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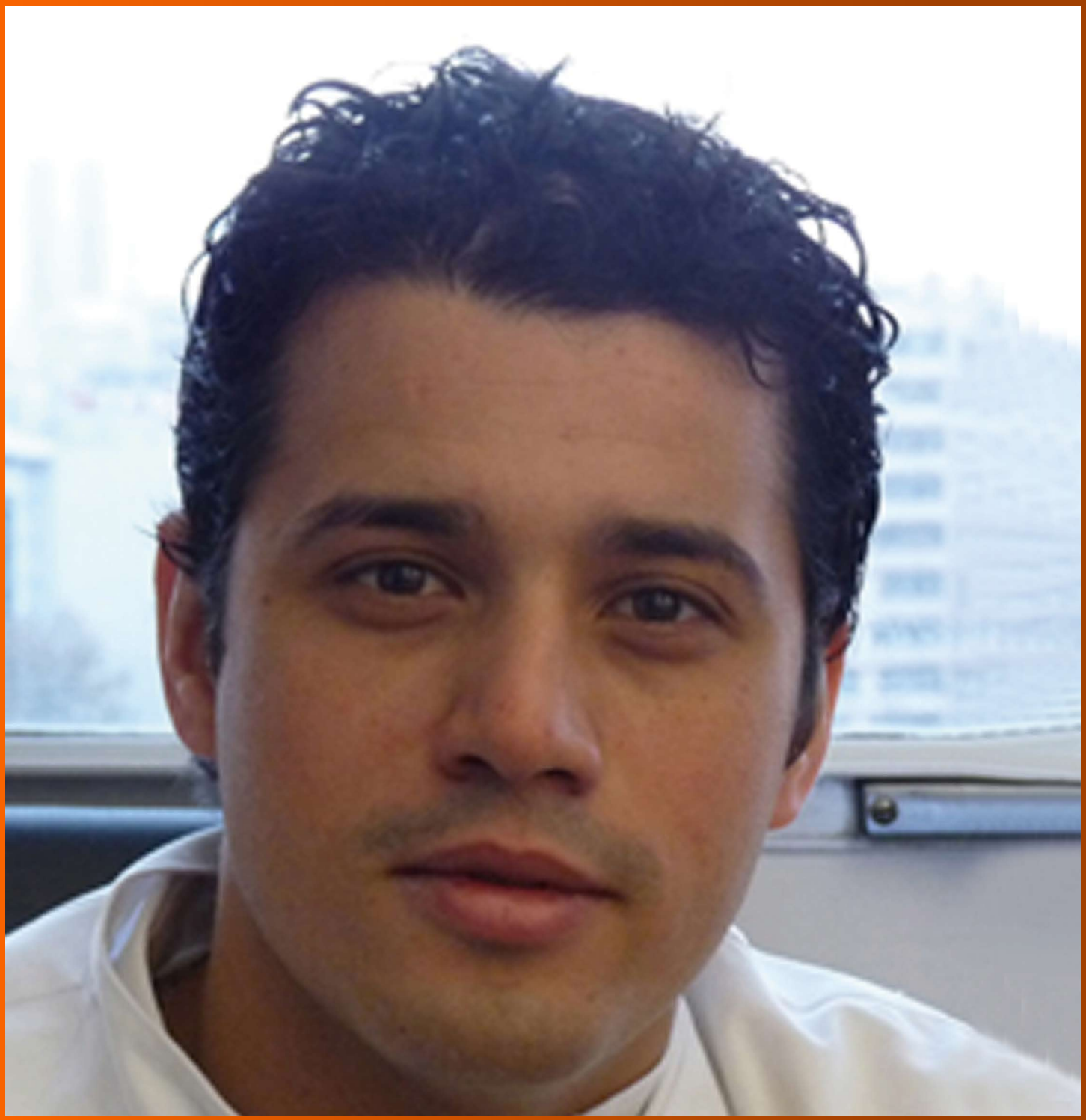

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Clinical significance of lymphadenectomy in patients with gastric cancer

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Abstract

Approximately thirty percent of patients with gastric cancer undergo an avoidable lymph node dissection with a higher rate of postoperative complication. Comparing the D1 and D2 dissections, it was found that there is a significant difference in morbidity, favoured D1 dissection without any difference in overall survival. Subgroup analysis of patients with T3 tumor shows a survival difference favoring D2 lymphadenectomy, and there is a better gastric cancer-related death and non-statistically significant improvement of survival for node-positive disease in patients with D2 dissection. However, the extended lymphadenectomy could improve stage-specific survival owing to the stage migration phenomenon. The deployment of centralization and application of national guidelines could improve the surgical outcomes. The Japanese and European guidelines enclose the D2 lymphadenectomy as the gold standard in R0 resection. In the individualized, stage-adapted gastric cancer surgery the Maruyama computer program (MCP) can estimate lymph node involvement preoperatively with high accuracy and in addition the Maruyama Index less than 5 has a better impact on survival, than D-level guided surgery. For these reasons, the preoperative application of MCP is recommended routinely, with an aim to perform "low Maruyama Index surgery". The sentinel lymph node biopsy (SNB) may decrease the number of redundant lymphadenectomy intraoperatively with a high detection rate (93.7%) and an accuracy of 92%. More accurate stage-adapted surgery could be performed using the MCP and SNB in parallel fashion in gastric cancer.

Key words: Gastric cancer; Surgery; Lymphadenectomy; Sentinel node biopsy; Maruyama computer program

Core tip: Comparing the D1 and D2 dissections, it was found that there is a significant difference in postoperative morbidity and mortality, favoured D1

dissection without any difference in overall survival. The implementation of centralization and application of national guidelines could improve the surgical outcomes. More accurate stage-adapted surgery could be performed using the Maruyama computer program and sentinel lymph node biopsy in parallel fashion in gastric cancer.

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INTRODUCTION

In most cases, modern, optimal treatment of patients with different neoplasms can be achieved with a stage adapted, combined modality therapy according to international protocols. In case of solid tumors, the lymph node (LN) involvement and its exact number is the most important prognostic factor. Adjuvant chemotherapy, as well as the oncological outcome is terminated by the tumor-node-metastasis stage. Preoperative imaging techniques provide a much more accurate determination of the T and M stage than that of the N stage. The correct status of LN metastases can be obtained only by histology following an optimally extended node dissection. The removal of further LNs on the other hand, increases operative time, the rate of complications, and if negative may be considered unnecessary.

Almost three hundred thousand patients with gastric adenocarcinoma do not have LN metastasis in the one million new cases each year[1,2]. The depth of tumor invasion[3,4], the metastatic LN status and R0 resection are the most important independent prognostic factors for overall and disease free survival (OS, DFS)[5-7]. Moreover, a lot of study proved that LN metastasis is an independent risk factor for local recurrence as well as the time interval between radical gastrectomy and hepatic metastasis in patients after R0 resection[8-10].

The aim of this review is to report the latest issues from 2014 according to lymphadenectomy in gastric cancer and compare these results with earlier studies.

LN INVOLVEMENT

Successful estimation of LN involvement may help to define which patients would or would not benefit from an extended LN dissection in association with gastrectomy[11]. However, preoperative diagnostic tools have a low sensitivity and specificity for defining these patient subpopulations. Sensitivity, specificity and accuracy of spiral computer tomography for detection of pathologic LN involvement are 73.1%, 50.0% and 84.2%, respectively[11,12]. Endoscopic ultrasonography has an accuracy of 68.6%, with a sensitivity and specificity of 66.7% and 73.7%, respectively[11,13]. The real problem of these imaging procedures is that exclusion of endoscopic ultrasonography, only the size of the LNs is taken into account.

In association with T stage, LN involvement can be found in 15% of patients with carcinoma confined to the mucosa, whereas LN metastases were detected in 23.4%, 48.2%, and 69.8% of patients with carcinoma invading the submucosa, muscularis propria and serous layer, respectively[14] Gertler *et al*[15] showed that not only infiltration of the submucosa but also lymphatic vessel invasion, multifocal tumor growth, younger patient age and poor tumor differentiation were associated with nodal disease. Besides T stage, LN involvement can also be influenced by tumor size. The overall accuracy of tumor size for preoperative N staging was 82.13%[16]. The incidence of LN metastasis in patients with a cancer size of 3-5 cm is 64.9%, 80% in patients with a cancer size of 5-7 cm and 84.3% in patients with a cancer size of > 7 cm[14]. Additionally, early gastric cancer (EGC) has nodal metastases in 38.9% in poorly differentiated or undifferentiated types of tumor, in 41.7% with Lauren diffuse type and in 33.3% with a size larger than 3 cm[17]. Yang *et al*[18] found that venous invasion, submucosal invasion or antral tumor location were independent predictors for LN metastasis in multivariate analysis. The rates of LN metastasis were 1.1% for patients with one or no predictor and 17.8% for those with two or more predictors[18].

While the prognostic significance of macrometastasis in the LNs is obvious, the role of lymphovascular invasion (LVI) or micrometastasis (MM) is controversial. Lee *et al*[19] confirmed that the recurrence-free survival is lower in N0/LVI(+) patients than in N0/LVI(-) patients, however they did not find any effect of LVI+ on overall survival. The incidence of LN MM is lower than 10% in patients with node negative EGC[20] but it is higher in histologically diffuse type tumors[21]. The presence of MM influenced DFS, although the OS analysis revealed no significant difference between MM-positive and MM-negative patients[19]. The reverse transcriptase polymerase chain reaction proved to be the most sensitive method in the detection of MM[22].

Meanwhile, a multivariate survival analysis concluded that the number of examined LN (eLN) was an independent predictor of overall survival of patients with node-negative gastric cancer. According to the cut-point analysis, T2-T4 patients with 11-15 eLN had a significantly longer mean OS than those with 4-10 eLN or 1-3 eLN. Patients with ≤ 15 eLN were more likely to experience locoregional and peritoneal recurrence than those with > 15 eLN[23]. However, this trend was not observed when the number of examined LN exceeded 30[24].

These results are potentially associated with the elimination of MM in negative LNs[25]. Based on these findings, LVI and MM should be considered in postoperative management of gastric cancer[19].

Table 1 The primary and revised results of prospective randomized trials comparing D1 to D2 dissection

Ref.	Morbidity (%)		Mortality (%)		Splenectomy (%)		Pancreatectomy (%)		RR (%)		OS (%)	
	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2
Dent[32]	13.6[a]	38[a]	0	0	0	0	0	0			81	76
British[33,36]	28[a]	46[a]	6.5[a]	13[a]	27	9	4[1]	57[1]	NS	NS	35	33
Dutch[34,37]	24[a]	43[a]	4[a]	10[a]	11	37	3	30	43	47	45	47
Dutch - 15 yr[38]									22[a]	12[a]	21	29
Taiwanese[39,44]	7.3[a]	17.1[a]	0	0	3	1	1[1]	13[1]	50.6	40.3	53.6[a]	59.5[a]
Italian[35,43]	12	17.9	3	2.2	6.8	9.0	1.5	1.5			66.5	64.2

[a]$P < 0.05$. [1]Pancreato-splenectomy. RR: Relapse risk; OS: Overall survival; NS: Non-significant.

LYMPHADENECTOMY

D1 vs D2 lymphadenectomy

The adequate extension of lymphadenectomy differs significantly between East Asian and Western countries. Extended lymphadenectomy (D2) is the standard of care in Japan and South Korea, while for example, the majority of United States patients receive at most a limited lymphadenectomy (D1)[26,27]. This controversy may originate from different factors. First, the incidence of gastric cancer is significantly higher in Asia than in European Union, or in the United States[27,28].

Second, centralization of treatment has not yet been solved in the latter regions; 80% of Medicare patients with gastric cancer in the United States go through surgery in centers performing less than 20 procedures per year[29] and there is a significant number of low-volume surgeons performing less than two cases annually[30,31].

Table 1 shows the primary and revised results of prospective randomized trials (RCT) comparing D1 to D2 lymphadenectomy in association with postoperative morbidity, mortality, frequency of splenectomy and pancreatectomy and long term oncological outcomes such as relapse risk and overall survival (OS). The three earliest studies found a higher morbidity and mortality rate following extended LN dissection of patients with gastric cancer when compared to those undergoing D1 dissection only[11,32-34]. These higher rates were related mostly to splenectomy and pancreatectomy. Although Dent *et al*[32] did not perform resection of these organs, this study should be evaluated with reservations because of the small series size. Furthermore, limited surgical experience could explain these results. The quality control of lymphadenectomy was inadequate, as the non-compliance rate (absence of LNs from more than two LN stations that were supposed to be harvested) was 51% in the D2 group in the Dutch trial[34,35] and, in the extended group of the British trial, the dissection of LN station no.7 was 63.5%, and was less than 50% in station no.8 and no.9[36].

Moreover, extended LN dissection did not have any effect on oncological outcomes. The relapse risk and survival were similar in these studies. Only the revision of the Dutch trial showed better survival in advanced disease in the D2 group, after 11-year follow-up[37]. The 15-year follow-up results revealed that cancer-related death rates were lower (37% *vs* 48%) with a lower rate of local recurrence in the D2 lymphadenectomy group (Table 1)[38]. Subgroup analysis of this trial demonstrated significantly higher survival for females (35% *vs* 21%) and in stage Ⅱ disease (33% *vs* 15%) in the D2 arm. The 15-year survival in patients without pancreatico-splenectomy was significantly higher with D2 than D1 dissection (35% *vs* 22%)[38].

