STAT3 in gastric cancer cells. The miR-125b-5p inhibitor or STAT3 overexpression could reverse propofol-attenuated malignant phenotypes of gastric cancer cells.

Research conclusions

Propofol induced ferroptosis and inhibited malignant phenotypes of gastric cancer cells by regulating the miR-125b-5p/STAT3 axis.

Research perspectives

Propofol may serve as a potential therapeutic candidate for gastric cancer therapy.

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ORIGINAL ARTICLE

Basic Study

BRAF^{V600E} mutant colorectal cancer cells mediate local immunosuppressive microenvironment through exosomal long noncoding RNAs

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Author contributions: Jia YT made substantial contributions to the conception and design of the study; Zhi J searched and reviewed published articles and wrote the manuscript; Jia XJ and Yan J collected patient data and prepared the tables; Zhi J, Jia XJ, Yan J and Wang HC performed the relevant experiments in the manuscript, statistically analyzed the data, and prepared the figures; Feng B and Xing HY critically reviewed the article and made revisions to the manuscript; all authors approved the final version.

Institutional review board statement: The study was reviewed and approved by the Hebei General Hospital Institutional Review Board (approval No. 202134).

Conflict-of-interest statement: The authors report no conflicts of interest for this manuscript.

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Abstract

BACKGROUND

BRAF^{V600E} mutated colorectal cancer (CRC) is prone to peritoneal and distant lymph node metastasis and this correlates with a poor prognosis. The BRAF^{V600E} mutation is closely related to the formation of an immunosuppressive microenvironment. However, the correlation between BRAF^{V600E} mutation and changes in local immune microenvironment of CRC is not clear.

AIM

To explore the effect and mechanism of BRAF^{V600E} mutant on the immune microenvironment of CRC.

METHODS

Thirty patients with CRC were included in this study: 20 in a control group and 10 in a treatment group. The density of microvessels and microlymphatic vessels, and M2 subtype macrophages in tumor tissues were detected by immunohistochemistry. Screening and functional analysis of exosomal long noncoding RNAs (lncRNAs) were performed by transcriptomics. The proliferation and migration of human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were detected by CCK-8 assay and scratch test,

Data sharing statement: No additional data are available.

Country/Territory of origin: China

Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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respectively. The tube-forming ability of endothelial cells was detected by tube formation assay. The macrophage subtypes were obtained by flow cytometry. The expression of vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β1, VEGF-C, claudin-5, occludin, zonula occludens (ZO)-1, fibroblast activation protein, and α-smooth muscle actin was assessed by western blot analysis. The levels of cytokines interleukin (IL)-6, TGF-β1, and VEGF were assessed by enzyme-linked immunosorbent assay.

RESULTS
BRAF^{V600E} mutation was positively correlated with the increase of preoperative serum carbohydrate antigen 19-9 ($P < 0.05$), and with poor tumor tissue differentiation in CRC ($P < 0.01$). Microvascular density and microlymphatic vessel density in BRAF^{V600E} mutant CRC tissues were higher than those in BRAF wild-type CRC ($P < 0.05$). The number of CD163^{+}M2 macrophages in BRAF^{V600E} mutant CRC tumor tissue was markedly increased ($P < 0.05$). Compared with exosomes from CRC cells with BRAF gene silencing, the expression of 13 lncRNAs and 192 mRNAs in the exosomes from BRAF^{V600E} mutant CRC cells was upregulated, and the expression of 22 lncRNAs and 236 mRNAs was downregulated ($P < 0.05$). The biological functions and signaling pathways predicted by differential lncRNA target genes and differential mRNAs were closely related to angiogenesis, tumor cell proliferation, differentiation, metabolism, and changes in the microenvironment. The proliferation, migration, and tube formation ability of HUVECs and HLECs induced by exosomes in the 1627 cell group (HT29 cells with BRAF gene silencing) was greatly reduced compared with the HT29 cell group ($P < 0.05$). Compared with the HT29 cell group, the expression levels of VEGF-A, bFGF, TGF-β1, and VEGF-C in the exosomes derived from 1627 cells were reduced. The expression of ZO-1 in HUVECs, and claudin-5, occludin, and ZO-1 in HLECs of the 1627 cell group was higher. Compared with the 1627 cell group, the exosomes of the HT29 cell group promoted the expression of CD163 in macrophages ($P < 0.05$). IL-6 secretion by macrophages in the HT29 cell group was markedly elevated ($P < 0.05$), whereas TGF-β1 was decreased ($P < 0.05$). The levels of IL-6, TGF-β1, and VEGF secreted by fibroblasts in the 1627 cell group decreased, compared with the HT29 cell group ($P < 0.05$).

CONCLUSION
BRAF^{V600E} mutant CRC cells can reach the tumor microenvironment by releasing exosomal lncRNAs, and induce the formation of an immunosuppressive microenvironment.

Key Words: Colorectal cancer; BRAF^{V600E} mutant; Exosome; Long noncoding RNA; Immunosuppressive microenvironment

Core Tip: This study revealed that BRAF^{V600E} mutant colorectal cancer (CRC) cells could lead to more angiogenesis and lymphoangiogenesis in the microenvironment by releasing exosomal long noncoding RNAs, inducing the formation of an immunosuppressive microenvironment. Our findings provide a hypothesis for finding new therapeutic strategies for BRAF^{V600E} mutant CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors, and ranks third in morbidity and mortality globally[1]. In China, societal and lifestyle changes have tended to increase the morbidity and mortality of CRC[2]. BRAF^{V600E} gene mutation accounts for approximately 10% of patients with metastatic CRC (mCRC), and is a point mutation at nucleotide 1799 of exon 15 (T mutated into A), resulting in a change of the encoded amino acid 600, valine replaced by glutamate (V600E)[3,4]. BRAF^{V600E} mutation can continuously activate the RAS-RAF-MEK-ERK signaling pathway, promote tumor cell proliferation and migration, and induce angiogenesis, thereby reducing tumor cell apoptosis[5-8]. Clinical data reveal that BRAF^{V600E} mutation frequently occurs in elderly women, and the related pathological type is mostly mucinous adenocarcinoma with a high level of tissue differentiation. Around 20% of patients have accompanying microsatellite instability, and most of them develop right colon cancer originating from serrated adenomas[4,9]. Compared with wild-type BRAF, patients with BRAF^{V600E} mutant CRC are prone to peritoneal metastasis and distant lymph node metastasis with a poor prognosis. Generally, the median survival time is < 12 mo. The effective treatment rate reaches only approximately 20%, even with three-drug chemotherapy and targeted combination therapy[10,11]. It is therefore important to explore the mechanism of BRAF^{V600E} mutant CRC with lymph node and peritoneal metastasis and to discover effective therapeutic strategies. This may be applicable to start with its specific immune microenvironment.

The tumor microenvironment (TME) is the local environment facilitating tumor growth and proliferation[12]. In thyroid cancer, the proportion of mast cells in BRAF^{V600E} mutant TME is markedly increased compared with wild-type BRAF, which may be involved in mediating the formation of the immunosuppressive microenvironment, suggesting that BRAF^{V600E} mutation affects the TME[13]. Multiple cells (namely, endothelial cells, fibroblasts, and immune cells) and extracellular components (namely, cytokines, growth factors, hormones, and extracellular matrix) in the TME can induce tumor angiogenesis and lymphangiogenesis and promote chronic inflammation, thereby creating a local immunosuppressive microenvironment, which plays a vital role in tumor occurrence, invasion, and metastasis and drug resistance[14]. Tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) are the principal components of the TME. Among them, M2-type macrophages have an immunosuppressive effect. They can promote tumor cell proliferation, infiltration, and metastasis, and their expression level is intimately associated with patient prognosis [15,16]. Meanwhile, CAFs have undergone substantial changes in morphology, proliferation activity, motility, and secretory function, which can facilitate tumor proliferation, invasion, metastasis, and angiogenesis, and their expression level is closely linked to tumor stage and poor prognosis[17]. There is no current research investigating the relationship between BRAF^{V600E} gene mutation and the formation and functional changes of blood vessels, lymphatic vessels, TAMs, and CAFs in the CRC local immune microenvironment.

Exosomes are a type of cystic microvesicle secreted by cells, and the secretion process is active. They are able to carry specific biologically active molecules including lipids, miRNA, and long noncoding RNAs (lncRNAs) into the corresponding target cells and mediate substance transportation and information exchange intercellularly [18]. LncRNAs belong to a class of single-stranded RNA molecules that do not encode proteins. They play a role *via* transcription, post-transcription, and translation, and participate in tumor occurrence, invasion, and metastasis. Exosomes secreted by tumor cells can modify the TME through lncRNAs, thereby promoting the development of tumors[19,20]. Liang *et al*[21] noted that the expression of exosome-derived lncRNA RPPH1 in CRC was markedly upregulated. It can prevent ubiquitination by binding to TUBB3 and induce epithelial-mesenchymal transition, and interact with TAMs to promote the polarization of TAMs to M2 subtype, thereby accelerating tumor progression. However, there is still a lack of relevant investigations on whether BRAF^{V600E} mutant CRC cells affect the TME through the release of exosomal lncRNAs.

The present study aimed to investigate the influence of BRAF^{V600E} mutation in CRC on the surrounding immune microenvironment, and to elucidate whether BRAF^{V600E} mutant CRC cell-derived exosomes participate in the formation of an immunosuppressive microenvironment. It is hoped that the results will provide a novel therapeutic strategy for BRAF^{V600E} mutant CRC.

MATERIALS AND METHODS

General information

Data of ten BRAF^{V600E} mutant CRC patients who underwent surgical treatment at the Hebei General Hospital from September 2014 to June 2019 were collected. Twenty BRAF wild-type CRC patients were selected as controls. There were 18 male and 12 female patients. The age range was 27-79 years, with an average of 57.57 ± 2.13 years and median of 57 years. The specimens were obtained with informed consent obtained from the patients and under the approval of the Ethics Committee of the Fourth Hospital of Hebei Medical University. The inclusion criteria were: (1) Patients with a pathological diagnosis of CRC, in whom those identified as having BRAF^{V600E} mutation were included in an experimental group, and those identified as having wild-type BRAF were included in a control group; (2) Untreated patients; and (3) Undergoing first surgical treatment for primary CRC. The exclusion criteria were: (1) History of malignant tumors; (2) Current primary tumors in other regions; and (3) Patients with incomplete pathological data.

Cells and reagents

The human colon cancer cell line HT29 (BRAF^{V600E} mutant) was purchased from the Cell Bank of the Chinese Academy of Sciences. The human colon cancer cell line 1627 was obtained from Shanghai GenePharma Company, which was a HT29 cell strain with the BRAF gene being silenced. Human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were purchased from ScienCell, San Diego, CA, United States. Human monocytic leukemia cells (THP-1 cells) and human embryonic lung fibroblasts (MRC-5 cells) were obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. McCoy's 5A, RPMI 1640, minimal essential medium, and fetal bovine serum (FBS) were all purchased from Gibco (NY, United States).

Induction of macrophages

THP-1 cells in the logarithmic phase of growth were obtained and placed in a 15-mL centrifuge tube, and centrifuged at 1000 r/min for 5 min. The supernatant was discarded. Following addition of fresh RPMI 1640 culture medium, the cells were resuspended and inoculated in a 12-well plate with 5 × 10^5 cells per well, supplemented with pharmaceutical manufacturers association (PMA) solution (dissolved in DMSO) (Cayman, MI, United States) at a final concentration of 100 ng/mL, and cultured in a constant temperature incubator for 48 h. The cells were ultimately placed under a biological microscope (Nikon, Tokyo, Japan) and the cell morphology and adhesion were observed.

Immunohistochemistry

The postoperative tissue samples of CRC patients were collected, embedded in paraffin, and cut into 5-mm sections. After deparaffinization and hydration, antigen retrieval, and incubation in 3% H_2O_2 for 10 min, the corresponding antibodies were added, including rabbit anti-human CD31 monoclonal antibody (1:2000, Arigo, Hsinchu City, Taiwan), rabbit LYVE-1 polyclonal antibody (1:200, Arigo), mouse anti-human CD68 monoclonal antibody (1:100, BD Biosciences, NJ, United States), rabbit anti-human CD163 monoclonal antibody (1:100, HUABIO, Hangzhou, China), and rabbit anti-human α-smooth muscle actin (SMA) monoclonal antibody (1:100, HUABIO). 3,3-diaminobenzidine (DAB) color development was performed for microscopic observation. A double-blind reading method was adopted by two pathologists. The sections were initially visualized under low magnification (× 100) to determine three fields where cells were most densely distributed. The number of positive cells was counted under high magnification (× 400), and the average number was calculated.

Extraction and identification of exosomes

Cell supernatant was collected and centrifuged at 480 × *g* for 5 min, followed by 2000 × *g* for 10 min to remove cell debris. The supernatant was collected and centrifuged at 10000 × *g* for 30 min to remove macrovesicles. The supernatant was collected again and centrifuged at 100000 × *g* for 2 h. The supernatant was discarded and the exosomes were resuspended in 200 mL of PBS solution, and stored in a freezer at -80 °C for later use. The morphology of exosomes was identified with a transmission electron microscope (Hitachi, Tokyo, Japan). At room temperature, 10 mL of exosome suspension was dripped on a 2-nm-pore-diameter copper mesh using a pipette and

allowed to stand for 2 min. The liquid was absorbed dry using absorbent paper. A quantity of 30 mL 3% tungsten phosphate was dripped onto the copper mesh for negative staining for 5 min, and the liquid was absorbed dry using absorbent paper. After drying, photographs were taken under a transmission electron microscope.

Western blot analysis

The cell samples were removed from the -80 °C freezer and supplemented with 200 mL of RIPA lysis solution for 20 min to extract the total cell proteins. The BCA protein concentration determination kit was used to quantify the proteins. The proteins were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes at 250 mA, and blocked with bovine serum albumin for 2 h. The blocked PVDF membranes were placed directly into the freshly prepared primary antibody working solution at 4 °C overnight. Primary antibodies used included rabbit anti-human CD9 monoclonal antibody (1:1000, Abcam, Cambridge, United Kingdom), rabbit anti-human CD63 monoclonal antibody (1:5000, Abcam), rabbit anti-human BRAF monoclonal antibody (1:1000, Arigo), rabbit anti-zonula occludens (ZO)-1 polyclonal antibody (1:1000, GenTex, Gentex, United States), rabbit anti-claudin-5 polyclonal antibody (1:1000, GenTex), rabbit anti-occludin polyclonal antibody (1:1000, HUABIO), rabbit anti-transforming growth factor (TGF)-1 polyclonal antibody (1:1000, Bioss, Beijing, China), rabbit anti-vascular endothelial growth factor (VEGF)-C polyclonal antibody (1:800, Bioworld, MN, United States), rabbit anti-basic fibroblast growth factor (anti-bFGF) polyclonal antibody (1:600, Bioss), mouse anti-human VEGF-A monoclonal antibody at a concentration of 6 mg/mL (Abcam), rabbit anti-human fibroblast activation protein (FAP) monoclonal antibody (1:1000, Abcam), and rabbit anti-human α-SMA monoclonal antibody (1:1000, Abcam). Following membrane washing on the next day, the corresponding secondary antibody was incubated, and chemiluminescence was detected with ECL substrate. After developing and fixing, the film was scanned using a scanner, and Tanon 1600 software was used for gray scale analysis and quantification.

CCK-8 assay

Cells in the logarithmic phase of growth were selected and seeded in a 96-well plate at 5×10^3 cells/well with a volume of 100 mL per well, and cultured at 37 °C in a 5% CO_2 incubator. After 6 h of inoculation, the experimental group was treated with 10 mL exosomes, and the control group with 10 mL PBS solution, and then cultured in the incubator again. Exosomes were cocultured with HUVECs for 24, 48, and 72 h, and cocultured with HLECs for 12, 24 and 36 h; 10 mL of CCK-8 reagent (DOJINDO, Kyushu, Japan) was added to each well and incubated at 37 °C with 5% CO_2 for 1 h. Absorbance at 560 nm was measured using a microplate reader (ThermoFisher Scientific, MA, United States).

Scratch test

HUVECs or HLECs were plated in six-well plates. When the cells were evenly spread, they were scratched using a 200-μL pipette tip. PBS solution was used to rinse once for the removal of the suspended cells. To each well, 2 mL of FBS-free culture medium was added. The experimental group was supplemented with 20 μL of exosomes, and the control group with 20 μL of PBS solution. Both groups were cultured at 37 °C in a 5% CO_2 incubator. The cells were observed and photographed under a microscope at 0 and 24 h, respectively, to detect the scratch healing. Cell migration rate was calculated as [(scratch area at 0 h - scratch area at 24 h)/scratch area at 0 h] × 100%.

Tube formation assay

After freezing and thawing the Matrigel matrix (BD Biosciences, United States) containing low levels of growth factors at 4 °C, the homogenate was mixed well with a precooled pipette tip and packaged into precooled Eppendorf tubes. Matrigel matrix was diluted with serum-free medium at a ratio of 1:3, and the 96-well plate was placed on an ice pack. Fifty microliters of diluted Matrigel matrix was added to each well and left to stand at 37 °C for 30 min. Cells were seeded in a 96-well plate at 10^4 cells/well and supplemented with 100 μL cell suspension in each well. The experimental group was treated with 10 μL of exosomes, and the control group with 10 μL PBS solution, and then cultured at 37 °C in a 5% CO_2 incubator. After culturing for 24 h, the cells were visualized and photographed under a low magnification (× 100) microscope and the tube formation ability of endothelial cells was observed.

Flow cytometry

THP-1 cells were induced with PMA for 48 h and the culture medium was discarded following three cycles of washing with PBS. Trypsin (0.5 mL) was supplied to each well for digestion, and 1.5 mL of culture medium containing 10% FBS was utilized to terminate the digestion. The cells were then collected and centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and the cells were resuspended in PBS and centrifuged again to obtain the precipitate. Mouse anti-human CD68 monoclonal antibody (5 μL; BD Biosciences) and rabbit anti-human CD163 monoclonal antibody (5 μL; BD Biosciences) were added and incubated at 4 °C in the dark for 30 min. Following two cycles of washing with PBS, the cells were centrifuged at 1000 r/min for 5 min, resuspended in 300 μL PBS, and loaded on a flow cytometer (Beckman Coulter, CA, United States) for determination.

Enzyme-linked immunosorbent assay

After cells were cultured for 48 h, the medium was collected and centrifuged at 1000 r/min for 5 min. An enzyme-linked immunosorbent assay (ELISA) kit (MULTI SCIENCES, Hangzhou, China) was used to detect the expression of interleukin (IL)-6, TGF-β1, and VEGF in the culture medium. IL-6 antibody, TGF-β1 antibody, VEGF antibody, and horseradish peroxidase-labeled streptavidin were all diluted 1:100.

Extraction and identification of exosomal RNA

AllPrep RNA/LncRNA kit (Qiagen, Hilden, Germany) was used to extract total RNA from exosomes. The purity of RNA was determined using QubitRNA kit (Thermo Fischer Scientific). The RNA concentration was accurately quantified utilizing Qubit. The integrity of RNA was assessed using Agilent 2100.

Transcriptome library preparation, sequencing, and data analysis

Exosome samples of HT29 and 1627 cells were initially obtained and total exosomal RNA was extracted. Ovation Solo RNA kit (NugEN) was used for library construction of exosomal lncRNAs. The constructed library was sequenced through the Illumina Hiseq 4000 platform where the double-terminal 250-300 nt transcripts were generated. The raw data in Fastq format were processed using perl to obtain sequencing data. By logging in to the TopHat2 system, the sequenced data transcripts were aligned and analyzed in light of the reference genome. Cufflinks software was used to compare the analyzed data for transcript splicing and the transcriptome was obtained. Quantitative analysis was performed on the transcript. Finally, the transcript data set was retrieved using the RefSeq database as an mRNA data set. Cuffmerge software was used to merge the obtained transcripts after splicing, delete the transcripts with unclear mid-strand direction, and obtain a mRNA transcript data set. LncRNAs were ultimately screened from the combined transcript set.

Prediction of lncRNA target genes and GO and KEGG enrichment analysis

The target genes of lncRNAs were predicted through cis-acting and trans-acting, including annotating the function of its target gene mRNA. The GO seq software and KOBAS software were used to enrich differential lncRNA target genes and differential mRNAs in the three processes of biological process, cell component, and molecular function. The biological functions involved in differential lncRNA target genes and differential mRNAs were analyzed. KOBAS software was used to enrich the differential lncRNA target genes and differential mRNAs with KEGG pathways. The signal transduction pathways involved in differential lncRNA target genes and differential mRNA were also analyzed.

Statistical analysis

SPSS 21.0 software was used for statistical analyses. Enumerative data are expressed as percentages and analyzed using the χ^2 test or Fisher's exact test. The enumerative data are expressed as the mean ± SD. The two groups of enumerative data were tested by two independent samples *t*-tests. One-way analysis of variance was applied for multiple groups of samples. $P < 0.05$ was considered statistically significant.

RESULTS

Relationship between BRAF^{V600E} mutation and clinicopathological parameters

We collected and analyzed the clinical data of 10 BRAF^{V600E} mutant and 20 BRAF wild-type CRC patients. In CRC, BRAF^{V600E} mutation was positively correlated with the increase of preoperative serum carbohydrate antigen (CA)19-9 ($P < 0.05$), and it was correlated with poor tumor tissue differentiation ($P < 0.01$). However, no correlation was revealed with gender, age, location, mucous tissue, T stage, TNM stage, lymph node metastasis, nerve invasion, vascular tumor thrombus, preoperative carcinoembryonic antigen (CEA) level, or preoperative platelet count of the patients (Table 1).

BRAF^{V600E} mutation promotes formation of microvessels and microlymphatic vessels in tumor tissues and increases infiltration of M2 macrophages

To explore the influence of BRAF^{V600E} mutation on the TME, we identified the formation of tumor blood vessels and lymph vessels in BRAF^{V600E} mutation and BRAF wild-type CRC tissues. Microvascular density (MVD) and microlymphatic vessel density (MLVD) in BRAF^{V600E} mutant CRC tissues were higher than those in BRAF wild-type CRC ($P < 0.05$) (Figure 1A and Table 2). The number of CD163^{+}M2 macrophages in BRAF^{V600E} mutant CRC tumor tissue was markedly increased ($P < 0.05$) (Figure 1B and Table 3), whereas the number of CD68^{+}M1 macrophages was not significantly different (Figure 1B and Table 3). Additionally, the density of fibroblasts exhibited no significant difference (Figure 1C and Table 4). These results suggest that BRAF^{V600E} mutation promotes the formation of microvessels and microlymphatic vessels in tumor tissues, increases the infiltration of M2 macrophages, and induces an immunosuppressive microenvironment.

BRAF^{V600E} mutant CRC cells can reach the TME by releasing exosomal lncRNAs, and induce formation of an immunosuppressive microenvironment

We performed transcriptomics analysis on exosomes derived from BRAF^{V600E} mutant CRC cells and those with BRAF gene silencing. Compared with the exosomes from cells with BRAF gene silencing, the expression of 13 lncRNAs and 192 mRNAs in the exosomes of BRAF^{V600E} mutant CRC cells was upregulated, and the expression of 22 lncRNAs and 236 mRNAs was downregulated ($P < 0.05$) (Figure 2A). Using cluster analysis charts, the distribution of differentially expressed lncRNAs and mRNAs in exosomes of BRAF^{V600E} mutant CRC cells and those with BRAF gene silencing was further exhibited (Figure 2B).

To illustrate the biological functions of differential lncRNAs and mRNAs and the signaling pathways involved, we conducted GO enrichment analysis and KEGG signaling pathway analysis. GO enrichment analysis included three aspects of biological process, cell component, and molecular function. Differential lncRNA target genes and mRNAs presented similar biological functions, and they are mainly involved in nucleic acid metabolism, macromolecular metabolism, nitride metabolism, and RNA metabolism. Cellular components include mostly the formation of nuclei, organelles, and nucleosomes. Molecular functions involve regulation of cell adhesion, cytoskeletal remodeling, gene expression regulation, and protein binding (Figure 2C).

KEGG results indicated that the target genes of differential lncRNAs were mainly involved in the p53 pathway, ErbB pathway, steroid synthesis pathway, actin cytoskeleton regulation pathway, pyruvate metabolism pathway, cell cycle regulation pathway, and the pathway of protein processing in the endoplasmic reticulum (Figure 2D). Differential mRNAs are mainly involved in the VEGF pathway, mammalian target of rapamycin pathway, mitogen-activated protein kinase pathway, and the pathway of protein processing in the endoplasmic reticulum (Figure 2D). The biological functions and signaling pathways predicted by differential lncRNA target genes and differential mRNAs coincided with each other. Furthermore, it was closely related to angiogenesis, tumor cell proliferation, differentiation, metabolism, and changes in the microenvironment, suggesting that BRAF^{V600E} mutant CRC cells could reach the TME by releasing exosomal lncRNAs, and induce formation of an immunosuppressive microenvironment through mRNAs.

BRAF^{V600E} mutant CRC cell-derived exosomes promote proliferation, migration, and tube formation of HUVECs and HLECs, and induce angiogenesis and lymphoangiogenesis

Exosomes derived from HT29 and 1627 cells were cocultured with HUVECs or HLECs to detect cell proliferation. OD values of HUVECs in the HT29 group and 1627 group

Table 1 Relationship between BRAF^{V600E} mutation and clinicopathological parameters in colorectal cancer

Variable	Cases (%)	BRAF^{V600E} mutation		*P* value
		Yes (%)	No (%)	
Gender				
Male	18 (60)	6 (60)	12 (60)	0.326
Female	12 (40)	4 (40)	8 (40)	
Age (yr)				
≥ 65	10 (33.3)	4 (40)	6 (30)	0.440
< 65	20 (66.7)	6 (60)	14 (70)	
Location				
Right colon	9 (30)	2 (20)	7 (35)	0.345
Left colon	9 (30)	5 (50)	4 (20)	
Rectum	12 (40)	3 (30)	9 (45)	
Mucous tissue				
Positive	6 (20)	1 (10)	5 (25)	0.326
Negative	24 (80)	9 (90)	15 (75)	
Differentiated degree				
High/moderate differentiation	20 (66.7)	3 (30)	17 (85)	0.005[a]
Poor differentiation	10 (33.3)	7 (70)	3 (15)	
T stage				
T2	4 (13.3)	0	4 (20)	0.211
T3	3 (10)	2 (20)	1 (5)	
T4	23 (76.7)	8 (80)	15 (75)	
TNM stage				
I + II	13 (43.3)	2 (20)	11 (55)	0.074
III + IV	17 (56.7)	8 (80)	9 (45)	
Lymph node				
Positive	17 (56.7)	8 (80)	9 (45)	0.074
Negative	13 (43.3)	2 (20)	11 (55)	
Nerve invasion				
Positive	7 (23.3)	4 (40)	3 (15)	0.143
Negative	23 (76.7)	6 (60)	17 (85)	
Vessel carcinoma embolus				
Positive	3 (10)	1 (10)	2 (10)	0.652
Negative	27 (90)	9 (90)	18 (90)	
CA19-9 (U/mL)				
High	12 (40)	7 (70)	5 (25)	0.024[a]
Normal	18 (60)	3 (30)	15 (75)	
CEA (ng/mL)				
High	11 (36.7)	3 (30)	8 (40)	0.452
Normal	19 (63.3)	7 (70)	12 (60)	
PLT count (× 10^9/L)				
High	12 (40)	5 (50)	7 (35)	0.344

Normal	18 (60)	5 (50)	13 (65)

[a]*P* < 0.05.

PLT: Platelets; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9.

Table 2 Relationship between BRAF^{V600E} mutation and microvascular density/microlymphatic vessel density in colorectal cancer

BRAF^{V600E} mutation	MVD		MLVD	
	mean ± SD	*P* value	mean ± SD	*P* value
+	38.53 ± 17.11	0.030	9.67 ± 5.63	0.0001
-	27.54 ± 9.54		3.81 ± 2.06	

MVD: Microvascular density; MLVD: Microlymphatic vessel density.

Table 3 Relationship between BRAF^{V600E} mutation and tumor-associated macrophages in colorectal cancer

BRAF^{V600E} mutation	CD68$^+$ macrophage		CD163$^+$ M2 macrophage	
	mean ± SD	*P* value	mean ± SD	*P* value
+	18.43 ± 13.53	0.664	8.33 ± 5.93	0.040
-	20.96 ± 15.34		3.67 ± 3.02	

Table 4 Relationship between BRAF^{V600E} mutation and cancer-associated fibroblasts in colorectal cancer

BRAF^{V600E} mutation	CAFs		*P* value
	Low expression (cases)	High expression (cases)	
+	2	8	1
-	3	17	

CAF: Cancer-associated fibroblast.

at 24 h were 1.215 ± 0.032 and 0.986 ± 0.046, respectively; 1.563 ± 0.035 and 1.200 ± 0.163 at 48 h, respectively; and 1.661 ± 0.031 and 1.369 ± 0.020 at 72 h, respectively. At 24, 48 and 72 h, HUVEC proliferation induced by exosomes in the 1627 cell group was reduced compared with the HT29 cell group ($P < 0.05$), but there was no significant difference in HUVEC proliferation between the 1627 cell group and control group (Figure 2A). The OD values of HLECs in the control group, HT29 cell group, and 1627 cell group at 12 h were 0.477 ± 0.006, 0.526 ± 0.007, and 0.500 ± 0.004, respectively; 0.622 ± 0.003, 0.728 ± 0.010, and 0.680 ± 0.010 at 24 h, respectively; and 0.644 ± 0.006, 0.725 ± 0.009, and 0.682 ± 0.014 at 36 h, respectively. At 12, 24, and 36 h, the proliferation of HLECs induced by exosomes in the 1627 cell group was greatly reduced compared with the HT29 cell group ($P < 0.05$) and the proliferation of HLECs in the 1627 cell group was greater than that of the control group ($P < 0.05$) (Figure 3A).

Light microscopy indicated that the average migration rate of HUVECs in the HT29 cell group was 82.863% ± 3.095% and that of the 1627 cell group was 45.067% ± 2.895% at 24 h after scratch. The average migration rate of HLECs in the HT29 cell group was 42.393% ± 0.247%, and that of the 1627 cell group was 23.327% ± 1.434%. Compared with the 1627 cell group, the migration of HUVECs and HLECs induced by exosomes in the HT29 cell group was substantially elevated ($P < 0.01$) (Figure 3B).

After 24 h of incubation, the average number of tube formations by HUVECs in the HT29 cell group and 1627 cell group was 30.625 ± 0.925 and 12.750 ± 1.887, respectively, at low magnification. The average number of tube formations by HLECs in the HT29 cell group and 1627 cell group was 26.750 ± 2.016 and 15.375 ± 1.413, respectively, at low magnification. Compared with the 1627 cell group, the tube formation ability of HUVECs and HLECs induced by exosomes in the HT29 cell group

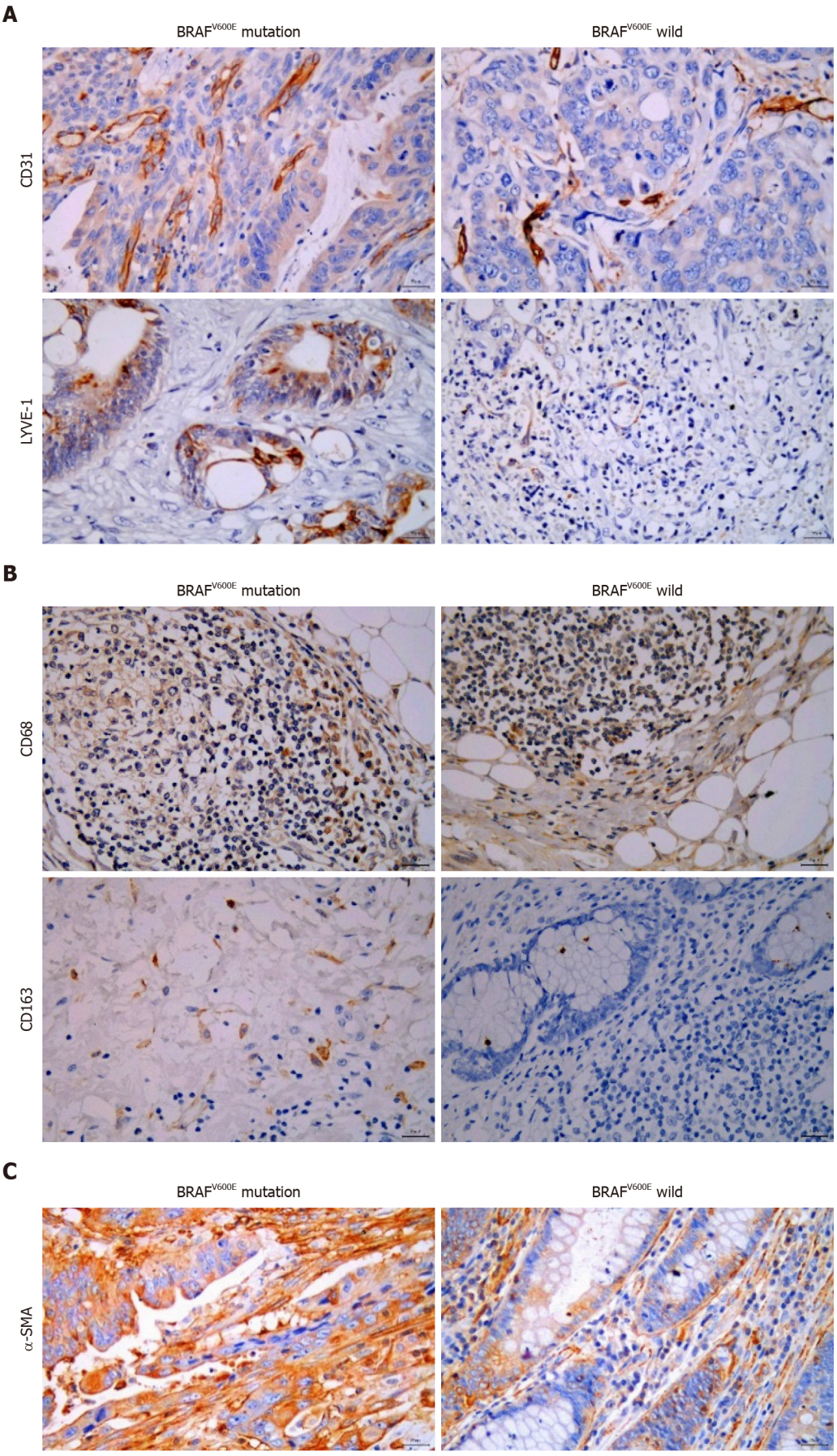


Figure 1 Relationship between BRAF^{V600E} mutation and microvascular density, microlymphatic vessel density, tumor-associated macrophages, and cancer-associated fibroblasts. A: Expression of CD31 and lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) in

BRAF^{V600E} mutant and BRAF wild type colorectal cancer (CRC) tissues (CD31 labeled microvascular density, and LYVE-1 labeled microlymphatic vessel density; immunohistochemistry, × 400); B: Expression of CD68 and CD163 proteins in BRAF^{V600E} mutant and BRAF wild type CRC tissues (CD68 labeled TAMs, and CD163 labeled M2 subtype macrophages; immunohistochemistry, × 400); C: Expression of α-SMA protein in BRAF^{V600E} mutant and BRAF wild type CRC tissues (α-SMA labeled CAFs; immunohistochemistry, × 400).

was markedly higher than that in the 1627 cell group ($P < 0.01$) (Figure 3C).