The two latest randomized trials from the 21st century did not present significant differences in postoperative mortality between the D1 and D2 group[35,39]. The morbidity rate was higher with D2 lymphadenectomy in the Taiwanese trial (which compared D1 to D3 dissection; however their D3 lymphadenectomy is similar to the current definition of D2 dissection). The Italian study did not show this difference and proved that D2 dissection could be performed safely without splenectomy and distal pancreatectomy, with comparable mortality and morbidity to those with D1 dissection in specialized centers[39,40]. These rates are comparable to the Japan Clinical Oncology Group (JCOG) 9501 trial and the nationwide Japanese registry where the mortality was less than 2% after D2 dissection[41,42]. Neither did the latter study find any survival benefit from the extended lymphadenectomy[43]. Subgroup analyses showed a 5-year disease-specific survival benefit for patients with pathological tumor 1 (pT1) disease in the D1 group (9% *vs* 83% for the D2 group; $P = 0.015$), and for patients with pT2-4 status and positive LNs in the D2 group (59% *vs* 38% for the D1 group; $P = 0.055$). However, the non-compliance rate was 33.6%[43]. It was concluded that the contamination (over-extensive nodal dissection) (18%) and the higher rate of stage IA disease in the D1 group and of stage Ⅳ in the D2 arm, apparently nullified the effect of correct extended dissection[41,43]. The other randomized trial from Taiwan proved a better ($P = 0.041$) survival with D2 dissection[44].

The results of these recent studies call attention to the importance of the learning curve and the necessity of standardized procedures with routine preservation of the spleen and pancreas in experienced centers[40].

Besides the RCT, the latest meta-analysis found significant differences in morbidity, anastomotic leakage, pancreatic leakage, reoperation rates, wound infection, pulmonary complications and postoperative mortality, all of which favoured D1 dissection. The conclusion was that there is no difference in OS when comparing the D1 and D2 arm. Subgroup analysis of patients

with T3 tumor shows a survival difference favoring D2 lymphadenectomy (25.9% *vs* 11.5%), and there is a trend towards a lower risk of gastric cancer-related death among patients having a D2 dissection with preservation of the spleen or pancreas and non-statistically significant improvement of survival for node-positive patients[40,45]. Unfortunately, the main problem of meta-analysis was that it was not possible to match patient groups for treatment with age, sex, type of gastrectomy, pathological stage, tumor location, co-morbidity, treatment strategies, surgeon experience, hospital case volume and extent of LN dissection, all of which affect postoperative complications and overall survival rates[40].

Keeping this in mind, the comparison of oncological outcomes of D1 and D2 dissections in association with different T and N stages could be problematic due to the concept of stage migration. The reason for this is that a limited lymphadenectomy can not represent the adequate staging of LN involvement. Conversely, extended lymphadenectomy could improve stage-specific survival due to the stage migration phenomenon. Furthermore, Xu *et al*[46]demonstrated that it is necessary to examine at least 16 LNs for accurate pathological examination of gastric cancer, even in node-negative gastric cancer patients[25], and Datta *et al*[47], who analyzed the data of more than 22000 patients found that the examination of 15 or more LN is a reproducible prognostic factor for gastric cancer outcomes in the United States and should continue to serve as a benchmark for the quality of care.

In addition to the quality of surgery, the pathologist plays a large role in the proper identification and examination of the extracted LN[48].

EXTENSION OF LYMPHADENECTOMY BEYOND SUGGESTED LIMITS

The latest issue of the Japanese Gastric Cancer Association treatment guideline contains the standard lymphadenectomies regarding the type of gastric resection: Total gastrectomy with D2: D1 (Nos.: 1-7) + Nos. 8a, 9, 10, 11p, 11d, 12a; distal gastrectomy with D2: D1 (Nos. 1, 3, 4sb, 4d, 5, 6, 7) + Nos. 8a, 9, 11p, 12a; pylorus-preserving gastrectomy with D1+: D1 (Nos. 1, 3, 4sb, 4d, 6, 7) + Nos. 8a, 9; and in proximal gastrectomy with D1+: D1(Nos. 1, 2, 3a, 4sa, 4sb, 7) + Nos. 8a, 9, 11p[49].

In the field of tumor-location specific LN involvement recent studies can be divided into 2 cohorts depend on the position of the gastric tumor (proximal *vs* middle and distal).

Proximal gastric cancer

The frequency of metastasis in station no.4d, 5 and 6 LNs in patients with proximal gastric cancer is more than 10%[14,50]. The incidence of station no.10 LN metastasis is 11.82% in upper third advanced gastric cancer (AGC). The estimated OS were 46% and 37% regarding station no.10 dissection or not, which was not statistically significant. Authors suggest high-quality studies with larger sample sizes to determine the clinical significance of no.10 LN removal[51]. Following an 18 mo follow-up of 108 patients Li *et al*[52] concluded that routine no.10 lymphadenectomy may be unnecessary for advanced, upper third gastric cancer without serosal invasion, unless T3 tumors are located in the greater curvature.

Middle and distal gastric cancer

LN metastasis in station no.2 LNs from distal gastric cancer is only 1.0%, while the metastasis in station no.4 LNs is more than 20%. Since station no.11p is immediately adjacent to stations no.7 and no.9, in the case of distal gastric cancer, station no.11d should be preserved; however, both no.11p and no.11d stations should be removed in cases of proximal gastric cancer[14]. According to Japanese gastric cancer treatment, as station no.14v is closely adjacent to station no.6, station no.14v LNs should also be removed if suspicion of metastasis to the LNs in station no.6 arises[14,49].

As the LN metastasis rate in station no.7 was similar to that of perigastric LNs in 570 patients with advanced distal gastric tumor it is reasonable to include LNs in the no.7 station in the D1 LN dissection[53]. Evaluating LN involvement after total gastrectomy, Galizia found that the incidence of nodal involvement of stations no.10, no.11d, and no.12a was 5%, and the 5-year DFS rate was zero; they concluded that modified D2 lymphadenectomy confers the same oncologic adequacy as standard D2 lymphadenectomy, with a significant reduction of postoperative morbidity[54].

During investigation of LN involvement of the hepatoduodenal ligament (HDLN) a logistic regression analysis showed that no.5 and no.12a LN metastases were associated with a 6.9 and 11.3 fold increase respectively, for risk of no.12p and no.12b LN metastases. In addition, significant differences in 5-year OS of patients with and without no.12p and no.12b LN metastases were observed[55]. However, the clinical significance of removing these LN was not evaluated. Analyzing the data of 1872 patients, LN involvement in station no.12 was 3.6% whereas HDLN metastasis was not a significant factor for survival in multivariate analysis and the 5-year survival rate of 41 patients with HDLN metastasis without distant metastasis at any other site was significantly higher than that among 120 patients with stage Ⅳ disease without HDLN metastasis. It is suggested that the inclusion of HDLN in the distant metastatic LN group in gastric cancer is inappropriate and that the seventh American Joint Committee on Cancer criteria for node grouping should be revised[56].

The incidence of no. 14v LN metastasis was 5.0% in 1661 patients who underwent curative resection for middle or lower third gastric cancer. In clinical stages Ⅰ and Ⅱ, no.14v LN dissection did not affect overall survival; in contrast, no.14v LN dissection was an independent prognostic factor in patients with clinical stage Ⅲ/Ⅳ

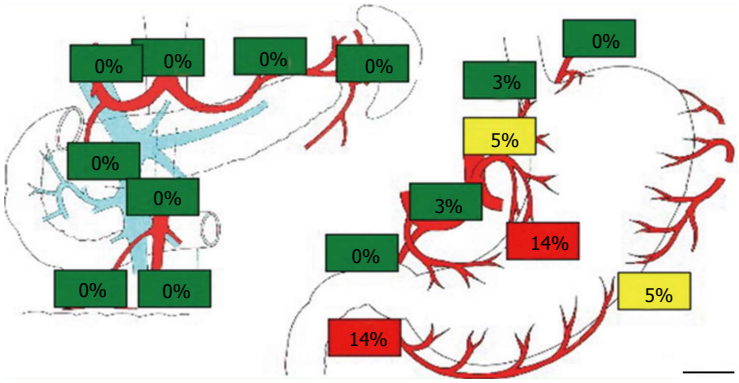

Figure 1 Prediction of lymph node involvement by the Maruyama computer program in a 65-year-old male patient. The tumor histology was well differentiated adenocarcinoma, showing muscular mucosa involvement, early cancer type 2B. The lesion was found in the anterior wall in the lower third of the stomach and had a maximal diameter of 30 mm.

gastric cancer[57].

Involvement of no.13 nodes is defined as M1 in the current version of the Japanese classification. However, excision of this LN may be an option in a potentially curative gastrectomy for tumors invading the duodenum[49].

Para-aortic nodal dissection

The Japanese authors do not perform randomized trials comparing D1 and D2 dissection, because they have excellent operative and oncological outcomes with extended lymphadenectomy. The majority of trials with "super-extended" lymphadenectomy are being performed in Asia. The incidence of para-aortic lymph nodes (PALN) metastases was 8.5% in the JCOG 9501 multi-institutional randomized trial, which proved that D2 lymphadenectomy plus para-aortic nodal dissection (PAND) did not increase the OS (70.3% *vs* 69.2%) or 5-year recurrence-free survival (61.7% *vs* 62.6%) in curable gastric cancer *vs* D2 lymphadenectomy[58]. Robertson strengthened this finding in a prospective randomized trial from Hong Kong[59]. Junfeng *et al*[60] found 6 risk factors to predict the involvement of PALN: Tumor in the upper third, tumor size over 5 cm, tumor penetrating deeper than T2, tumor in stage N2 and N3, tumor regarded as the poorly differentiated type and the Borrman 3, 4 type by macroscopic classification. Additionally, the metastasis of lower lymph nodal stations may be predictors of the positive PALN especially no.1, no.3, no.7 and no.9 stations which evidently had higher odd ratios than the others[60]. de Manzoni *et al*[61] did not find a significant difference in the cumulative incidence of recurrence between D2 and D3 lymphadenectomy in the analysis of 568 patients. The risk of recurrence was higher after D3 than after D2 (45.1% *vs* 35.3%, $P = 0.078$) in the intestinal histotype while the pattern was reversed in the mixed/diffuse histotype (48.3% *vs* 61.5%, $P = 0.084$)[61].

In overall 5-year survival Zhang *et al*[62] could not demonstrate a significant difference between patients underwent D2 plus PAND surgery and those underwent D2 surgery. He suggests that this "over-extended" dissection should only be recommended for T3-4 and N2 stage gastric tumor and should not be utilized for EGC and total gastrectomy[62].

So, the D2 lymphadenectomy is the gold standard in R0 resection by the Japanese[49] and European guidelines[27].

The American NCCN guidelines recommend a D1+ or a modified D2 LN dissection, the latter performed by experienced surgeons in high-volume centers[27,63]. To support this, the deployment of centralization and implementation of national clinical guidelines in Denmark resulted in a decrease in mortality from 8.2% to 2.4% and the proportion of patients with at least 15 LNs removed has increased from 19% to 76%[64].

MARUYAMA COMPUTER PROGRAM

The Maruyama computer program (MCP) was developed and first published in 1989[65]. It was later improved as the Windows-based program WinEstimate v. 2.5 using a database of 4302 primary gastric cancer patients treated at the National Cancer Center Hospital in Tokyo between 1968 and 1989[66,67]. This program calculates the expectation (%) of lymph-node involvement in station No. 1-16. The calculation required the following prognostic factors: Age, gender, position of the tumor, Bormann's classification or EGC classification, depth of infiltration and histological type (Figure 1)[68]. MCP was validated in Japanese patients and the program was able to predict LN involvement in 94%[69]. In the European region the accuracy of MCP was 91% in Slovenian patients[67] and 83.4% for stations 1 to 6 and 81.6% for stations 7 to 12 in Italian patients[70]. The sensitivity for LN detection was high (97%-100%) in a German study of 222 patients but a specificity as low as 20% was found for perigastric LNs (stations 1 to 6)[68,71]. Similarly in an Italian study, where the sensitivity increased to 100% with a lower cut-off level, the specificity decreased to 26%. Better prediction of LN metastases may be feasible with the artificial neural network using the following parameters: Bormann classification, depth of tumor infiltration, size, location of tumor, and LN metastases in station 3. These increased accuracy from 66% to

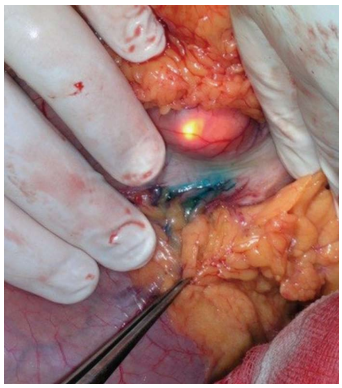

Figure 2 Sentinel lymph node mapping following submucosal marking by an endoscopist.