Western blot analysis indicated that compared with the HT29 cell group, the expression of VEGF-A, bFGF, TGF-β1, and VEGF-C proteins in the exosomes derived from 1627 cells was reduced (Figure 3D). However, the expression of ZO-1 in HUVECs and that of claudin-5, occludin, and ZO-1 in HLECs in the 1627 cell group were higher than those in the HT29 cell group (Figure 3E and F). Neither the 1627 nor the HT29 group failed to display occludin and claudin-5 protein. This suggests that exosomes derived from BRAF^{V600E} mutant CRC cells promote angiogenesis and lymphoangiogenesis.

Exosomes derived from BRAF^{V600E} mutant CRC cells promote polarization of macrophages to M2 subtype and enhance secretory function of macrophages and fibroblasts

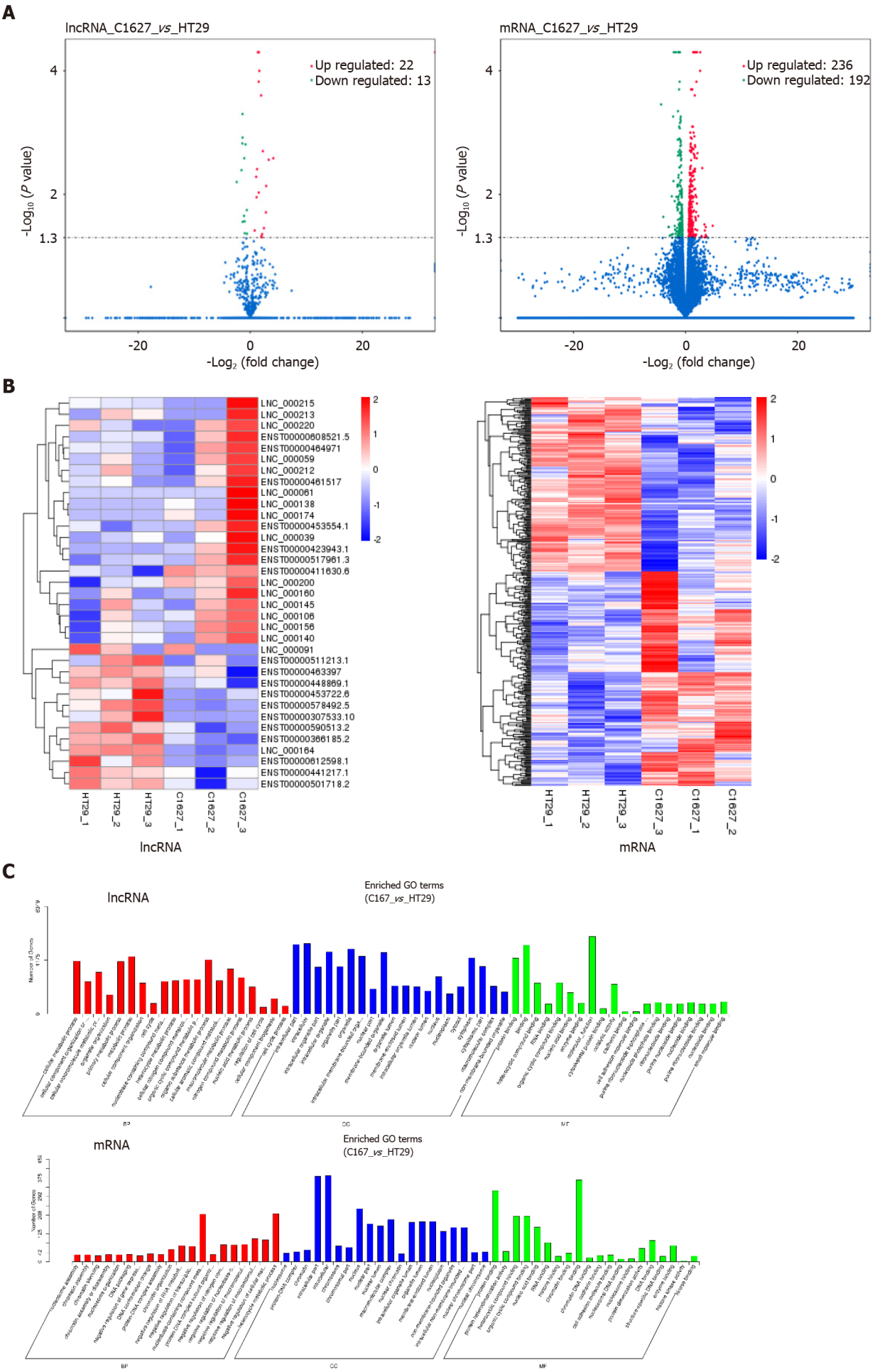
The exosomes derived from HT29 and 1627 cells were cocultured with macrophages. Flow cytometry revealed that the exosomes of the HT29 cell group promoted the expression of CD163 in macrophages compared with the control group and 1627 cell group ($P < 0.05$) (Figure 4A). ELISA indicated that IL-6 secreted by macrophages in the HT29 cell group was markedly elevated ($P < 0.05$), whereas TGF-β1 was decreased ($P < 0.05$) (Figure 4B). Western blot analysis demonstrated that there was no significant difference in the expression of fibroblast FAP and α-SMA in the control, HT29 cell, and 1627 cell groups (Figure 4C). Conversely, the levels of IL-6, TGF-β1, and VEGF secreted by fibroblasts in the 1627 cell group decreased, compared with the HT29 cell group ($P < 0.05$) (Figure 4D). Exosomes derived from BRAF^{V600E} mutant CRC cells promoted polarization of macrophages to M2 subtype and enhanced the secretory function of fibroblasts.

DISCUSSION

This study demonstrated that BRAF^{V600E} mutant CRC generates a unique immune microenvironment. Compared with BRAF wild type CRC, there was more angiogenesis and lymphoangiogenesis in the microenvironment. Meanwhile, the polarization of macrophages to M2 subtype was more obvious, and the immunosuppression was more prominent. Exosomes derived from BRAF^{V600E} mutant CRC cells could induce this change. Further analysis indicated that exosomes derived from BRAF^{V600E} mutant CRC cells were rich in certain lncRNAs and mRNAs, which might link to these alterations. Our findings suggested that for this particular CRC, it might be worthwhile to try to investigate the TME.

It is currently argued that BRAF^{V600E} mutation is of vital significance in predicting the prognosis of CRC patients[22]. Several studies have indicated that BRAF^{V600E} mutation is not only correlated to poor tissue differentiation, but also to gender, advanced stage, high T stage, right colon, lymph node metastasis, mucous tissue, and high levels of platelets and CEA[22,23]. Conversely, our results were inconsistent with those reported in the literature. We recognized that BRAF^{V600E} mutation was only associated with poor tissue differentiation and increased preoperative CA19-9 levels, which might have been caused by the small sample size.

However, we analyzed the TME of such patients. The results demonstrated that the MVD and MLVD in BRAF^{V600E} mutant CRC tissues were higher than those in BRAF wild type tissues, and the number of CD163^{+} M2 macrophages increased substantially. In papillary thyroid carcinoma, BRAF^{V600E} mutation upregulates the expression of VEGF-A in cancer cells and promotes angiogenesis, and increases the expression of VEGF-C in cancer cells and promotes lymphangiogenesis, whereas silencing BRAF gene can reverse these effects[24]. Under normal circumstances, HCT116 colon cancer cells can activate the VEGF signaling pathway, promote the proliferation, migration, and angiogenesis of vascular endothelial cells, and facilitate tumor metastasis by releasing exosomes[25]. Exosomes derived from CRC cells can also promote TAMs to release VEGF-C, enhance proliferation of lymphatic endothelial cells, and induce


A
lncRNA_C1627_vs_HT29
Up regulated: 22
Down regulated: 13
-Log10 (P value)
-Log2 (fold change)
mRNA_C1627_vs_HT29
Up regulated: 236
Down regulated: 192
B
lncRNA
mRNA
HT29_1
HT29_2
HT29_3
C1627_1
C1627_2
C1627_3
C
lncRNA
Enriched GO terms
(C167_vs_HT29)
mRNA
Enriched GO terms
(C167_vs_HT29)

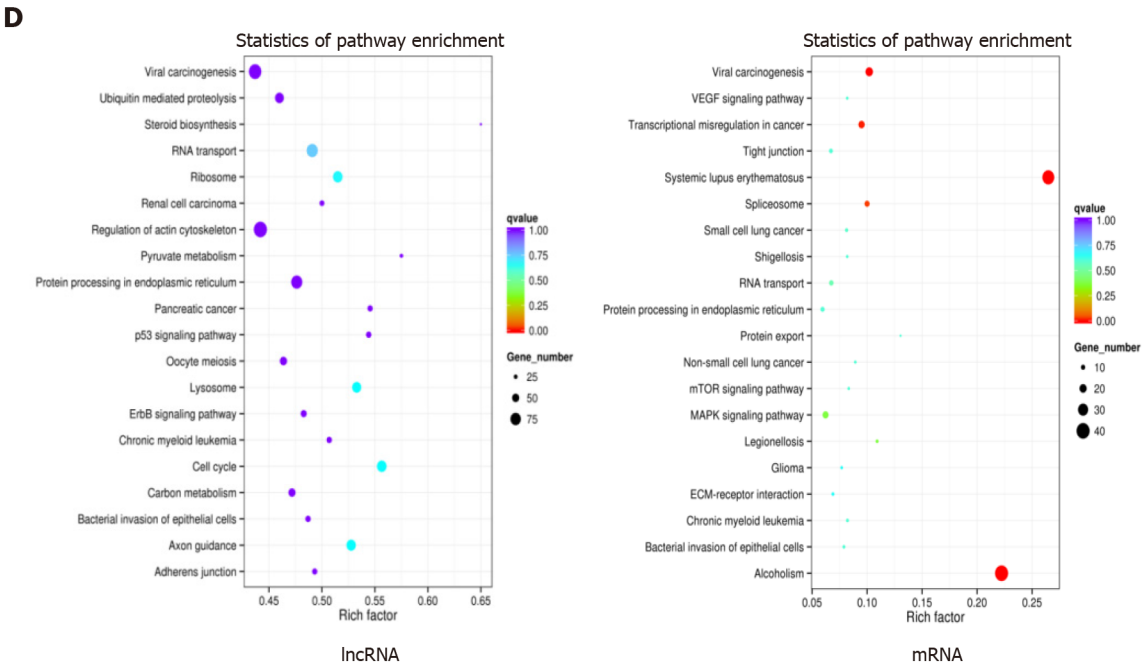
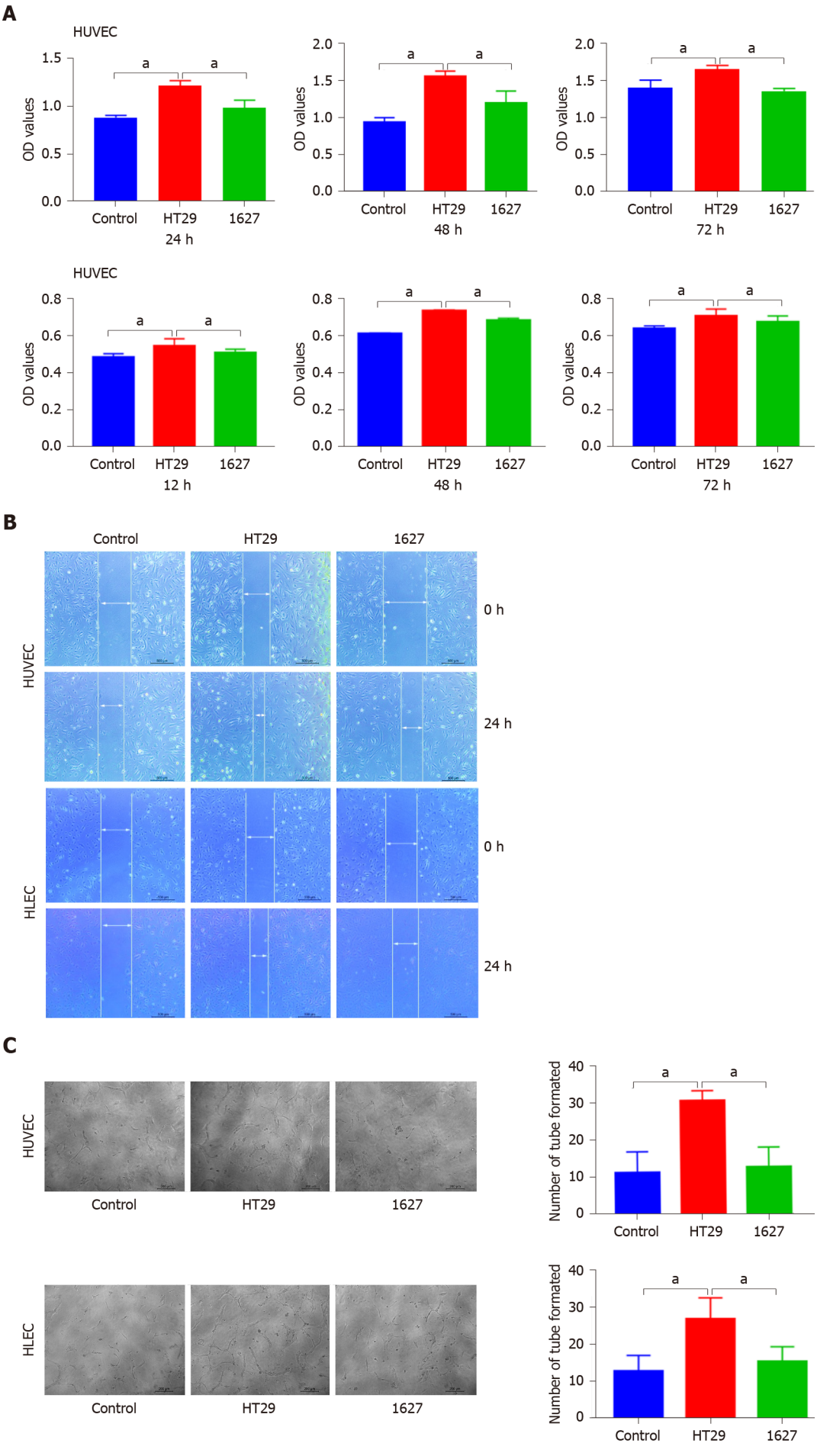


Figure 2 Screening and functional analysis of differentially expressed long noncoding RNAs and mRNAs in exosomes of BRAF^{V600E} mutant colorectal cancer cells and those with BRAF gene silencing. A: Transcriptomics analysis of differentially expressed long noncoding RNAs (lncRNAs) and mRNAs in the exosomes of HT29 and 1627 cells. Volcano maps are shown (the x axis represents the multiple of difference, and the y axis *P* values); B: Hierarchical cluster analysis of differentially expressed lncRNAs and mRNAs (red represents upregulated expression, and blue represents downregulated expression); C: GO enrichment analysis of differentially expressed lncRNA target genes and mRNAs; D: KEGG pathway analysis of differentially expressed lncRNA target genes and mRNAs. LncRNA: Long noncoding RNA.

lymphangiogenesis[26]. However, our results indicate that BRAF^{V600E} mutation promotes angiogenesis and lymphoangiogenesis in the microenvironment.

Simultaneously, BRAF^{V600E} mutation affected cellular components in the microenvironment. We identified that there was no difference in the content of CD68^{+} macrophages in BRAF^{V600E} mutant and BRAF wild-type CRC tissues, and the number of M2 macrophages was markedly higher than that of BRAF wild-type tissues. This is in agreement with the results reported in the literature. Compared with BRAF wild type tumor, there is no difference in CD68^{+} macrophages in BRAF^{V600E} mutant thyroid cancer, whereas M2 subtype macrophages increase[27]. Furthermore, TAMs have been positively related to lymph node and peritoneal metastasis in ovarian cancer, gastric cancer, and other tumors[28-30]. CAFs play an essential role in tumor development and metastasis. Our findings indicated no difference in the content of CAFs in CRC tissues of BRAF^{V600E} mutant and BRAF wild-type CRC. We hypothesized that BRAF^{V600E} mutant CRC might promote lymph node and peritoneal metastasis through other mechanisms, but the specific mechanism needs further exploration. This harsh microenvironment may lead to a worse prognosis and even result in resistance to traditional treatments.

Recent research has drawn a link between exosomes and intercellular communication, which are responsible for transporting specific RNA transcripts to target organs, participating in substance exchange in the distant environment, regulating immune function, and promoting tumor angiogenesis, invasion, and metastasis, thereby directly or indirectly affecting the progression and outcome of tumors[31]. We analyzed the expression profiles of exosomal lncRNAs and mRNAs in HT29 cells and 1627 cells using high-throughput sequencing, screening out differentially expressed lncRNAs and mRNAs. GO enrichment analysis and KEGG signaling pathway analysis were performed on differentially expressed lncRNA target genes and mRNAs. The findings indicated that differential lncRNA target genes were enriched in functions related to cell components, proliferation, metabolism, and migration, which were in agreement with functions of differential mRNA enrichment. The lncRNAs and mRNAs secreted by tumor cells were transported to target cells by exosomes. On the one hand, a microenvironment suitable for tumor cell metastasis (*i.e.*, premetastatic niche) could be created at a distance; on the other hand, it could also directly act on other tumor cells, altering their characteristics, and even change the metabolic programming of the tumor, thereby ultimately accelerating the invasion, metastasis,


A
HUVEC
OD values
Control
HT29
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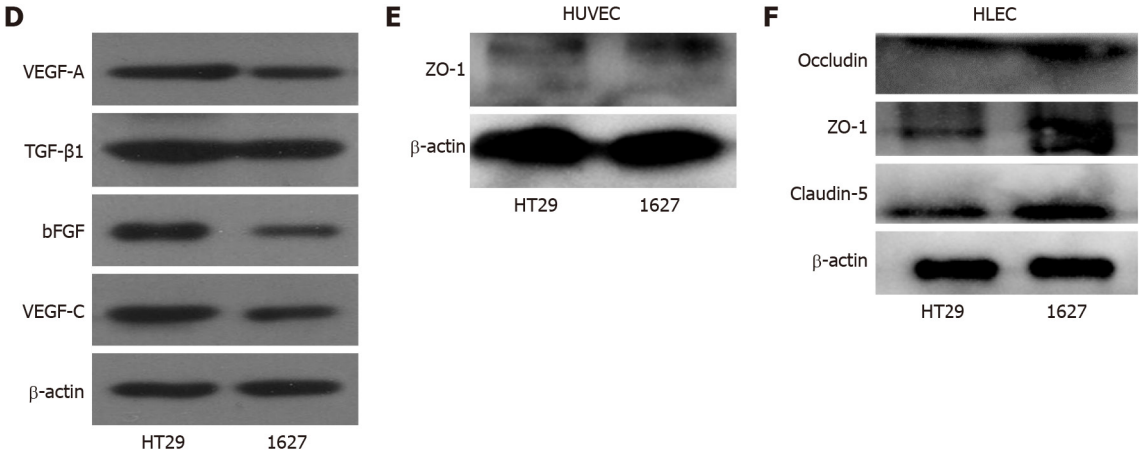

Figure 3 Effect of BRAF^{V600E} mutant colorectal cancer-cell-derived exosomes on the proliferation, migration, tube formation, and protein expression of human umbilical vein endothelial cells and human lymphatic endothelial cells. A: Detection of changes in proliferation of human umbilical vein endothelial cells (HUVECs) induced by exosomes derived from HT29 and 1627 cells at 24, 48, and 72 h by CCK8 assays. Changes in the proliferation of human lymphatic endothelial cells (HLECs) induced by exosomes derived from HT29 and 1627 cells were detected at 12, 24, and 36 h (the control group was treated with PBS); B: Detection of effects of HT29 and 1627 cell-derived exosomes on the migration of HUVECs and HLECs by scratch test (× 50); C: After 24 h of incubation, the effect of HT29 and 1627 cell-derived exosomes on tube formation ability of HUVECs and HLECs was observed (× 100); D: ELISA was used to detect expression of VEGF-A, TGF-β1, bFGF, and VEGF-C proteins in exosomes derived from HT29 and 1627 cells; E: ELISA was used to detect the effect of exosomes derived from HT29 and 1627 cells on expression of ZO-1 proteins in HUVECs; F: ELISA was used to detect the effect of exosomes derived from HT29 and 1627 cells on expression of ZO-1, occludin, and claudin-5 proteins in HLECs. $^{a}P < 0.05$. HUVECs: Human umbilical vein endothelial cells; HLECs: Human lymphatic endothelial cells.

and drug resistance of tumors. The expression of the exosome-derived lncRNA RPPH1 in CRC was markedly upregulated and it simultaneously interacted with TAMs to promote the polarization of TAMs to M2 subtype, thereby accelerating tumor progression[21]. Gao *et al*[32] have found that the lncRNA 91H in tumor-cell-derived exosomes can increase the expression of heterogeneous nuclear ribonucleoprotein K, and markedly enhance the migration and invasion of CRC cells. The above evidence suggests that BRAF^{V600E} mutant CRC cells can reach the TME by releasing exosomal lncRNAs, and participate in tumor angiogenesis and tumor cell proliferation, differentiation, and metabolism through mRNAs, which are closely associated with the TME.

To explore the effect of exosomes secreted by BRAF^{V600E} mutant cells on the surrounding immune microenvironment, we cocultured exosomes derived from two colon cancer cell lines (HT29 and 1627) with HUVECs or HLECs. The exosomes derived from BRAF^{V600E} mutant CRC cells promoted proliferation, migration, and tube formation of endothelial cells, and induced angiogenesis and lymphoangiogenesis. Silencing BRAF gene generated corresponding inhibitory effects. BRAF^{V600E} mutation can promote the expression of matrix metalloproteinase-2 and VEGF-A in malignant melanoma cells, mediate angiogenesis, and enhance the invasiveness of tumor cells. However, BRAF gene deletion leads to a lack of VEGF-A, inhibiting angiogenesis and minimizing the permeability between endothelial cells[33]. Additional research has also proposed that BRAF^{V600E} mutant thyroid cancer cells can promote angiogenesis and lymphoangiogenesis by releasing VEGF-A and VEGF-C into the TME. Zelboraf can reduce the contents of these factors in the TME and inhibit angiogenesis and lymphoangiogenesis, thereby minimizing distant metastasis.

The expression of VEGF-A, bFGF, TGF-β, and VEGF-C proteins in exosomes derived from BRAF^{V600E} mutant CRC cells was also increased. Tight junction proteins ZO-1, claudin-5, and occludin participate in the formation of the endothelial barrier, affect cell permeability, and play an vital role in regulating the proliferation, migration, and tube formation of endothelial cells[34-36]. This study found that exosomes derived from BRAF^{V600E} mutant CRC cells could inhibit the expression of ZO-1, claudin-5, and occludin in HLECs and ZO-1 in HUVECs. Due to the low concentrations of claudin-5 and occludin in HUVECs, this study failed to detect the effect of silencing BRAF gene on the expression of claudin-5 and occludin proteins in HUVECs. Despite that inhibition of ZO-1, claudin-5, and occludin can restrain the growth of endothelial cells, it may not be enough to resist BRAF^{V600E} mutation for the promotion of vascularization (namely, increased expression of VEGF-A, bFGF, TGF-β, and VEGF-C proteins). Our study suggests that exosomes derived from BRAF^{V600E} mutant CRC cells promote the expression of VEGF-A, bFGF, TGF-β, and VEGF-C to facilitate

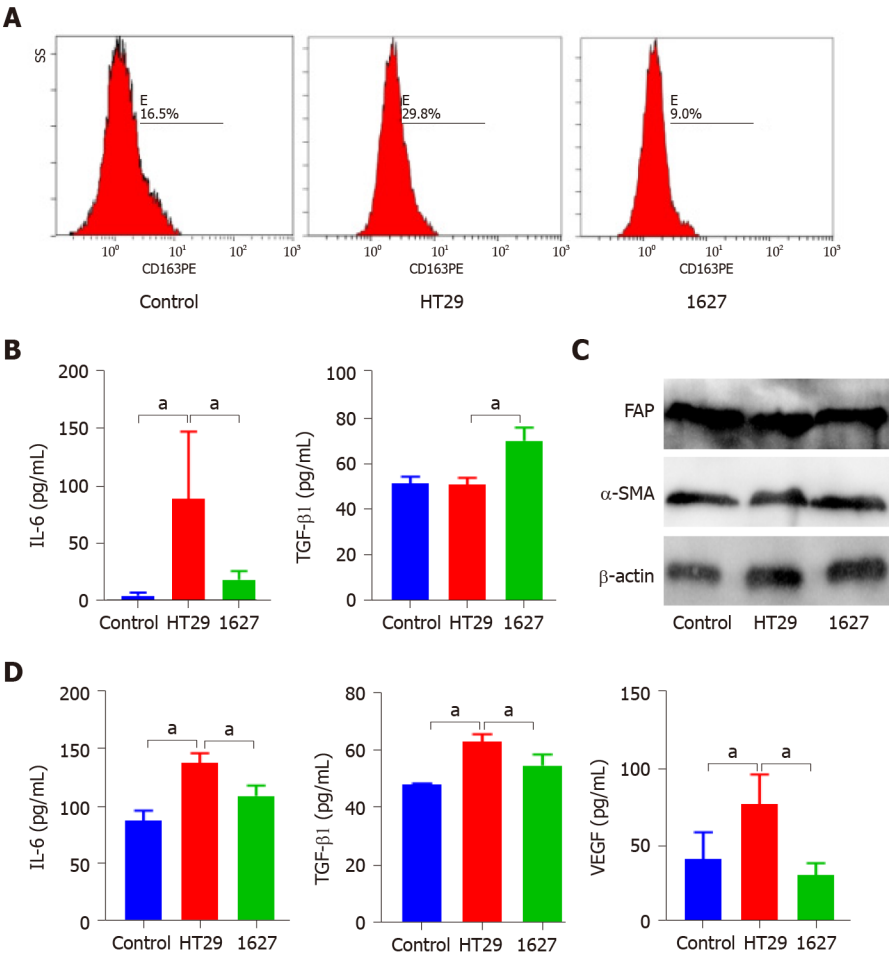

Figure 4 Effect of exosomes derived from BRAF^{V600E} mutant colorectal cancer cells on the phenotype and secretion function of tumor-associated macrophages and cancer-associated fibroblasts. A: Effect of silencing BRAF gene on expression of CD163 in macrophages induced by exosomes derived from BRAF^{V600E} mutant colorectal cancer cells; B: ELISA was used to detect expression of interleukin (IL)-6 and transforming growth factor (TGF)-β1 in the supernatant of THP-1 cells; C: Western blotting was used to detect expression of FAP and α-SMA in MRC-5 cells; D: ELISA was used to detect expression of IL-6, TGF-β1, and vascular endothelial growth factor in the supernatant of MRC-5 cells. $^{a}P < 0.05$. VEGF: Vascular endothelial growth factor; IL: Interleukin; TGF: Transforming growth factor.

angiogenesis and lymphangiogenesis.

We cocultured exosomes derived from HT29 and 1627 cells with macrophages and found that exosomes derived from BRAF^{V600E} mutant CRC cells enhanced the polarization of macrophages to M2 subtype. M1 subtype macrophages secrete IL-6, and M2 subtype macrophages secrete TGF-β1[37]. IL-6 is highly expressed in diverse malignant TMEs, and it can promote tumor invasion, distant metastasis, and angiogenesis, and participate in tumor resistance[38]. Exosomes derived from BRAF^{V600E} mutant colon cancer cells can promote the secretion of IL-6 by macrophages and after silencing the BRAF gene, exosomes can inhibit the secretion of IL-6 by macrophages. Some researchers have discovered that exosomes secreted by hepatoma promote the secretion of IL-6 by macrophages, whereas exosomes secreted by melatonin-treated hepatocellular carcinoma can inhibit the secretion of IL-6 by macrophages[39]. TGF-β1 inhibits tumor proliferation and induces apoptosis in the early stage of tumor development. Conversely, when in the advanced stage, it promotes the development and metastasis of the tumor by promoting epithelial–mesenchymal transition, regulating the microenvironment and the immune system[40,41]. Some studies have found that BRAF^{V600E} mutant CRC-derived exosomes can inhibit macrophages from secreting more TGF-β1. Therefore, we speculated that exosomes derived from BRAF^{V600E} mutant CRC cells could promote the polarization of macrophages to M2 subtype, increase the secretion of IL-6, and reduce the secretion of TGF-β1, thereby facilitating distant metastasis.

The cell component with the highest content in the microenvironment is CAFs, which highly express α-SMA and FAP, and secrete IL-6, TGF-β1, and VEGF. Meanwhile, they assist tumor cells in immune escape, and promote angiogenesis,

tumor invasion, and metastasis[42,43]. The present study indicated that after exosomes derived from BRAF^{V600E} mutant CRC cells were cocultured with fibroblasts, the expression of α-SMA and FAP in fibroblasts did not change markedly. Instead, they promoted the secretion of IL-6 and TGF- β1 and VEGF by CAFs. It was suggested that BRAF^{V600E} mutation had little effect on the number of CAFs, mainly affecting their function. Some researchers have found that exosomes derived from hepatocellular carcinoma can promote the differentiation of hepatic astrocytes into CAFs. The activated CAFs secrete cytokines VEGF, TGF-β, and IL-6, and promote angiogenesis and liver metastasis[44,45].

We selected only one BRAF^{V600E} mutant CRC cell line, and only performed *in vitro* experiments. The differentially expressed lncRNAs were not verified. Nevertheless, our research demonstrated that BRAF^{V600E} mutant CRC had a unique immune microenvironment, which might be induced by the release of exosomes rich in certain lncRNAs. Therefore, in the future, we can consider to reshape the immune microenvironment, combined with traditional treatment, to treat this specific type of CRC.

CONCLUSION

Our study showed that, compared with wild type BRAF, BRAF^{V600E} mutation led to more angiogenesis and lymphoangiogenesis in the microenvironment. Meanwhile, the polarization of macrophages to M2 subtype was more obvious, and the immunosuppression was more prominent. Further analysis indicated that exosomes derived from BRAF^{V600E} mutant CRC cells were rich in certain lncRNAs and mRNAs, which might be linked to these alterations. This provides a hypothesis for finding new therapeutic strategies for BRAF^{V600E} mutant CRC.

ARTICLE HIGHLIGHTS

Research background

BRAF^{V600E} gene mutation accounts for approximately 10% of patients with metastatic colorectal cancer (CRC). Compared with CRC patients with wild-type BRAF, patients with BRAF^{V600E} mutant CRC are prone to peritoneal metastasis and distant lymph node metastasis with a poor prognosis. Previous findings suggest that BRAF^{V600E} mutation affects the tumor microenvironment (TME).

Research motivation

BRAF^{V600E} mutation is involved in the formation of the immunosuppressive microenvironment in thyroid cancer. However, the influence and the related mechanism of BRAF^{V600E} mutation in CRC on the surrounding immune microenvironment are not clear.

Research objectives

The study aimed to determine the influence of BRAF^{V600E} mutation in CRC on the surrounding immune microenvironment, elucidating whether BRAF^{V600E} mutant CRC cell-derived exosomes participate in the formation of an immunosuppressive microenvironment.