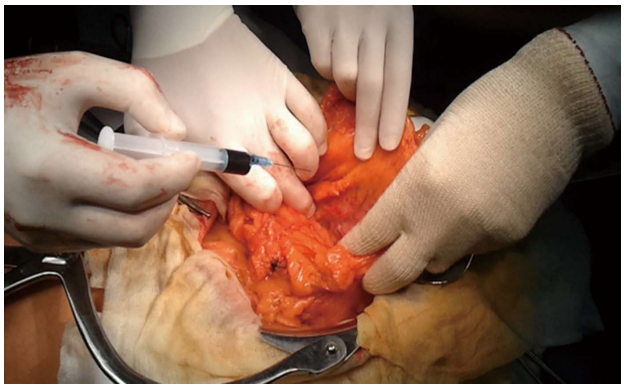

Figure 3 Subserosal marking by a surgeon.

93%[72].

Our study demonstrated a similar degree of reliability of MCP to those cited above, with 90.2% of sensitivity, 63.3% of specificity and 78.4% of accuracy. The rate of false negatives was 9.8%[73]. These studies demonstrate that the results of the computerized prediction of LN metastases are superior to those of the standard pre-operative imaging techniques.

Another advantage of the MCP is that it can determine long term oncological results. Hundahl defined the Maruyama Index (MI) at first in 2002 as a measure of unresected regional nodal disease in gastric cancer using the data of the Intergroup 0116 trial and he proved it is an independent predictor of survival[74,75]. Peeters *et al*[76] reanalyzed the data of the Dutch D1-D2 trial using univariate and multivariate analyses and showed that the MI is an independent predictor of overall survival (P = 0.016, HR = 1.45) and relapse risk (P = 0.010, HR = 1.72). It was concluded that the MI is a quantitative yardstick for assessing the adequacy of lymphadenectomy in gastric cancer patients[75,76]. Later, Hundahl evaluated autopsy findings from the Dutch D1-D2 trial and showed that MI < 5 or a low MI for surgery is associated with enhanced regional control and survival[76,77]. Dikken *et al*[78] proved the prognostic significance of low MI in a 2-year survival rate (82% *vs* 59%), as did Sachdev, who demonstrated that lower MI correlated with better survival, as a continuous (P < 0.02) and categorical (P < 0.04) variable[79].

Overall these results suggest that a Maruyama Index less than 5 has a better impact on survival, than D-level guided surgery. For these reasons, the preoperative application of MCP is recommended routinely, with an aim to perform "low Maruyama Index surgery". In addition, the application of MCP to predict LN involvement can influence the indication for neoadjuvant chemo-therapy, and furthermore a "high Maruyama Index" could indicate the necessity for postoperative oncological treatment.

SENTINEL LYMPH NODE BIOPSY

While the MCP calculates the probability of LN involvement preoperatively, the concept of sentinel lymph node biopsy (SNB) can determine the existence of LN metastases intraoperatively. The first potentially affected LN, the sentinel lymph node (SLN), reliably reflects the status of the nodes in the second and third line, which is supported by data of numerous publications. If the SLN contains tumor deposit(s), extended dissection is warranted, but if findings are negative, the patient could be spared additional complications associated with extended dissection. However, the method of dye/tracer injection and the tracer's selection is controversial. Some authors use dye alone (patent blue, indocyanine green, isosulfan blue)[11,80-82], Kitagawa *et al*[83] handle 99m Tc colloid, and Aikou *et al*[84] uses the combination of these tracers. The latest systematic review concluded that the SLN's identification rate is the same with the dual or single mapping method[85]. It is eminent that body-mass index (BMI) affects the sentinel LN detection rate[86]. The Hungarian study proved that the identification of sentinel LNs in obese patients can be difficult owing to the feathering of blue dye in the fatty tissues[11]. This was concluded as the only patient in whom marking did not occur had a BMI significantly higher than average (26.8 *vs* 22.8)[11]. Then again, the application of blue dye for SNB has a beneficial side effect, as it significantly increased the number of harvested LN and the ratio of the number of the harvested LN per time[87]. To avoid quick dispersal to multiple LNs Kong applied ICG/poly-γ-glutamic acid complex, which remained longer than diluted ICG in animal models[88].

Yaguchi, Lee and Tóth have compared the subserosal to the submucosal labeling method (Figures 2 and 3) without any significant difference and they suggest the endoscopic injection of a tracer in cases of non-palpable tumors and/or laparoscopic procedures[89-91].

The cardinal problem in the SNB concept is the intraoperative false negative rate. The JCOG 0302 trial called attention to the importance of the learning curve and the inadequacy of the pick-up method. The demand for only five patients per institute provided an insufficient learning period which presented a 46% false negative rate[92].

Lee *et al*[93] proved that the removal of entire nodal basins can significantly decrease this rate against the pick-up method, and Kumagai *et al*[94] called attention to the opportunity of introducing the one-step nucleic acid

amplification test for the intraoperative diagnosis of LN metastasis with similar results to postoperative 2-mm-interval histological examination.

Miyashiro *et al*[95] demonstrated that an extensive surgical experience is necessary for application of SNB concept and standardization of SLN mapping technique, using improved tracer, and guideline to evaluate the positiveness of SLN specimen should be planned to incorporate SNB in routine practice[96]. Recent studies and the latest meta-analysis of SLN mapping have shown a high detection rate (93.7%) and an accuracy of 92%[97] and suggest that the SNB concept could be suitable for tumors following endoscopic resection[98] and could represent a new era of sentinel node navigation surgery in EGC[99,100]. Moreover, its success rate did not correlate to tumor grade[101].

Based on the results of the largest prospective multicenter trial from Japan with an identification rate of 97.5% and an accuracy of 99%[102], a phase Ⅲ multicenter trial for individualized surgery for EGC based on SLN mapping has been commenced in the Eastern Asian countries. The long-term results of these studies will be available between 2018 and 2020.

CONCLUSION

The latest RCT comparing D1 and D2 dissections represents a higher surgical quality (more contamination, less non-compliance, low morbidity and mortality rate) than previous trials[43]. This could lead to a trend towards the execution of the less limited D1 lymphadenectomy for more experienced and well-trained surgeons, and hopefully the results of western surgeons will achieve a level similar to those of the Asian surgical outcomes in the near future.

On the other hand, the era of multimodal treatment and the increase in elderly patients with serious comorbidities indicates the necessity of a stage- and patient-adapted, individualized surgery in gastric cancer. It was conceived at an expert panel, also: "A D2 lymphadenectomy is preferred for curative-intent resection in advanced, non-metastatic gastric cancer; in patients with EGC or substantial comorbidities, a D1 lymphadenectomy is more appropriate"[103]. The Japanese guidelines enclose that the AGC should be treated with D2 lymphadenectomy. D1 or D1+ should be recommended as a choice for EGC. D1+ can be an alternate for D2 in high-risk patients[104]. Inokuchi *et al*[105] suggested that the presence of heart or liver disease is a significant risk factor for postoperative morbidity in patients who undergo laparoscopic gastrectomy. Although it did not reduce complications, insufficient LN dissection (for example, D1+ for advanced gastric cancer) might be permissible in high-risk patients as it had no negative impact on gastric cancer-specific survival. More accurate stage-adapted surgery could be performed using the MCP and SNB in parallel fashion.

It is generally accepted that metastases in the SLNs warrant a D2 lymphadenectomy. The authors analyzed the relevance of MCP in sentinel node positive patients in an earlier study[73]; while the efficiency of SNB method is superior to MCP, the positive predictive value of MCP and SNB was proven equivalent in the sentinel node positive group and the accuracy of MCP in these cohort of patients was 10% higher. For these reasons it would be interesting to find the appropriate combination of these techniques in the future and we suggest using them simultaneously in the operating room.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Role of *Helicobacter pylori* in gastric cancer: Updates

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Abstract

Helicobacter pylori (*H. pylori*) infection is highly prevalent in human, affecting nearly half of the world's population; however, infection remains asymptomatic in majority of population. During its co-existence with humans, *H. pylori* has evolved various strategies to maintain a mild gastritis and limit the immune response of host. On the other side, presence of *H. pylori* is also associated with increased risk for the development of various gastric pathologies including gastric cancer (GC). A complex combination of host genetics, environmental agents, and bacterial virulence factors are considered to determine the susceptibility as well as the severity of outcome in a subset of individuals. GC is one of the most common cancers and considered as the third most common cause of cancer related death worldwide. Many studies had proved *H. pylori* as an important risk factor in the development of non-cardia GC. Although both *H. pylori* infection and GC are showing decreasing trends in the developed world, they still remain a major threat to human population in the developing countries. The current review attempts to highlight recent progress in the field of research on *H. pylori* induced GC and aims to provide brief insight into *H. pylori* pathogenesis, the role of major virulence factors of *H. pylori* that modulates the host environment and transform the normal gastric epithelium to neoplastic one. This review also emphasizes on the mechanistic understanding of how colonization and various virulence attributes of *H. pylori* as well as the host innate and adaptive immune responses modulate the diverse signaling pathways that leads to different disease outcomes including GC.

Key words: Cag pathogenicity island; Gastric cancer; Gastric mucosa; *Helicobacter pylori*; Type Ⅳ secretion system

Core tip: Although the incidence and mortality of gastric cancer (GC) is declining in recent decades but it still remains a major threat in developing countries as compared to developed one. Among various etiological agents, *Helicobacter pylori* (*H. pylori*) play a detrimental role in development of GC. Through this review we focus on the recent progress in the field of research on *H. pylori* induced GC and providing the brief insight into *H. pylori* pathogenesis, the role of major virulence factors of *H. pylori* that modulates the host environment

and transform the normal gastric epithelium to neoplastic one.

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INTRODUCTION

In 1984 Marshall and Warren[1] identified *Helicobacter pylori* (*H. pylori*) from gastric biopsy culture. In 1994, *H. pylori* was recognized as definite carcinogen by International agency for research on cancer. *H. pylori* induced gastric cancer (GC) is accountable for 5.5% of global cancer burden[2].

H. pylori is spiral shaped, gram-negative, microaerophilic, flagellated human pathogen that successfully colonizes gastric mucosa of majority of individuals[3]. Epidemiologically, the *H. pylori* infection is exists all over the world, but colonization rates vary considerably; high in developing compared to the developed world[4]. *H. pylori* acquisition thought to occurs in early childhood. Fecal-oral or oral-oral were considered as possible route of *H. pylori* transmission[4,5]. *H. pylori* urease is among the various virulence factors that aids in colonizing the highly acidic environment of stomach *via* breakdown of urea into ammonia, generating hospitable locale for its colonization[6] (Figure 1). Among the majority of *H. pylori* infected individuals only a small percentage of colonized individuals develop severe clinical disease such as GC. Determing factors responsible for variation in clinical outcomes of *H. pylori* infection are still not well studied. For a longer period of time association between *H. pylori* and GC was debatable. A study from Japan on 1526 patients gives a clear evidence that *H. pylori* infection is significantly associated with risk of developing GC[6]. Proof that *H. pylori* has an influence on early stages of gastric carcinogenesis is demonstrated by randomized prospective studies which shows association between *H. pylori* eradication and reduction of premalignant tumors[7,8]. Research on experimentally challenged Mongolian gerbils, provide evidence concerning *H. pylori* eradication with attenuation of developmental process related to GC progression[9,10]. Together these studies authenticate that *H. pylori* plays a key role in development of GC and indicate that *H. pylori* eradication provide protection against *H. pylori*-induced GC. Interaction among environmental factors, host genetic polymorphism and bacterial virulence attributes collectively influence the clinical outcome of *H. pylori* infections[11].

This review aims to highlight recent progress in *H. pylori* pathogenesis, especially the bacterial and host factors that are involved in the host-pathogen interaction during persistent colonization. It also highlights the host immune response towards *H. pylori* colonization and its effect on diverse clinical outcomes, especially on advancement leading to GC.

EPIDEMIOLOGY OF GC

GC is a multifactorial disease. Correa's model describes array of event beginning from chronic active gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and eventually leads to GC[12] (Figure 2). Risk factors for the development of the GC include interaction among the pathogen, environmental and host-related factors[13]. World Health Organization recognized *H. pylori* as class Ⅰ carcinogen in 1994. GC is identified as the fifth most common malignancy and third leading cause of cancer-related morbidity globally, constituting 9.7% of all cancer-related mortality[14]. Highest age-standardized mortality rate (ASMR) is predicated for Eastern Asia (28.1 per 100000 in men, 13.0 per 100000 in women), the lowest ASMR in North America (2.8 and 1.5 per 100000, respectively)[15]. Studies reported high mortality rates are from East Asia, Central and Eastern Europe, Central and South America[15]. Developing countries have high burden of GC compared to the developed world and GC accounts for approximately 70% of both new cases and deaths[16]. Categorizing on basis of gender, 466900 cases of males were reported from developing as compared to 173700 cases from developed countries and for females the corresponding disease load was 247000 and 102000 cases, respectively. GC is associated with age incidence; commonly occurs in age group of 55 to 80 years, rare among young individual. Frequency of GC rates are two fold higher in males than females[17].

Over past decades in western nations, GC has considerably declined. The possible reasons behind this reduction include fall in *H. pylori* prevalence accompanied by better hygienic practices and innovative medical diagnostic facility. Despite the decline in GC incidence in developed world, the scenario of developing world is diverse. GC incidence and mortality rate remain very high in the developing nations, particularly in regions of East Asia and South America[17]. It is expected that if appropriate measures are not implemented the number of estimated GC cases are likely to increase in future.

PATHOLOGICAL DIFFERENTIATION

Majority of gastric malignant tumors are adenocarcinomas. Histologically Lauren categorized gastric adenocarcinoma in intestinal and diffuse subtypes. Intestinal type adenocarcinoma is event dependent, start from chronic atrophic gastritis to intestinal metaplasia to dysplasia and finally carcinoma. Intestinal type adenocarcinoma is more frequent in developing world, common in male, and associated with age incidence, whereas diffuse type occurs more often in younger patients having family history of cancers, more frequent in females, background of atrophic gastritis is not prerequisite condition for its occurrence[18,19]. Anatomical site of origin is another way of differentiation of gastric adenocarcinoma. Tumors

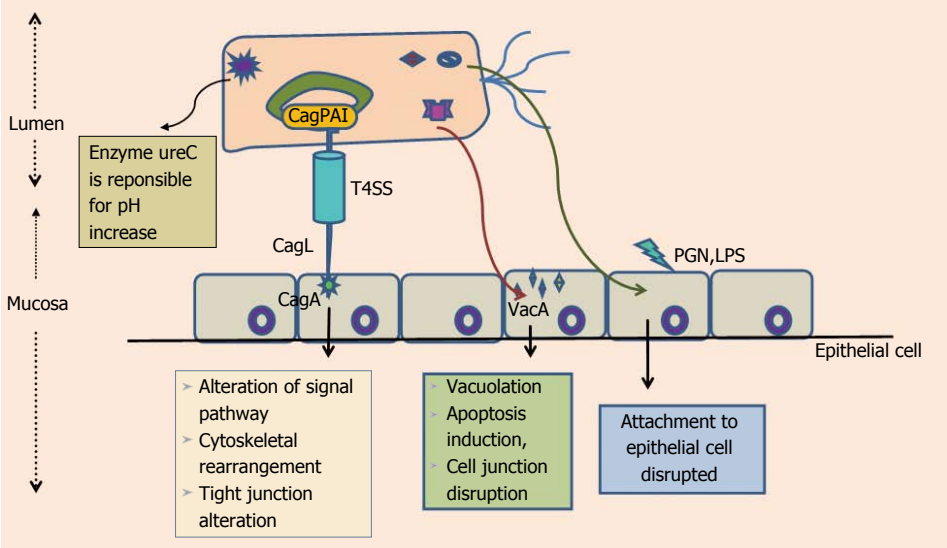

Figure 1 Interaction between *Helicobaceter pylori* type Ⅳ secretion system and virulence determinants such as CagA, CagL, lipopolysaccharides, pepetidoglycan and vacuolating cytotoxin gene, with mucosal epithelial cells, resulting in alteration of signal pathways, cell polarity disruption and vaccuolation, which ultimately leads to death. T4SS: Type Ⅳ secretion system; LPS: Lipopolysaccharides; PGN: Pepetidoglycan; VacA: Vacuolating cytotoxin; CagPAI: Cag pathogenicity island.

arising in the cardia region of the stomach are said to be proximal, and those from body and antrum (non-cardia region) as distal. Histological subtypes represent etiological and epidemiological differences between the two tumor sub sites. Globally GC incidence is declining. However, studies show rise in incidence of cardia carcinoma which may be partly due to more accurate reporting and fall in incidence of distal cancers[20].

H. PYLORI AS A RISK FACTOR FOR GC

Colonization of the stomach by *H. pylori* causes development of gastritis. *H. pylori* is truly an "opportunistic" bacterium that uses various well defined virulence factors as tool for attachment and persistent colonization of human gastric mucosa. The possible transmission route is fecal-oral, but contaminated food or water are also reported[21,22]. The most likely sources are person-to-person contact in families and/or exposure to a common source of infection such as contaminated water or food as supported by majority of data[23]. This notion is supported by studies of children in custodial care where the prevalence of infection is higher than expected and from studies of crowded families in which there is at least one infected child[24].

Before attachment of *H. pylori* to gastric epithelium, it has to first cross the thick mucus layer by adhering to the mucosal surface. This is aided by the presence of unipolar sheathed flagella, which allows *H. pylori to* quickly move from inhospitable low pH of gastric lumen to surface epithelium where pH is high and favorable for its successful colonization despites efforts made by the host to get rid of this bacterium. Non-motile mutant *H. pylori* strains fail to colonize the stomach of gnotobiotic piglets[25,26]. In majority of infected individuals colonization results in development of inflammatory and immune responses against *H. pylori*, but in some subjects *H. pylori* infection becomes chronic and leads to induction of gastric inflammation which can eventually lead to destruction of normal gastric glands and their replacement by intestinal-type epithelium resulting in atrophy of gastric mucosa.

The risk for atrophic gastritis depends on pattern as well as extent of distribution of chronic active inflammation. The individuals with lower acid output show a higher tendency towards atrophy[27]. Reduction in gland size and level of intestinal metaplasia were associated with rise in GC risk by 5- to 90-folds depending on the extent and severity of atrophy[28].

Increased odds ratios were evident from case-control studies that aimed to seriously study the signs of earlier *H. pylori* infection in GC patients and controls for development of non-cardia GC in presence of *H. pylori* infection[29]. This fact is supported by data from animal models including Mongolian gerbil model, in which *H. pylori* infection induces atrophic gastritis and GC[30-32]. A small number of subjects for research purposes were deliberately infected with pathogenic *H. pylori* strain and individuals developed acute inflammation of gastric mucosa with neutrophilic infiltration[33,34]. Volunteers after several decades when exposed repeatedly to intragastric pH-electrodes contaminated with *H. pylori* developed conditions called "epidemic hypochlorhydria"[34]. Such hypochlorhydric gastritis can either resolve spontaneously or change into chronic gastritis.

ROLE OF HOST GENETICS

H. pylori infection results in three possible outcomes. First is corpus-predominant gastritis beginning from

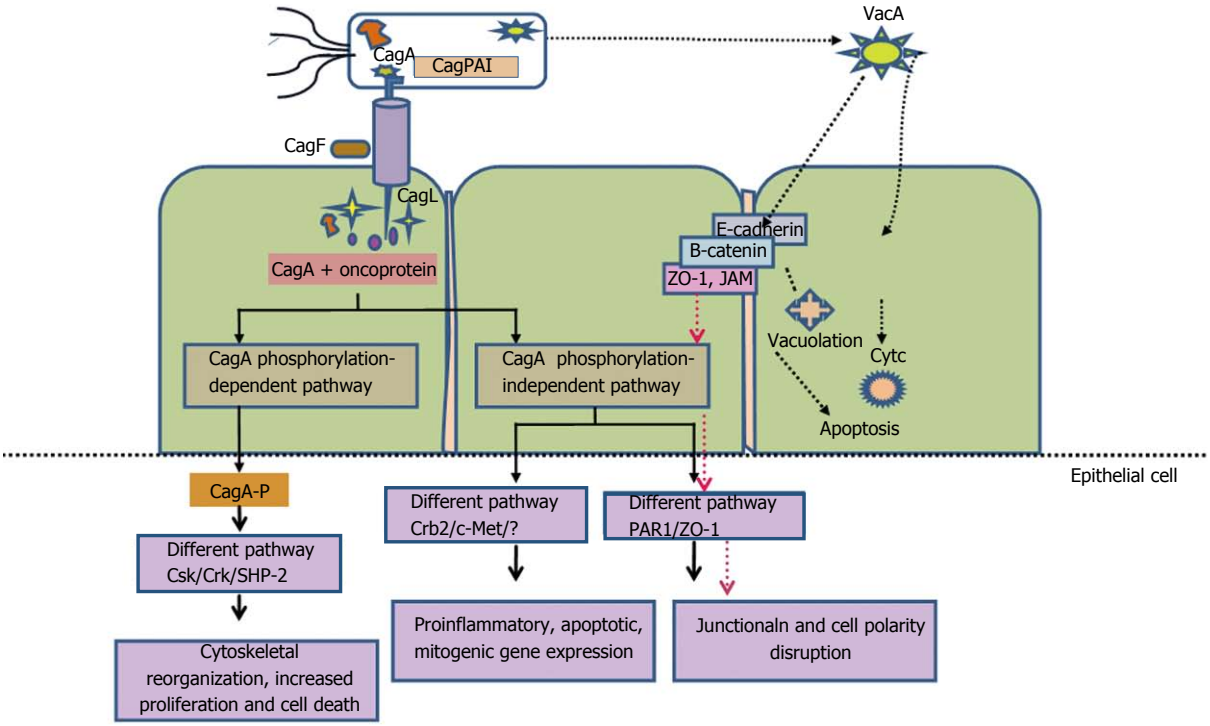

Figure 2 Combination of host, bacterial and environmental factors, which act in a synergetic way, resulting in development of precancerous cascade that ultimately leads to development of gastric cancer. ZO-1: Zonula occludens 1; CagPAI: Cag pathogenicity island; JAM: Junctional adhesion molecule; VacA: Vacuolating cytotoxin; Cytc: Cytochrome.

atrophic gastritis to hypochlorhydria and finally to GC. Second type results in a pangastritis having slightest impact on the host gastric acid production. Duodenal ulcer is third outcome, where an antrum-predominant gastritis leads to hyperchlorhydria. There arises controversy that infections of *H. pylori* can predispose to two equally exclusive situations. The possible explanation why some people are more expected to develop GC phenotype when compared with others may be due to disparity among individual host response to *H. pylori* infections (Figure 1). Initial evidence for the importance of host genetic polymorphisms was reflected in the study where a rise in incidence of atrophic gastritis and hypochlorhydria was evident from relatives of *H. pylori* induced GC patients than controls[35].

Pro-inflammatory cytokine like interleukin-1beta (IL-1β) act as a powerful negative regulator of acid secretion. *IL-1β* gene is now considered as a potential contender for host genetic polymorphisms that may elevates GC risk. Individuals possessing *IL-1β* gene cluster polymorphisms have 2–3-folds increased risk of non-cardia cancer[36,37] (Figure 2). Elevated levels of TNF-α in gastric mucosa of *H. pylori* infected individuals were evident from numerous studies. However, down regulation of anti-inflammatory cytokine IL-10, that suppresses the level of pro-inflammatory cytokines including IL-1β, TNF-α and interferon-γ (IFN-γ) is also reported[36].

The risk associated with GC development in *H. pylori* infected individuals upsurges 27-folds in individuals with three or four polymorphisms[38]. This evidently illustrates that interaction between host genetics and environment plays a key role in progression of GC, by regulating hosts adaptive immune response resulting in transformation of normal gastric mucosa to neoplastic one.

IL-8

Higher expression of chemokine IL-8 and polymorphism (promoter region) has been reported in studies and linked with increased risk for GC[39]. Study on Caucasian populations proved that relationship among functional polymorphism within Toll like receptor 4, risk of GC and decrease in production of anti-inflammatory cytokine IL-10[40]. These studies reflect that host genetic polymorphisms are capable of modulating the innate immune response which results, severe inflammation and premalignant lesions in *H. pylori* infected individuals (Figure 2). These studies raises a query that whether *H. pylori* strain characteristics are responsible for increasing cancer risk employed by host genotypes, needs to be studied further. Odds ratios for non-cardia GC were highest for individuals with elevated IL-1β expression, colonized by *H. pylori* vacAs1-type strains[41].

It is evident from case-control studies that *H. pylori* successfully form a vital equations with host by its ability to send and receive signals from its hosts[42,43]. Only certain *H. pylori* strains enhance the possibility of carcinogenesis because the equilibrium is likely different for each colonized individual. For example, individual infected with CagA strains leads to severe gastritis, which results in rise of proinflammatory cytokines levels that are responsible for both amplifying the mucosal inflammatory response as well as reducing the acid

production. This creates a milieu encouraging growth of *H. pylori* that promote inflammation and continually produce oxidative stress, thus augmenting risk for transformation of normal mucosa to neoplastic through series of events (Figure 2).

Cyclooxygenase

H. pylori triggers numerous forms of proinflammatory cyclooxygenase (COX) enzymes. Production of endoperoxide from arachidonic acid is brought by COX enzymes. Enzymes prostaglandin synthases produces prostaglandins and various eicosanoids from endoperoxide[44]. Important role is played by prostaglandins in regulating physiologic processes for instance immunity and development. Two COX isoforms (COX-1 and COX-2) have been categorized on the basis of variances in expression characteristics and inhibition profiles for nonsteroidal anti-inflammatory drugs (NSAIDs). COX-2 expression is inducible while COX-1 is constitutively expressed in cells and tissues[45-47]. Expression of COX-2 can be stimulated by proinflammatory cytokines, growth factors such as TNF-α, IFN-γ and IL-1. COX-2 expression are raised in *H. pylori* infected human gastric mucosa, gastric premalignant and malignant lesions[47-49]. Inhibitors of COX (aspirin and NSAIDs) are associated with reduced risk of non-cardia GC[50]. Numerous studies demonstrate substantial role of COX-2-generated products involved in promoting neoplasia. Mechanisms like apoptosis inhibition, regulation of expression of cell surface adhesion, and production of promoting factors of neoplasia leads to malignancy[51,52] (Figure 2).