Research methods

CRC patients were divided into either a control group or a treatment group. The formation of microvessels and microlymphatic vessels and M2 subtype macrophages in tumor tissues were detected by immunohistochemistry. Screening and functional analysis of exosomal long noncoding RNAs (lncRNAs) were performed by transcriptomics. The proliferation and migration of human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were detected by CCK-8 assays and scratch test, respectively. The tube-forming ability of endothelial cells was assessed by tube formation assay. The macrophage subtypes were obtained by flow cytometry. The expression of vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β1, VEGF-C, claudin-5, occludin, zonula occludens (ZO)-1, fibroblast activation protein (FAP), and α-smooth muscle actin was assessed by Western blot analysis. The levels of cytokines interleukin (IL)-6, TGF-β1, and VEGF were assessed by ELISA.

Research results

BRAF^{V600E} mutation was positively correlated with a poor prognosis in CRC ($P < 0.01$). Microvascular density and microlymphatic vessel density in BRAF^{V600E} mutant CRC tissues were higher than those in BRAF wild-type CRC ($P < 0.05$). The number of CD163^{+} M2 macrophages in BRAF^{V600E} mutant CRC tumor tissue was markedly increased ($P < 0.05$). Compared with exosomes from CRC cells with BRAF gene silencing, the expression of 13 lncRNAs and 192 mRNAs in the BRAF^{V600E} mutant CRC cell exosomes was upregulated, and the expression of 22 lncRNAs and 236 mRNAs was downregulated ($P < 0.05$). The biological functions and signaling pathways predicted by differential lncRNA target genes and differential mRNA were closely related to angiogenesis, tumor cell proliferation, differentiation, metabolism, and changes in the microenvironment. The proliferation, migration, and tube formation ability of HUVECs and HLECs induced by exosomes in the 1627 cell group (HT29 cells with BRAF gene silencing) was greatly reduced compared with the HT29 cell group ($P < 0.05$). Compared with the HT29 cell group, the expression levels of VEGF-A, bFGF, TGF-β1, and VEGF-C in the exosomes derived from 1627 cells were reduced. The expression of ZO-1 in HUVECs, and claudin-5, occludin, and ZO-1 in HLECs of the 1627 cell group was higher. Compared with the 1627 cell group, the exosomes of the HT29 group promoted the expression of CD163 in macrophages ($P < 0.05$). IL-6 secretion by macrophages in the HT29 cell group was markedly elevated ($P < 0.05$), whereas TGF-β1 was decreased ($P < 0.05$). The levels of IL-6, TGF-β1, and VEGF secreted by fibroblasts in the 1627 cell group decreased, compared with the HT29 group ($P < 0.05$).

Research conclusions

BRAF^{V600E} mutant CRC cells can reach the TME by releasing exosomal lncRNAs, inducing the formation of an immunosuppressive microenvironment.

Research perspectives

The study will provide a novel therapeutic strategy for BRAF^{V600E} mutant CRC.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatocellular carcinoma surveillance and quantile regression for determinants of underutilisation in at-risk Australian patients

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Conflict-of-interest statement: The

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Abstract

BACKGROUND

While clinical guidelines recommend hepatocellular carcinoma (HCC) surveillance for at-risk individuals, reported surveillance rates in the United States and Europe remain disappointingly low.

AIM

To quantify HCC surveillance in an Australian cohort, and assess for factors associated with surveillance underutilisation.

METHODS

All patients undergoing HCC surveillance liver ultrasounds between January 1, 2018 to June 30, 2018 at a tertiary hospital in Melbourne, Australia, were followed until July 31, 2020, or when surveillance was no longer required. The primary outcome was the percentage of time up-to-date with HCC surveillance (PTUDS). Quantile regression was performed to determine the impact of factors associated with HCC surveillance underutilisation.

RESULTS

Among 775 at-risk patients followed up for a median of 27.5 months, the median PTUDS was 84.2% (IQR: 66.3%-96.3%). 85.0% of patients were followed up by specialist gastroenterologists. Amongst those receiving specialist care, quantile

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regression demonstrated differential associations at various quantile levels of PTUDS for several factors. Older age at the 25th quantile (estimate 0.002 per percent, P = 0.03), and cirrhotic status at the 75th quantile (estimate 0.021, P = 0.017), were significantly associated with greater percentage of time up-to-date. African ethnicity (estimate -0.089, P = 0.048) and a culturally and linguistically diverse (CALD) background (estimate -0.063, P = 0.01) were significantly associated with lower PTUDS at the 50th quantile, and again for CALD at the 75th quantile (estimate -0.026, P = 0.045).

CONCLUSION
While median PTUDS in this Australian cohort study was 84.2%, awareness of the impact of specific factors across PTUDS quantiles can aid targeted interventions towards improved HCC surveillance.

Key Words: Liver cirrhosis; Hepatitis, viral, human; Carcinoma, hepatocellular; Liver neoplasms; Early detection of cancer; Population surveillance

Core Tip: This study evaluated the uptake of liver cancer screening in a cohort of high-risk Australians, and found that on average, patients were up-to-date with their surveillance for 84.2% of the study time period. Certain factors, such as absence of cirrhosis, younger age, African ethnicity and a non-English speaking background were associated to varying degrees with lower time up-to-date with hepatocellular carcinoma screening.

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INTRODUCTION

Globally, hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and is the fourth leading cause of cancer-related mortality[1-3]. Over the last three decades in Australia, there has been a 306% increase in age-standardised incidence of liver cancer, and a corresponding 184% increase in attributable mortality rates[4]. These concerning escalations are mirrored in recent reports from the United States, and the age-adjusted worldwide incidence of HCC stands at 10.1 cases /100,000 person-years[5,6].

While curative treatment options offer 5-year survival rates in excess of 70%, these are only feasible at early disease stages, often when HCC is clinically silent[1,7].A meta-analysis of cohort studies demonstrated improved early-stage detection and associated survival with HCC screening in at-risk populations[8]. This is particularly accepted in the non-cirrhotic population, for whom surgical resection is often a viable and life-saving intervention for early HCC[9]. As such, international professional societies recommend that at-risk patients undergo HCC surveillance with liver ultrasound (USS) every 6 months, with or without concurrent alpha fetoprotein (AFP) measurements[10,11].

The effectiveness of HCC surveillance relies on consistent clinical uptake of screening, particularly as sensitivity rates of ultrasonography for the detection of HCC are reported to be as low as 60%, thus requiring serial scans to ensure maximum yield [6]. However, several cohort studies have supported longstanding concerns of HCC surveillance underutilisation in clinical practice, with two recent meta-analyses reporting overall pooled adherence rates of 24% and 52%[3,12]. Research into adherence to surveillance is limited by the marked heterogeneity in the definitions of adherence in the literature[3]; studies vary from requiring perfect 6-monthly

adherence, to allowing leeway periods of up to 12 mo between imaging, raising the possibility of lost interim early-stage HCC detection[3].

To better incorporate both frequency of screening and quantity of imaging performed, the concept of 'percentage of time up-to-date with surveillance' (PTUDS) has been proposed as a more robust, continuous metric to standardise the measurement of adherence to HCC surveillance[3,7]. This retrospective cohort study aimed to quantify the percentage of time up-to-date with HCC screening in an Australian cohort of 'at-risk' patients, and to identify determinants associated with higher adherence to surveillance.

MATERIALS AND METHODS

Study design

This retrospective cohort study at a large tertiary hospital in Melbourne, Australia captured all at-risk patients identified by radiology records as undergoing a liver ultrasound scan (USS) for HCC surveillance within the Austin Hospital radiology department between 1 January 2018 to 30 June 2018. All USS were requested by gastroenterologists, infectious disease physicians and nurse practitioners who manage patients deemed at-risk of HCC in outpatient specialty clinics. Most at-risk patients at our centre were managed in physician-led specialty clinics. A proportion of consenting patients, who were felt to be at low risk of progressive liver disease, were primarily managed in a nurse-led, HCC screening clinic. Eligible patients for the nurse-led clinic were patients not requiring treatment for chronic hepatitis B, and patients with well-compensated cirrhosis from a disease aetiology that was considered adequately treated. All patients referred to the nurse-led clinic were previously seen by physicians and physician oversight was available if needed.

At-risk patients for HCC development were defined according to AASLD guidelines, and included those with cirrhosis irrespective of aetiology, and patients with chronic hepatitis B and additional HCC risk factors (i.e. family history of HCC, African descent, Asian males over 40 years old, Asian females over 50 years old)[10]. Our institution also performs surveillance for patients with chronic hepatitis C and advanced fibrosis (F3 and above), as HCC incidence in this demographic may surpass the threshold for cost-effective screening[6]. Patients who did not meet this criteria or had a documented history of HCC within the last 2 years were excluded.

Patients were followed-up from the index USS until July 31, 2020, or when surveillance was no longer required. Reasons for this included HCC diagnosis (after which patients were diverted into our liver cancer clinic), death, receipt of an orthotopic liver transplant, or when surveillance was no longer deemed appropriate, such as cases of elderly multi-comorbid patients with limited life expectancy. Follow-up periods were also truncated for patients who were discharged to other medical services. In these cases, follow-up end-date was documented as date of the formal discharge letter written by attending clinicians to the patient and/or their primary care physician. This acknowledged the possibility of subsequent external imaging and follow-up.

This study was approved by the Austin Health Research Ethics Committee and carried out in line with the National Statement on Ethical Conduct in Human Research (2007).

Data collection

Patient demographics, clinical history, laboratory investigations and imaging results were extracted from their electronic medical records at the time of inclusion. Diagnoses of chronic hepatitis B were verified by record review of positive hepatitis B surface antigen or HBV DNA tests at least 6 mo apart. Confirmation of chronic hepatitis C occurred if there was evidence of positive hepatitis C antibody or viral RNA. All cirrhosis diagnoses were verified by review of clinical records by a consultant gastroenterologist, where cirrhosis was confirmed using a combination of clinical, biochemical, radiological and histological findings. Scanned medical records were also reviewed to retrieve externally performed liver imaging.

Outcomes of interest

The primary outcome was adherence to HCC surveillance imaging. Accepted imaging modalities included targeted liver USS, or contrast-enhanced CT or MRI, given their routine use in clinical practice when USS images are inadequate. Adherence was defined as the percentage of time up-to-date with screening (PTUDS, %), calculated as

the percentage of an individual's total follow-up time in which they were within 6 mo of an accepted HCC surveillance test. This endpoint was chosen as it accounted for both number and timing of surveillance imaging performed during the screening period. Following surveillance imaging, patients were credited as having 6 mo of 'time up-to-date'. Any subsequent imaging that occurred within that 6-month period resulted in a re-set of the 6-month interval clock from the date that the new imaging was performed (Figure 1). AFP levels were recorded, but were not included in the primary outcome, given AFP testing is not considered a stand-alone surveillance test.

Patient demographics

Demographic variables, based on literature-reported factors, were also extracted from the database and assessed for potential associations with uptake of HCC surveillance. These variables of interest included age, gender, ethnicity, primary language spoken, aetiology of liver disease, cirrhosis status and MELD score.

Statistical analysis

Descriptive statistical analysis was reported as proportions (%) for categorical variables, and means (with standard deviations) or medians (with IQRs) for continuous variables, depending on the distribution of values. Comparative analysis was conducted using the Student t-test and Wilcoxon rank-sum tests. Univariable and multivariable quantile regression analysis was then performed to assess for factors associated with PTUDS, particularly variables with significant associations at different PTUDS quantiles. Quantile regression modelling was chosen given the non-parametric distribution of the outcome variable relative to covariates, its flexibility in assessing the relationship between determinants of surveillance and PTUDS at the upper and lower tails, and the lack of stringent model assumptions required for valid inference. The median (50^{th}), 25^{th}, and 75^{th} quantiles were selected to provide a description of covariate associations across the range of the distribution of PTUDS, whilst avoiding the effect of extreme outliers. Bootstrapping was used to calculate 95% confidence intervals around the estimates. Both univariate and multivariate models were constructed separately for each of the selected PTUDS quantiles to determine if there were differential covariates associations across the distribution.

Data analysis was performed using Statistical Analysis Software (SAS) v.9.4 and R Software v.4.0.3 using the quantreg package[13,14].

RESULTS

Baseline characteristics

We identified 838 patients who underwent liver USS with radiology record requests for HCC surveillance within the 6-month recruitment period. On further review, 22 patients were excluded due to prior liver transplantation, 1 due to recent history (< 2 years) of HCC, 9 who did not meet criteria for screening (incorrectly classified as cirrhotic) and 31 with chronic hepatitis B who did not meet guideline criteria for surveillance. 775 patients were included in the final analysis. Baseline characteristics are detailed in Table 1.

The mean age of patients was 60.0 (standard deviation 12.2) years. The majority were male (59.0%), Caucasian (57.0%) and English-speaking (73.9%), with cirrhosis the most common indication for HCC surveillance (55.3%). Of those with cirrhosis, the median MELD score at inclusion was 9. The majority of patients were followed up in hepatology clinics (58.5% general liver, 16.3% pre-transplant, 10.3% nurse-led). The median follow-up time was 27.5 mo (IQR: 26.0-29.0 months). The most commonly used screening modality was ultrasound (86.9%), followed by contrast-enhanced CT liver (9.4%) and contrast-enhanced MRI (3.7%). AFP was performed in conjunction to imaging at least once in 91.9% of patients.

Twenty patients had an interim orthotopic liver transplant, 22 died and 41 were discharged from clinic surveillance, either due to service nonattendance (36%), transfer of care to other institutions (50%), or when surveillance was deemed no longer clinically appropriate (14%). HCCs developed in 22 patients (2.8%) over the course of the study. Of these 22 HCCs, 14 (64%) were detected at an early stage.

Primary outcome

The median PTUDS overall was 84.2% (IQR: 66.3%-96.3%). 13.2% of patients were up-to-date for less than 50% of their surveillance time period, representing average

Table 1 Baseline Characteristics of 775 at-risk patients participating in hepatocellular carcinoma surveillance, 2018-2020

Characteristic		*n* = 775 (%)	mean ± SD	Median (IQR)
Age (years)			60.0 ± 12.2	
Sex	Male	457 (59.0)		
	Female	318 (41.0)		
Ethnicity	Caucasian	442 (57.0)		
	Asian	249 (32.1)		
	African/Middle-Eastern	68 (8.8)		
	Unreported	16 (2.1)		
Primary language spoken	English	573 (73.9)		
	Non-English	202 (26.1)		
Indication for hepatocellular carcinoma surveillance	Cirrhosis	429 (55.3)		
	Chronic HBV	343 (44.3)		
	Chronic HCV with Advanced Fibrosis	3 (0.4)		
Aetiology of Cirrhosis, *n* = 429	HBV hepatitis	58 (13.5)		
	HCV hepatitis	136 (31.7)		
	Alcoholic hepatitis	103 (24.0)		
	NASH	64 (14.9)		
	Non-viral, non-alcoholic, non-NASH cirrhosis[1]	68 (15.9)		
HBV anti-viral use, *n* = 385	Yes	206 (53.5)		
	No	179 (46.5)		
Specialty care clinic	General hepatology[2]	453 (58.5)		
	Pre-transplant	126 (16.3)		
	Nurse-led surveillance	80 (10.3)		
	Non-liver[3]	76 (9.8)		
	Unspecified	40 (5.2)		
MELD score, *n* = 429				9 (7-13)

[1]Includes autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, cryptogenic cirrhosis.
[2]Includes general liver and outreach clinics.
[3]Includes clinics such as infectious diseases, renal, general medicine where patients received specialist care.
HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; SD: Standard deviation.

screening intervals of less frequently than 12-monthly (see Figure 2). Only 40% of patients spent at least 90% of their follow-up period up-to-date with surveillance.

Univariable analyses

Older age (≥ 60 years old), cirrhotic status, Non-Asian ethnicity and English-speaking as the primary language correlated with higher continuous adherence to surveillance (see Table 2). Sex and the receipt of anti-viral therapy in chronic hepatitis B patients did not correlate with differences in surveillance adherence, nor did MELD score in cirrhotics.

Continuous adherence to HCC screening varied by aetiology of liver disease. Patients with non-alcoholic steatohepatitis (NASH) had higher median PTUDS (90.9%) compared to those with viral hepatitis (82.3%, P = 0.003) and alcohol-induced liver disease (83.8%, P = 0.07). Those with non-viral, non-alcoholic and non-NASH disease also had statistically higher continuous surveillance adherence (median PTUDS 90.8%) than their counterparts whose aetiologies were viral hepatitis (P = 0.0008), or alcohol (P = 0.07). There were no significant differences in median PTUDS between the patients whose HCCs were detected earlier (n = 14), than in those with late HCC detection (n = 8) (100% *vs* 95.9%, P = 0.73).

Table 2 Comparison of surveillance determinants associated with greater continuous hepatocellular carcinoma surveillance among 775 at-risk patients for hepatocellular carcinoma

Factor	Hepatocellular carcinoma surveillance adherence (PTUDS)		
	< 60 yr	60 yr	*P* value
Age	80.6%	86.8%	< 0.001
	Non-cirrhotic	Cirrhotic	
Cirrhosis status	80.1%	87.3%	< 0.001
	Non-Asian	Asian	
Ethnicity	85.1%	81.2%	0.04
	English	CALD	
Primary language spoken	85.7%	80.2%	0.03
	Male	Female	
Sex	83.3%	85.6%	0.58
	Treatment naïve	Anti-viral therapy	
Hepatitis B treatment status	79.4%	82.1%	0.07

PTUDS: Percentage of time up-to-date with hepatocellular carcinoma surveillance; CALD: Culturally and linguistically diverse background.

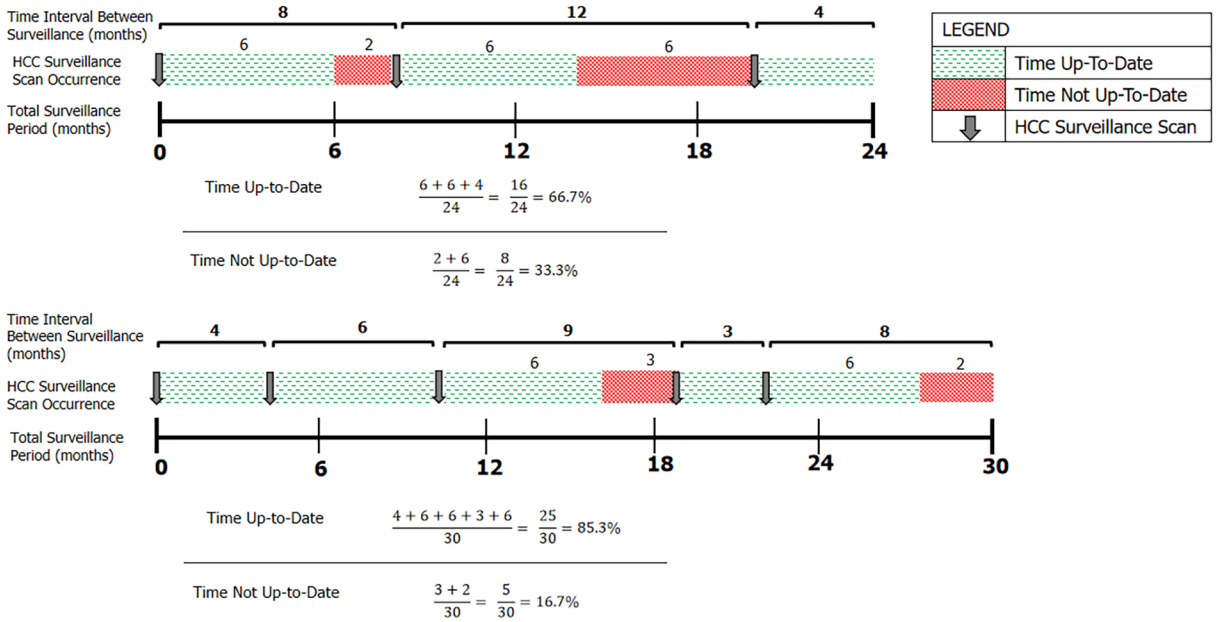

Figure 1 Scenario calculations of percentage of time up-to-date with surveillance.

Analysis demonstrated significant differences in surveillance adherence based on clinic setting, with highest PTUDS for patients attending liver transplant clinics (median PTUDS 97.4%). This was significantly greater than general hepatology clinics (median PTUDS 82.4%, $P < 0.001$). Of note, patients attending nurse-led screening clinics achieved continuous surveillance adherence rates that surpassed general hepatology clinics (median PTUDS 90.0%, $P = 0.0001$). This was not reflected in the performance of non-hepatology clinics, which reported the lowest continuous surveillance adherence rates (median PTUDS 62.3%).

Multivariable analysis

Multiple quantile regression analysis demonstrated that age and clinic type (subspecialty hepatology care *vs* other) had differential effects across different PTUDS quantiles. Older age (≥ 60 years) increased PTUDS across all quantiles, but this

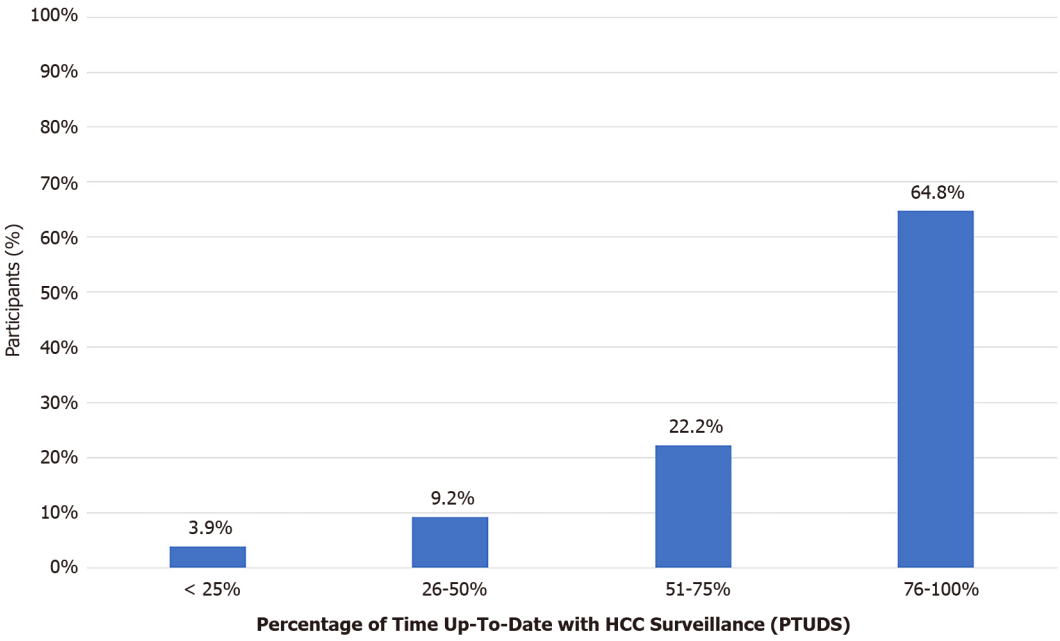

Figure 2 Distribution of participants within percentage of time up-to-date with hepatocellular carcinoma surveillance categories.

increase was only significant at lower quantiles (25th quantile parameter estimate (PE) = 0.003, P = 0.01; 50th quantile PE = 0.002, P < 0.001). Non-liver-specific clinic care, compared with subspecialty hepatology care, was associated with lower PTUDS across all quantiles (P < 0.001).

The results from subgroup quantile regression analysis of patients attending liver-specific specialty clinics (85.0% of all patients) are demonstrated in Table 3. Primary language spoken, ethnicity, and cirrhosis status were shown to variably affect PTUDS across different quantiles. The association between older age and increased PTUDS was maintained only in the 25th quantile (P = 0.03). Patients with cirrhosis had increased PTUDS compared to their non-cirrhotic counterparts at the 75th quantile (P = 0.02). However, African ethnicity and culturally and linguistically diverse (CALD) backgrounds were both significantly associated with lower PTUDS at the 50th quantile (P = 0.048 and P = 0.01). CALD background was also a strong predictor of lower PTUDS at the 75th quantile (P = 0.045).

DISCUSSION

In this Australian retrospective cohort study of HCC surveillance utilisation in 775 at-risk patients, we found that average time up-to-date with 6 monthly screening was 84.2% over a median follow-up of over 2 years. However, less than half (40.8%) of patients spent at least 90% of their surveillance period up-to-date with screening, which represents a concerning proportion of patients at risk of delayed diagnosis of HCC. Younger age, non-cirrhotic status, African ethnicity and CALD background were variably associated across PTUDS quantiles with significantly decreased time up-to-date with HCC surveillance on quantile regression analysis.

Our study is one of the few that presents adherence to HCC surveillance using a continuous outcome measure, contrasting with versions of the binary categorisation of 'adherent' and 'non-adherent' of other studies[7,15-19]. Depending on definitions used, these all-or-nothing classifications potentially enforce too stringent or too lax restrictions on surveillance intervals, and distort true interpretation of HCC surveillance application in real-time clinical practice. This study has described more accurate quantification of adherence and thus better identifies patients at risk.

Given heterogeneity in the published definitions of adherence, comparisons of surveillance uptake are limited. However, Goldberg *et al*[7] reported an analysis of patients with cirrhosis, applying the PTUDS measure to characterise adherence using United States Veterans Health Administration data. This national study found a median PTUDS for surveillance by USS only as 10% (IQR: 0%-29%)[7]. The vast discrepancy in median PTUDS compared with our finding of 84.2% is likely

Table 3 Quantile regression parameter estimates and 95% confidence intervals of factors associated with hepatocellular carcinoma surveillance adherence for the 25th, 50th and 75th quantiles of percentage of time up-to-date with surveillance

	QR Estimates (95%CI)					
	25th	*P* value	50th	*P* value	75th	*P* value
Variables						
Age	0.002 (0.000, 0.004)	0.03				
African ethnicity			-0.089 (-0.177, -0.001)	0.048		
CALD			-0.063 (-0.110, -0.016)	0.01	-0.026 (-0.052, -0.001)	0.045
Cirrhotic status					0.021 (0.004, 0.039)	0.02

CALD: Culturally and linguistically diverse background.

multifactorial. First, Goldberg's study encompassed patients followed-up in both community or specialist practices, and found that number of specialist visits was the strongest predictor for greater surveillance participation[7]. Adherence rates for those in non-specialist care have historically been low, with international and limited local data showing optimal surveillance uptake of only 8.8%-27%[3,20,21]. Specialist input may serve as a surrogate marker for greater frequency of reminders for test follow-up, may select for a group of patients more engaged with healthcare, or indicate more unwell patients undergoing imaging for reasons other than surveillance[22]. In particular, hepatology care as compared to other subspecialty providers may better identify the at-risk cohort eligible for HCC screening, therefore having 85% of patients in this study engaged in hepatology clinics may explain our comparatively high PTUDS. Furthermore, our study recruited patients based on attendance at an initial HCC screening scan, which likely also represents a more informed and engaged subset of all patients requiring HCC surveillance. Our findings are, however, similar to pooled adherence surveillance estimates of 73.7% for patients enrolled in specialist care from a recent meta-analysis, associating specialist care with higher likelihood of HCC surveillance[3].

In addition to specialist gastroenterologist/hepatologist care, this study provided insight into other factors correlated with utilisation of HCC surveillance. The high continuous surveillance adherence found in nurse-led clinic-directed care lends support to the effectiveness of this model of care shown in two cohort studies[23,24]. In keeping with several prior studies, we found lower rates of surveillance in younger patients[7,19,25,26]. Studies assessing adherence by cirrhosis status echoed our finding of screening under-utilisation in patients without cirrhosis. This is potentially attributable to a higher perceived HCC risk with cirrhosis stage by patients and providers, suggesting under-recognition of screening eligibility in this group[12,22,27]. Targeted education of providers, particularly primary care physicians who follow most high-risk patients nationally, on screening guidelines that include non-cirrhotic chronic hepatitis B patients, may improve surveillance rates. Receipt of anti-viral therapy, revealed in other studies to increase the likelihood of surveillance adherence, was not significant on multivariable analysis in our cohort of chronic hepatitis B patients[16,22].

Several studies have reported lower surveillance rates in non-Caucasians, with African American/Black patients significantly less likely to receive surveillance[7,28]. African ethnicity in our cohort was similarly associated with lower PTUDS at the 50th quantile, a concerning finding given higher rates of HCC and younger age at risk within this population[21,29]. This may correlate with our lower screening uptake in patients whose primary language was not English, although to our knowledge, no studies have yet reviewed the impact on HCC screening uptake in patients whose care is provided in a primary language that is not their native tongue. However, other cancer surveillance studies have also identified language as a key barrier in CALD patients' understanding of screening rationale and participation[30,31]. An Australian qualitative study into colorectal screening uptake within culturally and linguistically diverse groups suggested that language barriers hindered otherwise willing participation[30]. A fatalistic view on cancer diagnoses, or a fear of bad luck due to cultural beliefs, were also identified as potentially modifiable barriers to screening adherence, and suggest that culturally-tailored and language-appropriate resources may need to be employed to target diverse populations[30].

In our study, a total of 22 HCCs were detected, of which 14 were early stage and amenable to curative treatment. The annual incidence rate of HCC in our cohort was 1.3 per 100 person-years. This low incidence of HCC may be due to a high proportion in our cohort of people with treated HCV, and HBV without cirrhosis, representing patients with a relatively lower incidence rate of HCC[32]. We did not find a significant difference in overall adherence in patients with HCCs detected at an earlier compared to a later stage. While our study was not powered to answer this question, it provides interesting insight into the effect of surveillance in a cohort with adherence rates closer to the ideal, and highlights the need for quality assessment of surveillance imaging.

Strengths of this study included use of the continuous variable 'percentage of time up-to-date with surveillance' to quantify adherence to HCC screening, and quantile regression modelling to describe covariate effects across the spectrum of PTUDS[3,28]. Our data also reflects contemporary clinical practice, with confirmation of the 'at-risk' history of our surveyed population. This is also the first Australian study of a large cohort, and offers a perspective on surveillance uptake in a population not hindered by costs of screening tests. Prior observational studies have raised concerns that patient-level barriers, such as costs of surveillance investigations, may contribute to poorer surveillance receipt[22,33]. While we were unable to assess socioeconomic factors, as this data was unavailable, concerns regarding financial burden of surveillance measures are likely alleviated by their cost-free nature in Australia's healthcare system. Finally, we accounted for patient receipt of alternate, non-ultrasound imaging. This reflects a more realistic practice of clinicians accepting contrast-enhanced CT or MRI liver imaging as appropriate surveillance studies, thus obviating the performance of additional ultrasonography.

Findings from our study should be interpreted within its limitations. As with retrospective study designs, our study is limited by missing data, unmeasured confounders and selection bias. Our analysis involved patients at a single healthcare centre, and may not be generalised to other practice environments, particularly primary care settings. Secondly, patient enrolment based on an initial HCC surveillance USS attendance in a 6 mo period does not incorporate eligible but non-adherent patients, nor those qualifying for screening but who have not been identified by clinicians as at risk of HCC. As such, our adherence rates may be overestimated towards a well-informed, more inherently adherent and correctly identified cohort. However, this large study provides valuable current insight into maintenance of HCC surveillance in patients already identified to be at risk.

CONCLUSION

In conclusion, average time-up-to-date with HCC surveillance in an at-risk Australian cohort was 84.2% following an index screening test performed for the purpose of surveillance. Subspecialty care was associated with higher subsequent adherence to surveillance imaging. Conversely, younger age, non-cirrhotic status, African ethnicity, and CALD background, were variably associated with significantly reduced PTUDS across different PTUDS quantiles. Further research into patient and system barriers towards HCC screening will provide further information regarding provider and patient factors to better guide development of appropriate and targeted interventions to increase adherence to HCC surveillance.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) surveillance rates reported from the United States and Europe remain low, despite published clinical guideline recommendations. Surveillance patterns in Australia, which has the benefit a universal healthcare program, have not been clearly delineated.

Research motivation

Patients, and evaluate factors associated with greater uptake of HCC cancer screening. In incorporating both frequency of screening and quantity of imaging performed, we aimed to have a more continuous way of standardising 'adherence'. Identification of determinants associated with higher HCC screening adherence aims to guide further

areas for intervention.

Research objectives

As stated above, the objectives were characterising continuous HCC surveillance adherence. This method provides a way of standardising 'adherence', and thus allows for equal comparison between different studies evaluating the concept of HCC screening adherence.

Research methods

This was a retrospective cohort study that incorporated data electronic medical records to obtain patient demographics, clinical history, lab investigations and radiological imaging results. Data analysis was both on the univariate and multivariate level. In particular, quantile regression was performed for the non-parametric outcome variable, and provides greater description of covariate associations across the range of the outcome variable.

Research results

Follow-up of 775 at-risk patients demonstrated that median time-up-to-date with HCC surveillance was 84.2%. However, different patient factors, affected HCC surveillance adherence variably across different ranges of the outcome variable percentage of time up-to-date with HCC surveillance (PTUDS). At the 25th quantile/percentile for PTUDS, older age was associated with greater HCC surveillance. At the 50th quantile, African ethnicity had lower HCC surveillance. At the 75th quantile, cirrhotic status was associated with greater adherence to surveillance. Those of culturally and linguistically diverse backgrounds had lower continuous HCC surveillance rates at both the 50th and 75th quantiles. The ramifications of these findings and identified determinants affecting HCC surveillance participation in other settings, including the primary care setting, are less clear. However, they remain very important areas for further research. In particular, addressing the impact of ethnicity and cultural and linguistic backgrounds on screening uptake may well have beneficial consequent effects in other areas of healthcare.

Research conclusions

The study suggests specific patient and systemic factors that contribute to participation in HCC surveillance. These factors include younger age, non-cirrhotic status, African ethnicity and coming from a culturally and linguistically diverse background, which all are variably associated with lower percentage of time up-to-date with HCC surveillance.

Research perspectives

Future research should be directed at determining interventions aimed at the factors identified in this study to be associated with reduced HCC screening adherence. Those that improve participation in HCC surveillance may well benefit from widespread implementation to improve earlier diagnosis of HCCs.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of tumor regression grading systems for locally advanced gastric adenocarcinoma after neoadjuvant chemotherapy

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Author contributions: Liu ZN, Wang YK and Li ZY designed this study; Wang YK and Liu ZN enrolled patients and collected clinical data; Zhang L and Sun Y reviewed the samples; Liu ZN and Ying XJ conducted statistical analysis; Liu ZN is responsible for data visualization; Liu ZN and Wang YK drafted this article; All authors read and approved the final manuscript; Liu ZN, Wang YK and Zhang L contributed equally to this work.

Institutional review board statement: The Ethics Committee of Peking University Cancer Hospital approved this study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later

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Abstract

BACKGROUND

Current tumor regression grade (TRG) evaluations are based on various systems which brings confusion for oncologists and pathologists when interpreting results. The recent six-tier system (JGCA2017-TRG) recommended by the Japanese Gastric Cancer Association (JGCA) is worth investigating, as four-tier TRG systems are favored in various parts of the world.

AIM

To compare the predictive accuracies of five published TRG systems.

METHODS

Data were retrospectively collected from patients with locally advanced gastric cancer (LAGC) who underwent neoadjuvant chemotherapy followed by D2 Lymphadenectomy between January 2005 and January 2014 at our institution. Outcomes were overall survival (OS) and disease-free survival (DFS), which were evaluated separately using the following TRG systems: JGCA2017, JGCA, Becker, AJCC/CAP, and Mandard.

RESULTS

All five published TRG systems were independent predictors for OS and DFS. Concordance indices of the JGCA2017, JGCA, Becker, AJCC/CAP-TRG, and Mandard systems were 0.651/0.648 0.652/0.649, 0.693/0.695, 0.688/0.685, and

versions. Informed consents were obtained from all patients for being included in the study. This study does not involve animal study.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All authors declare no conflict of interest.

Data sharing statement: The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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0.674/0.675 for OS and DFS, respectively. The four-tier Becker system showed the highest c-index, which was significantly greater than that of the six-tier JGCA2017 and five-tier JGCA systems ($P < 0.05$ in OS and DFS). When residual tumor percentages were reset as: "no residual tumor", < 10%, < 100%, and "no response", the rearranged cutoff values achieved a maximum c-index with 0.728 for OS and 0.737 for DFS, which was superior to the other five systems.

CONCLUSION

The newly introduced six-tier JGCA-TRG system cannot increase prognostic stratification. The four-tier Becker system is more suitable for LAGC patients. A population-based study is warranted to define the optimal criterion for TRG in LAGC patients.

Key Words: Gastric cancer; Neoadjuvant chemotherapy; Tumor regression grade; Survival; Concordance index

Core Tip: Current Tumor regression grade (TRG) evaluations are based on various systems bringing confusion to oncologists and pathologists when interpreting results in similar clinical contexts. On the other hand, the recent six-tier system tumor regression grade (JGCA2017-TRG) recommended by Japanese Gastric Cancer Association (JGCA) is investigational. This is the first report of the use of the c-index to evaluate predictive accuracies of five published TRG systems in gastric cancer. With a satisfying sample size, our results gave clinicians a better understanding of the TRG, especially the residual tumor percentage, in gastric cancer and furthermore alleviates the oncologists and pathologist's workload.

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INTRODUCTION

Although surgical resection is the mainstay therapy of locally advanced gastric cancer (LAGC), neoadjuvant chemotherapy (NACT) has now been widely adopted for LAGC in Europe and most recently in China due to the solid evidence that it reduces the risk of recurrence and improves overall survival[1-3]. Theoretical benefits of NACT are downstaging the primary tumor, increasing the R0 resection rate, and treatment of potential micrometastases.

The effects of NACT on the tumor can be histopathologically evaluated in subsequent resection specimens by applying pathological tumor regression grading (TRG) systems. There are currently more than five commonly used TRG systems for GC across the world with different principles, different layers, and different cutoff values [4,5]. These various practices in TRG evaluation place a large burden on oncologists and pathologists and make it hard to interpret results from different systems in similar clinical contexts. Pathologists may also be required to be familiar with more than one TRG system in daily practice[4].

Currently, most pathologists favor four-tier TRG systems in gastrointestinal cancer. There are the Becker system and the American Joint Committee on Cancer (AJCC)/College of American Pathologists (CAP) system, as these have superior inter-rater agreement with no loss of discriminatory ability[4,6]. In October 2017, the 15th Japanese Classification of Gastric Carcinoma proposed a new six-tier pathological regression evaluation for GC based on its previous Japanese Gastric Cancer Association (JGCA) TRG system[7]. This added the following sub-groupings of JGCA-TRG grade 2 (residual tumor 1%-33%): grade 2a (residual tumor 10%-33%) and 2b (residual tumor <

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10%) according to the result of JCOG1004-A[8,9]. This new classification did not draw much attention in Western countries, only in East Asia[10]. However, as both the JGCA and AJCC/CAP criteria obtained good consistency in Chinese patients, extensive validation is warranted to verify the adjustment in the new JGCA-TRG system [11]. Furthermore, an optimized histopathological evaluation system for predicting patient prognosis is urgently needed to resolve this contentious issue.

Therefore, the present study sought to validate the utility of the new JGCA-TRG system (JGCA2017-TRG). This was achieved by comparing JGCA2017-TRG with different TRG systems and exploring meaningful cutoff values of residual tumor percentage based on a current dataset comprising 413 LAGC patients who received D2 Lymphadenectomy following NACT.

MATERIALS AND METHODS

Patients

Data were obtained from a retrospective database of all patients receiving NACT followed by curative gastrectomy at the Peking University Cancer Hospital and Institute ("The Institute") from January 1, 2005 to January 1, 2014.

The inclusion criteria included: (1) Proven diagnosis of gastric adenocarcinoma by preoperative pathology; (2) No signs of distant metastasis at first visit; (3) Complete perioperative medical record and documentation of NACT in the Institute; and (4) Curative gastrectomy with D2 Lymph node resection performed at the Institute.

The exclusion criteria were as follows: (1) Insufficient record of clinicopathological information; (2) Patients who received radiotherapy or targeted therapy before surgery; (3) Specimen information was not available; (4) Patients with R1/R2 resection or suspected of having metastasis when surgery was performed; (5) Non-adenocarcinoma diagnosis based on postoperative histological findings (except for complete response cases); (6) Remnant gastric cancer; and (7) Died within 30 d post-surgery.

Regimen and radical surgery

Except for eight patients with logistic reasons, *e.g.*, poor economic status or severe adverse events, all patients received at least two cycles of chemotherapy. In summary, 364 patients received platin-based doublet regimens, 25 patients received Taxol-based doublet regimens, and 24 patients received Taxol-platin-based triplet regimens. Supplementary Table 1 describes the detailed dosing regimens.

To assess the influence of the treatment duration, three 14 d cycles of FOLFOX or POS were regarded as two 21 d cycles of treatment. Dosage reduction or withdrawal was applied in cases of severe adverse events during chemotherapy; this was determined by the clinician according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0, as in our previous study[12]. After two to three chemotherapy cycles, the antitumor effect was evaluated using abdominal computed tomography (CT). In most cases, two or three alignment cycles were performed. The therapy was prematurely terminated in cases of disease progression. Otherwise, gastrectomy or continued NACT was considered after obtaining informed consent and approval from patients. Subtotal or total gastrectomy plus D2 Lymphadenectomy was performed according to the JGCA guideline[13].

Histopathological examinations

The pathological preparation of the surgical specimens was commenced immediately after the operation. After recording the localization, measurement, and complete inclusion of visible tumor or suspected tumor areas, a surgeon identified the lymph node groups in the specimen. They were dissected and labeled separately from the main stomach specimen. Generally, the stomach tissue was fixed in 10% neutral buffered formalin overnight and then embedded in paraffin wax. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin (H&E) for microscopic examination, all according to standard procedures. The histological patterns, degrees of differentiation, the extent of tumor invasion, number of regional lymph node metastases, and lymphovascular invasion (LVI), were recorded in each patient's pathology report. This information was then integrated according to the 8th AJCC Cancer Staging Manual and World Health Organization pathologic classifications by two oncologists (Liu ZN and Wang YK)[14,15].

All normal sections were stained with H&E and preserved in paraffin. From December 2017, two designated pathologists (Zhang L and Sun Y) were responsible for reviewing the extent of tumor regression. All patients' H&E slides were re-

examined using bright-field fluorescence microscopy for discrimination between necrotic or heat-fixed tissue and viable tissue. The extent of regressive tumors was evaluated and recorded according to: (1) The amount of viable tumor *vs* fibrotic tissue, which ranged from a total lack of tumor regression to complete response with no viable tumor identified; and (2) The percentage of the viable residual tumor, which was calculated by dividing the viable residual tumor area by the total tumor area. Tumor regression grades were then allocated according to the JGCA2017, JGCA, Becker, AJCC/CAP, and Mandard systems (Table 1)[7,8,16-18]. As for the tumor regression grade, the JGCA2017 criteria example for each grade is shown in Figure 1. The review task ended in December 2019.

Data collection

In addition to histopathological features, other included patient characteristics were age, sex, body mass index (BMI), American Society of Anesthesiologists score (ASA), ECOG performance status, tumor location, tumor diameter (on short axis), type of resection, type of NACT regimens, complications grade by Clavien-Dindo classification, NACT cycles, survival time, and survival status[19]. The follow-up methods were described in our earlier study[20]. Disease-free survival (DFS) was calculated from the date of surgery to the date of recurrence or metastasis.

Statistical analysis

Continuous variables were summarized as the median (IQR) and were compared across groups using the Kruskal-Wallis test. Categorical variables were analyzed using the Chi-squared test. The relationships between clinical and pathological factors and long-term DFS and OS were assessed using univariate log-rank tests and a multivariate Cox proportional hazard model. Tumor or treatment characteristics that achieved a P value < 0.10 in univariate analysis were included in the multivariate analysis. The prognostic strength and the discrimination ability of each TRG system were assessed using the concordance index (c-index ± SE), with a concordance index of 1 indicating perfect prediction and 0.5 indicating no discrimination. The c-index was calculated and compared using the "survcomp" R package[21]. Testing for trends was based on various statistical hypotheses when necessary. For all analyses, $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SE STATA (Stata Statistical Software, release 15.1; Stata Corp, College Station, TX, United States).

RESULTS

Patient characteristics

A total of 413 patients met the inclusion criteria and were included in this study (Figure 2). All achieved total tumor clearance (R0). The patients had a median age of 61 years (range 24-82) and were predominantly male (73.61%). Tumor localization was proximal (including esophagogastric junction Siewert III) in 166 cases, body in 51 cases, distal in 170 cases, and 26 patients had tumor involvement in the whole stomach (linitis plastica). Most patients received preoperative therapy of 5-Fu-based oxaliplatin doublet regimen (88.14%) and 105 patients did not receive adjuvant treatment after complete resection (25.42%). The demographic data of these patients are shown in Table 2, stratified by the JGCA2017-TRG system.

Tumor regression assessment

According to the JGCA system, 26 cases were grade 0 (6.30%), 205 were grade 1a (49.64%), 78 were grade 1b, 68 were grade 2 (16.46%) including 29/39 (7.02%/9.44%) in grades 2a/2b according to the JGCA2017 classification, and 36 patients were grade 3 (8.72%; Table 2, Supplementary Table 2). Similarly, the subgroup frequencies according to the Becker, AJCC/CAP, and Mandard systems are presented in Supplementary Table 3-6, respectively. Significant differences were found in the ypT, ypN, ypTNM, and LVI stages in all five systems. The correlation coefficients of ypT were 0.619, 0.587, 0.662, 0.639, and 0.616 for the JGCA017, JGCA, Becker, AJCC/CAP and Mandard systems, respectively. On the other hand, no statistical significance was found between the NACT regimen and the TRG grade or between the duration of NACT and the TRG grade in any system.

Table 1 Criteria of five tumor regression grading systems

TRG system	Description
JGCA/JGCA2017-TRG	
0	No response
1a	67%-99% residual tumor/tumor bed
1b	34%-66% residual tumor/tumor bed
2/2a	10%-33% residual tumor/tumor bed
/2b	< 10% residual tumor/tumor bed
3	Complete response
Becker-TRG	
1a	Complete response
1b	< 10% residual tumor/tumor bed
2	10%-50% residual tumor/tumor bed
3	> 50% residual tumor/tumor bed
AJCC/CAP-TRG	
0	No residual tumor
1	Single cells or rare small groups of cancer cells
2	More than single cells or rare small groups of cancer cells with evident tumor regression
3	Extensive residual tumor or no response
Mandard-TRG	
1	No residual tumor
2	Rare residual tumor
3	Fibrosis outgrowing residual tumor
4	Residual tumor outgrowing fibrosis
5	No response

TRG: Tumor regression grade; JGCA: Japanese Gastric Cancer Association; AJCC: American Joint Committee on Cancer; CAP: College of American Pathologists.

Survival analysis and performance evaluation

The median follow-up was at 62 mo, with an IQR of 4.5 to 210 mo. At the final follow-up, 209 patients had recurrence, and 200 died due to cancer. Kaplan-Meier curves for OS and DFS based on each system are presented in Figures 3 and 4. In the univariate analyses, all five regression classification systems had prognostic relevance (Table 3). Although all five systems revealed statistical trends towards an increase in the risk of OS and DFS (P_{trend} < 0.001), JGCA2017 grade 2a showed a higher OS risk compared with grade 1b despite no statistical intergroup significance (HR: 1.06; 95%CI: 0.59-1.89; P = 0.855). The C-index for the six-tier JGCA2017, five-tier JGCA, four-tier Becker, four-tier AJCC/CAP, and five-tier Mandard systems was 0.651 ± 0.027, 0.652 ± 0.027, 0.693 ± 0.033, 0.688 ± 0.031, and 0.674 ± 0.028, respectively, for OS, and 0.648 ± 0.028, 0.649 ± 0.028, 0.695 ± 0.034, 0.685 ± 0.031, and 0.675 ± 0.028, respectively, for DFS. The four-tier Becker system had the highest c-index and was statistically significantly more accurate in predicting survival and recurrence than the six- or five-tier JGCA systems (Becker *vs* JGCA2017, P = 0.006 for OS, P = 0.002 for DFS; Becker *vs* JGCA, P = 0.007 for OS, P = 0.003 for DFS). The c-indices were comparable between the Becker and AJCC/CAP systems (P = 0.397 for OS and P = 0.273 for DFS), and between the Becker and Mandard systems (P = 0.148 for OS and P = 0.136 for DFS), while the predictive ability of the four-tier AJCC/CAP system was more accurate than the five-tier Mandard system for OS (P = 0.039) under similar evaluation principles.

Multivariate analysis for overall OS and DFS were then performed, including features that were related to poorer survival prognosis in univariate analysis (P <

Table 2 Clinical and demographic characteristics of the study population

Characteristics	*n* (%)
No. of patients	413
Age, median (IQR), yr	61 (54-67)
BMI, median (IQR), (kg/m^2)	23.04 (20.83-25.10)
Male	304 (73.61)
ASA score	
1	102 (24.70)
2	255 (61.74)
3	56 (13.56)
ECOG	
0	229 (55.45)
1	168 (40.68)
2	16 (3.87)
Location	
Upper	166 (40.19)
Middle	51 (12.35)
Lower	170 (41.16)
Diffuse	26 (6.30)
Diameter (cm)	3.0 (1.5-4.0)
Differentiation	
Well	27 (6.54)
Moderate	176 (42.86)
Poor	209 (50.61)
Mucinous or signet cell	85 (20.58)
LVI	132 (31.96)
Cycles of treatment	2 (2-3)
ypT	
ypT0	37 (8.96)
ypT1	25 (6.05)
ypT2	55 (13.32)
ypT3	66 (15.98)
ypT4	230 (55.69)
ypN	
N0	169 (40.92)
N1	64 (15.50)
N2	70 (16.95)
N3	110 (26.63)
ypStage	
pCR	32 (7.75)
I	57 (13.80)
II	116 (28.09)
III	208 (50.36)

Total gastrectomy	180 (43.58)
Regimen	
Platin-based	364 (88.14)
Taxol-based	25 (6.05)
Triplet	24 (5.81)
Adjuvant chemotherapy	308 (74.58)
Postoperative complications	
Grade 0-1	277 (67.07)
Grade 2	78 (18.89)
Grade 3-4	58 (14.04)
JGCA2017-TRG	
Grade 3 (no residual)	36 (8.72)
Grade 2b (< 10%)	39 (9.44)
Grade 2a (10%-33%)	29 (7.02)
Grade 1b (34%-66%)	78 (18.89)
Grade 1a (67%-99%)	205 (49.64)
Grade 0 (no response)	26 (6.30)
JGCA-TRG	
Grade 3 (no residual)	36 (8.72)
Grade 2 (< 33%)	68 (16.46)
Grade 1b (34%-66%)	78 (18.89)
Grade 1a (67%-99%)	205 (49.64)
Grade 0 (no response)	26 (6.30)
Becker-TRG	
1a (no residual)	36 (8.72)
1b (< 10%)	39 (9.44)
2 (10%-50%)	65 (15.74)
3 (> 50%)	273 (66.10)
AJCC-TRG	
0 (complete response)	36 (8.72)
1 (moderate response)	48 (11.62)
2 (minimal response)	89 (21.55)
3 (poor response)	240 (58.11)
Mandard-TRG	
1 (complete response)	36 (8.72)
2 (Fibrosis + scattered tumor cells)	48 (11.62)
3 (Fibrosis predominance + tumor cells)	89 (21.55)
4 (Tumor cells preponderance + fibrosis)	214 (51.82)
5 (No response)	26 (6.30)

BMI: Body mass index; ASA: American Society of Anesthesiologists; ECOG: Eastern Cooperative Oncology Group; LVI: Lymphovascular invasion; NACT: Neoadjuvant chemotherapy; TRG: Tumor regression grade.

Table 3 Univariate analyses for overall survival and progression-free survival using a Cox proportional hazards model

Variables	OS		DFS	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age				
≤ 65				
> 65	1.12 (0.84-1.51)	0.439	1.12 (0.84-1.49)	0.4351
BMI				
≤ 23.9	1.39 (1.04-1.87)	0.027	1.31 (0.98-1.74)	0.065
> 23.9				
Gender				
Male				
Female	1.14 (0.84-1.56)	0.393	1.06 (0.78-1.45)	0.690
ASA score				
1-2				
3	1.00 (0.67-1.49)	0.993	0.97 (0.65-1.43)	0.874
ECOG				
0				
1-2	1.29 (0.98-1.70)	0.073	1.30 (0.99-1.71)	0.056
Location				
Diffuse *vs* Upper	3.32 (2.07-5.31)	< 0.001	2.97 (1.86-4.73)	< 0.001
Diffuse *vs* Middle	2.63 (1.51-4.56)	< 0.001	2.28 (1.32-3.94)	0.003
Diffuse *vs* Lower	3.94 (2.45-6.35)	< 0.001	3.52 (2.19-5.65)	< 0.001
Diffuse	1.00		1.00	
Diameter (cm)				
≤ 5				
> 5	2.79 (2.00-3.88)	< 0.001	2.99 (2.17-4.13)	< 0.001
Differentiation				
Well-Moderate				
Poor	1.43 (1.08-1.90)	0.012	1.51 (1.15-1.99)	0.003
Histology				
Non-mucinous				
Mucinous or signet cell	1.86 (1.39-2.53)	< 0.001	1.77 (1.31-2.40)	< 0.001
Lymphovascular invasion				
No				
Yes	2.75 (2.08-3.64)	< 0.001	2.91 (2.21-3.83)	< 0.001
ypT				
ypT0-2				
ypT3-4	3.54 (2.35-5.36)	< 0.001	3.66 (2.44-5.49)	< 0.001
ypN				
ypN0				
ypN+	3.50 (2.50-4.90)	< 0.001	3.59 (2.58-4.98)	< 0.001
Resection type				
Subtotal				

Total	1.79 (1.35-2.36)	< 0.001	1.74 (1.32-2.28)	< 0.001
Cycle of NACT				
≤ 2				
> 2	1.18 (0.89-1.56)	0.247	1.18 (0.90-1.55)	0.233
NACT regimen				
Platin-based	1.00		1.00	
Paclitaxel-based	1.10 (0.62-1.92)	0.752	1.27 (0.75-2.15)	0.373
Triplet drug	1.05 (0.59-1.89)	0.862	1.03 (0.57-1.84)	0.930
Adjuvant chemotherapy				
Received				
Not received	1.36 (1.00-1.85)	0.050	1.18 (0.87-1.60)	0.286
Complications				
Clavien-dindo 0-2				
Clavien-dindo 3-4	1.15 (0.78-1.69)	0.491	1.11 (0.76-1.63)	0.585
JGCA2017-TRG				
Grade 3 (no residual)	1.00		1.00	
Grade 2b (< 10%)	8.97 (2.06-39.02)	0.003	8.75 (2.01-38.09)	0.004
Grade 2a (10%-33%)	13.55 (3.11-58.93)	0.001	14.03 (3.23-61.06)	< 0.001
Grade 1b (34%-66%)	12.83 (3.10-53.18)	< 0.001	14.05 (3.40-58.09)	< 0.001
Grade 1a (67%-99%)	15.15 (3.74-61.42)	< 0.001	15.55 (3.84-62.97)	< 0.001
Grade 0 (no response)	20.24 (4.67-87.68)	< 0.001	21.15 (4.88-91.67)	< 0.001
JGCA-TRG				
Grade 3 (no residual)	1.00		1.00	
Grade 2 (< 33%)	10.79 (2.59-45.05)	0.001	10.79 (2.58-45.05)	0.001
Grade 1b (34%-66%)	12.83 (3.10-53.18)	< 0.001	14.04 (3.40-58.05)	< 0.001
Grade 1a (67%-99%)	15.15 (3.74-61.42)	< 0.001	15.54 (3.84-62.93)	< 0.001
Grade 0 (no response)	20.24 (4.67-87.66)	< 0.001	21.18 (4.89-91.78)	< 0.001
Becker-TRG				
1a (no residual)	1.00		1.00	
1b (< 10%)	8.98 (2.06-39.06)	0.003	8.74 (2.01-38.05)	0.004
2 (10%-50%)	12.19 (2.92-50.87)	0.001	12.72 (3.05-53.06)	< 0.001
3 (> 50%)	15.50 (3.84-62.62)	< 0.001	16.15 (4.00-65.22)	< 0.001
AJCC-TRG				
0 (complete response)	1.00		1.00	
1 (moderate response)	10.46 (2.46-44.48)	0.001	10.31 (2.42-43.90)	0.002
2 (minimal response)	11.21 (2.71-46.34)	0.001	11.67 (2.83-48.22)	0.001
3 (poor response)	16.31 (4.03-65.97)	< 0.001	16.94 (4.19-68.49)	< 0.001
Mandard-TRG				
1 (complete response)	1.00		1.00	
2 (Fibrosis + scattered tumor cells)	10.46 (2.46-44.48)	0.001	10.33 (2.43-43.95)	0.002
3 (Fibrosis predominance + tumor cells)	11.20 (2.71-46.30)	0.001	11.66 (2.82-48.16)	0.001
4 (Tumor cells preponderance + fibrosis)	15.85 (3.91-64.19)	< 0.001	16.48 (4.07-66.71)	< 0.001
5 (No response)	20.27 (4.68-87.81)	< 0.001	21.22 (4.90-91.96)	< 0.001

BMI: Body mass index; ASA: American Society of Anesthesiologists; ECOG: Eastern Cooperative Oncology Group; NACT: Neoadjuvant chemotherapy; TRG: Tumor regression grade; HR: Hazard ratio; DFS: Disease-free survival; OS: Overall survival.

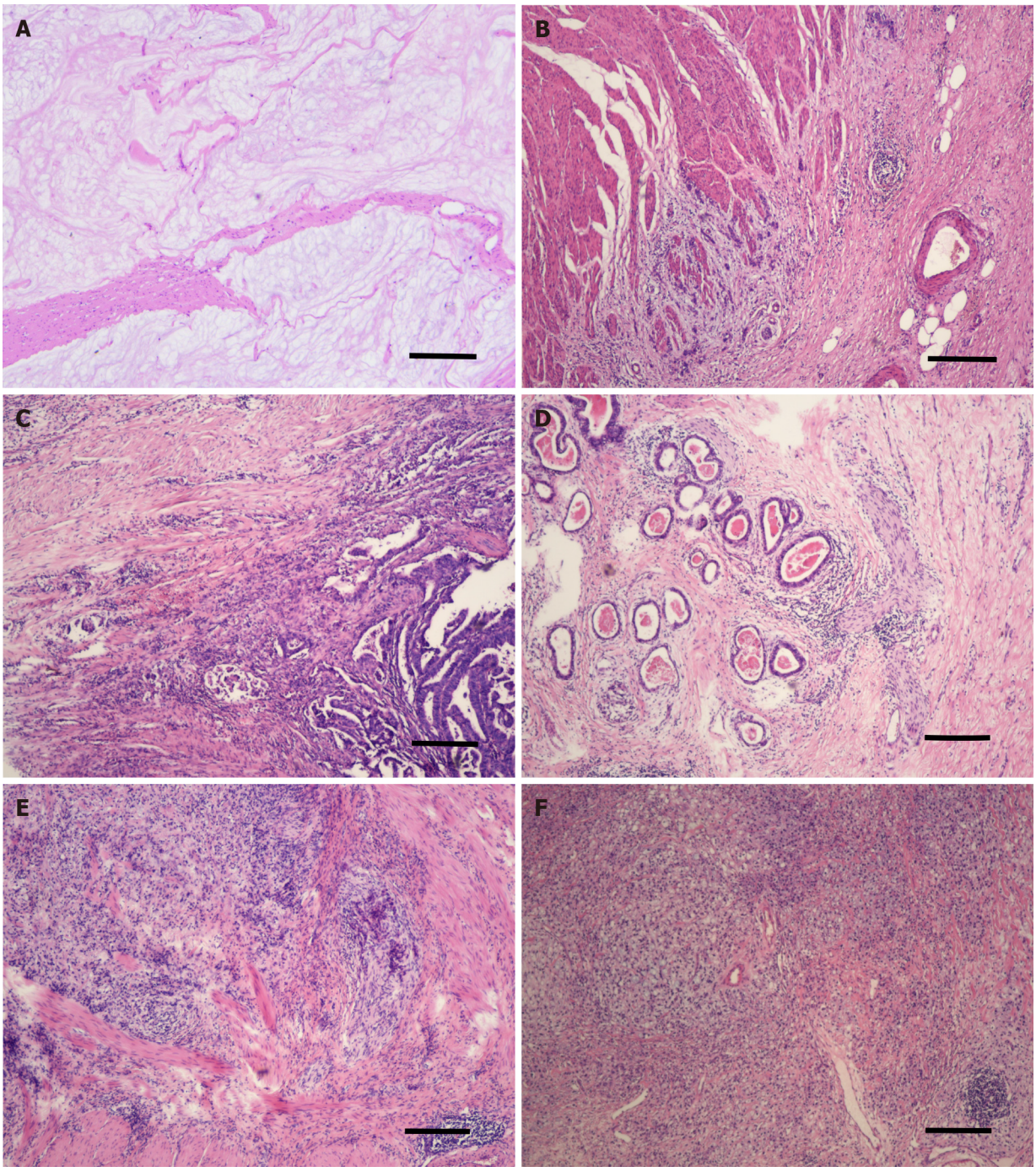

Figure 1 Tumor regression grading according to 15th Japanese Classification of Gastric Carcinoma criteria. A: Grade 3 (complete regression); B: Grade 2b (5% residual tumor); C: Grade 2a (30% residual tumor); D: Grade 1b (50% residual tumor); E: Grade 1a (70% residual tumor); F: Grade 0 (No response) (original magnification 20×).

0.10): BMI, ECOG, tumor location, diameter in short axis, differentiation, histology type, LVI, resection type, adjuvant chemotherapy, and ypT and ypN stages. After adjusting for potential confounders in the multivariate Cox regression model, BMI, histology type, LVI, and the ypN stage were independent predictors for OS, while LVI and the ypN stage were independent risk factors for DFS. All five TRG systems showed significant differences when setting the "complete response" group as a reference (Table 4). However, the increase in the hazard ratio was not entirely in accord with the increase in the TRG grade in the JGCA2017, AJCC/CAP, and Mandard

Table 4 Multivariate Cox hazards regression model for the predictable risk of overall survival and disease-free survival in different covariate inclusion in whole patients

	Whole patients (*n* = 413)			
Covariates	OS		DFS	
	HR	*P* value	HR	*P* value
BMI ≤ 23.9	1.37 (1.01-1.87)	0.045	1.28 (0.95-1.72)	0.109
ECOG > 0	1.19 (0.87-1.61)	0.271	1.18 (0.88-1.60)	0.272
Linitis plastica	1.74 (0.97-3.13)	0.063	1.30 (0.74-2.30)	0.362
Diameter > 5 cm	1.20 (0.77-1.88)	0.426	1.43 (0.93-2.19)	0.102
Poorly differentiated	1.16 (0.85-1.57)	0.345	1.24 (0.92-1.66)	0.160
Mucinous or signet cell	1.45 (1.03-2.05)	0.036	1.32 (0.94-1.84)	0.111
Lymphovascular invasion	1.53 (1.12-2.10)	0.008	1.61 (1.18-2.19)	0.002
ypT3-4	1.45 (0.92-2.28)	0.113	1.52 (0.97-2.37)	0.065
ypN+	1.96 (1.35-2.85)	< 0.001	1.94 (1.34-2.82)	< 0.001
Total gastrectomy	1.30 (0.94-1.79)	0.118	1.23 (0.90-1.69)	0.202
Without AC	1.35 (0.98-1.87)	0.066	Not included	NA
JGCA2017-TRG (Model 1)				
Grade 3 (no residual)	1.00		1.00	
Grade 2b (< 10%)	4.69 (1.04-21.08)	0.044	4.50 (1.00-20.27)	0.050
Grade 2a (10%-33%)	5.48 (1.19-25.23)	0.029	5.50 (1.20-25.26)	0.028
Grade 1b (34%-66%)	5.32 (1.22-23.30)	0.026	5.73 (1.32-24.88)	0.020
Grade 1a (67%-99%)	6.69 (1.55-28.96)	0.011	6.22 (1.44-26.81)	0.014
Grade 0 (no response)	8.60 (1.87-39.58)	0.006	8.44 (1.84-38.76)	0.006
JGCA-TRG (Model 2)				
Grade 3 (no residual)	1.00			
Grade 2 (< 33%)	5.00 (1.15-21.78)	0.032	4.90 (1.13-21.30)	0.034
Grade 1b (34%-66%)	5.27 (1.21-23.05)	0.027	5.67 (1.30-24.61)	0.021
Grade 1a (67%-99%)	6.63 (1.53-28.66)	0.011	6.16 (1.43-26.52)	0.015
Grade 0 (no response)	8.48 (1.84-39.00)	0.006	8.31 (1.81-38.15)	0.006
Becker-TRG (Model 3)				
1a (no residual)	1.00		1.00	
1b (< 10%)	4.74 (1.05-21.30)	0.043	4.57 (1.02-20.57)	0.047
2 (10%-50%)	5.11 (1.16-22.51)	0.031	5.13 (1.17-22.49)	0.030
3 (> 50%)	6.77 (1.57-29.14)	0.010	6.64 (1.55-28.46)	0.011
AJCC-TRG (Model 4)				
0 (complete response)	1.00		1.00	
1 (moderate response)	5.39 (1.22-23.78)	0.026	5.34 (1.21-23.50)	0.027
2 (minimal response)	5.01 (1.15-21.85)	0.032	5.05 (1.16-21.93)	0.031
3 (poor response)	6.72 (1.56-28.97)	0.011	6.53 (1.52-28.05)	0.012
Mandard-TRG (Model 5)				
1 (complete response)	1.00		1.00	
2 (Fibrosis + scattered tumor cells)	5.37 (1.22-23.68)	0.026	5.31 (1.21-23.39)	0.027
3 (Fibrosis predominance + tumor cells)	4.95 (1.14-21.60)	0.033	4.98 (1.15-21.62)	0.032

4 (Tumor cells preponderance + fibrosis)	6.44 (1.49-27.87)	0.013	6.26 (1.45-26.93)	0.014
5 (No response)	8.44 (1.83-38.86)	0.006	8.41 (1.83-38.60)	0.006

BMI: Body mass index; ECOG: Eastern Cooperative Oncology Group; AC: Adjuvant chemotherapy; TRG: Tumor regression grade; HR: Hazard ratio; DFS: Disease-free survival; OS: Overall survival.