H. PYLORI VIRULENCE FACTORS

Cag pathogenicity island

H. pylori have genetically heterogeneous genome. A number of *H. pylori* virulence factors are supposed to play an essential role in diverse clinical outcome of *H. pylori* infections. The Cag pathogenicity island (CagPAI) is a 40-kb region, consisting of 32 genes, flanked by 31-bp direct repeats. CagPAI is an island consisting of virulence genes, which are acquired by horizontal transfer. CagPAI encodes a type Ⅳ secretion system (T4SS) that is responsible for the entrance of a most remarkably investigated *H. pylori* virulence determinant effector protein CagA[53-55] (Figure 1). Positive association of CagA was found with peptic ulcer disease[56,57]. Due to its association with several gastroduodenal pathologies, initially CagA was considered as an indicator for presence of the entire CagPAI but as research speeded up, studies demonstrated that despite its presence, CagPAI intactness and clinical outcome varied.

More or less 70% of *H. pylori* strains from western world and nearly 100% of East Asian strains express virulent protein CagA[54,58,59]. Majority of *H. pylori* strains induces superficial gastritis but the risk for chronic gastritis, atrophic gastritis, metaplasia, and non-cardia GC with intact CagPAI is much higher compared to those that lacked it[56,57,60-66]. Among 32 genes of CagPAI, 18 genes are thought to code for structural parts of a T4SS, this system is responsible for exporting peptidoglycans and cagA into host gastric epithelial cells, *via* forming a pilus like assembly connecting bacterial and host epithelial membrane (Figure 1).

CagA

CagA is terminal gene product of the CagPAI. Classifying *H. pylori* strains on the basis of presence and absence of cagA into cagA-positive and cagA-negative strains. After the *H. pylori* attachment to epithelial cell, CagA is internalized through T4SS apparatus. After translocation, CagA is tyrosine phosphorylated at glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motif, *i.e.*, EPIYA motif which is associated with cell morphological changes known as "the hummingbird phenotype," which results in increased cellular migration[67-71].

Polymorphic region of CagA, has been identified within the carboxy-terminal and distinguished by different amino acid sequences. Till date, four distinct EPIYA motifs (EPIYA-A, -B, -C and D) are known[72,73]. EPIYA-A and -B motifs are present in strains all over the world, whereas EPIYA-C is specific to western world (Europe, North America, and Australia). Variation in number of EPIYA-C sites occurs, while majority of CagA proteins contain a single EPIYA-C site (A-B-C type). The level of phosphorylation of EPIYA-C sites is greater than EPIYA-A and EPIYA-B sites. Risk for development of GC is found to be associated with the number of cagA EPIYA-C in western strains[74]. EPIYA-D motif is exclusive to East Asian strains (from Japan, South Korea, and China), and strains possessing this motif produces higher level of IL-8 from gastric epithelial cells as compared to strains harboring western A-B-C-type CagA[72,75].

CagA phosphorylation-dependent host cell signaling

Kinase families of Abl and Src are responsible for phosphorylation of CagA into phospho-CagA. Interaction between phosphorylated CagA and various intracellular effectors, triggers an eukaryotic tyrosine phosphatase (SHP-2), which results in continuous stimulation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), Crk adaptor[76] and C-terminal Src kinase in a tyrosine phosphorylation-dependent manner. In East Asian A-B-D types, negative response is induced by interactions of phospho-CagA with C-terminal Src kinase resulting down regulation of Src signaling[77] (Figure 3).

Experimental studies on cell lines revealed that CagA internalization give rise to "hummingbird phenotype". These alterations are characterized by cell elongation and cell scattering[69,78]. Additional study also indicates that interplay among phosohorylated CagA, dephosphorylation of SHP-2 and down-regulation of focal adhesion kinase, causes cell elongation[69,79]. A different mechanism of cell elongation by phosphorylated CagA is by making a defect in cell retraction; yet the signaling molecules prerequisite for this phenotype remain vague[80].

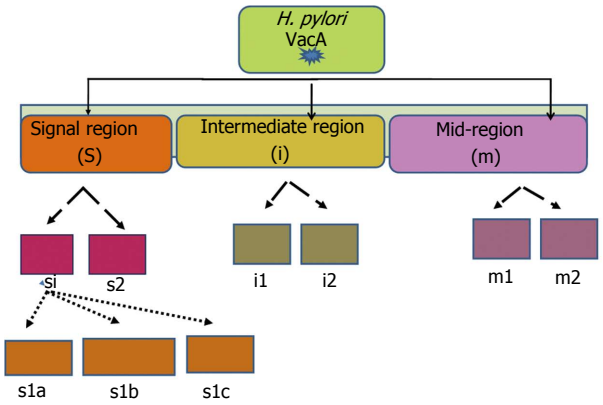

Figure 3 Schematic representation of multiple pathways of *Helicobacter pylori* pathogenesis involved type Ⅳ secretion system and internalization of virulence determinants like CagA and oncoproteins; CagA-phosphorylation dependent and CagA-phosphorylation independent pathways leads to cytoskeletal reorganization, increase proinflammatory and mitogenic gene expression. Another major virulent factor, VacA is responsible for alteration of junction and cell polarity by binding with tight junction molecules such as E-cadherin, ZO. VacA also causes mitochondrial membranes depolarization, Cytc release from mitochondria to cytosol and caspase-3 activation followed by cell apoptosis. T4SS: Type Ⅳ secretion system; VacA: Vacuolating cytotoxin; ZO: Zonaoccludans; Cytc: Cytochrome; *H. pylori*: *Helicobacter pylori*.

Phosphorylated CagA obstructs the enzymatic activity of c-Src, which leads to tyrosine dephosphorylation of actin binding proteins such as cortactin, ezrin, and vinculin, ultimately results in cell elongation[81-83] (Figure 3).

CagA phosphorylation-independent host cell signaling

Non-phosphorylated CagA have a different way of exerting effects within the cell. CagA translocation without phosphorylation leads to aberrant catenin activation, apical-junctional complex disruption and cellular polarity loss[84-89]. Relation between non-phosphorylated CagA and epithelial tight junction scaffolding proteins, zonula occludens 1 and junctional adhesion molecule A, results in imperfect association of tight junctions at located sites of bacterial attachment. Additional molecules includes E-cadherin, hepatocyte growth factor receptor c-Met, phospholipase C gamma (PL), adaptor protein Grb2, and kinase partitioning defective 1b/microtubule affinity-regulating kinase 2 (PAR1b/MARK2) resulting in mitogenic responses, interruption of cell-cell junctions and cell polarity destruction[84,87,88,90] (Figure 3). Recent study revealed that CagA directly binds to the cell polarity regulator such as PAR1b/MARK2. This binding prevents kinase PAR1b/MARK2 activity and deregulates the formation of mitotic spindle by cells which affects cell polarity[88,91].

Studies on transgenic mice revealed the correlation between CagA and oncogenesis by showing that CagA expression led to gastric epithelial cell proliferation and neoplastic changes. However, the following modifications were not detected in mice expressing phosphorylation-resistant CagA[52].

Presence of contradictory documentation on functionality of CagA as a bacterial oncoprotein in mammals exists besides solid proof provided by animal models. Pathological alterations described for transgenic CagA mice followed by absence of inflammation, which reflects disparity to what is seen in humans[52]. Although CagA act as oncoprotein, it remains to be explored why only few individuals inhabited by CagA-positive *H. pylori* develop GC. Recent study demonstrate that *H. pylori* prompts the presence of a host phospholipid, phosphatidylserine where CagA can explicitly interact and gain entry into the cells[92]. Focus of future research should be to define the exact mechanism of CagA internalization in gastric epithelial, factor responsible for regulation of this process and when during chronic infection CagA delivery in human epithelial cells.

VacA

Another important *H. pylori* virulence gene is vacuolating cytotoxin (VacA), which encodes a bacterial toxin (VacA) that induces series of cascades leading apoptosis of epithelial cells *via* induction of cytoplasmic vacuoles (Figure 4). VacA is found throughout the *H. pylori* strains. The diverse polymorphic form of VacA are related with clinical outcomes[93]. Considerable genetic variations are found in: The s (signal) region with alleles s1a, slb, slc, or s2; the m (middle) region with m1 or m2 alleles; and the i (intermediate) region with type i1 or i2 alleles (Figure 4).

H. pylori strains having combination VacAs1/m1 or vacAs1/m1/i1 are associated with increased risk of progression to premalignant lesion and GC than vacA s2/m2 or vacAs2/m2/i2 strains[94] (Figure 5).

OTHER RISK FACTORS

Besides *H. pylori*, the following other environmental factors are considered to contribute in the pathogenesis of GC.

Diet

The variations in GC incidence are due to environmental inputs, particular in dietary pattern. Previous accumulating studies have been indicated that downward trend in GC occurrence. This may be due to the advent of widespread refrigeration of foodstuff and reduction in dependency on food preservation. In addition, other studies have suggested that a preventive role of diet containing fresh vegetables and fruits. However, data from European prospective study failed to show an overall association between fresh fruits and vegetables intake and GC risk[95]. Recent studies being conducted on this field revealed that a significant association between total dietary vegetables contents (onion and garlic intake) and intestinal GC subtypes.

Additional studies are required for demonstration of positive association between *H. pylori* eradication and prevention of cancer. The controversy related to point of no return in case of atrophy and metaplasia is still debatable. Proposed studies on side effects and expenses of such preventive measures are required in future for proper management and treatment of GC, therefore GC prevention remains a key part of research on *H. pylori*.

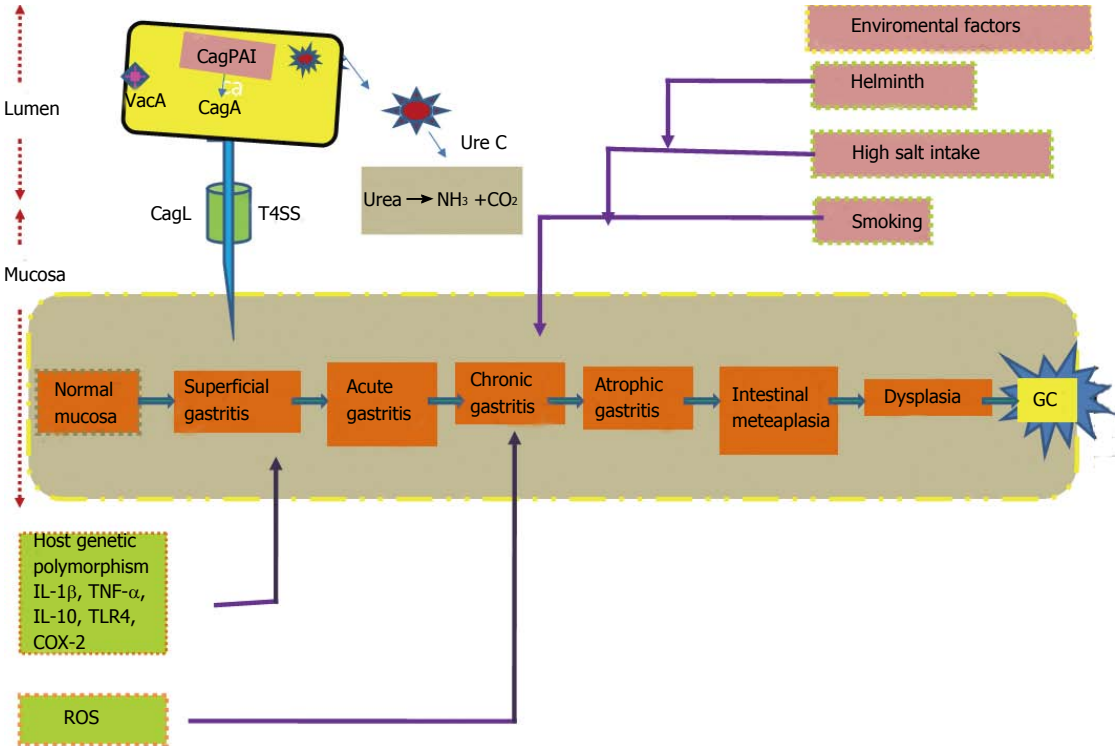

Figure 4 Representation of *Helicobaceter pylori* major virulence factor, vacuolating cytotoxin containing three domain 1: signal sequence(S) 2: middle region (m) 3: recently identified intermediate region (i) s, m and i region are further stratified into the subtypes s1, s2, m1, m2 and i1, i2 respectively. TLR4: Toll like receptor 4; T4SS: Type Ⅳ secretion system; VacA: Vacuolating cytotoxin; CagPAI: Cag pathogenicity island; GC: Gastric cancer; ROS: Reactive oxygen species.