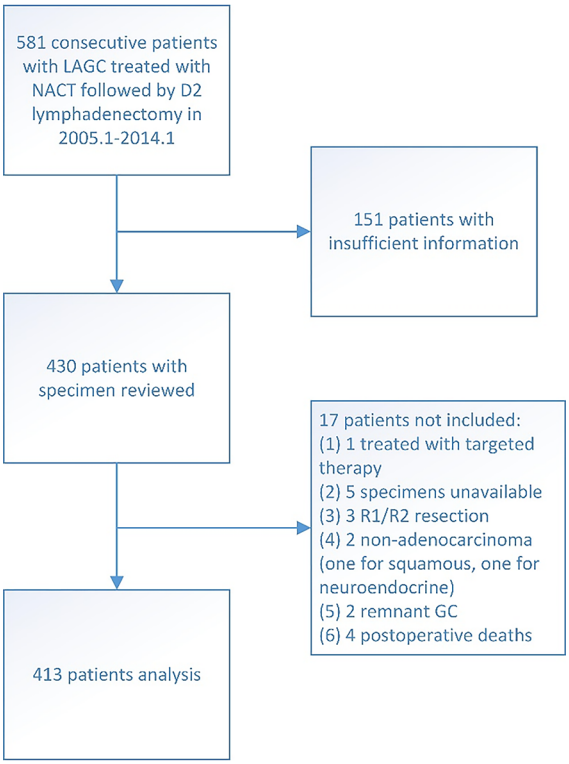

Figure 2 Selection of patients for inclusion.

systems. In fact, the intergroup differences were not statistically significant when the "complete response" group was absent in each system. Only a marginal difference was found between JGCA2017-TRG grade 0 (no response) *vs* 2b (< 10%) for OS (HR: 1.84; 95%CI: 0.90-3.75; $P = 0.096$) and DFS (HR: 1.87; 95%CI: 0.92-3.83; $P = 0.085$).

Rearranged cutoff values based on current residual tumor percentage

According to the previous analysis, a comparison of the five systems revealed the Becker system to enable the best prognostic differentiation between subgroups across the whole patient cohort. The AJCC/CAP system, although having the second-highest c-index, did not provide better intergroup discrimination in multivariate analysis. According to the JGCA2017 criteria, two cutoff values of residual tumor percentage - 10% and 100% - were of more clinical significance than any other commonly used cutoff percentages except for total regression. Despite the intergroup differences being marginal, a higher c-index of 0.728 ± 0.035 for OS and 0.737 ± 0.035 for DFS, could be achieved based on the following rearranged residual tumor percentage cutoffs: 0 (no residual tumor; reference), < 10% (HR: 4.61; 95%CI: 1.02-20.73; $P = 0.047$ for OR; HR: 4.46; 95%CI: 0.99-20.08; $P = 0.051$ for DFS), 10-99% (HR: 5.98; 95%CI: 1.40-25.63; $P = 0.016$ for OS; HR: 5.93; 95%CI-25.29; $P = 0.016$ for DFS), no response (HR: 8.36; 95%CI: 1.82-38.44; $P = 0.006$ for OS; HR: 8.33; 95%CI: 1.82-38.23; $P = 0.006$ for DFS). There was a significant difference in the prognostic ability for DFS ($P = 0.046$) and a borderline significance for OS ($P = 0.073$) between the rearranged cutoffs and the Becker system (Table 5).

DISCUSSION

Neoadjuvant chemotherapy followed by surgery and adjuvant chemotherapy is the

Table 5 The pairwise comparison of C-indexes between different tumor regression grade based on Cox regression for overall survival

	JGCA2017	JGCA	Becker	AJCC/CAP	Mandard	Modified
Overall survival						
JGCA2017	1.000	0.308	0.006	0.018	0.053	< 0.001
JGCA		1.000	0.007	0.021	0.063	< 0.001
Becker			1.000	0.397	0.148	0.073
AJCC/CAP				1.000	0.039	0.062
Mandard					1.000	0.005
Modified						1.000
Disease-free survival						
JGCA2017	1.000	0.320	0.002	0.021	0.033	< 0.001
JGCA		1.000	0.003	0.025	0.040	< 0.001
Becker			1.000	0.273	0.136	0.046
AJCC/CAP				1.000	0.112	0.024
Mandard					1.000	0.002
Modified						1.000

JGCA: Japanese Gastric Cancer Association; AJCC: American Joint Committee on Cancer; CAP: College of American Pathologists.

current standard treatment for LAGC[1,2]. Although the benefit of this multimodality treatment was first confirmed by the MAGIC trial in 2006, the use of NACT had been adopted in GC for 30 years[22,23]. To assess the treatment response, despite the widespread use of the TRG system for gastrointestinal tract tumors, the response rates are always poor in GC compared with esophageal or colorectal cancer[5]. This might be due to the lack of chemoradiation and sensitive regimens in preoperative settings. Due to the currently limited preoperative therapies and the limited number of responsive patients, findings on the value of TRG prognostic systems in LAGC are varied. Additional complexities arise when the study contexts are based on different TRG systems, especially on the comparison between TRG and ypTNM systems as independent predictors of patients survival[24-29]. Becker *et al*[16] investigated 480 patients with LAGC undergoing surgical resection and found TRG 2-3 grade (10%-100% residual tumor) to be an independent risk factor for patient OS; this reinforced the efficacy of the Becker TRG system. Ikoma *et al*[25] reviewed 356 LAGC patients receiving D0-D2 Lymphadenectomy following NACT or NACRT, finding that the residual tumor < 50% group was associated with a shorter OS but not as an independent predictor[25]. And Derieux first proved the predictive value of the Mandard system in GC, observing a poorer OS and DFS in patients with a high proportion of residual cancer cells (Mandard TRG 4) and no response (Mandard TRG 5)[29].

Therefore, when verifying the prognostic value of the histological response, considerable work should be done on determining an optimal tumor response classification for GC. Currently, two major principles are common to these systems for grading tumor regression: (1) estimating residual tumor in relation to fibrotic changes, *e.g.*, the Mandard, AJCC/CAP, and Dworak systems[17,18,30]; and (2) proportioning the residual tumor in relation to the previous tumor site, *e.g.*, the Becker and JGCA system[16,31]. Although both are semiquantitative principles, the use of different systems reveals great regional disparities. The estimation of residual tumor is considered to be easier than considering therapy-induced fibrosis by the majority of pathologists[4], which potentially means a better inter-rater consistency for the residual tumor percentage method[32,33]. Most recently, an international survey was conducted and summarized preferences for using various TRG systems in gastrointestinal cancer among 173 global pathologists[4]. According to the published results, the AJCC/CAP and Mandard systems were widely adopted in North America and Europe, respectively. However, the questionnaires from East Asia - one from Japan and the other from Korea - accounted for only two of the 173 valid responses, with no input from China[34]; it is doubtful whether these two contributions could fully picture the three countries that

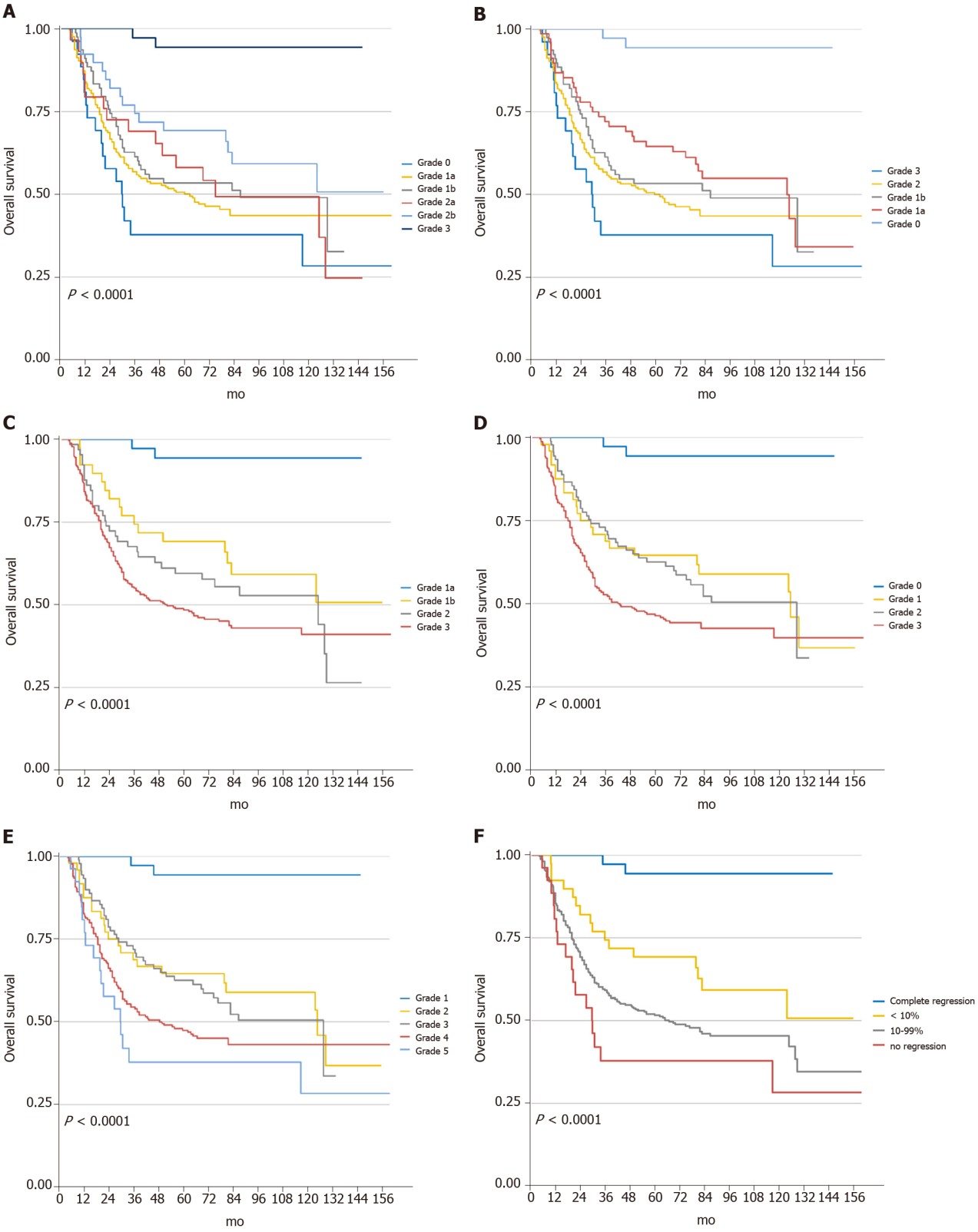

Figure 3 Kaplan-Meier curves for overall survival of five tumor regression grade systems. A: JGCA2017-tumor regression grade (TRG); B: JGCA-TRG; C: Becker-TRG; D: AJCC/CAP-TRG; E: Mandard; F: Rearranged cutoff values. *P* value stands for log-rank test.

account for approximately one-third of the worldwide GC population. Overall, global diversity leads to obstacles in the comparison of experiments using different standards.

A comparison of different histological response systems for GC was conducted by Zhu *et al*[28]. This study included 192 patients and found that five TRG systems - including Mandard, JGCA, AJCC/CAP, Becker, and China - were not independent predictors for patient survival. Although the predictive abilities of each system were

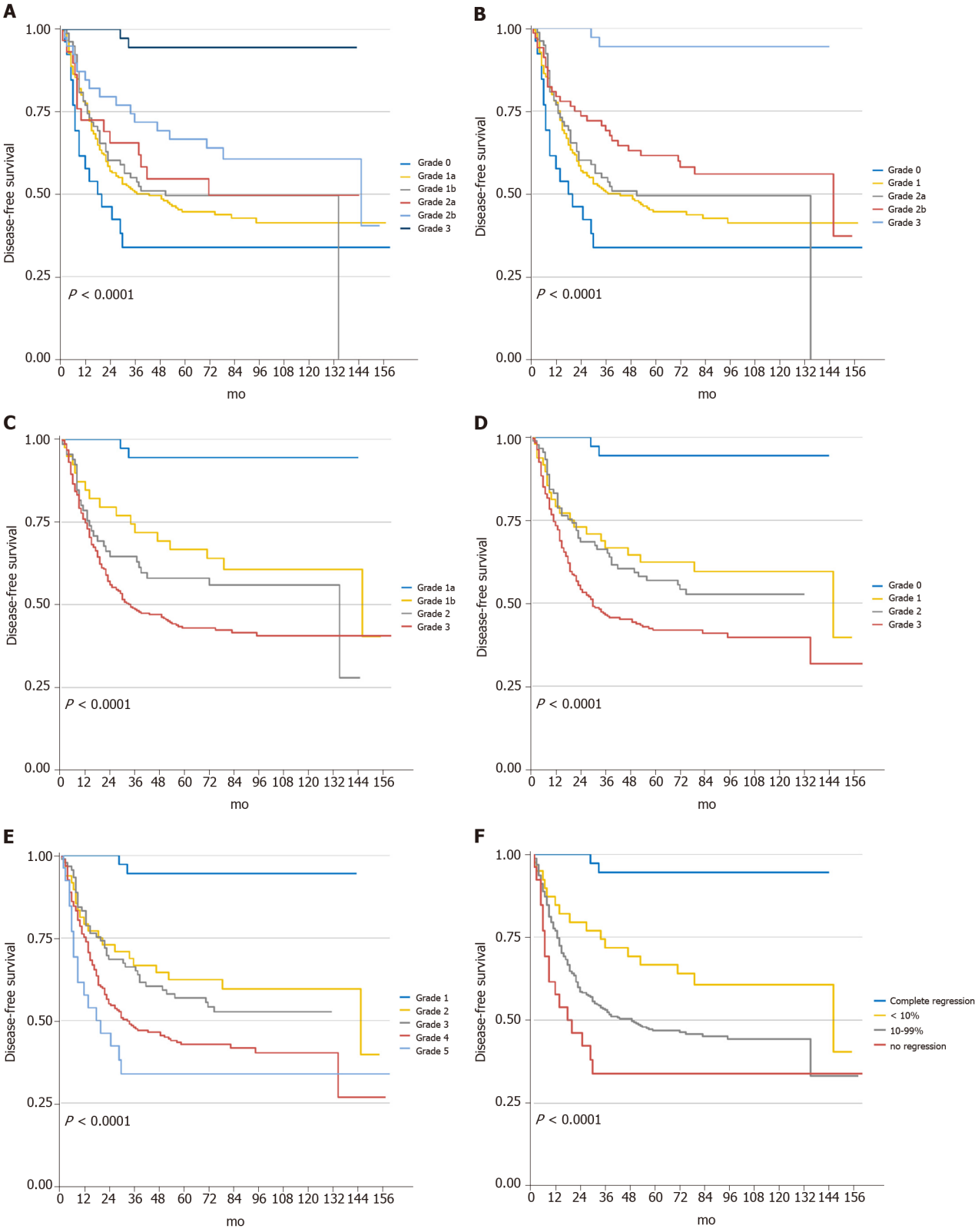

Figure 4 Kaplan-Meier curves for progression-free survival of five tumor regression grade systems. A: JGCA2017-tumor regression grade (TRG); B: JGCA-TRG; C: Becker-TRG; D: AJCC/CAP-TRG; E: Mandard; F: Rearranged cutoff values. *P* value stands for log-rank test.

not measured, the Mandard and JGCA systems were recommended due to their superior prognosis prediction abilities. This was because a higher hazard ratio was discovered in the "no response" patients. In JCOG1004-A, 173 patients who received surgery following NACT were stratified according to different residual tumor cutoff percentages of 10%, 33%, 50%, and 67%. The 10% cutoff was found to be the best predictor of survival for various pathological types[9]. While this 10% cutoff finding was remarkable and coincided with Becker's cutoff method (described above)[35],

whether the current five- or six-tier JGCA standard provided the optimal discrimination value was not further investigated.

In the present study, c-index analysis was used to compare the discrimination value of five TRG systems including the most recent JGCA2017-TRG system. Both the five-tier JGCA and the six-tier JGCA2017 systems scored significantly lower c-indexes than the four-tier AJCC/CAP and Becker systems. Because both the JGCA2017 and the JGCA have overlapped measuring spacing compared with the four-tier Becker system, the results of the present study indicated that five- or six-tier grading systems performed no better (and even worse) than four-tier systems in evaluating GC patients. The c-index comparison suggested that the four-tier Becker system had the best predictive value for GC patients. Because of their relatively wide measuring distance, four-tier systems based on residual percentages also mean a lower workload, easier understanding of protocols, and less inter-observer disagreement propagation[4, 36].

On the other hand, based on the JCGA2017 criteria, the present study revealed that grade 2b (1%-10%) was likely to predict longer OS and DFS than grade 0 (no response). Interestingly, when the percentages of residual tumor were reset to "no residual tumor", < 10%, < 100%, and "no response", the c-index of the rearranged cutoff values scored significantly higher than the Becker system for patient survival. Similar results using these revised cutoffs were reported by Zhu *et al*[28], wherein an overt higher HR was observed for grade 3 among the other JGCA grades, and by Becker *et al*[35] who demonstrated the independent predictive ability of the Becker system by using a cutoff of < 10% residual tumor. The results of the present study suggested that among moderate-to-poor (residual tumor 10-99%) responders, the response rate may not have a decisive impact on hazard stratification because NACT or chemotherapy only accounted for a small part of improving the prognosis among significant covariates in this group of GC patients. Meanwhile, a complete or subtotal response (0%-10%) often indicated a fairly good sensitivity to chemotherapy and vice versa for non-responders (no regression), who cannot receive any benefit but toxicity. Although the non-responders only accounted for 6.3% of the total patient number, it is suggested that this "break off both ends" approach provides a way for screening chemosensitivity and predicting prognosis in GC patients. However, a larger sample size is required to verify this proposal.

There were some limitations to this study. First, it was restricted by its single-center retrospective nature. Second, although histopathology was performed by two pathologists with over 10 years of experience, analysis of the inter- and intra-observer variability of the actual TRG classification was not conducted. Third, despite the involvement of many covariates, the macroscopic information may not be sufficient. According to JCOG1004-A, the TRG cutoff standard may not be recommended for Bormann type IV patients, for which a current dataset is not available[9]. Furthermore, this study did not consider intestinal and diffuse types according to the Lauren classification, which are thought to be independent prognostic factors for survival[37]. Statistically, collinearity between the TRG and ypT categories is inevitable but would have affected the multivariable analysis results: the Pearson's coefficients with ypT were 0.619, 0.587, 0.662, and 0.639 for the JGCA2017, JGCA, Becker, and AJCC/CAP systems, respectively. To reduce the impact of multicollinearity, studies with an increased sample size are warranted.

CONCLUSION

In conclusion, it was demonstrated that although all five TRG systems could be used as independent predictors for LAGC patient survival, the six-tier JGCA-TRG system did not increase prognostic stratification but may reduce the reproducibility and increase the working load on histological response evaluation. Patient survival can be effectively discriminated by the Becker system using the residual tumor percentage rather than by estimating the fibrosis/residual tumor ratio. Apart from when using the Becker classification, the group of non-responders with no regression was predicted to have a poorer prognosis. A large population-based study is still required to find the optimal criteria and validate the boundary settings of current TRG systems for LAGC patients.

ARTICLE HIGHLIGHTS

Research background

The tumor regression grade systems for gastric cancer (GC) are various, while the most suitable one is yet to be known.

Research motivation

We aimed to investigate the most accurate criteria for TRG in predicting patient's prognosis.

Research objectives

To collect 413 locally advanced GC (LAGC) patient's clinical data and their post-treatment pathological samples after neoadjuvant chemotherapy treatment.

Research methods

This is a retrospectively clinical study in which the LAGC patient's specimens were reviewed by two pathologists and the TRG grades were revalued. Then, the predictive abilities of five TRG criteria were assessed and statistically compared based on survival/risk prediction model.

Research results

The four-tier Becker system showed the highest predictive ability, among the five common TRG criteria. The TRG criteria could achieve an optimal prediction when the residual tumor percentages were reset as: "no residual tumor", < 10%, < 100%, and "no response".

Research conclusions

The four-tier Becker system is more suitable and should be recommended for LAGC patients.

Research perspectives

A population-based study is warranted to define the optimal criterion for TRG for GC.

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ORIGINAL ARTICLE

Retrospective Study

Clinical features of intracerebral hemorrhage in patients with colorectal cancer and its underlying pathogenesis

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Author contributions: Deng XH and Liang ZJ conceived and designed the study; Deng XH collected the data and drafted the manuscript; Li J, Chen SJ, Xie YJ, Zhang J, Cen GY and Song YT helped to collect the data; Li J and Chen SJ analyzed the results; Liang ZJ critically revised the manuscript and provided financial support for this work; all the authors have read the manuscript and approved the final version.

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Abstract

BACKGROUND

The diagnosis of both cancer and intracerebral hemorrhage (ICH) in the same patient is not uncommon, but the clinical features and pathogenesis of patients with colorectal cancer (CRC) and ICH are still not well known.

AIM

To investigate the clinical features and underlying pathogenesis of ICH in patients with CRC.

METHODS

A retrospective review of CRC patients complicated with ICH from three centers between January 2014 and December 2020 was performed. Clinical data such as laboratory examinations, imaging features, prognosis, and underlying pathogenesis were analyzed.

RESULTS

Of 16673 identified CRC patients, 20 (0.12%) suffered from ICH. There were 13 males and 7 females, with an average age (mean ± SD) of 68.45 ± 10.66 years. Fourteen patients (70%) had distant metastases and most patients (85%) showed an elevation of one or more cancer biomarkers. The hemorrhagic lesions in 13 patients (65%) were in the intracerebral lobe. Four patients were completely dependent and 4 died within 30 days after hemorrhage. Intratumoral hemorrhage (50%) and coagulopathy (50%) accounted for the majority of hemorrhages.

CONCLUSION
Patients with ICH and CRC often have clinical features with lobar hemorrhage, distant metastases and poor prognosis. Intratumoral hemorrhage and coagulopathy are the main causes of ICH in patients with CRC.

Key Words: Colorectal cancer; Intracerebral hemorrhage; Clinical features; Pathogenesis

Core Tip: The association between cancer and intracerebral hemorrhage (ICH) has long been studied, however little attention has been paid to the hemorrhagic cerebrovascular events in patients with colorectal cancer (CRC). CRC has been reported to increase the risk of ICH. The present study retrospectively analyzed the clinical data of patients with CRC and ICH, and indicated that intratumoral hemorrhage and coagulopathy were the main causes of ICH in CRC patients.

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INTRODUCTION

Intracerebral hemorrhage (ICH) and cancer are both common disorders, and are significant causes of morbidity and mortality worldwide in the elderly. It is reported that ICH accounts for almost half of cerebrovascular events in cancer patients[1,2] and significantly aggravates their condition and prognosis[3,4]. Several population-based studies have demonstrated that multiple cancers are associated with an increased risk of ICH[5,6]. Despite the fact that traditional vascular risk factors for ICH are commonly observed in cancer patients, current studies have revealed that ICH in some patients shows clear-cut distinctions by its unique characteristics and pathogenesis. ICH can manifest as direct or indirect effects of cancer, namely cancer-related ICH. Cancer-related ICH might result from primary or metastatic brain malignancies, coagulopathy or systemic effects of oncological therapy[1,7,8]. However, it may lead to a variety of clinical features and pathogenesis of cancer-related ICH due to the complexity and diversity of cancers.

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in the world [9,10]. It has been estimated that CRC burden rose to approximately 1.9 million new cases and 0.9 million deaths worldwide in 2020[10]. However, there are few detailed reports in the literature that focus on the hemorrhagic cerebrovascular events in CRC patients. The purpose of this study was to investigate the clinical features and underlying pathogenesis of ICH in patients with CRC.

MATERIALS AND METHODS

The present study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. CRC patients with ICH were recruited from the First Affiliated Hospital and the Affiliated Cancer Center of Guangxi Medical University, and the Affiliated Yuebei People's Hospital of Shantou University Medical College between January 2014 and December 2020. The diagnostic criteria for ICH were based on the 2015 Guidelines for the Diagnosis and Treatment from the American Heart Association[11]. The diagnosis of active CRC followed the definition of active cancer in the study by Lee *et al*[12]. Patients who met the following criteria were included: (1) Diagnosis of CRC within 6 mo before enrollment, any treatment for CRC within the previous 6 mo, or recurrent or metastatic CRC; (2) Presence of clinical symptoms, such as sudden onset of unconsciousness, headache, hemiplegic paralysis, slurred speech, or other focal neurological deficits; and (3) The

the study are published on the home page of the First Affiliated Hospital of Guangxi Medical University.

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presence of ICH on intracranial computed tomography (CT) or magnetic resonance imaging (MRI) scan and susceptibility-weighted imaging, which could explain the symptoms. The exclusion criteria were as follows: (1) Combined with other systemic malignancy; (2) Presence of cerebral infarction and other central nervous system complications; (3) CRC diagnosed > 5 years ago, with no evidence of recurrence or metastasis; and (4) Patients with incomplete records (Figure 1). The selected cases were reviewed and determined by a panel which consisted of an oncologist, a neurologist and a neuroradiologist who were all blind to the study.

Collection of clinical data

General demographic data such as age and gender were obtained. Vascular risk factors for ICH including hypertension, diabetes mellitus, hypercholesterolemia, tobacco and alcohol consumption, aneurysm, arteriovenous malformation and stroke history were also documented. Moreover, data on CRC, such as pathological types of cancer cells, metastasis, prior and current treatment and information related to acute ICH, including onset form, cardinal symptoms and signs, and hemorrhagic locations were recorded. In addition, routine blood examination, blood biochemistry, coagulation indices, plasma D-dimer, cancer biomarkers, electrocardiography, Doppler echocardiography, cervical vascular Doppler, head and neck CT angiography, head CT, head MRI and magnetic resonance angiography were also carried out. The National Institute of Health Stroke Scale (NIHSS) was used to evaluate the severity of focal neurological deficits. To minimize the effects of CRC progression on physical activities, patients' functional prognosis on the 30th day after hemorrhage was measured using the modified Rankin Scale (mRS), and a mRS score > 3 was regarded as a poor prognosis[13]. According to a research study by Navi and colleagues on ICH [2], coagulopathy was identified if any of the following parameters were fulfilled: platelets < 100 × 10^9/L, international normalized ratio (INR) > 1.5, activated partial thromboplastin time (APTT) > 45 s, prothrombin time (PT) > 15 s, and disseminated intravascular coagulation (DIC) (fibrinogen < 200 mg/dL and D-dimer > 290 ng/dL).

Statistical analysis

All statistical analyses were undertaken using SPSS 20.0 software (IBM, Inc., Armonk, NY, United States). Quantitative data were shown as mean ± SD and qualitative data were expressed as frequency and percentages.

RESULTS

A total of 16673 patients with CRC were identified, and 20 patients (0.12%) with ICH met the inclusion criteria. Of these 20 patients, 13 were male (65%) and 7 were female (35%). The average age (mean ± SD) was 68.45 ± 10.66 years (range 47-82). All patients were pathologically confirmed to have adenocarcinoma. Ten patients had vascular risk factors, including tobacco use (30%), hypertension (30%), diabetes mellitus (10%), alcohol abuse (5%), hypercholesterolemia (15%), stroke history (10%) and coronary or kidney disease (5%). No aneurysms or arteriovenous malformations were identified in these patients. None of the patients were on therapeutic anticoagulation or antiplatelet agents at the time of hemorrhage. Coagulopathy was observed in 10 patients. Thrombocytopenia was present in 6 patients (30%). Six patients (30%) had a prolonged PT value, 2 (10%) had a prolonged APPT value, and 3 (15%) displayed INR values greater than 1.5. DIC was documented in 1 patient. Most patients (17/20, 85%) had at least one elevated cancer biomarker. Six patients (30%) showed hepatic dysfunction, while 14 patients (70%) did not. Fourteen patients (70%) exhibited distant metastases, intracranial metastasis occurred in 10 patients (50%) and hepatic/osseous metastases occurred in 8 patients (40%), when ICH developed. Prior to hemorrhage, 13 patients (65%) had received oncological therapy while 7 patients (35%) had not (Table 1).

ICH occurred in 2 patients (10%) before cancer diagnosis and 18 patients (90%) after cancer diagnosis. The most frequent hemorrhagic lesion was in the cerebral lobe, occurring in 13 patients (65%). Fewer lesions were found in the basal ganglia (4/20, 20%), cerebellum (2/20, 10%), and brainstem (1/20, 5%). The etiologies of hemorrhage were ascribed to intratumoral hemorrhage (10/20, 50%), coagulopathy (10/20, 50%), both intratumoral hemorrhage and coagulopathy (5/20, 25%), hypertension (4/20, 20%), and trauma (1/20, 5%). Fifteen patients (75%) received hemorrhage targeted treatment and 5 (25%) withdrew treatment. The mean NIHSS score was 10.25 ± 7.59 (range 2-25) on the day of hemorrhage onset. On the 30th day after hemorrhage, 7 patients (35%) were completely independent, 5 (25%) were partially independent, 4

Table 1 Demographics of colorectal cancer patients with intracerebral hemorrhage

Characteristics	mean ± SD/*n* (%)
Age, yr	68.45 ± 10.66
Gender	
Male	13 (65)
Female	7 (35)
Vascular risk factors	10 (50)
Tobacco	6 (30)
Hypertension	6 (30)
Diabetes mellitus	2 (10)
Hypercholesterolemia	3 (15)
Alcohol abuse	1 (5)
Stroke history	2 (10)
Coronary or kidney disease	1 (5)
Coagulopathy	10 (50)
PLT < 100 × 10^9/L	6 (30)
PT > 15 s	6 (30)
APTT > 45 s	2 (10)
INR > 1.5	3 (15)
DIC	1 (5)
Elevated cancer biomarkers	17 (85)
CEA > 5 ng/mL	16 (80)
CA125 > 35 U/mL	4 (20)
CA153 > 31.3 U/mL	2 (10)
CA199 > 37 U/mL	9 (45)
Hepatic dysfunction	
Yes	6 (30)
No	14 (70)
Distant metastasis	
Intracranial metastasis	10 (50)
Hepatic/osseous metastasis	8 (40)
Metastasis of other organs	7 (35)
None	6 (30)
Cancer treatment before hemorrhage	
Surgery alone	2 (10)
Chemotherapy	3 (15)
Both of the above	8 (40)
No treatment	7 (35)

PLT: Platelet; PT: Prothrombin time; APTT: Activated partial thromboplastin time; INR: International normalized ratio; DIC: Disseminated intravascular coagulation; CEA: Carcinoembryonic antigen; CA125: Carbohydrate antigen 125; CA153: Carbohydrate antigen 153; CA199: Carbohydrate antigen 199.

(20%) were completely dependent, and 4 (20%) died (Table 2). Among the 4 patients who died, the cause of death was fatal ICH induced by DIC in one patient, brain metastasis and intratumoral hemorrhage in one patient, and aspiration pneumonia in

Table 2 Data on intracerebral hemorrhage

Characteristics	*n* (%)
Location of hemorrhage	
Lobe	13 (65)
Basal ganglia	4 (20)
Brainstem	1 (5)
Cerebellum	2 (10)
Intratumoral hemorrhage	10 (50)
Treatment of hemorrhage	
Conservative treatment	14 (70)
Surgery	1 (5)
Withdrawal of treatment	5 (25)
NIHSS score on the day of hemorrhage onset	
NIHSS (0-5)	7 (35)
NIHSS (6-10)	5 (25)
NIHSS (11-20)	3 (15)
NIHSS (> 20)	5 (25)
mRS score 30 d after hemorrhage	
Completely independent (mRS = 0-1)	7 (35)
Partially independent (mRS = 2-3)	5 (25)
Completely dependent (mRS = 4-5)	4 (20)
Death (mRS = 6)	4 (20)
Etiology	
Intratumoral hemorrhage	10 (50)
Coagulopathy	10 (50)
Both of the above	5 (25)
Hypertension	4 (20)
Trauma	1 (5)
Time interval between CRC diagnosis and hemorrhage	
ICH onset before cancer diagnosis	2 (10)
ICH onset after cancer diagnosis	
< 6 mo	8 (40)
6-12 mo	5 (25)
1-5 yr	4 (20)
5-10 yr	1 (5)

ICH: Intracerebral hemorrhage; CRC: Colorectal cancer; NIHSS: National Institute of Health Stroke Scale; mRS: Modified Rankin Scale.

the other two patients (Figure 2).

DISCUSSION

ICH is a well-known potential complication of cancer[1,2]. A nationwide follow-up study from Sweden reported that compared with a non-cancer population, the overall risk of hemorrhage after diagnosis of cancer within 6 mo, 6 mo to 1 year and 1 year to

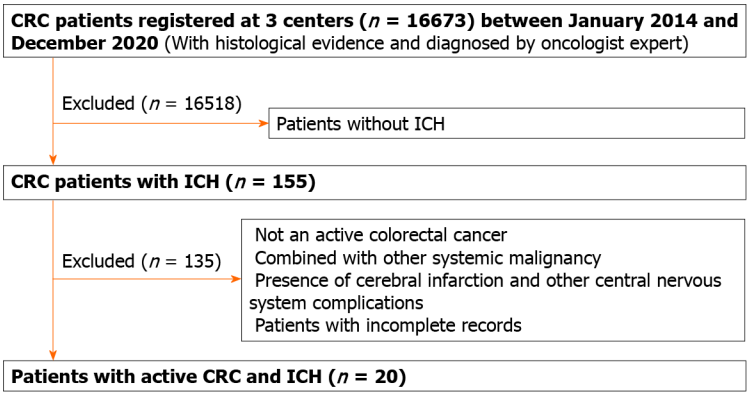

Figure 1 Patient enrollment flowchart. CRC: Colorectal cancer; ICH: Intracerebral hemorrhage.