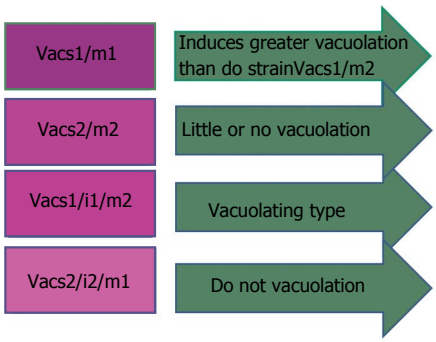

Figure 5 Vacuolating cytotoxin of *Helicobacter pylori* may have any combination of signal sequence and mid region with different virulence activities as stated above. Vac: Vacuolating cytotoxin.

Salt

H. pylori is not the only the culprit for the development of GC; other influential causes include host polymorphisms and environmental elements (Figure 2). High dietary salt intake was found to be uniformly been associated with an increased risk of GC[12,96]. Two studies, one study from Japan and other a case-control study from South Korea stated that *H. pylori*-infected subjects taking high-salt diet had an greater risk of GC than those with lower levels of salt[97,98]. Association between the frequency of *H. pylori* infection and amount of dietary salt intake is reported in another study[99].

Research on Mongolian gerbils had shown that the *H. pylori* presence and usage of a more salt containing diet applied concerted effects on development of precancerous satge[100,101]. Additional study on *H. pylori*-infected gerbils demonstrates that there is a positive association between level of severity of gastric inflammation and rate of proliferation of epithelial cells in gerbils consuming high-salt diet than those consuming a normal diet[100]. Similar studies on gerbils infected with *H. pylori,* when treated with carcinogen (N-methyl-N-nitroso urea) shows that higher frequency of GC related with animals consuming high-salt diet as compared to animals with a normal diet[101,102].

Mechanisms behind the high-salt diet increases the risk of development of GC in humans remains unclear. Among various explanations, one plausible hypothesis is that salt may lower the threshold for malignant transformation by altering the physiology of gastric epithelium thus allowing entry of carcinogens into gastric tissue and resulting in damage to gastric mucosa. Another possibility is that high salt intake might be regulating the gene expression in *H. pylori*. Two independent studies suggested that consumption of excess amount of salts in diet leads to higher expression of *H. pylori* virulence factors[103,104].

Dietary antioxidants

Many studies had proved the antioxidants present in food in green vegetables and fruits plays a preventive role against progression of GC[105]. There is scarcity on studies on association of *H. pylori* infection with nutritive elements in gastric carcinoma. A case-control study

recommended that consistent excessive consumption of vitamin C and carotene might be able to curtail the casual for developing GC in subjects having infection of *H. pylori*[106].

A randomized study on population susceptible for GC development demonstrated that combination of vitamin C and carotene dietary supplements and *H. pylori* eradication increases the preneoplastic lesions regression at 6 years of follow-up; at another 6 years follow-up lacking dietary supplements, the protective role of vitamin C and carotene gradually end up[7]. These results were also validated by other studies[95,106]. A similar study from Hawaii proof that consumption of fresh vegetable among *H. pylori* infected individuals provided a little protection against GC occurrence[107]. On the contrary, other studies fail to provide a positive association between *H. pylori* infection and plasma vitamin C level, with risk of GC incidence[108]. Additional research is required to determine whether antioxidants are capable of providing protection against GC among *H. pylori* infected patients.

Cigarette smoking

It is evident from various studies that cigarette smoking is associated with risk of developing GC in *H. pylori* infected subjects. In Japan, cigarette smoking and *H. pylori* infection together are considered as potential threat for developing GC[109]. Swedish and German population-based case control studies also demonstrated combination of cigarette smoking and infection by CagA positive *H pylori* strains increased the risk of developing GC (Figure 2). Los Angeles study also reported a tendency toward increased risk of GC in smokers[59,110]. On collectively analyzing studies, it emerges that there exists relationship between *H. pylori* infection and smoking with increased risk of developing GC.

Helminth infection

H. pylori co-infection with helminths may have some impact in disease pathogenesis. Reduced Th1 response associated with higher levels of Th2 cytokines was reported in one study[111]. Another study on Colombian children from a coastal region having infection of both helminths and *H. pylori,* showed a higher Th2 associated IgG1 response[112]. Further studies are needed to assess the impact of *H. pylori* and helminthes co-infection in disease pathogenesis.

FOCUS OF FUTURE ENDEAVORS

Gastric cancer remains a major threat to mankind. Improvement in living standards, increase awareness in sanitation and hygiene practices, reduction in intake of salted food products and advent of refrigeration in households resulted in measurable decline both in incidence of *H. pylori* infection and GC. Although both *H. pylori* infection and GC are showing decreasing trends in the developed world, they still remain a major threat to human population in the developing countries. Therefore, there is a need for improvement in early diagnosis, identification of risk factors, and development of preventive strategies and initiation of timely therapeutic interventions, especially focused for the developing countries. Further, it remains to be investigated why a small fraction of individuals colonized by *H. pylori* develop GC, and future research should focus on bacterial, host genetics, environmental and dietary factors.

There is need to formulate clear cut recommendation for screening and timely intervention of high risk population with family history of GC. Whether all high-risk areas should undergo routine screening of *H. pylori* infection is still questionable. Since the patients having atrophic gastritis or dysplasia in the gastric mucosa are at increased risk of developing GC, there is a need for special recommendations including endoscopic surveillance for such patients.

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TOPIC HIGHLIGHT

2016 Pancreatic Cancer: Global view

Endoscopic approach to the diagnosis of pancreatic cystic tumor

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Abstract

Because of the aging of the population, prevalence of medical checkups, and advances in imaging studies, the number of pancreatic cystic lesions detected has increased. Once these lesions are detected, neoplastic cysts should be differentiated from non-neoplastic cysts. Furthermore, because of the malignant potential of some neoplastic pancreatic cysts, further differentiation between benign and malignant cysts should be made regardless of their size. Although endoscopic ultrasound (EUS) has a very high diagnostic performance for pancreatic cystic lesions among the various imaging modalities, EUS findings alone are insufficient for the differentiation of pancreatic cysts and diagnosis of malignancy. In addition, cytology by EUS-guided fine-needle aspiration (FNA) has a high specificity but a low sensitivity for diagnosing malignancy in pancreatic cystic tumors. The levels of amylase, lipase, and tumor markers in pancreatic cystic fluid are considered auxiliary parameters for diagnosis of benign and malignant cysts, and a definitive diagnosis of malignancy using these parameters is difficult. Thus, in addition to EUS, cytology by EUS-FNA, and cystic fluid analysis, new techniques based on EUS-guided through-the-needle imaging, such as confocal laser endomicroscopy and cystoscopy, have been explored in recent years.

Key words: Endoscopic ultrasound; Endoscopic retrograde cholangiopancreatography; Endoscopic ultrasound-needle aspiration; Pancreatic cystic tumor; Cytology

Core tip: The number of pancreatic cystic lesions detected has increased. Neoplastic cysts should be differentiated from non-neoplastic cysts. Further differentiation between benign and malignant cysts should be made regardless of their size. In addition to endoscopic ultrasound (EUS), cytology by EUS-fine-needle aspiration, and cystic fluid analysis, new techniques based on EUS-guided through-the-needle imaging, such as confocal laser endomicroscopy and

cystoscopy, have been explored in recent years. We reviewed an endoscopic approach to the diagnosis of pancreatic cystic tumor.

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INTRODUCTION

Because of the aging of the population, prevalence of medical checkups, and advances in imaging studies, the number of incidentally detected pancreatic cystic lesions has increased. Pancreatic cystic lesions include a variety of entities, including non-neoplastic pancreatic pseudocysts, such as those resulting from pancreatitis, and retention cysts, as well as neoplastic pancreatic cysts and solid tumors with cystic degeneration. As differential diagnosis of these lesions is important in the consideration of therapeutic strategies[1], it is essential to differentiate between neoplastic pancreatic cysts, including intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), and serous cystic neoplasm (SCN), and to further determine whether they are benign or malignant[1].

Diagnostic imaging modalities used in the evaluation of pancreatic cystic lesions include abdominal ultrasound (US), contrast-enhanced computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP), endoscopic ultrasound (EUS), and endoscopic retrograde pancreatography (ERP). US is a non-invasive method but is affected by the presence of gastrointestinal gas, making the evaluation of the entire pancreas difficult. Although CT is superior in depicting solid lesions, radiation exposure and allergic reactions to contrast media, limit its application. MRCP is superior in depicting pancreatic cystic lesions, while EUS is highly valued, as it provides high image resolution despite the presence of gastrointestinal gas, allowing close observation of the entire pancreas. Although ERP is superior in depicting details of the pancreatic duct and allows a pathologic diagnosis by cytology of the pancreatic juice at same time, attention should be paid to pancreatitis as a potential complication of endoscopic retrograde cholangiopancreatography (ERCP). At present, the lesions are comprehensively diagnosed by a combination of these methods. In recent years, EUS, EUS-guided fine-needle aspiration (FNA), contrast-enhanced EUS, and other modalities of interventional EUS, have been especially useful in the accurate differentiation of pancreatic cystic tumors[1,2].

TRANSPAPILLARY DIAGNOSIS

A transpapillary approach is significant for the diagnosis of either, main-duct or branch-duct type of IPMNs formed in the pancreatic duct[3]. This approach allows to demonstrate the presence of mucus, and is also effective in the diagnosis of concurrent pancreatic ductal carcinoma. However, for the diagnosis of SCNs and MCNs, which generally do not communicate with the pancreatic duct, the transpapillary diagnostic approach not only lacks significance but may also causes pancreatitis after ERCP. IPMN are pancreatic cystic tumors in which transpapillary diagnosis is significant.

Pancreatic juice cytology

As the pancreatic juice in IPMNs is viscous and often difficult to aspirate, pancreatic juice cytology is used to improve the diagnostic performance of ERCP by allowing the collection of pancreatic juice *via* an implanted endoscopic naso-pancreatic drainage tube. Branch-duct type IPMN, which communicates with the main pancreatic duct, is well indicated for this technique because mucus-containing abundant tumor cells are found in the main pancreatic duct.

IPMNs are high mucous-producing and often well-differentiated adenocarcinomas, even when they are cancerous. Therefore, the diagnosis of this type of tumors using pancreatic juice cytology is difficult. To overcome these limitations, the genetic analysis of pancreatic juice is being studied to aid the objective evaluation of malignancy. Such studies show that tumor markers, including carcinoembryonic antigen (CEA), telomerase activity, matrix metalloproteinase activity, human telomerase reverse transcriptase, mRNA, sonic hedgehog, K-ras, and p-53, present in pancreatic juice may be useful in the assessment of cancer risk in patients undergoing ERP, while complementing pancreatic juice cytology findings[4-10].

EUS DIAGNOSIS

The differential diagnosis of pancreatic cystic lesions can be made by focusing on EUS findings, *i.e.*, size, number, overall cyst shape, state of cyst walls, and features of cystic contents, as well as the presence of underlying lesions[11]. Sedlack *et al*[12] classified 34 resected pancreatic cystic lesions into two groups: A group of benign pancreatic cysts, including simple cysts, pseudocysts, and SCNs, and a group of malignant or malignant potential lesions including MCNs, IPMNs, neuroendocrine tumors with necrotic lesions, and cystic adenocarcinomas. Comparison of the diagnostic performance between the 2 groups showed that EUS had a sensitivity, specificity, and diagnostic accuracy of 91%, 60% and 72%, respectively. Song *et al*[13] evaluated 75 pancreatic cysts (58 neoplastic pancreatic cysts and 17 pancreatic pseudocysts) using EUS, and showed that, while intracystic debris and pancreatic parenchymal changes were characteristic EUS findings of pancreatic pseudocysts, the presence of septa and nodes were typical of neoplastic pancreatic cysts. Song *et al*[13] reported that although EUS is useful in the diffe-

rential diagnosis of pancreatic cystic lesions, it might be insufficient on its own, to completely differentiate pancreatic cysts. In addition, in a multicenter study conducted by Brugge[14] to evaluate the performance of EUS in the diagnosis of pancreatic cyst malignancy, low sensitivity, specificity, and diagnostic accuracy values of 56%, 45% and 51%, respectively, were observed. Moreover, Ahamad *et al*[15] demonstrated that the diagnostic accuracy of EUS for pancreatic cysts and non-cystic lesions varied from 40% to 93% among 8 endoscopists, indicating that experience and skills influence the diagnostic performance of this method.

DIFFERENTIATION OF PANCREATIC CYSTIC LESIONS USING CONTRAST-HARMONIC EUS

Differentiation between neoplastic (IPMNs, MCNs, and SCNs) and non-neoplastic pancreatic cystic lesions is important. Although there are sporadic reports on the use of B-mode imaging for pancreatic cystic lesions diagnosis[16,17], reports on similar studies using contrast-harmonic (CH)-EUS are limited. However, because CH-EUS clearly depicts the internal structure and shape of lesions, it appears to be useful for picking up the characteristic imaging findings of each lesion. Compared to conventional B-mode imaging, CH-EUS facilitates pancreatic duct observation by depicting it as a structure without blood flow. In consequence, communication between a lesion and the pancreatic duct, an important aspect for differentiation of pancreatic cystic lesions, can be easily confirmed. In cases of IPMN in which a structure is observed in the dilated pancreatic duct, differentiation between a mucinous mass or tumor resulting from papillary growth by B-mode imaging, is often difficult. However, the CH mode allows their differentiation according to the presence or absence of blood flow.

EUS-FNA DIAGNOSIS

In Japan, because of a reported incident of peritoneal metastasis caused by EUS-FNA for IPMN[18], doctors have become reluctant to perform the procedure. However, EUS-FNA is commonly used for the diagnosis of pancreatic cystic tumors worldwide, as well as for the evaluation of pancreatic cystic fluid, in terms of its nature (mucinous or serous), cytology, and measurement of CEA/amylase levels[19].

The nature of the cystic fluid collected by EUS-FNA is important for differentiation of pancreatic cystic tumors. IPMNs and MCNs, or SCNs should be suspected if the fluid is mucinous, or serous, respectively.

The cytology of pancreatic cystic tumors by EUS-FNA, has a high specificity for diagnosis of malignancy similar to that of ERP, albeit with a low sensitivity. Moreover, in cases of multilocular cysts, sufficient specimens may not be collected due to the small diameter of each cyst or high viscosity of the cystic fluid, which limits its aspiration with a puncture needle. The inability to collect sufficient amounts of cells seems to be the cause of the low sensitivity. The rate of successful collection of specimens required for cytology is reported to be approximately 80%, and the differential diagnostic accuracy for pancreatic cysts ranges from 13%-96%[12,15,20-26]. In addition, the diagnosis of malignancy has a specificity of 86%-100% and a sensitivity of 25%-88%. The international guidelines for the differential diagnosis between benign and malignant lesions, therapeutic strategies, and follow-up procedures of main-duct and branch-duct type IPMNs were revised in 2012. According to the revised guidelines, the cytological assessment of especially worrisome features (main pancreatic duct diameter of 5-9 mm and absence of either nodes or growth in main-duct and branch-duct type, respectively) is important. The results of a meta-analysis showed that, despite the high specificity and diagnostic accuracy of cytology, its sensitivity is low, with a possibility of misdiagnosing malignant lesions as benign, concluding that cytology needs to be complemented by the additional measurement of CEA, carbohydrate antigen (CA) 19-9, micro-RNA, *etc*[27].

Amylase, CEA, and CA19-9 levels in cystic fluid are highly useful for IPMNs, MCN, and SCN differentiation. Amylase levels in cystic fluid are high in IPMNs because they communicate with the pancreatic duct. By contrast, as MCNs and SCNs do not communicate with the pancreatic duct, their amylase levels are typically low. In addition, a cut-off amylase value in cystic fluid set at 250 U/L, has a sensitivity and specificity of 44% and 98%, respectively, for excluding pancreatic pseudocysts from the diagnosis of pancreatic cystic lesions[28].

CEA levels in cystic fluid are useful for differentiation between MCN (including IPMN) and SCN. A CEA cut-off value in cystic fluid of 192 ng/mL, had a 79% diagnostic accuracy for MCN, which was higher than that of 59% using diagnostic imaging by EUS[22]. In a cyst containing $\geqslant$ 800 ng/mL of CEA in cystic fluid, or diagnosed as malignant by cytology, the specificity for diagnosing the cyst as MCN was 98%-100%. Moreover, a CEA level in cystic fluid $\leqslant$ 5 ng/mL had a 95% specificity for the diagnosis of a pancreatic cyst as benign, of which, 6% were, however, MCNs[28].

A CA19-9 level in cystic fluid $\leqslant$ 37 U/mL has an accuracy and specificity for diagnosing a pancreatic cystic tumor as benign of 46% and 94%, respectively. CA19-9 is useful for complementing diagnosis of benign and malignant pancreatic cystic tumors[28].

Thus, analysis of amylase, CEA, and CA19-9 levels in cystic fluid improves the ability to differentiate mucinous from serous pancreatic cystic tumors. Because malignant SCN is rare, its reliably diagnosis is important. However, levels of amylase, CEA, and CA19-9 in cystic fluid are reportedly not helpful for differentiation of cancer among MCN[29].

Various attempts have been made to improve the diagnosis of malignancy in pancreatic cystic tumors. As

a reason for the low sensitivity of cystic fluid cytology is the scarcity of cell components in cystic fluid, attempts to collect more cells have been reported. These include, abrasion of cystic wall by brushing[30]. Abrasion/puncture of cystic wall with the tip of a puncture needle while cystic fluid is aspirated[31], and direct biopsy of cystic wall with miniature biopsy forceps that can be passed through a puncture needle[32]. Although of cystic fluid specimens collected by all of these techniques contain more cell components than those collected by conventional aspiration, they have failed to improve the diagnostic performance for malignancy. This is attributed to the fact that the grade of atypism is not always consistent in the cystic wall itself. If target biopsy of nodular lesions can be performed, diagnostic performance may be improved.

Procedural accidents

While serious complications or procedural accidents associated with EUS-FNA for pancreatic cystic lesions have not been reported, pancreatitis (0.5%-4%)[33], cyst infection (< 1%)[20,33,34], and intracystic hemorrhage (< 1%)[15,20,35], rarely occur. Cyst infections can be prevented by infusion of antibiotics before EUS-FNA or oral administration of antibiotics for 2 to 5 d after puncture, while EUS-FNA can be safely performed using a 22-gauge puncture needle[15,33].

EUS-GUIDED THROUGH-THE-NEEDLE IMAGING OF PANCREATIC CYSTIC TUMORS

Confocal laser endomicroscopy

In many reports, confocal laser endomicroscopy (CLE) has been described as useful for virtual biopsy and provides images similar to pathological images during endoscopic observation[36]. There are a CLE device that incorporates an endoscope and probe-based CLE (pCLE) in which a probe is inserted through the forceps channel of the endoscope for observation. These devices are reported to be useful for detailed examination of the gastrointestinal tract before therapeutic endoscopy.

Needle-based CLE

A prototype device (Cellvizio AQ-Flex-19®, Mauna Kea Technologies, Paris, France) with a diameter smaller than that of pCLE has been developed. This device can be inserted in an EUS-FNA 19-gauge needle and used to perform EUS-guided needle-based CLE (nCLE) for the diagnosis of pancreatic cysts.

The *in vivo* CLE Study in the Pancreas With Endosonography of Cystic Tumors trial[37], compared the findings of EUS-guided nCLE with those of pathological analysis. When the findings of nCLE were classified into 3 categories, *i.e.*, epithelial structure, non-epithelial structure, and intracystic floating components, an abnormal epithelial structure, mainly including papillary projections, was a characteristic finding of mucinous tumors. In addition, nCLE of IPMNs revealed dark aggregates with high cell density in areas suspected of dysplasia, while blood vessels, which are non-epithelial structures, were seen as white bands in other areas. SCNs, only showed non-epithelial structures, whereas no epithelial structure was observed. Although the specificity of the findings of EUS-guided nCLE was 100%, the sensitivity was low, with a value of 57.9%. According to a report indicating that findings reflecting hypervascular patterns of cystic walls and septa of SCNs are useful, there was no technical problem, whereas it was difficult to puncture lesions of the pancreatic head with a 19-gauge needle[38].

Cystoscopy

Cystoscopy is a diagnostic procedure in which a pancreatic cystic tumor is punctured with a 19-gauge FNA needle, and a SpyGlass probe made of optic fiber directly is inserted into the pancreatic cyst to observe cystic contents and the nature of the cystic wall. According to cystoscopy, the cystic fluid in IPMNs and MCNs is mucus. Regarding the cystic wall, IPMNs have papillary projections or communicate with the pancreatic duct, while MCNs have a smooth cystic wall. However, the cystic fluid of SCNs is clear, while the cystic wall is smooth and has abundant blood vessels.

Combination of cystoscopy and nCLE

In the Diagnosis of Pancreatic Cysts: EUS-guided Through-the-needle Confocal Laser-induced Endomicroscopy and Cystoscopy Trial (DETECT study)[39], the contribution of the cystoscopy and nCLE combination to further improve diagnostic performance, was evaluated. For the diagnosis of mucinous cysts, the specificity of both cystoscopy and nCLE was 100%, whereas their sensitivity was also relatively favorable with values of 71% and 77%, respectively. Furthermore, when these 2 modalities were combined, the specificity remained at 100%, and the sensitivity was elevated to 88%, indicating an improved diagnostic performance. However, in terms of diagnosis of malignancy, the image quality of cystoscopy and nCLE decreased as the diameter of a probe reduced. Therefore, the image quality of this technique is insufficient at present.

CONCLUSION

We have described the endoscopic diagnosis of pancreatic cystic tumors. While the diagnosis of benign and malignant cysts is especially important, the diagnostic performance of endoscopy is still insufficient. Further advances, mainly in EUS technology are thus awaited in the future.

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2016 Pancreatic Cancer: Global view

Advancement in treatment and diagnosis of pancreatic cancer with radiopharmaceuticals

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Abstract

Pancreatic cancer (PC) is a major health problem. Conventional imaging modalities show limited accuracy for reliable assessment of the tumor. Recent researches suggest that molecular imaging techniques with tracers provide more biologically relevant information and are benefit for the diagnosis of the cancer. In addition, radiopharmaceuticals also play more important roles in treatment of the disease. This review summaries the advancement of the radiolabeled compounds in the theranostics of PC.

Key words: Pancreatic cancer; Diagnosis; Therapy; Radiopharmaceuticals; Positron emission tomography

Core tip: This review describes the development of radiopharmaceuticals in diagnosis and therapy of pancreatic cancer. We herein discuss the role of the radiolabeled compounds in the preoperative diagnosis, staging, post-therapeutic monitoring, prognosis and the treatment of the disease.

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INTRODUCTION

Pancreatic cancer (PC) is a major health problem due to low 5-year survival rate[1-3]. Surgery is the only curative treatment but less than 20% of cases are suitable to

be respectable during diagnosis for the late onset of the symptoms[4-6]. Therefore, suitable diagnosis and staging is essential for management of the disease.

Computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS), *etc*., provide information regarding tumor size, location, and morphology, which can be used for initial staging, tumor evaluation and follow-up. However, it also remain suboptimal in the preoperative diagnosis and may hamper the treatment. The discrimination between benign and malignant lesions are still challenging with these methods[7,8].

Molecular imaging techniques are important tool capable of providing high sensitive non invasive and quantitative images of various cancer[9-11]. Radiopharmaceuticals is a key factor in the non-invasive molecular imaging technique which enables specific cellular and molecular processes to be functionally visualized. The development of molecular imaging agents target for specific biomarkers could provide more sensitive and specific cancer detection.

Meanwhile, a number of compounds labeled with therapy radionuclides have been employed for cancer treatment through intratumoral administration[12-15]. Compared with traditional high-dose external radiation, intratumoral administration delivers more radioactivity to the tumor than the normal structure[16].

Here, we review the pertinent literatures and the advancement in treatment and diagnosis of PC with radiopharmaceuticals was discussed.

SMALL MOLECULE TRACERS FOR TUMOR IMAGING

^{18}F-fluorodeoxyglucose

Over the past decade, positron emission tomography (PET) is an important molecular imaging methods in various malignancies[17-20]. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is an analogue of glucose. After injected into the body, it is actively transported *via* glucose transporters (GLUT) into cells, then phosphorylated by hexokinase in the same pathway as glucose. However, unlike normal glucose, the reactions of ^{18}F-FDG do not proceed further and the corresponding product remains in the cells[21,22]. Overexpression of GLUT-1 and hexokinase-Ⅱ has been reported in PC[23]. In patients with PC, several studies have demonstrated that ^{18}F-FDG PET/CT was an important key factor for in staging, detecting postoperative recurrence, and evaluating the response to treatment[24-28]. The recent typical researches and interest findings were listed in the follow.

Preoperative diagnosis: Ergul *et al*[29] compared the values of ^{18}F-FDG PET/CT, multidetector row computed tomography (MDCT), MRI and EUS in the diagnosis and management of the tumor. It revealed that sensitivity of PET/CT were equal to EUS (100%) and higher than those of MDCT and MRI. Meanwhile, Specificity of MDCT was significantly lower than PET/CT. It suggested that ^{18}F-FDG PET/CT is an useful imaging techniques for management of the disease[29].

Maximum standardized uptake value (SUVmax) reflects tumor aggressiveness as a marker of tumor glucose metabolism. Hu *et al*[30] found that the SUVmax of benign lesions significantly lower than that of malignant tumors (2.9 ± 2.0 *vs* 6.3 ± 2.4 respectively). A positive correlation between the SUVmax and Ki-67 was existed. It suggested that the SUVmax of ^{18}F-FDG can be applied in the differential diagnosis and can also benefit for monitoring the proliferative status of PC[30].

Nagamachi *et al*[31] compared ^{18}F-FDG PET/CT and ^{18}F-FDG PET/MRI fusion image in diagnosing tumor. ^{18}FDG-PET/MRI fusion image significantly improved accuracy. Results showed that this image technique was useful in differentiating diagnosis[31].