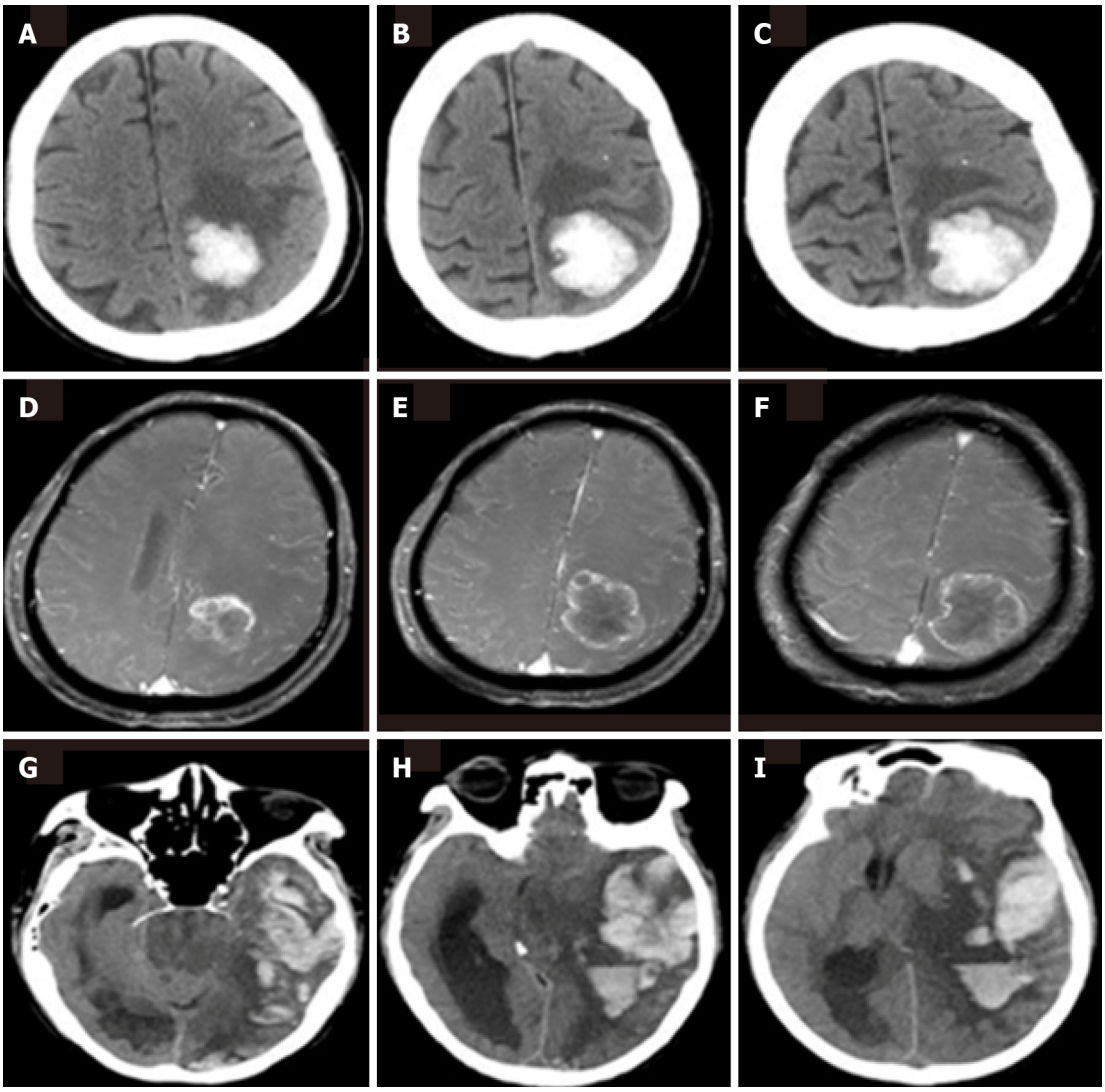

Figure 2 Neuroimaging findings. A-F: Neuroimages of a 76-year-old patient with active colorectal cancer (CRC). Brain computed tomography axial views, showing a hemorrhagic lesion in the left parietal lobe (A-C); brain enhanced magnetic resonance views, showing a metastatic tumor in the same location (D-F); G-I: Neuroimages of a 65-year-old patient with active CRC and disseminated intravascular coagulation, showing a massive hemorrhage in the left temporal and parietal lobe.

5 years was 2.2, 1.4 and 1.3, respectively[6], indicating that cancer could increase the incidence of ICH and that cancer-related ICH theoretically exists. Patients with CRC also had a significantly increased risk of hemorrhage[6]. CRC-related ICH may also theoretically exist. In this study, although only 0.12% of CRC patients developed ICH,

the incidence of hemorrhage was still much higher than that in the general population. Traditional pathogenesis were observed in the etiology of ICH in CRC patients, but more attention should be paid to the direct or indirect role of CRC itself.

In a retrospective review, cancer patients with ICH were more likely to present with coagulopathy, a lobar hemorrhage location, and poor short-term prognosis when compared with non-cancer patients[14]. In the present study, hemorrhagic lesions were more frequent in the cerebral lobe, occurring in 13 of 20 patients, and the short-term prognosis was poor, as disability and death occurred in 8 of these 20 patients within 30 d after hemorrhage onset. Coagulopathy was identified in half of the patients. In some respects, the results of this study were consistent with the research stated above. In addition, most patients developed distant metastases and had significantly elevated cancer biomarkers when ICH occurred, which was another characteristic in CRC patients with ICH.

Although ICH is a late complication in most cancer patients, it may precede cancer diagnosis. It is worth noting that 2 patients were newly diagnosed with CRC during hospitalization for ICH, suggesting that ICH might be the first manifestation of CRC. Cancers with ICH as the initial symptom include choriocarcinoma, leukocythemia, and medulloblastoma[15-17]. How to quickly identify insidious cancer in patients with ICH is still a challenge. Therefore, it is necessary to conduct cancer associated examinations, including CRC associated examinations, in patients with unexplained hemorrhage.

ICH in patients with cancer often arises from unique mechanisms which are uncommon in the general population. A clinical series of 208 cancer patients with intracranial hemorrhage found that intratumoral hemorrhage and coagulopathy were the main causes, and that hypertension, the most common cause of ICH in the population, accounted for only a small proportion[2]. In the present study, ICH was caused by hypertension in a small number of patients but was mostly caused by intratumoral hemorrhage and coagulopathy, which was similar to the above results, indicating that intratumoral hemorrhage and coagulopathy might be the main pathogenesis of ICH in patients with CRC.

Pathophysiologically, factors favoring intratumoral hemorrhage include tumor necrosis, aberrant neovascularization, vascular infiltration with rupture, imbalances in the fibrinolytic cascade, and overexpression of vascular endothelial growth factor (VEGF) and metalloproteinases[1-2,18,19]. Tumor cells can directly invade blood vessels and destroy their integrity, resulting in vascular rupture. Overexpression of VEGF in tumor cells can stimulate neovascularization and increase microvessel density. However, the fine structure of newly formed blood vessels induced by VEGF tends to have high permeability and fragility leading to hemorrhagic events[19]. Abnormal high expression of metalloproteinases can degrade extracellular matrix proteins which maintain basement membrane structural/functional integrity, and cause considerable damage to capillary integrity leading to hemorrhage[19]. Overexpression of VEGF in CRC cells has been confirmed, and anti-VEGF therapies have been proved to be effective for metastatic CRC[20,21]. The expression and activity of metalloproteinases are high in CRC cells[22], and matrix metalloproteinase inhibitors might be a new way of treating metastatic CRC.

Coagulopathy is typically related to a complex interplay of multiple mechanisms, including thrombocytopenia, coagulation factor abnormalities or both. The etiology of thrombocytopenia can be diverse and multifactorial in cancer patients. Systemic chemotherapy is the most common cause of bone marrow suppression and thrombocytopenia[23]. In patients with solid tumors, thrombocytopenia is potentially associated with bone marrow metastasis of malignant cells and secondary myelofibrosis [24]. Metastatic malignant cells can interfere with the hematopoietic microenvironment and inhibit hematopoiesis. Myelofibrosis may be induced by a series of cytokines secreted by immune system cells as part of the inflammatory response to tumors. In addition, DIC, thrombotic thrombocytopenic purpura, immune-mediated thrombocytopenia and heparin-induced thrombocytopenia all contribute to increased platelet destruction in cancer patients[24]. In this study, some patients had been treated with chemotherapy before hemorrhage, and hence it is reasonable to consider chemotherapy as a significant risk factor. Some patients had osseous metastases, and it was speculated that their thrombocytopenia was related to bone marrow metastasis. Abnormalities in coagulation factors generally result from hepatic dysfunction, vitamin K deficiency or DIC. Hepatic dysfunction in some patients may be related to a primary or metastatic liver tumor, and chemotherapy. The production of coagulation factors may be affected in patients with obvious hepatic dysfunction, which may lead to a deficiency in coagulation factors. Patients with advanced cancer often suffer from severe malnutrition due to insufficient food intake, and the shortage of multiple

nutrients, especially vitamin K, further aggravates their coagulopathy. DIC is the most serious form of coagulopathy. The combination of excessive consumption of coagulation factors and platelets and primary fibrinogenolysis account for the high risk of bleeding during the pathological process of DIC. It is reported that CRC can also present with DIC during treatment[25,26]. In the present study, some patients had liver metastases, obvious hepatic dysfunction and poor nutrition, which may have resulted in coagulation factor deficiency. One patient developed DIC during treatment, resulting in fatal ICH.

CA125, CA153, CA199 and CEA are widely used as common cancer biomarkers in the clinic, and are well known carcinoma mucins. Previous studies revealed that carcinoma mucins were overexpressed by malignant cells and were shown to play a multifaceted role in the initiation, progression, metastasis and subsequent colonization of multiple malignancies[27,28]. In a clinical study of lung cancer-related ICH, elevated plasma CEA and CA199 Levels were independent risk factors for ICH in patients with active lung cancer[13]. The researchers hypothesized that elevated cancer markers could activate platelets and lead to increased platelets, hypercoagulability, and eventually thrombotic events in the early stage of cancer; however, in the later stage, elevated cancer markers could lead to decreased platelets due to consumption, coagulopathy, and finally hemorrhagic events. Significantly elevated cancer biomarkers were observed in most patients in this study, but the role of these biomarkers in ICH requires further research.

The pathogenesis of ICH induced by CRC is complex and has not been fully elucidated. To date, there is no feasible method to effectively prevent and treat ICH in patients with CRC. In view of the pathophysiological changes such as thrombocytopenia and abnormalities of coagulation factors, it is possible to take active measures, including increasing nutrition supply, and supplementing platelets and fresh plasma, to improve the coagulation state in the early stage. These measures may delay or reduce the occurrence of ICH in patients with CRC, but should be confirmed in future studies.

One of the limitations of this study is its retrospective nature; thus, information bias may exist. Although clinical data were collected from three centers, the total number of selected cases was relatively small. Prospective population-based cohort studies are warranted to better elucidate the clinical characteristics and pathogenesis of CRC patients with ICH.

CONCLUSION

Patients with ICH and CRC often have clinical features with lobar hemorrhage, distant metastases and poor prognosis. Intratumoral hemorrhage and coagulopathy are the main causes of ICH in patients with CRC. More clinical trials are needed to validate these findings in the future.

ARTICLE HIGHLIGHTS

Research background

Many studies have confirmed that cancer can increase the risk of intracerebral hemorrhage (ICH). However, most previous studies were conducted on multiple cancers, and few focused on a specific cancer. The clinical characteristics and mechanisms of ICH in colorectal cancer (CRC) patients have not been fully elucidated.

Research motivation

There are few reports on hemorrhagic cerebrovascular events in patients with CRC.

Research objectives

This retrospective study aimed to investigate the clinical features and underlying pathogenesis of ICH in patients with CRC.

Research methods

A retrospective review of 20 patients (13 males and 7 females) with CRC and ICH from three centers between January 2014 and December 2020 was conducted. The clinical data of the patients such as vascular risk factors, laboratory results, neuroimaging and

underlying pathogenesis were analyzed.

Research results

The average age (mean ± SD) of the patients was 68.45 ± 10.66 years. Fourteen patients (70%) had distant metastases and most patients (85%) had an elevation of one or more cancer biomarkers. The hemorrhagic lesions in 13 patients (65%) were in the intracerebral lobe. Four patients were completely dependent and 4 died within 30 days after hemorrhage. Intratumoral hemorrhage (50%) and coagulopathy (50%) accounted for the majority of hemorrhages.

Research conclusions

Patients with ICH and CRC often have clinical features with lobar hemorrhage, distant metastases and poor prognosis. Intratumoral hemorrhage and coagulopathy are the main causes of ICH in patients with CRC.

Research perspectives

The detailed mechanism of ICH in CRC patients requires further elucidation. Prospective population-based studies are needed to confirm these findings in the future.

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ORIGINAL ARTICLE

Prospective Study

Anatomic resection improved the long-term outcome of hepatocellular carcinoma patients with microvascular invasion: A prospective cohort study

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Author contributions: Zhou JM analyzed and interpreted the patient data and wrote the manuscript; Zhou CY managed the patients, including recruiting patients, performing operations, and following-up with the patients; Zhang ZW and Chen XP designed the experiment and modified the manuscript; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

The long-term effect of anatomic resection (AR) is better than that of non-anatomic resection (NAR). At present, there is no study on microvascular invasion (MVI) and liver resection types.

AIM

To explore whether AR improves long-term survival in patients with hepatocellular carcinoma (HCC) by removing the peritumoral MVI.

METHODS

A total of 217 patients diagnosed with HCC were enrolled in the study. The surgical margin was routinely measured. According to the stratification of different tumor diameters, patients were divided into the following groups: ≤ 2 cm group, 2-5 cm group, and > 5 cm group.

RESULTS

In the 2-5 cm diameter group, the overall survival (OS) of MVI positive patients was significantly better than that of MVI negative patients (P = 0.031). For the MVI positive patients, there was a statistically significant difference between AR and NAR (P = 0.027). AR leads to a wider surgical margin than NAR (2.0 ± 2.3 cm *vs* 0.7 ± 0.5 cm, P < 0.001). In the groups with tumor diameters < 2 cm, both AR and NAR can obtain a wide surgical margin, and the surgical margins of AR are wider than that of NAR (3.5 ± 5.8 cm *vs* 1.6 ± 0.5 cm, P = 0.048). In the groups with tumor diameters > 5 cm, both AR and NAR fail to obtain wide surgical margin

original anonymous dataset is available on request from the corresponding author at zhiweizhangtjh@163.com.

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(0.6 ± 1.0 cm *vs* 0.7 ± 0.4 cm, $P = 0.491$).

CONCLUSION
For patients with a tumor diameter of 2-5 cm, AR can achieve the removal of peritumoral MVI by obtaining a wide incision margin, reduce postoperative recurrence, and improve prognosis.

Key Words: Microvascular invasion; Hepatocellular carcinoma; Anatomic resection; Surgical margin; Recurrence; Surgery

Core Tip: The prognosis of anatomic resection is better than that of non-anatomic resection with diameters from 2 to 5 cm. For tumor diameters smaller than 2 cm and larger than 5 cm, anatomic resection is not superior to non-anatomic resection. Anatomic resection can achieve the removal of peritumoral microvascular invasion by obtaining a wide incision margin. Both anatomic resection and non-anatomic resection can obtain wide surgical margins in the group with tumor diameters smaller than 2 cm. Both anatomic resection and non-anatomic resection failed to obtain wide surgical margins in the diameter larger than 5 cm group.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and its high mortality makes it the second leading cause of cancer death[1]. Although the poor prognosis of HCC has improved significantly over the last decade due to increased knowledge of HCC behavior, improvements in staging systems, and multiple therapeutic options compared with other malignancies, HCC still has a high mortality rate[2]. The prognosis of HCC remains very poor due to the high incidence of recurrence and metastasis, and the 5-year recurrence rate after curative treatment remains high (70%), with 15% of HCC patients developing extrahepatic metastasis[3]. One important reason is that tumor cells are able to penetrate the microvasculature, disseminate through the bloodstream to other sites, and form metastatic tumors. Studies have suggested that microvascular invasion (MVI) in HCC is one of the most significant risk factors for recurrence and metastasis in HCC following curative surgical resection[4]. MVI is defined as clusters of cancer cells observed microscopically in vessels located in the tumor capsule and surrounding liver parenchyma[5]. Previous research reported that the incidence of MVI ranged from 15% to 57% in HCC specimens and was associated with tumor size, levels of alpha fetoprotein (AFP), and typical image features[6]. Even for patients with HCC, the presence of MVI increases the risk of recurrence and dramatically shortens long-term survival[7,8]. The main reason for this is that the residual microthrombosis results in early recurrence. A safe surgical margin is a prerequisite for the complete removal of residual microtumor thrombosis. In HCC, invasion of the portal vein and intrahepatic and distant metastases are frequently observed. Resection of the portal vein invaded by the tumor is one method to decrease the risk of recurrence. Previous research has reported that both anatomic resection (AR) and non-anatomic resection (NAR), with a sufficient margin, can reduce the risk of early recurrence and improve the prognosis of HCC[9,10]. A meta-analysis has shown that anatomical hepatectomy is more effective than non-anatomical hepatectomy[11]. We defined AR as the complete removal of at least one Couinaud segment containing the focus and the portal vein in the drainage area of the lesion. A complete tumor plus the rim of non-neoplastic liver parenchyma was considered a non-anatomic resection[12]. Studies have shown that although the width

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of the resection margin does not influence the postoperative recurrence rates after liver resection, a wide margin is associated with a lower recurrence risk in patients with venous invasion or microsatellites[12,13]. Studies have shown that not all patients are suitable for AR, and controversy remains over the superiority of AR compared to NAR. In general, AR guarantees a wider surgical margin. However, a wider surgical margin means that more healthy liver tissue has to be removed. Almost all patients with HCC have liver cirrhosis, and the excessive removal of non-neoplastic liver parenchyma can lead to liver dysfunction and the morbidities of ascites, jaundice, and hypoalbuminemia. When the tumor is enormous and the remaining liver tissue is insufficient, AR may not be appropriate[14]. When the liver volume is insufficient, a surgical margin of at least 5 mm should be secured by NAR whenever possible[15]. Therefore, NAR still plays an important role in hepatectomy. Previous studies have shown that AR should be performed when the size of HCC ranges from 2 to 5 cm[10]. However, whether AR should be recommended when the diameter is less than 2 cm or greater than 5 cm remains controversial. In addition, whether anatomical hepatectomy improves the prognosis of patients with hepatocellular carcinoma with microvascular invasion regardless of tumor size is unclear. Therefore, the aim of the study is to determine whether AR improves long-term survival in patients with hepatocellular carcinoma (HCC) by removing microvascular invasion (MVI).

MATERIALS AND METHODS

Patient selection

We consecutively enrolled 217 patients who underwent AR or NAR from November 2016 to November 2018 at the Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The surgical margin was routinely measured. A pathological specimen of each patient was promptly sent to the pathology department and a detailed pathology report was issued. The pathological characteristics of our data, such as MVI, were derived from the report. The flow diagram of the enrolled patients is displayed in Figure 1. According to the stratification of different tumor diameters, patients were divided into the following groups: ≤ 2 cm group, 2-5 cm group, and > 5 cm group.

Patient eligibility

The inclusion criteria were: (1) Definitive pathological diagnosis of HCC based on the World Health Organization criteria; (2) Curative resection, defined as complete macroscopic removal of the tumor with negative (R0) margins; (3) No prior anticancer treatment; and (4) Aged between 18 and 80 years. The exclusion criteria were: (1) Distant metastasis; (2) Portal vein tumor thrombosis (PVTT); and (3) Child–Pugh C liver disease. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology, and the number of the approval was TJ-IRB20181101. Informed consent was obtained from each patient included in the study.

Terminology

Completely removing at least one Couinaud segment containing the focus and portal vein in the drainage area of the lesion was defined as an AR. A complete tumor plus a rim of non-neoplastic liver parenchyma was considered an NAR.

Surgical procedure

All surgeries were accomplished by a team who was able to professionally implement a hepatectomy. Patients were placed in supine position and under general anesthesia. The surgical principles were followed according to the corresponding the Union for International Cancer Control TNM classification. Intraoperative ultrasonography was routinely used in all patients to assess the number and size of the tumors, and their relation to nearby vascular structures. Proper hepatic vascular control techniques, including the selective inflow occlusion (SIO) maneuver and intermittent Pringle maneuvers (IPs), were used to reduce bleeding during liver resection. The SIO maneuver is described by the following procedure: dissecting the portal vein, proper hepatic artery, right and left hepatic arteries, and bile ducts followed by continuously blocking the hepatic artery in the tumor bearing lobe with a bulldog clamp. IPs encircling the hepatoduodenal ligament were performed with cycles of clamping and

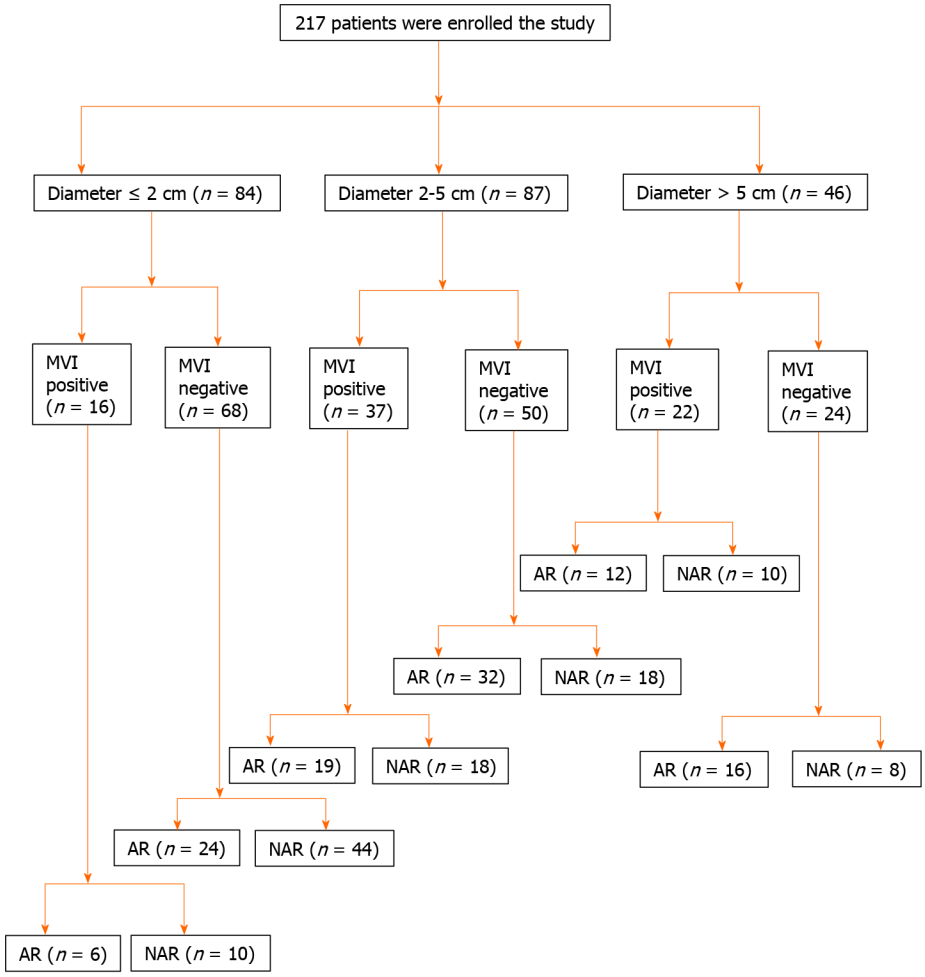

Figure 1 Flow diagram for the study. AR: Anatomic resection; MVI: Microvascular invasion; NAR: Non-anatomic resection.

unclamping times of 15 min and 5 min, respectively.

Follow-up and tumor recurrence

The patients were surveilled every 1 mo with ultrasonography and AFP during the first 6 mo after surgery and every 3 mo thereafter. Patients were scheduled to have a computerized tomography (CT) scan every 6 mo and a magnetic resonance imaging (MRI) every year. Recurrence was diagnosed by computed tomography scans, magnetic resonance imaging, digital subtraction angiography, and elevated serum AFP level. We reviewed the governmental death registration and performed telephone follow-ups. Patients were excluded if they were not followed up as required, or their governmental data were incomplete. Follow-up was terminated on May 31, 2021. Patients lost to follow-up and with missing data were prematurely excluded. Ultimately, 217 eligible patients were enrolled in the study. Death was the primary endpoint and the diagnosis of intrahepatic recurrence and/or extrahepatic metastasis was the secondary endpoint.

Study design

First, all patients were randomized to receive standard anatomic or non-anatomic resection. After surgery, we measured the surgical margin and identified microvascular invasion. We divided them into three groups based on tumor size to compare the effects of anatomic and nonanatomic hepatectomy in subgroups. In addition, the relationship between tumor size and surgical margin was further analyzed.

Statistical analysis

Data are presented as the mean ± SD. The overall survival (OS) was analyzed using Kaplan-Meier survival curves and a log-rank test. Student's *t*-tests were used for comparison between groups where appropriate. A χ^2 test was used for comparison between groups where appropriate. $P < 0.05$ was considered statistically significant.

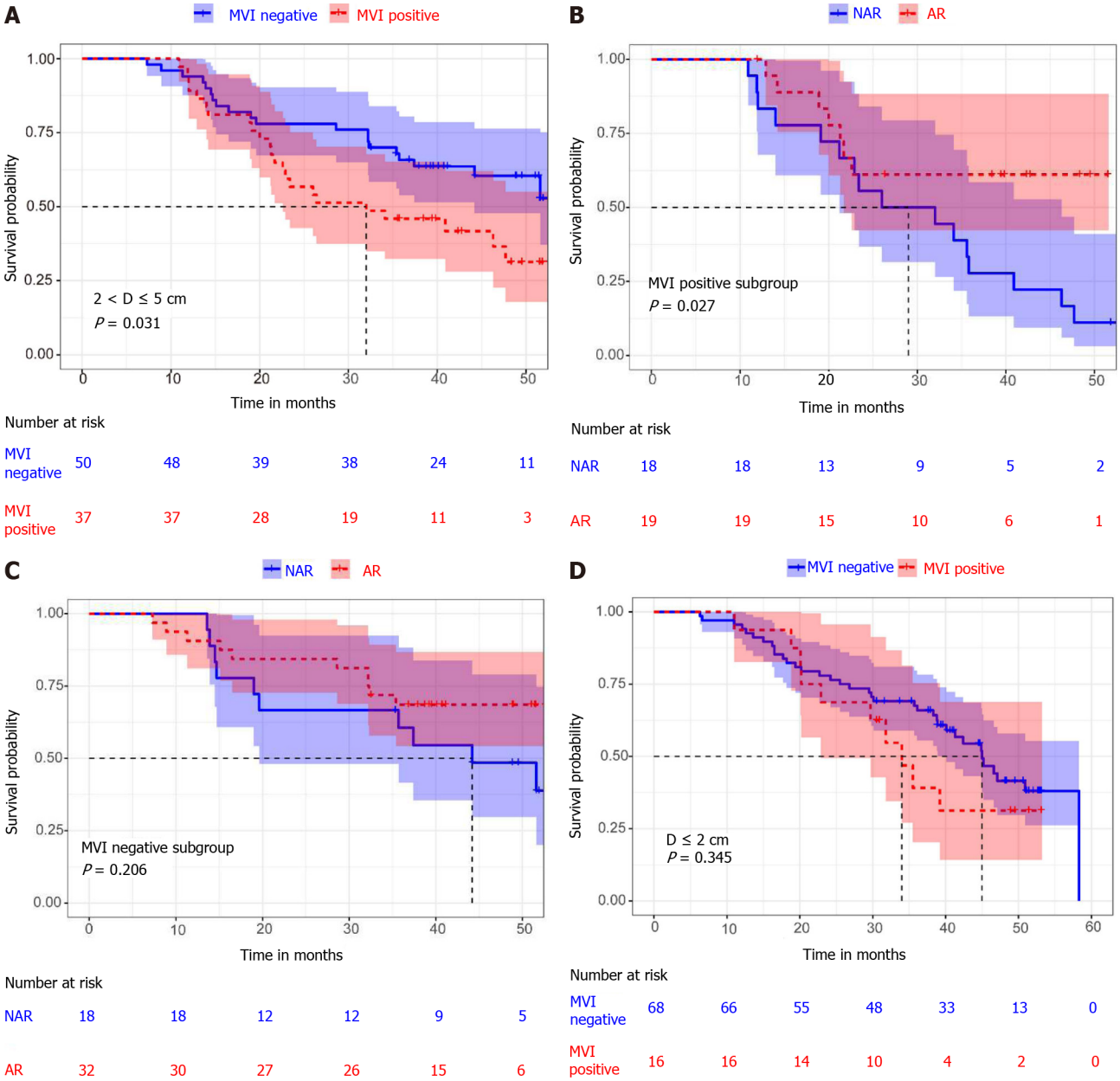

Figure 2 The long-term outcome of hepatocellular carcinoma with different tumor diameters. A: Kaplan-Meier analysis of the overall survival (OS) in microvascular invasion (MVI) positive and MVI negative patients with a tumor diameter 2-5 cm; B: In the group with a tumor diameter of 2-5 cm, Kaplan-Meier analysis of the OS in receiving MVI positive anatomic resection (AR) and non-anatomic resection (NAR) patients; C: In the group with a tumor diameter of 2-5 cm, Kaplan-Meier analysis of the OS in receiving AR and NAR patients with MVI negative. D: Kaplan-Meier analysis of the OS in MVI positive and MVI negative patients with a tumor diameter of less than 2 cm.

Statistical analyses were performed with SPSS version 19.0.

RESULTS

Patient characteristics

The mean follow-up time was 45.2 ± 6.3 mo (median: 46.0 mo; range: 30.6-53.4 mo). The cumulative survival rate for all patients was 90%, 57%, and 39% at 1, 3, and 5 years. All of the category boundaries were defined by the clinical guideline or recognized criterion when continuous variables were categorized. Table 1 demonstrates the clinical and tumor characteristics of the 217 patients with HCC. The mean patient age was 52.6 ± 12.4 years (range: 21–74 years). The patients were 90.8% (197/217) male and 9.2% (20/217) female. In total, 86.6% (178/217) were positive for hepatitis B virus (HBV) infection and 10 patients were positive for the hepatitis C virus (HCV). Overall, 42.4% (92/217) were Barcelona Clinic Liver Cancer (BCLC) 0+A, and

Table 1 Clinical characteristics of 217 patients

Clinical characteristics	Value
Age, yr, (mean ± SD)	52.6 ± 12.4
Sex, *n* (%)	
Male	197 (90.8)
Female	20 (9.2)
ALT (U/mL)	30.2 ± 14.3
AST (U/mL)	38.6 ± 32.0
TBiL (μmol/L)	15.8 ± 9.6
Child-Pugh score, *n* (%)	
A	209 (96.3)
B	8 (3.7)
Hepatitis virus, *n* (%)	
HBV	178 (82.0)
HCV	10 (4.6)
No	29 (13.4)
Liver cirrhosis, *n* (%)	
No	43 (19.8)
Yes	174 (80.2)
ICG-R15 (%)	6.6 ± 3.8
AFP (ng/mL), *n* (%)	
≤ 400	144 (66.4)
> 400	73 (33.6)
Tumor diameter (cm)	4.9 ± 3.5
No. of tumor, *n* (%)	
Singe	157 (72.4)
Multiple	60 (27.6)
Microvascular invasion, *n* (%)	
Yes	75 (34.6)
No	142 (65.4)
BCLC stage, *n* (%)	
0+A	92 (42.4)
B+C	125 (57.6)
Operation method, *n* (%)	
Open	56 (25.8)
Laparoscopic	161 (74.2)
Operation time (min)	171 ± 40
Blood loss (mL)	160 ± 180
Blood transfusion, *n* (%)	
No	189 (87.1)
Yes	28 (12.9)
Hepatic vascular occlusion, *n* (%)	
No	64 (29.5)

Yes	153 (70.5)

AFP: Alpha fetoprotein; ALT: Alanine transaminase; AST: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer staging system; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ICG R15 (%): Indocyanine green retention rate at 15 min; SD: Standard deviation; TBiL: Total bilirubin.

34.6% (75/217) were MVI positive. Eight patients (3.7%) had a hepatic function of Child-Pugh score B and received short-term liver protective therapy before surgery. The clinical and pathological characteristics of the AR and NAR groups are summarized in Table 2. There were no significant differences in age, sex, Child-Pugh class, *etc.* between the two groups.

Comparison of types of hepatectomy with prognosis in the 2-5 cm tumor diameter subgroup

The 217 included patients were divided into three groups: diameter ≤ 2 cm (84/217), 2-5 cm (87/217) and > 5 cm (46/217). In the diameter 2-5 cm group, there was a statistically significant difference between MVI positive and MVI negative patients (median OS 32.0 mo, 95% confidence interval (CI): 13.3-50.8 mo *vs* not reached, $P = 0.031$) (Figure 2A). For the MVI positive patients, there was a statistically significant difference between AR and NAR (median OS not reached *vs* 29.0 mo 95%CI: 8.0-43.9 mo, $P = 0.027$) (Figure 2B). However, for the MVI negative patients, there were no statistically significant differences between those who underwent AR and NAR (median OS not reached *vs* 44.2 mo, 95%CI: 27.0-61.5 mo, $P = 0.206$) (Figure 2C). This suggests that AR improved OS only in MVI positive patients, but not in MVI negative patients in the diameter 2-5 cm group. In addition, we compared the surgical margin of AR and NAR in the 2-5 cm diameter group. We found that AR led to a wider surgical margin than NAR (2.0 ± 2.3 cm *vs* 0.7 ± 0.5 cm, $P < 0.001$) (Table 3). We speculated that AR can achieve a wide enough surgical margin, which can remove MVI in advance, reducing the risk of postoperative recurrence and improving the prognosis.

Comparison of types of hepatectomy with prognosis in the subgroup with a tumor diameter less than 2 cm

Among patients with a diameter less than 2 cm, there was no statistically significant difference between MVI positive and MVI negative patients (median OS 34 mo 95%CI: 27.6-40.4 mo *vs* 45.0 mo 95%CI: 39.4-50.6 mo, $P = 0.345$) (Figure 2D). In addition, there was no statistically significant difference in overall survival between patients who received AR and NAR, whether MVI positive or MVI negative (median OS 32.4 mo 95%CI: 13.0-46.3 mo *vs* not reached, $P = 0.097$; median OS 46.6 mo, 95%CI: 32.8-60.4 mo *vs* 41.3 mo, 95%CI: 34.6-48.1 mo, $P = 0.869$) (Figure 3A and B). By comparing the surgical margins of AR and NAR patients, we found that although the AR margins were wider than those of NAR patients, the margins of both were greater than 1 cm (3.5 ± 5.8 cm *vs* 1.6 ± 0.5 cm, $P = 0.048$) (Table 3; Figure 4B). If a surgical margin of 1 cm is ensured, it can be clinically regarded as a R0 resection. Thus, for patients with a tumor of less than 2 cm in diameter, both AR and NAR can achieve a wide surgical margin to ensure the removal of MVI.

Comparison of types of hepatectomy with prognosis in the subgroup with a tumor diameter larger than 5 cm

In the group with a diameter > 5 cm, the prognosis of MVI positive patients was significantly worse than that of MVI negative patients (median OS 24.0 mo, 95%CI: 15.7-32.4 mo *vs* not reached, $P = 0.004$) (Figure 3C). However, there was no statistically significant difference in overall survival between patients who received AR and NAR, whether MVI positive or MVI negative, (median OS 27.2 mo, 95%CI: 21.9-32.5 mo *vs* 20.1 mo, 95%CI: 5.6-28.8 mo, $P = 0.428$; median OS not reached *vs* 38.3 mo, 95%CI: 19.5-60.5 mo, $P = 0.714$) (Figures 3D and 4A). In addition, there were no statistically significant differences between AR and NAR in surgical margins and the margins of both were less than 1 cm (0.6 ± 1.0 cm *vs* 0.7 ± 0.4 cm, $P = 0.491$) (Table 3; Figure 4B). For patients with tumors larger than 5 cm in diameter, neither AR nor NAR could obtain a wide enough surgical margin to ensure the removal of MVI.

Table 2 Clinical characteristics of anatomic resection and non-anatomic resection

Clinical characteristics	AR (*n* = 103)	NAR (*n* = 114)	*P* value
Age, yr (mean ± SD)	54.1 ± 12.5	51.3 ± 12.2	0.095
Sex, *n* (%)			0.812
Male	93 (90.3)	104 (91.2)	
Female	10 (9.7)	10 (8.8)	
ALT (U/mL)	30.5 ± 14.5	29.8 ± 14.2	0.721
AST (U/mL)	36.3 ± 28.9	40.6 ± 34.6	0.332
TBiL (μmol/L)	17.0 ± 10.5	15.9 ± 9.0	0.098
Child-Pugh score, *n* (%)			0.154
A	103 (100)	106 (93.0)	
B	0 (0)	2 (7.0)	
HBsAg, *n* (%)			0.622
Positive	88 (85.4)	100 (87.7)	
Negative	15 (14.6)	14 (12.3)	
Liver cirrhosis, *n* (%)			0.841
No	21 (20.4)	22 (19.3)	
Yes	82 (79.6)	92 (80.7)	
AFP (ng/mL), *n* (%)			0.446
< 400	71 (68.9)	73 (64.0)	
≥ 400	32 (31.1)	41 (36.0)	
ICG-R15 (%)	5.9 ± 3.5	5.4 ± 3.1	0.578
BCLC stage, *n* (%)			0.867
0 + A	46 (44.7)	46 (40.4)	
B + C	57 (55.3)	68 (59.6)	
Operation method, *n* (%)			0.423
Open	24 (23.3)	32 (28.1)	
Laparoscopic	79 (76.7)	82 (71.9)	
Operation time (min)	171.4 ± 48.2	165.5 ± 45.3	0.335
Blood loss (mL)	210 ± 233	170 ± 175	0.233
Blood transfusion, *n* (%)			0.774
No	89 (86.4)	100 (87.7)	
Yes	14 (13.6)	14 (12.3)	
Hepatic vascular occlusion, *n* (%)			0.314
No	27 (26.2)	37 (32.5)	
Yes	76 (73.8)	77 (67.5)	
Largest tumor size, (cm)	5.1 ± 3.6	4.7 ± 3.3	0.507
No. of tumors, *n* (%)			0.884
Single	75 (72.8)	82 (71.9)	
Multiple	28 (27.2)	32 (28.1)	
Tumor encapsulation, *n* (%)			0.051
No	57 (55.3)	66 (57.9)	
Yes	46 (44.7)	48 (42.1)	

Satellite lesion, *n* (%)			0.822
Yes	9 (8.7)	9 (7.9)	
No	94 (91.3)	105 (92.1)	
Tumor differentiation stage, *n* (%)			0.943
Edmondson I II	51 (49.5)	57 (50.0)	
Edmondson III IV	52 (50.5)	57 (50.0)	
Microvascular invasion, *n* (%)			0.845
Yes	37 (35.9)	38 (33.3)	
No	66 (64.1)	76 (66.7)	

AFP: Alpha fetoprotein; ALT: Alanine transaminase; AST: Aspartate aminotransferase; AR: Anatomic resection; BCLC: Barcelona Clinic Liver Cancer staging system; HBsAg: Hepatitis B surface antigen; ICG R15 (%): Indocyanine green retention rate at 15 min; NAR: Non-anatomic resection; TBiL: Total bilirubin.

Table 3 The surgical margin of different tumor diameter groups

Tumor diameter	Surgical margin (cm)		
	AR	NAR	*P* value
D ≤ 2 cm	3.5 ± 5.8	1.6 ± 0.5	0.048
2 cm < D ≤ 5 cm	2.0 ± 2.3	0.7 ± 0.5	< 0.001
D > 5 cm	0.6 ± 1.0	0.7 ± 0.4	0.491

AR: Anatomic resection; D: Diameter; NAR: Non-anatomic resection.

DISCUSSION

At present, surgeons consider hepatectomy and liver transplantation the optimal therapies to improve prognosis in HCC; however, tumor recurrence is still an important cause of death in patients[16]. Previous research has demonstrated that microvascular invasion is a vital risk factor for the prognosis of HCC patients after curative hepatectomy[17]. As long as a surgical margin of 1 cm is ensured, it can be clinically regarded as an R0 resection[18]. The margin of microvascular invasion is generally no more than 1 cm. Therefore, an R0 resection enables the complete removal of the liver tissue invaded by the microvascular thrombosis. In contrast, positive margins were associated with a worse prognosis[19,20]. Previous studies have shown that a wider surgical margin has been associated with a better prognosis among patients with HCC[21-23]. In addition, a previous study has shown that AR led to a better OS than NAR. In a multivariable analysis, an AR was one of the prognostic factors[9]. AR can reduce the risk of tumor residues and recurrence due to the elimination of venous tumor thrombosis within the resected domain, when at least one complete Couinaud segment and the portal vein in the drainage area of the lesion are removed[24,25]. However, almost all patients with HCC have liver cirrhosis and excessive removal of non-neoplastic liver parenchyma can lead to liver dysfunction and the morbidities of ascites, jaundice, and hypoalbuminemia.

Our data indicated that AR improved OS only in MVI positive patients, but not in MVI negative patients in the 2-5 cm diameter group. We speculated that AR can achieve a wide enough surgical margin, which can remove MVI in advance, reduce the risk of postoperative recurrence, and improve the prognosis. For patients with a tumor diameter of less than 2 cm, both AR and NAR can obtain a wide surgical margin to ensure removal of MVI. Therefore, patients with a diameter less than 2 cm, both AR and NAR, can achieve a good prognosis. In other words, an R0 resection enabled the removal of the liver tissue invaded by the MVI regardless of whether AR or NAR was chosen by the surgeon. This suggests that AR is not necessary for tumors with a diameter of less than 2 cm, as long as sufficient surgical margin is ensured. However, in the > 5 cm group, both AR and NAR cannot guarantee sufficient surgical margin, which is one of the reasons why tumors with a diameter of more than 5 cm have a

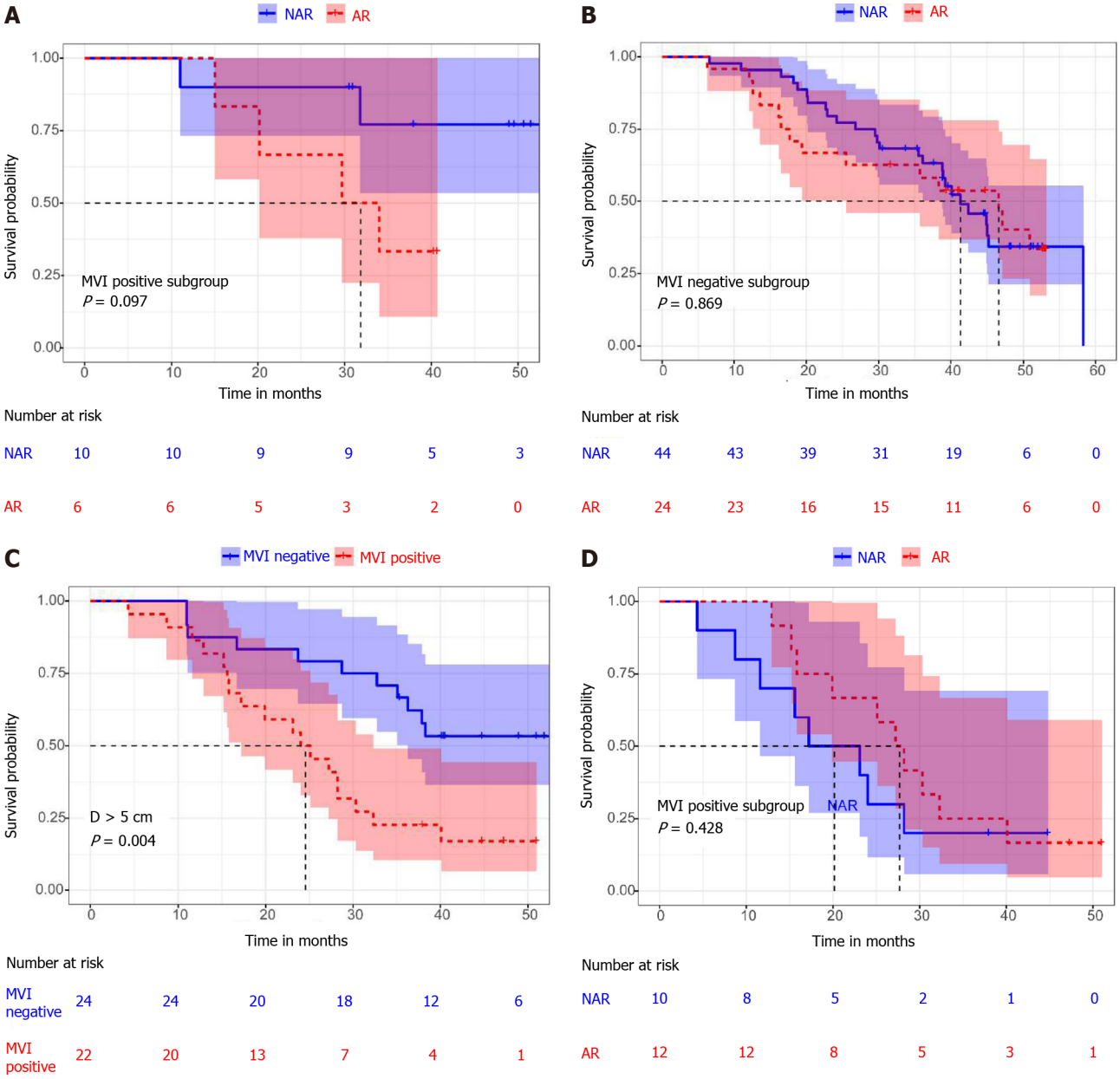

Figure 3 The long-term outcome of hepatocellular carcinoma with different tumor diameter. A: In the group with a tumor diameter of less than 2 cm, Kaplan-Meier analysis of the overall survival (OS) in receiving anatomic resection (AR) and non-anatomic resection (NAR) patients with microvascular invasion (MVI) positive; B: In the group with a tumor diameter of less than 2 cm, Kaplan-Meier analysis of the OS in patients receiving AR and NAR patients who were MVI negative; C: Kaplan-Meier analysis of the OS in MVI positive and MVI negative patients with tumor diameters larger than 5 cm; D: In the group with a tumor diameter of larger than 5 cm, Kaplan-Meier analysis of the OS in MVI positive patients receiving AR and NAR.

worse prognosis than tumors with a diameter of less than 2 cm. We believe that the surgeon needs to consider whether the residual liver volume and liver function reserve are sufficient when faced with a very large tumor. Therefore, for tumor diameters larger than 5 cm, the width of the resection margin should be increased appropriately when a sufficient liver volume and a good liver function can be ensured.

Limitations

The limitations of this study are the relatively small samples, short follow-up time, and a single study center cohort study. A multicenter clinical trial should be designed to further validate the prognostic significance of types of hepatectomy in HCC.

CONCLUSION

For patients with a tumor diameter of 2-5 cm, AR can achieve the removal of pe-

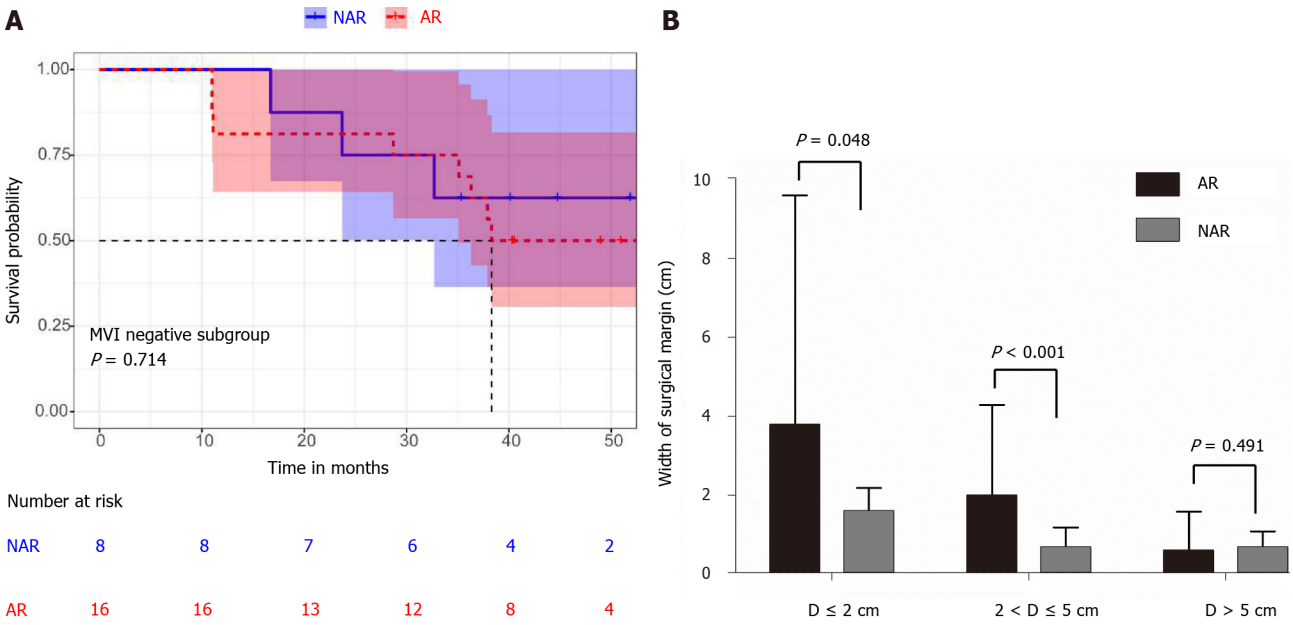

Figure 4 Kaplan-Meier analysis of the overall survival (A) and comparison of surgical margins in patients with different tumor diameters (B). A: In the group with a tumor diameter larger than 5 cm, Kaplan-Meier analysis of the overall survival in microvascular invasion negative patients receiving anatomic resection (AR) and non-anatomic resection (NAR); B: Comparison of surgical margins in patients receiving AR and NAR with different tumor diameters.

ritumoral MVI by obtaining a wide incision margin, reduce postoperative recurrence, and improve prognosis.

ARTICLE HIGHLIGHTS

Research background

At present, most studies suggest that anatomical resection is more effective than non-anatomical resection in the tumor diameter ranging from 2 cm to 5 cm. However, for tumors smaller than 2 cm and larger than 5 cm in diameter, the advantage of anatomic hepatectomy is not significant. Why is that? Does anatomic resection (AR) have an advantage over non-anatomic resection (NAR) in hepatocellular carcinoma (HCC) patients with microvascular invasion (MVI)?

Research motivation

Our study aimed to determine the effects of AR and NAR in different tumor diameter stratification. Further analysis shows that AR improves patient outcomes by obtaining a wider surgical margin.

Research objectives

This study compared the efficacy of AR and NAR in different tumor diameter subgroups in a prospective cohort study.

Research methods

First, all patients were randomized to receive standard anatomic or non-anatomic resection. After surgery, we measured the surgical margin and identified microvascular invasion. We divided them into three groups based on tumor size.

Research results

When the tumor is enormous and the remaining liver tissue is insufficient, AR may not be appropriate. For patients with a tumor diameter of 2-5 cm, AR can achieve the removal of peritumoral MVI by obtaining a wide incision margin, reducing postoperative recurrence and improving prognosis. For patients with a tumor of less than 2 cm in diameter, both AR and NAR can obtain a wide surgical margin to ensure the removal of MVI. AR should not be recommended for those patients. For patients with tumors larger than 5 cm in diameter, neither AR nor NAR could obtain a wide surgical margin to ensure removal of MVI.

Research conclusions

The doctor should ensure sufficient surgical margin on the premise of ensuring the safety of the operation. Therefore, for patients with a tumor diameter of 2-5 cm, AR should be strongly recommended.

Research perspectives

The study could guide doctors in their choice of surgical procedures. In general, AR guarantees a wider surgical margin. However, a wider surgical margin means that more healthy liver tissue has to be removed. Almost all patients with HCC have liver cirrhosis, and the excessive removal of non-neoplastic liver parenchyma can lead to liver dysfunction and the morbidities of ascites, jaundice, and hypoalbuminemia. When the tumor is enormous and the remaining liver tissue is insufficient, AR may not be appropriate.

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SYSTEMATIC REVIEWS

Minimally invasive surgical treatment of intrahepatic cholangiocarcinoma: A systematic review

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Abstract

BACKGROUND

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer and is characterized by an aggressive behavior and a dismal prognosis. Radical surgical resection represents the only potentially curative treatment. Despite the increasing acceptance of laparoscopic liver resection for surgical treatment of malignant liver diseases, its use for ICC is not commonly performed. In fact, to achieve surgical free margins a major resection and/or vascular and/or biliary reconstructions is often needed, as well as an associated lymph node dissection.

AIM

To review and summarize the current evidences on the minimally invasive resection of ICC.

METHODS

A systematic review of the literature based on the criteria predetermined by the investigators was performed from the 1st of January 2009 up to the 1st of January

Country/Territory of origin: Italy

Specialty type: Surgery

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

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2021 in 4 databases (PubMed, Scopus, Google Scholar, and Cochrane databases). All retrospective and prospective studies reporting on the comparative outcomes of open *vs* minimally invasive treatment of ICC were included. An evaluation of manuscripts quality was achieved using Methodological Index for Non-Randomized Studies criteria and Newcastle-Ottawa Scale.

RESULTS
After a systematic search 9 studies fulfilled the inclusion criteria. Among the all 3012 included patients, 2450 were operated by an open approach and 562 by a minimally invasive (laparoscopic) approach. Baseline characteristics, tumor characteristics, surgical outcomes and oncological outcomes were collected and analyzed, highlighting values with a statistical significant difference between patients treated with open or laparoscopic approach. Shorter hospital stay and lower intraoperative blood losses were reported by some Authors in minimally invasive surgery, on the contrary, in the open group there was a higher number of lymphadenectomies and a higher percentage of major hepatectomies.

CONCLUSION
Minimally invasive resection of ICC has some short-term benefits and it is safe and feasible only in selected centers with a high experience in laparoscopic approach for liver surgery. Minimally invasive surgery, actually, was considered mainly in patients with a tumor with a diameter < 5 cm, without invasion of main biliary duct or main vessel and no vascular or biliary reconstructions were planned. Further studies are needed to elucidate its impact on long term oncologic outcomes.

Key Words: Cholangiocarcinoma; Minimally invasive; Laparoscopic; Liver resection; Hepatectomy; Biliary neoplasm

Core Tip: Reports on the minimally invasive treatment of intrahepatic cholanciocarcinoma are scanty and no clear evidences on the feasibility, safety and oncological results are currently available. The aim of our study is to review and summarize the current evidences on the topic and to compare the short and long term outcomes to those of open surgical resection.

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a rare gastrointestinal malignancy arising from epithelial cells of the intrahepatic bile ducts (cholangiocytes) and accounts for 10%-15% of all primary liver cancers[1]. ICC is the second most common primary liver cancer after hepatocellular carcinoma and is characterized by an aggressive behavior and a dismal prognosis[2]. Its occurrence has progressively raised worldwide during the past decades with a reported increase of more than 165% in its incidence in the last 35 years in the western world population[3,4] (from 0.49 per 100000 in 1995 to 1.49 per 100000 in 2014 in the United States)[5]. Radical surgical resection represents the only potentially curative treatment of ICC. Regrettably, less than 40% of patients are eligible for surgery mainly due to late advanced disease at the time of diagnosis[6]. Considering the lack of effective and established chemotherapeutic options, both in adjuvant and first line setting, even after radical resection 50% to 60% of patients will experience a recurrence[7], with a 5 years overall survival of ICC reported to vary from 15% to 40 % after liver resection[8], strongly depending on the presence of poor

prognostic factors such as lymph nodes involvement, multiple nodules and vascular invasion[9]. Despite the fact that minimally invasive approach to primary and metastatic liver cancer is becoming a routine approach in selected patients, showing improved perioperative outcomes and similar oncological outcomes than open surgery for the treatment of both hepatocellular carcinoma (HCC)[10,11] and colorectal liver metastases (CRLM)[12,13], reports on the minimally invasive treatment of ICC are scanty and no clear evidences on the feasibility, safety and oncological results are currently available. In a recent systematic review on laparoscopic liver resection, published in 2016, among 9527 patients only 116 underwent laparoscopic hepatectomy for ICC[14]. These data strongly reflect the reluctance, even in highly specialized centers, to embrace the minimally invasive approach for ICC. This is probably connected to the necessity of performing loco-regional lymphadenectomy, which is a technically demanding procedure to perform by a minimally invasive approach, and it is also due to the fact that ICC treatment often requires major hepatectomies or vascular and/or biliary reconstruction to achieve a R0 resection. In addition, the Southampton guidelines consensus, despite strongly supporting the adoption of the laparoscopic approach for both HCC and CRLM, did not address the role of minimally invasive approach for the surgical management of ICC[15]. Therefore, updates on the current evidences on the minimally invasive treatment of ICC are urgently needed. The aim of this study is to review and summarize the current evidences on the topic.

MATERIALS AND METHODS

The present study was accomplished in accordance with the preferred reporting Items for systematic reviews and meta-analyses (PRISMA) guidelines[16]. A systematic review of the literature, based on criteria predetermined by the investigators, was independently performed by two authors (B.A. and P.R.) from the 1st of January 2009 up to the 1st of January 2021 in 4 databases (PubMed, Scopus, Google Scholar, and Cochrane databases) in order to maximize articles capturing. Discrepancy in data collection, synthesis and analysis were solved by consensus of all authors. All retrospective and prospective studies reporting on the comparative outcomes of open *vs* minimally invasive treatment of ICC were included. Search terms included: "cholangiocarcinoma", "intrahepatic", "laparoscopic", "surgery", "minimally invasive", "robotic surgery" "biliary neoplasm", "liver resection" and "hepatectomy".

The following Inclusion and exclusion criteria were applied.

Inclusion criteria

(1) English language studies including patients with histologically proved ICC; (2) Use of a minimally invasive surgical approach (laparoscopic or robotic) for liver resection of ICC; (3) Comparing open surgery to minimally invasive surgery (laparoscopic or robotic) for the surgical treatment of ICC; and (4) Studies reporting on at least one intraoperative, postoperative, and long-term oncological outcomes (operative time, intraoperative complications, estimated blood loss, blood transfusion rate, length of stay, R0 resection rate, lymph nodes retrieval, postoperative morbidity and mortality rate, disease free and overall survival rates).

Exclusion criteria

(1) Non-English studies; (2) Animal studies; (3) Non-comparative studies; (4) Abstracts, expert opinions, editorials, meta-analysis, reviews, and letter to the editors; (5) Studies reporting inadequate clinical data; and (6) Studies including mixed pathologies besides ICC; The evaluation of manuscript quality was conducted using the Methodological Index for Non-Randomized Studies criteria[17] and the Newcastle-Ottawa Scale[18] to assess the quality of nonrandomized studies in meta-analyses because of the non-randomized nature of selected papers.

RESULTS

Study inclusion

After systematic search 4835 manuscripts were selected for initial screening. Among them 1704 papers were duplicates and therefore excluded. Based on title, abstract and keywords, the Authors selected and analyzed the full-text version of 189 papers. Main reasons for the exclusion were the absence of patients treated both with laparoscopic

and open approach (n = 114) and the inclusion of other types of tumors besides ICC (n = 36). Further causes of exclusion were population treated with palliative intent or case series or absence of specific data on the post-operative outcomes. Two studies selected after full-text analysis were then excluded because more recent studies from the same authors presented additional updated data. This led to the final selection of 9 studies which fulfilled the inclusion criteria[19-27]. The search strategy flow diagram is shown in Figure 1. There were no randomized clinical studies found. All 9 selected papers were retrospective comparative studies and 7 of them were single center series, one a bi-institutional analysis[25] and one was based on data from a national database[23]. Geographical distribution of the selected papers was as follows: Italy and United Kingdom (1), United States (1), Germany (1), Japan (1), Korea (2) and China (3). Characteristics of the included manuscripts and their quality assessment are summarized in Table 1.

Among all the 3012 included patients 2450 were operated by an open approach and 562 by a minimally invasive (laparoscopic) approach.

Baseline characteristics

As regards patients' baseline characteristics no statistically significant differences were detected in terms of age, sex, body mass index and American Society of Anaesthesiologists score between laparoscopic and open groups in all manuscripts. Eight studies [20-22,24-28] analyzed the presence of at least one comorbidity and no statistical difference was reported between laparoscopic and open group. Detailed data are reported in Table 2.

Tumor characteristics

Tumor size was reported in all, except one[28], of the analyzed studies and in the study by Martin *et al*[23] a statistically significant difference between groups was highlighted with a smaller tumor diameter in the laparoscopic group when compared to the open group. Seven of the selected manuscripts[20-22,24-26,28] reported data on preoperative tumors, nodes and metastasis (TNM) staging and CA19.9 values with no differences between groups. CEA preoperative values were analyzed only in four studies[20,24,25,28] and no differences were found. Zhu *et al*[22], Ratti *et al*[25] and Kang *et al*[26] reported a smaller tumor size in the laparoscopic group but this difference was adjusted after propensity score matching. Kinoshita *et al*[24] found no difference in mean tumor size between the two groups but a higher percentage of patients in the open group had tumors bigger than 3 cm when compared to the laparoscopic group (71% *vs* 33%). Two[21,22] of the analyzed studies were focused on large (> 3 cm) or multinodular ICCs. All tumors characteristics were resumed in Table 3.

Operative outcomes

Operative time was analyzed in 8 out of 9 analyzed studies and only in the study by Zhu *et al*[22] there was a statistically significant difference in favor of the laparoscopic group. Intraoperative blood loss was reported by 7 studies and a statistically significant lower blood loss was found in the laparoscopic group in 4 of them[20,24,25,28].

With the exception of the national database based study by Martin *et al*[23], data on postoperative morbidity were reported in all manuscripts and a lower incidence of postoperative complications in the laparoscopic group was found in the studies by Ratti *et al*[25] and by Haber *et al*[27].

Laparoscopic approach significantly decreased postoperative hospital stay in four of seven study[25-28]. Days spent in intensive care unit were analyzed only by two studies[25,27] with no differences between open and laparoscopic approach.

As regards the type of liver resection, a statistically significant higher rate of major hepatectomies was reported in the open groups in the studies by Kang *et al*[26], Martin *et al*[23] and Lee *et al*[20]. Accomplishment of lymph nodes dissection was investigated by all analyzed studies and in 3 of them[23,25,26] was reported a higher rate of lymph nodes clearance in the open group but with no difference in nodal status. Six authors reported histopathological margin data with no difference between R0 and R1 in the two surgical approaches[20-23,25,26].

Detailed data are reported in Tables 2 and 3.

Oncological outcomes

Eight of the selected studies[20-22,24-28] reported comparative data on the oncological outcomes expressed as overall and disease free survival and none of them reported any differences between the open and the laparoscopic group. In the study by Martin

Table 1 Study characteristics and quality assessment

Ref.	Country	Type of study		LS	OS	NOS			Minors
						Selection	Comparability	Outcame/Exposure	
Wu *et al*[28], 2020	China	RetS-SC	Case control study	18	25	***	*	***	17
Haber *et al* [27], 2020	Germany	RetS-SC	Case control study	27	31	***	*	**	16
Kang *et al* [26], 2020	Korea	RetS-SC	Propensity score matching	30	61	***	**	***	18
Kinoshita *et al* [24], 2020	Japan	RetS-SC	Case control study	15	21	***	**	***	18
Ratti *et al*[25], 2020	United Kingdom-Italy	RetS-TC	Propensity score matching	104	104	***	**	***	19
Martin *et al* [23], 2019	United States	RetS-DB	Database	312	1997	**	*	**	15
Zhu *et al*[22], 2019	China	RetS-SC	Propensity score matching	20	63	***	**	***	19
Wei *et al*[21], 2017	China	RetS-SC	Case control study	30	20	***	**	***	19
Lee *et al*[20], 2016	Korea	RetS-SC	Case control study	14	23	***	**	***	20

LS: Number of patients treated with laparoscopic surgery; OS: Number of patients treated with open surgery; NOS: Newcastle-Ottawa Scale for assessing the quality of nonrandomised studies.A study can be awarded a maximum of one star (*) for each numbered item within the selection and exposure categories. A maximum of two stars can be given for comparability. MINORS: Methodological index for non-randomized studies; RetS: Retrospective study; SC: Single center; TC: Two centres; DB: Data base.

et al[23] the authors focused electively on the rate of administration of adjuvant treatments and found no differences related to the surgical approach.

As regards specific variables affecting survival Wu *et al*[28] identified high preoperative values of CA19.9, high TNM stage and a poor tumor differentiation as independent risk factor for worst overall survival (OS) and disease-free survival (DFS) while Kang *et al*[26] identified tumor size, nodularity and perineural invasion as independent factors correlated to lower DFSs.

Kinoshita *et al*[24], instead, found tumor size (diameter ≥ 3 cm), presence of vascular invasion and a high CA19.9 levels on preoperative exams to be associated with a poorer OS.

Lee *et al*[20], trying to avoid bias, analyzed OS and RFS in laparoscopic liver resection and open liver resection for all patients by stratifying them by the accomplishment of lymph nodes dissection and found no difference in between groups.

Finally, the pattern of recurrence was investigated only in 3 of the selected manuscripts[20,25,28] with no statistically significant differences between the open and the laparoscopic approach. Detailed data are reported in Table 3.

DISCUSSION

The current systematic review is focused on the comparative outcomes of open *vs* minimally invasive resection of ICC. In fact, even if laparoscopy proved to be an effective option for the treatment of both HCC and CRLM, offering the benefit of minimally invasiveness without compromising the oncological outcomes, reports on the operative and oncological outcomes of minimally invasive treatment of ICC are scanty and seldom reported. The uncommon adoption of the laparoscopic or robotic approach for ICC is related to various oncological and technical reasons. First, ICC has a relative low incidence when compared to others liver malignancies and due to its aggressive biological behavior is often diagnosed at an advanced stage not suitable for radical surgery which remains the only potentially curative treatment option[1]. Second, surgery for ICC is often characterized by a high degree of technical difficulty

Table 2 Preoperative and surgical data

Ref.	SA	NP	AGE	ASA				MayorH	Lymphadenectomy		OT	IOBL	CONV.
				I	II	III	IV		Yes	Number			
Wu *et al*[28], 2020	OS	25	61	19		6		13 (52%)	6 (32%)	6	300 (257-392)	**500 (350-750)**	N/A
	LS	18	64	15		3		6 (33%)	8 (33%)	6	305 (207-390)	**375 (275-500)**	0
Haber *et al*[27], 2020	OS	31	63	1	21	8	1	24 (78%)	29 (94%)	8	282 (112–947)	/	N/A
	LS	27	69	0	15	12	0	19 (70%)	23 (85%)	8	314 (125–439)	/	2
Kang *et al*[26], 2020	OS	61	68	/	/	/	/	**53 (88.3%)**	**46 (75.4%)**	/	343.2 ± 106.0	979.3 ± 864.4	N/A
	LS	30	65	/	/	/	/	**20 (66.7%)**	**9 (30%)**	/	375.2 ± 204.0	1396.7 ± 2568.9	6
Kinoshita *et al*[24], 2020	OS	21	68	/	/	/	/	15	7 (33%)	3	358 (150-634)	**500 (105-3710)**	N/A
	LS	15	65	/	/	/	/	5	6 (40%)	2	360 (221-802)	**150 (20-2500)**	0
Ratti *et al*[25], 2020	OS	209	62	20	58	26	0	38 (36.5%)	**92 (88.5%)**	7 (5–14)	230 ± 60	**350 ± 250**	N/A
	LS	114	60	22	56	26	0	35 (33.7%)	**87 (83.7%)**	8 (5–11)	270 ± 65	**150 ± 100**	0
Martin *et al*[23], 2019	OS	1997	64	/	/	/	/	**1338 (67%)**	**1210 (61.2%)**	/	/	/	N/A
	LS	312	65	/	/	/	/	**137 (44%)**	**312 (38.5%)**	/	/	/	/
Zhu *et al*[22], 2019	OS	63	56	/	/	/	/	43 (68.3%)	27 (42.9%)	/	**200 (140–320)**	400 (50–2000)	N/A
	LS	20	54	/	/	/	/	11 (55%)	8 (40%)	/	**225 (140–400)**	200 (50–1000)	2
Wei *et al*[21], 2017	OS	20	60.5	/	/	/	/	11 (55%)	11 (55%)	/	230 (125–420)	350 (50–1200)	N/A
	LS	12	61.5	/	/	/	/	7 (58.3%)	4 (33%)	/	212.5 (60–500)	350 (30–2000)	0
Lee *et al*[20], 2016	OS	23	59	0	20	2	1	**19 (82.6%)**	15 (65.2%)	6 (1–16)	330.0 (140–590)	**625 (250–2500)**	N/A
	LS	14	66	0	12	2	0	**7 (50%)**	5 (35.7%)	4 (1–12)	255.0 (140–480)	**325 (10–1500)**	0

Results for each Author are represented divided in two lines: Open surgery and laparoscopic surgery. LS: Laparoscopic surgery; OS: Open surgery; SA: Surgical approach; NP: Number of patients; Age are expressed in year; ASA: American Society of Anaesthesiologists physical status classification; MajorH: Major hepatectomy considered as equal or more than 3 resected segments; OT: Operation time expressed in minutes; IOBL: Intra-operative blood loss expressed in mL; Conv: Number of procedure converted from laparoscopic to open approach. In bold differences with a P value < 0.05.

associated with the need of performing an appropriate lymphadenectomy and, especially in centrally located tumors, a vascular or biliary reconstruction as well as a major hepatic resection are often needed to achieve clear surgical margins[29]. These technical issues have probably slowed down the diffusion of ICC as a valid indication for a minimally invasive approach. In fact, major hepatectomies, hepatic hilum

Table 3 Post-operative and oncological data

Ref.	SA	ICUS	HS	HM		30-d morbidity		90-d morbidity	mFU	OS	DFS
				R0	R1	Grade I-II	Grade III-IV				
Wu *et al*[28], 2020	OS	/	**9 (7-15)**	/	/	23	2	1	/	20	4
	LS	/	**6 (5-12)**	/	/	17	1	0	/	47.1	0
Haber *et al*[27], 2020	OS	1 (0–6)	**12 (5–33)**	23	8	**8**	**10**	0	/	/	/
	LS	1 (0–81)	**10 (3–94)**	24	3	**3**	**5**	2	/	/	/
Kang *et al*[26], 2020	OS	/	**18.3 ± 14.7**	/	/	/	23	0	16.8	81.2	42.5
	LS	/	**9.8 ± 5.1**	/	/	/	8	0	39.2	76.7	65.6
Kinoshita *et al*[24], 2020	OS	/	/	20	1	/	4	/	/	36	19
	LS	/	/	14	1	/	2	/	/	32	24
Ratti *et al*[25], 2020	OS	4 (3–10)	**6 (3–21)**	99	5	17	8	2	50	47	34
	LS	3 (1–5)	**4 (2–10)**	101	3	11	4	1	39	46	36
Martin *et al*[23], 2019	OS	/	/	1451	546	/	/	/	/	/	/
	LS	/	/	247	65	/	/	/	/	/	/
Zhu *et al*[22], 2019	OS	/	7 (3–33)	58	5	22	6	0	24	17	32
	LS	/	6 (3–9)	19	1	3	1	0	24	21	31
Wei *et al*[21], 2017	OS	/	11 (5–30)	19	2	10	3	0	12	32.7	27.9
	LS	/	14 (6–23)	12	0	3	2	0	17.5	56.3	43.8
Lee *et al*[20], 2016	OS	/	20 (9–63)	/	/	1	4	0	/	75.7	/
	LS	/	15 (9–29)	/	/	0	3	0	/	84.6	/

Results for each Author are represented divided in two lines: Open surgery and laparoscopic surgery. LS: Laparoscopic surgery; OS: Open surgery; SA: Surgical approach; ICUS: Intensive care unit stay in days; HS: Hospital stay in days; HM: Histopathological margins; mFU: Median follow-up in months; OS: Overall survival expressed in months after surgery; DFS: Disease-free survival expressed in months after surgery. In bold differences with a P value < 0.05.

lymphadenectomy and biliary reconstructions are technically demanding to perform by a minimally invasive approach. In addition, to safely perform such procedures an extensive learning curve is needed[30] and since now this has been unlikely to be accomplished outside high volume centers with a steady commitment to minimal invasiveness. Notwithstanding that, recently initial data on the comparative outcomes of open *vs* minimally invasive resection of ICC have been published in the literature. The interest on this topic is, in fact, increasing and the surgical treatment of ICC is becoming one of the latest field of implementation of minimally invasive liver surgery. In particular, all the selected articles for this systematic review have been published in the last 5 years thus reflecting the growing interest on the topic. Nevertheless, despite the accurate search strategy applied, the current systematic review confirmed the paucity of current evidences on the minimally invasive approach for ICC. No randomized comparative studies are currently available and only 9 comparative retrospective studies were retrieved from the systematic search. Although representative of the experience of few highly specialized centers for minimally invasive liver surgery, the analyzed studies proved without doubt the feasibility and safety of the laparoscopic approach to ICC in patients with a tumor diameter < 5 cm, without main biliary duct invasion, without large vascular invasion and in which biliary and vascular reconstructions were not needed. Results from the analyzed studies also confirmed the typical benefit of minimally invasiveness already demonstrated for the laparoscopic treatment of HCC and CRLM, even when dealing with ICC. In fact, several of the analyzed studies reported a benefit of the minimally invasive approach in terms of peri-operative outcomes when compared to the open approach. In details, four studies[20,24,25,28] reported a lower intraoperative blood loss associated with the minimally invasive approach even when dealing with radical lymph nodes clearance and this is probably related to magnified view and the meticulous dissection

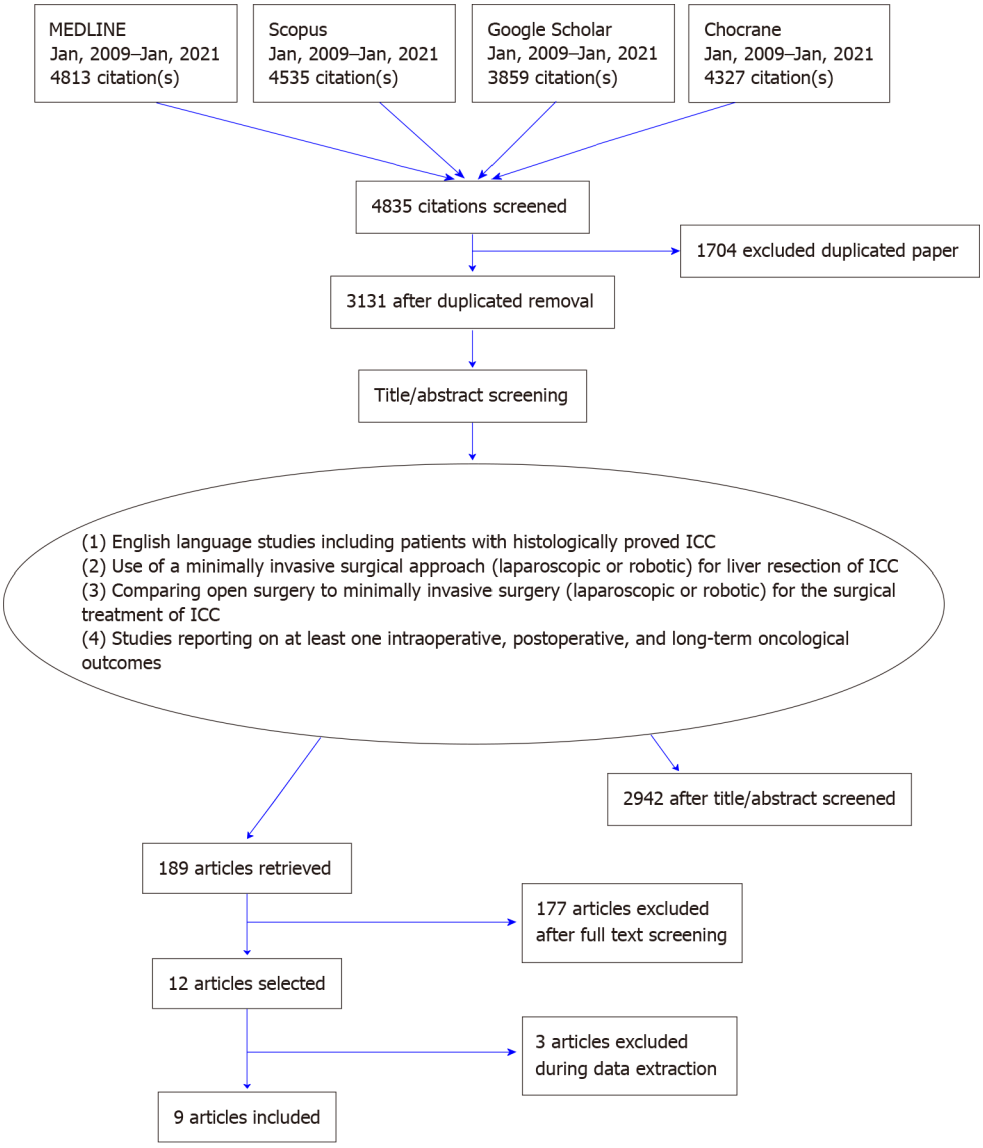

Figure 1 PRISMA flow-chart. Figure reported the diagram of our systematic review of the literature, performed in 4 databases from Jan 1, 2009 up to Jan 1, 2021. Search terms included: "cholangiocarcinoma", "intrahepatic", "laparoscopic", "surgery", "minimally invasive", "robotic surgery" "biliary neoplasm", "liver resection" and "hepatectomy". Inclusion criteria are in the big circle-box. Major reasons for exclusion were the absence of patients treated both with laparoscopic and open approach (n = 114) and the inclusion of other tumor types besides intrahepatic cholangiocarcinoma (n = 36). Further reasons for exclusion were population treated with palliative intent or case series or absence of specific data on the post-operative outcomes. This led to the final selection of 9 studies which fulfilled the inclusion criteria. ICC: Intrahepatic cholangiocarcinoma.

achievable by laparoscopy. Nevertheless, Kang *et al*[26] described a higher blood loss rate in the laparoscopic group, but these difference was not statistically significant (P value = 0.393). Furthermore, four of the analyzed studies[25-28] reported a shorter hospital stay associated to the laparoscopic approach, thus confirming the benefit of minimal invasiveness in terms of a faster recovery also in this setting. Finally, despite the relative initial experience, the studies by Ratti *et al*[25] and Haber *et al*[27] highlighted a benefit in terms of postoperative morbidity in favor of laparoscopy. However, the reported experiences are mainly focused on mass forming type ICC without vascular and biliary involvement (away from the liver plate) and, as highlighted by the large national database-based study by Martin *et al*[23], patients operated by laparoscopy had smaller tumor size when compared to those submitted to an open resection. In addition, a statistically significant higher rate of major hepatectomies was reported in the open groups in 3 of the analyzed studies[20,23,26]. This reflects the selection bias, which is to be expected when dealing with the appliance of laparoscopy to a new surgical indication. Indeed, the studies by Zhu *et al*[22] and Wei *et al*[21] were focused on large or multinodular ICCs and both confirmed positive results similar to those reported by studies with stricter selection criteria. The benefit of performing a lymphadenectomy for ICC is a debated issue. In fact, up to 40% of

resected patients can present with lymph nodes involvement[9] and several authors have highlighted a survival benefit in patients undergoing lymph nodes clearance associated to liver resections when compared to patients who did not[31]. On the contrary, discrepant studies reported no survival benefit and an increase in surgical morbidity associated with lymphadenectomy especially in case of patients with chronic liver disease[32,33]. Nevertheless, lymph nodes clearance for ICC is a crucial strategy for a correct staging of surgically resected patients and can both guides the administration of adjuvant chemotherapy and optimizes clinical risk stratification and prognostic outcomes. This factor is even more significant if we take into account the results of the BILCAP study which demonstrated the survival benefit of adjuvant gemcitabine for biliary tract cancers[34]. Indeed, the 8th edition of the American Joint Committee on Cancer (AJCC) guidelines recommends to perform lymphadenectomy with an optimal cut-off of six retrieved nodes for biliary tract cancers[35]. Is therefore to be expected that regional lymphadenectomy will be implemented in clinical practice and should be performed irrespectively from the open or minimally invasive surgical approach adopted. From the current systematic review, a certain under-employment of regional lymphadenectomy for ICC was highlighted. In fact, a lower rate of lymph nodes dissection in the laparoscopic group was reported in the studies by Kang *et al* [26] and Ratti *et al*[25]. These data are confirmed by the National Cancer Database analysis by Martin *et al*[23] which also highlighted that some form of nodal dissection was performed in only 58% of patients in the whole study cohort. Indeed, the vast majority of the published studies reports the initial experiences of selected high specialized centers and refers to a time preceding the AJCC guidelines diffusion and application. Therefore, after an initial learning curve, a major adherence to the guidelines it is likely to be accomplished. It is also to be expected that the accumulation of experience and the improvement of surgical techniques will probably promote the adoption of the minimally invasive approach for ICC.

In addition, the histopathological margin status is a crucial factor to be considered when comparing the minimally invasive approach to the standard open resection. In fact, an R0 margin represents the most significant predicting factor of oncological outcomes and results from our review show a superimposable rate of negative surgical margin in both approaches. This evidence together with the appropriateness of loco-regional lymphadenectomy and the reduced intraoperative blood loss reported in the majority of the analyzed studies, allow us to consider the laparoscopic approach non inferior to the open one in terms of operative outcomes. Therefore, is not surprising that the minimally invasive approach has been recently extended to the surgical treatment of hilar type cholangiocarcinoma[36] and gallbladder cancer[37,38]. These encouraging pivotal experiences seem to demonstrate the feasibility of minimally invasive surgery in a setting often requiring the completion of a major hepatic resection in association with loco-regional lymphadenectomy and the challenge of biliary reconstructions. It is therefore likely that in the very next future the surgical research in the field of minimally invasive surgery (MIS) for biliary cancer will be concentrated on hilar type tumors and on biliary duct resection (with the aid of Indocianyne green guidance) and reconstruction *via* duct to duct anastomosis or hepatico-jejunostomy. In addition, the implementation of the MIS approach for the surgical treatment of ICC is likely to be promoted by the diffusion of the robotic platforms. In fact, even if it has been demonstrated by the analyzed studies that an appropriate lymphadenectomy can be performed safely and effectively by laparoscopy, it requires advanced laparoscopic skills and a long learning curve. The application of the robotic platform in this setting, thanks to the higher dexterity achievable with the robotic instruments, which, with the endowrist system, have seven degrees of freedom, could facilitate an adequate surgical manipulation and the achievement of an appropriate lymph node clearance in a confined space such as the hepatic pedicle. The magnified high-resolution 3d stereoscopic view offered by the robotic platform is also an added value in defining the anatomical structures and can facilitate biliary reconstructions when needed. As regards the oncologic outcomes, the data are scanty and not conclusive. Some form of oncological data has been reported only by eight studies[20-22,24-28] and, even though no differences have been reported in terms of disease free survival and overall survival in this systematic review, a recent meta-analysis highlighted a possible trend towards a lower 5 years overall survival for patients treated with a laparoscopic approach for ICC when compared to those operated by open approach[39]. Therefore, the interpretation of the oncologic outcomes needs to be evaluated with extreme caution. In addition, no high quality evidences are currently available and thus the need for more qualified data is urgent.

CONCLUSION

In conclusion, the minimally invasive treatment of ICC is currently rarely performed but is rapidly gaining popularity. Currently available data seems to justify the implementation of the minimally invasive approach for ICC by demonstrating its safety and reproducibility and by confirming the well-known advantages of minimally invasiveness in term of perioperative outcomes also in this setting, as already proven for other liver neoplasms. Nevertheless, current evidences are based on few studies with a limited sample size and a short follow-up. In addition, selection criteria for the minimal invasive approach were highly restrictive (small tumors, generally < 3 cm, distant from the hilum and not requiring a biliary reconstruction) when compared to open series and, therefore, at high risk for selection bias. Dedicated study protocols and analysis of national and international registries are urgently needed to clarify the real role of minimally invasive surgery in the treatment of ICC and its impact on the long term oncologic outcomes.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholangiocarcinoma represents a very aggressive tumor with poor prognosis. Nowadays surgical open approach is still the gold standard treatment but minimally invasive surgery is gaining an important role. No randomized trials are available on this topic in scientific literature.

Research motivation

Our scientific group aim to contribute to the development of the scientific research on hepatobiliary minimally invasive surgery.

Research objectives

Our research had the objective to summarize and review the scientific evidences present in the literature on minimally invasive surgical approach for intrahepatic cholangiocarcinoma.

Research methods

We performed a systematic review of the literature between 01/01/2009 and 01/01/2021. Our research keywords were: "cholangiocarcinoma", "intrahepatic", "laparoscopic", "surgery", "minimally invasive", "robotic surgery" "biliary neoplasm", "liver resection" and "hepatectomy". We selected only papers comparing open and laparoscopic approach and reporting at least one intraoperative, postoperative or oncological outcomes.

Research results

We found 9 papers that fulfilled all inclusion criteria reporting data from 3012 patients with no differences in baseline characteristic. Almost all operative outcomes were in favor of laparoscopic groups (blood losses, operative time, hospital stay, post-operative complications) except for the number of lymphonodes retrieved (higher number of lymphonodes retrieved in the open groups). No statistical differences in oncological outcomes were reported.

Research conclusions

Our research demonstrates that very few studies investigated the role of minimally invasive surgery for intrahepatic cholangiocarcinoma. Currently available data in the Literature were not consistent enough to consider the laparoscopic approach to ICC as a standard of care but a steady implementation is likely to be realized in the next future.

Research perspectives

It is likely that soon the diffusion of robotic surgery and tailored surgery, will promote the diffusion of minimally invasive approach for intrahepatic cholangiocarcinoma and will help elucidating its role and the oncological outcomes.

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LETTER TO THE EDITOR

Gender differences in the relationship between alcohol consumption and gastric cancer risk are uncertain and not well-delineated

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Abstract

The role of alcoholic and other beverage consumption in the etiology of gastric cancer is unknown. Several studies have summarized and established a significant association between heavy alcohol consumption and gastric cancer risk, but evidence on alcohol-related cancer risk is conflicting.

Key Words: Alcohol; Gastric cancer; Meta-analysis; Gender and alcohol intake; Alcohol consumption

Core Tip: Alcohol consumption among adult men and women is consistently linked to an increased risk of gastric cancer. Its role as a confounding factor in the gastric cancer burden is frequently overlooked. Although many cancers are genetically determined, most cancers are caused by interactions between the host and environmental/lifestyle factors.

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TO THE EDITOR

Cancer is a complex disease that arises from interactions between genetic and environmental factors. There is overwhelming evidence that alcohol consumption affects cancer risk. According to the 4th edition of the European Code against Cancer, not drinking alcohol is better for cancer prevention[1]. We read the recent publication on "Sex as an effect modifier in the association between alcohol intake and gastric cancer risk" by Bae[2]. I sincerely appreciate the author for providing relevant information about the relationship between alcohol consumption and the risk of gastric cancer in men and women. It has been of great interest to us. In this systematic review and meta-analysis, the authors included ten Asian, seven European and three American cohort studies comprising 27 cohorts, making the study more robust.

The meta-analysis showed that overall alcohol intake increased the risk of gastric cancer with a summary risk ratio of 1.13 [95% confidence interval (CI): 1.04-1.23]. However, subgroup analysis by gender demonstrated higher relative risk (RR) in male cohorts (RR = 1.18, 95%CI: 1.06-1.32, I^2 = 55.5%) than in female cohorts (RR = 1.07, 95%CI: 0.96-1.19, I^2 = 0.0%). Several previous meta-analyses that have investigated the relationship between alcohol consumption and gastric cancer risk were inconclusive. Previous meta-analyses demonstrated no association between alcohol drinking and gastric cancer risk in overall and gender-stratified analyses[3]. In contrast to this, heavy alcohol consumption significantly increased the risk of gastric cancer in both men and women[4]. Subsequent meta-analyses indicated that alcohol consumption was associated with an increased risk of gastric cancer in men but not in women[5,6]. Recently, Kim *et al*[7] shown that high alcohol consumption of alcohol (≥ 20 g/d for women or ≥ 40 g/d for men) significantly increased the risk of gastric cancer. Further, large-scale meta-analyses by Han *et al*[8] found a protective effect of alcohol consumption in Europe and a significant harmful impact in men in America. That depends on other confounding factors, including age, education level, smoking status, and body mass index.

Although the systematic review and meta-analyses have linked gastric cancer risk with alcohol consumption, gender differences in the relationship between alcohol consumption and gastric cancer risk are uncertain and not well-delineated. Indeed, hormonal, genetic and environmental differences influence how men and women consume alcohol[9]. While men are more likely to drink excessive amounts of alcohol, women are more likely to abstain for long periods. Bae[2] has not considered the frequency of heavy and light alcohol drinking habits in men and women, which is the main factor that determines modulating effect of sex on the relationship between alcohol consumption and gastric cancer risk[2]. More research with critical confounding factors such as drinking intensity is needed to provide a more precise relationship between alcohol consumption and the risk of gastric cancer in men and women.

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LETTER TO THE EDITOR

Critical biomarkers of hepatocellular carcinoma in body fluids and gut microbiota

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer and one of the major causes of cancer-related death. The development of specific non-invasive or diagnostic markers from blood, urine and feces may represent a valuable tool for detecting HCC at an early stage. Biomarkers are considered novel potential targets for therapeutic intervention. It helps in the prediction of prognosis or recurrence of HCC, and also assist in the selection of appropriate treatment modality. We summarize the most relevant existing data about various biomarkers that play a key role in the progression of HCC.

Key Words: Hepatocellular carcinoma; Biomarker; Body fluids; Blood; Gut microbiota

Core Tip: Hepatocellular carcinoma (HCC) ranks fourth among the leading causes of cancer-related mortality. The development of specific noninvasive or diagnostic markers from blood, urine and feces may represent a valuable tool for detecting HCC at an early stage. Biomarkers help in the prediction of prognosis or recurrence, selection of appropriate treatment modality, and signify novel potential targets for therapeutic interventions. We summarize the most relevant existing data about various biomarkers involved in the progression of HCC.

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TO THE EDITOR

We were interested to read the review reported by Guan *et al*[1] that clearly emphasized the substantial role of biomarkers from different body fluids such as blood, urine and feces for the early detection of primary and recurrent hepatocellular carcinoma (HCC). From the study reports, detection of biomarkers through screening of body fluids or feces is regarded as beneficial due to the quick and easy extraction procedures, stability, proper time management, cost-effectiveness and accessibility in comparison with conventional screening methods. The review highlights the clinical significance of several diagnostic biomarkers of HCC, including proteins, metabolites, circulating nucleic acids, circulating tumor cells (CTCs), extracellular vesicles (EVs), and gut microbiota from blood, urine and feces.

A large pool of evidence suggests the presence of elevated serum blood levels of bilirubin, albumin, α-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-γ-carboxy prothrombin (DCP) at the time of diagnosis of HCC. These biomarkers exhibit a close relation with HCC staging and prognosis of overall survival and disease-free survival. Elevated levels of AFP in cases of liver injury above the reference range (400-500 ng/mL) can be considered crucial for the prognosis of HCC. AFP-L3 possesses better sensitivity but low specificity for the early detection of HCC. To its expression in small tumors (< 2 cm in diameter) of aggressive types, the prognosis of early-stage HCC is relevant if the AFP-L3 level is greater than 10% in comparison with AFP. DCP can be regarded as an excellent prognostic biomarker since it can differentiate nonmalignant cirrhosis and HCC with a specificity of 93% and sensitivity of 92% at a cut-off value of about 150 mAU/mL[2]. With disease progression, metabolic markers such as methionine, proline and ornithine increase, whereas the levels of pimelylcarnitine and octanoylcarnitine decrease[3]. The applicability of phenylalanyl-tryptophan and glycocholate as a superior biomarker was demonstrated in a multicenter cohort study that indicated its diagnostic accuracy of 86.0%-92.5% in HCC[4].

The progression of HCC involves invasion, migration, proliferation and metastasis. Studies have shown that drug resistance is mainly mediated through the functional activation of miRNAs. Clinicians can predict the overall survival of patients based on the expression of miRNA. Single miRNAs like miR-130b, miR-150, miR-182, miR-215 and miR-96 are considered key candidates among all miRNAs but the use of multiple miRNAs as promising biomarkers for the prediction of early as well as recurring HCC is recent[5]. CTCs play a significant role in the prediction of HCC recurrence, prognostic evaluation for surveillance, and promotion of suitable adjuvant therapy. CTCs are generally categorized as a small subpopulation of malignant cells secreted from primary malignant tissue and they are usually expressed at the aggressive malignancy stage; therefore, liquid biopsy of CTCs facilitates timely diagnosis of HCC [6]. Another important category of biomarker with a functional role in the prediction of HCC progression is EVs. Increased circulating levels of EVs have contributed to poor survival and disease-free survival in HCC patients. Despite their high capability of being absorbed into host cells, EVs are considered an efficient tool for targeted approaches. This is by the incorporation of therapeutic agents to improve therapeutic efficacy and reduce side effects. The incorporation of sodium/iodide symporter protein to EVs has been used as one of the systemic targeted approaches to cancer treatment with the promotion of cytotoxicity and radioiodine therapy[7].

Another potential category of biomarkers for HCC are urine-based. Among the biomarkers, higher levels of 8-oxodeoxyguanosine improve DNA repair mechanisms by overcoming oxidative DNA damage with a reduction in risk of developing HCC. Enhanced levels of 15-F2t-isoprostane are also correlated with the risks of HCC. Urinary proteins such as urinary DJ-1, chromatin assembly factor-1, heat shock protein 60 and orosomucoid, and metabolites such as ethanolamine, lactic acid, aconitinic acid, phenylalanine and ribose were found to be effective predictors for early HCC recurrence. Additionally, the overexpression of urinary trypsin inhibitor in HCC was revealed to be a risk factor for HCC recurrence[1]. In a study reported by Hann *et al* [8], detection of urinary markers such as TP53m, mSGTP and mRASSF1A were potential tools for the early detection of HCC recurrence (Figure 1).

Inflammation significantly decreases the expression of beneficial microflora which, in turn, enhances the risk of liver malignancy by accumulating harmful compounds.

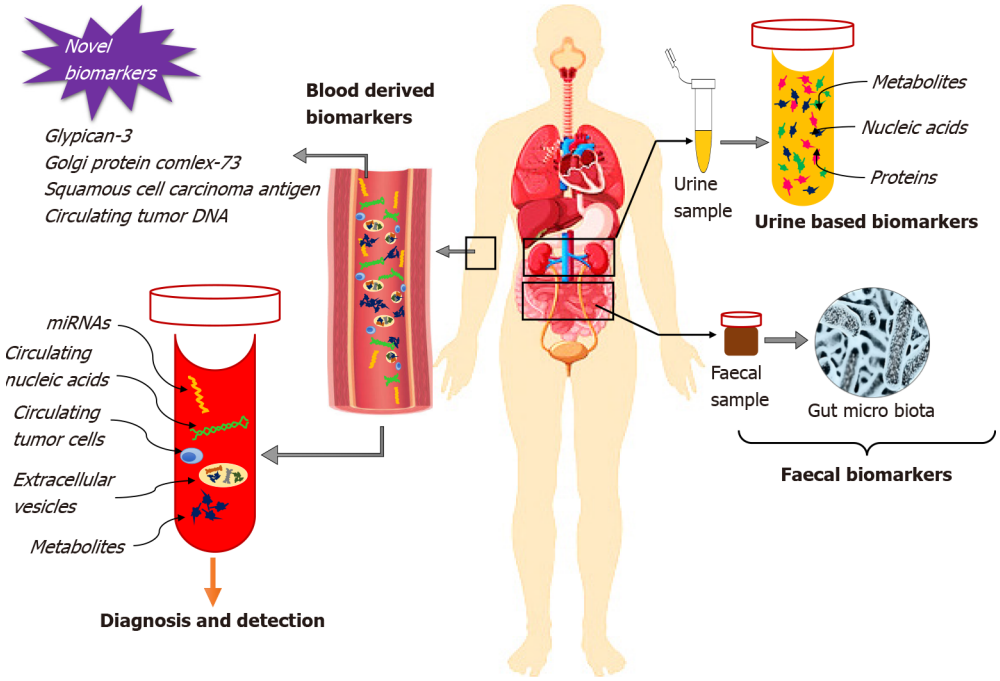

Figure 1 Pictorial representation of numerous biomarkers derived from different body fluids, namely, blood (serum), urine and feces. These biomarkers constitute a wide spectrum of proteins, nucleic acids and metabolites. Circulating tumor cells, miRNAs and gut microbiota which can be beneficial for the early detection, diagnosis and prognosis of hepatocellular carcinoma.

Translocated bacterial products such as lipopolysaccharides, peptidoglycans, muramyl-dipeptides and bacterial DNA from the infectious stage of the gut stimulate an inflammatory cascade by activation of signaling through Toll-like receptors (TLRs). Stimulation of interleukin-6, either directly or *via* the JAK/STAT3 pathway forces the gut microbiota to induce proliferation and progression of HCC. Gut microbiota can stimulate the generation of reactive free radical oxygen species indirectly *via* small molecular motifs derived from a pathogenic class of microbes by the activation of NADPH-oxidase (NOX1–NOX4). Microbial imbalance and enhancement of inflammation are directly correlated with fluctuating redox status. Modulation of farnesoid X receptor activation by gut microbiota enhances bile acid accumulation in the liver. This leads to damage of hepatocyte plasma membranes, resulting in activation of an inflammatory response and production of reactive oxygen species through stimulating the MAPK pathway. As a result, the secretion of inflammatory cytokines *via* the nuclear factor-B pathway is increased by induction of proliferation and immortalization of HCC cells directly or *via* the JAK/STAT3 pathway. Gut microbiota can exhaust the surveillance of the immune system within the tumor microenvironment of HCC through macrophage polarization *via* the activation of TLRs. This results in further diversification and progression of the tumor[9]. Additionally, several other biomarkers namely, glypican-3, Golgi protein complex-73, squamous cell carcinoma antigen and circulating tumor DNA are useful for early diagnosis of HCC, and might be clinically validated in the near future[10].

The supporting evidence gives an insight into novel biomarkers for early prediction and prevention of HCC. HCC accounts for almost 90% of primary liver malignancies and has a poor prognosis due to rapid metastasis and multidrug resistance. Diagnosis of HCC at an early stage is important for overcoming the hurdles associated with the disease[11]. To conclude, it is important to identify and develop promising biomarkers for early diagnosis and prognosis as well as therapy of HCC.

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