Zhang *et al*[32] reviewed 116 patients with pancreatic cystic tumors who had been treated with different imaging modalities. Compared with CT and EUS, PET had the best sensitivity, specificity and accuracy for detecting malignant cystic tumors[32].

When the conventional imaging modalities or biopsies are unavailable, PET also plays an important role in diagnosis of PC. Based on the ^{18}F-FDG uptake pattern, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for FDG-PET/CT in differentiating benign and malignant lesions were all greater than 85% respectively[33].

Diagnostic performance of diffusion-weighted MRI and ^{18}F-FDG PET/CT in the detection of pancreatic malignancy was also obtained by Wu *et al*[34]. When diagnosing patients with pancreatic malignancy, the sensitivity of PET/CT was higher than MRI but the specificity of the former was lower than the latter[34].

Staging: Wang *et al*[35] evaluate the value of ^{18}F-FDG PET/CT on the pre-operative staging of the disease. The sensitivity and accuracy of the imaging modality to detect distant metastasis especially metastatic lymph nodes are significantly higher than those of MDCT. It showed that the extra staging information PET/CT provided could be helpful for screen of surgery[35].

^{18}F-FDG PET/CT scans were performed at 17 patients in baseline and six weeks post-CRT. SUVmax significantly decreased during CRT (median pre- 8.0 and post- 3.6). It revealed that the baseline ^{18}F-FDG PET was benefit for definition of the biological target volume for non-uniform dose prescriptions[36].

Topkan *et al*[37] evaluated the impact of ^{18}F-FDG PET/CT restaging on management decisions and outcomes in patients with LAPC scheduled for concurrent CRT. According with PET/CT before therapy, these individuals were classified into non-metastatic (M0) and metastatic (M1) groups then received different treatment. Twenty-six point eight percent of distant metastases were detected *via* PET/CT not by conventional staging. Three additional regional lymph nodes were found by PET/CT restaging and the volumes of the tumors were larger

than CT-defined borders. The initial management decisions of 26 patients were changed through PET/CT.

Median overall survival (OS) and progression-free survival (PFS) of M0 patients were greater than those of M1 patients. These findings conformed that PET/CT-based restaging may benefit for screening patients suitable for CRT[37].

Post-therapeutic monitoring: Picchio *et al*[38] evaluated the role of ^{18}F-FDG PET/CT in screening patients with locally advanced PC for suitable treatment and monitoring the efficacy. Results showed that PET/CT play more important factors in designing the treatment plans for individual patient than conventional CT[38].

Kittaka *et al*[39] performed ^{18}F-FDG PET in patients classified as responders and nonresponders before and after preoperative CRT. A pre-CRT SUV > 4.7 was seen in 15 (71%) of 21 responders and in 6 (32%) of 19 nonresponders. A regression index > 0.46 was observed in 15 (71%) responders and 5 (26%) nonresponders. It showed that the SUV based on FDG-PET/CT is a useful implement for predicting the response of treatment[39].

To study whether FDG-PET parameters can predict relatively long-term survival in patients, Chang *et al*[40] assess the effect of coregistered ^{18}F-FDG PET in monitoring radiographically occult distant metastasis (DM) in patients with LAPC. Patients with a baseline standardized uptake value (SUV) < 3.5 and/or SUV decline ⩾ 60% had significantly better OS and PFS than those having none, even after adjustment for all potential confounding variables. ^{18}F-FDG PET can spare one-third of patients with occult DM from the potentially toxic therapy. ^{18}F-FDG PET parameters including baseline SUV and SUV changes may serve as useful clinical markers for predicting the prognosis in LAPC patients[40].

Prognosis: Several prognostic factors for PC recurrence have previously been reported including tumor size, T stage, lymph node metastasis, tumor differentiation, lymphovascular invasion, involvement of the surgical margin, and serum carbohydrate antigen 19-9 (CA19-9) level. Yamamoto *et al*[41] evaluated whether preoperative ^{18}F-FDG PET can predict the resectable PC. Among the patients, 34 cases with an SUVmax ⩾ 6.0 developed recurrence within half year, however only 3 patients with an SUVmax < 6.0 exhibited early recurrence. The median OS time of patients with a SUVmax < 6.0 was significantly greater than those of patients with an SUVmax ⩾ 6.0. Therefore, an SUVmax ⩾ 6.0 maybe a significant predictor of recurrence of PC[41].

The histopathological grade of differentiation is also one of the significant prognostic factors in the disease, especially in the patients with unresectable PC. It was found that a significant correlation of SUVs and pathologic grades existed by ^{18}F-FDG PET scans in 102 patients with histologically proven pancreas adenocarcinoma. It showed that ^{18}F-FDG SUV is related with histologic grade and might be competitive predictor for survival[42].

Xi *et al*[43] determined ^{18}F-FDG SUVmax in patients with PC at 1 h and 2 h post injection, and the retention index (RI) was defined as the percentage change between the values of two time points. It was found that there existed a significant positive correlation among RI and the tumor, node, and metastasis stage[43].

Shinoto *et al*[44] evaluated whether ^{18}F-FDG PET can be used as an indicator of preoperative carbon-ion radiotherapy (CIRT) for PC patients. SUVmax was significantly correlated with DMFS and OS. The DMFS and OS in high-SUVmax group were significantly lower than those in low SUVmax group. ^{18}F-FDG PET might be suitable for determining the indication of preoperative short-course CIRT for patients with resectable PC[44].

The prognostic role of ^{18}F-FDG PET/CT in the prediction of PFS and chemotherapeutic response in patients with locally advanced or metastatic PC was also investigated by Moon *et al*[45] PFS of the low SUVmax (< 6.8) group was significantly longer than those of the high SUVmax (⩾ 6.8) group. Resulted showed that SUVmax may be useful in independent predicting PFS of PC[45].

The prognostic value of volumetric parameters on preoperative ^{18}F-FDG PET/CT was assessed. Results revealed that metabolic tumor volume and total lesion glycolysis are independent prognostic factors for predicting RFS and OS. Thus, ^{18}F-FDG PET/CT can provide useful prognostic information for patients undergoing resection of PC with curative intent irrespective of neoadjuvant treatment[46].

Choi *et al*[47] evaluated the prognostic value of ^{18}F-FDG PET in patients with resectable PC. The OS and DFS were significantly longer in the low SUVmax group than those of high SUVmax group[47].

Hwang *et al*[48] reviewed retrospectively the medical records of 165 patients with a diagnosis of PC. Patients were allocated to high (> 4.1) and low (⩽ 4.1) SUV groups, and median survivals of these patients were 229 d and 610 d, respectively. Furthermore, SUVmax was found to be significantly related to survival in each stage. The median survival was also found to be significantly related to tumor size, site, serum level of CA19-9, distant metastasis, and type of treatment[48].

Epelbaum *et al*[49] evaluated the possibility of dynamic ^{18}F-FDG PET/CT parameters used as an indicator in the tumor. The OS of patients with a high ^{18}F-FDG influx was significantly lower than that of patients with a low ^{18}F-FDG influx (5 and 6 mo *vs* 15 and 19 mo respectively). Quantitative ^{18}F-FDG kinetic parameters in newly diagnosed PC correlated with the aggressiveness of disease[49].

Limitation: Although significant advances have been achieved in ^{18}F-FDG PET diagnostic technologies, it has some limitations in detecting cancer. Due to increased glycolytic metabolism, ^{18}F-FDG can also accumulate in the inflammatory cells[50]. As a result, it often yields false positive interpretations for PET. Kato *et al*[51] evaluated the efficacy of ^{18}F-FDG PET/CT for the differential diagnosis in 47 individuals. It showed that differentiation is difficult by^{18}F-FDG PET/CT due to overlapping in

SUVmax between the two diseases. In addition, elevated serum glucose levels may decrease the uptake in tumors for competitive inhibition, which decreased the sensitivity of ^{18}F-FDG PET in hyperglycemic patients[51]. Therefore, a numbers of other small molecule-based tracers were designed and developed for PET imaging of PC.

3-Deoxy-3-18F-fluorothymidine

A surrogate marker of DNA synthesis, 3-Deoxy-3-18F-fluorothymidine (^{18}F-FLT), is another potential tracer for visualization of proliferating tissues[52-55]. For differentiation of pancreatic tumors, ^{18}F-FLT PET showed a lower sensitivity but higher specificity than^{18}F-FDG PET/CT (70% *vs* 91% and 75% *vs* 50% respectively)[56].

RADIOLABELED PEPTIDES FOR PC IMAGING

Peptides and their derivatives have been successfully developed for the tracer due to favorable characteristics such as low antigenicity, high specificity, fast clearance from blood and rapid tissue penetration. Radiolabelled receptor-binding peptides have become important radiopharmaceuticals for diagnosis and therapy in tumor[57-61]. Recently, a few radiolabeled peptides have been successfully used for PC imaging. It may be a promising imaging strategy for PC diagnosis and treatment.

Radiolabeled RGD analogs

Angiogenesis is necessary for tumor growth and metastasis, and the integrin αvβ3 receptor plays an important role in promoting, sustaining, and regulating the angiogenesis[62]. *In vitro* analysis demonstrated that integrin αvβ3 receptor was expressed in 60% of invasive pancreatic ductal carcinomas and would be an excellent target for the early detection of malignant PC[63]. Radiolabeled Arg-Gly-Asp (RGD) peptides are widely used as integrin αvβ3 receptor imaging agents in various types of tumors[63]. Yoshimoto *et al*[64] employed ^{111}In-DOTA-c(RGDfK) for the early detection of PC in pancreatic carcinogenesis model. PC lesions as small as 3 mm in diameter as clearly were visualized after injection with the tracer. High tumor-to-normal pancreatic tissue radioactivity ratios were found by ARG analysis. There existed a significant relationship between the uptake of ^{111}In-DOTA-c(RGDfK) and αvβ3-integrin expression. It also found that the false-positive rate of ^{111}In-DOTA-c(RGDfK) was lower than that of ^{18}F-FDG. It revealed that SPECT with ^{111}In-DOTA-c(RGDfK) was benefit for the early accurate diagnosis of PC[64].

Trajkovic-Arsic *et al*[65] used ^{68}Ga-NODAGA-RGD PET for αvβ3 integrin receptor *in vivo* imaging of spontaneous pancreatic ductal adenocarcinoma (PDAC) occurring in mice. It showed that αvβ3 integrin is expressed in human and murine PDAC and can be detected by molecular imaging technologies in PDAC. This strategy can further be exploited for identification of patients with αvβ3 integrin positive and application of αvβ3 targeted therapies[65].

Aung *et al*[66] performed a preclinical evaluation of ^{64}Cu-RAFT-RGD in a clinically relevant orthotopic xenotransplantation model of PC. It was confirmed that the uptakes of ^{64}Cu-RAFT-RGD in tumor was greater than those of normal tissues. Meanwhile, the tumor to background uptake ratios of the tracers was higher than those of ^{18}F-FDG. It suggested that ^{64}Cu-RAFT-RGD PET imaging might be useful in the diagnosis of PC[66].

Radiolabeled exendin-4 analogs

Insulinomas are the most frequent hormone-active tumors of the pancreas arising from pancreatic β cells[67-69]. Recently, glucagon-like peptide-1 receptor (GLP-1R) was found to be massively overexpressed in gut and lung neuroendocrine tumors, especially insulinomas. It provides an attractive target for the cancers[70-72].

Several radioligands towards GLP-1 receptor have been developed for GLP-1R-positive tumor imaging. At first, the analog of native receptor ligand, GLP-1(7–36) amide, was labeled with ^{123}I and used for GLP-1R imaging. Although preclinical data showed ^{123}I-GLP-1(7–36) amide possessed high accumulation in a RINm5F insulinoma tumor, the low stability of the peptide due to rapid degrading of GLP-1 by the enzyme dipeptidyl peptidase Ⅳ (DPⅣ) limited its clinical use[73].

Exendin-4 arised from the salivary gland of the gila monster lizard and has a 53% amino acid homology with GLP-1. It is more resistant to the DPⅣ digestion and binds with great affinity to the GLP-1R[73]. ^{111}In- and ^{99m}Tc-labeled exendin-4 analogs have been evaluated for SPECT imaging of GLP-1R in rodents and humans, respectively, and promising results were obtained[74-77].

The sensitivity, imaging contrast and spatial resolution of PET was significantly higher than SPECT. In the past few years, exendin-4 analogs have been labeled with PET radionuclides for preclinical insulinomas imaging. Exendin-4 labeled with radio metals (^{68}Ga, ^{64}Cu) showed significant uptake in INS-1 insulinoma xenografts[78,79]. However, the substantial kidney uptake may limit their use in clinical practice due to high radiation exposure to the organs.

^{18}F is the commonly used isotope. It has nearly optimal nuclear decay characteristics and chemical properties for peptide-based receptor imaging studies. In the past few years, exendin-4 analogs have been modified with either a C-terminal or N-terminal cysteine to allow site-specific labeling with a maleimide-selective prosthetic reagent, ^{18}F-FBEM[80]. *In vivo* study showed that the INS-1 tumor uptake of ^{18}F-FBEM-Cys^{40}-exendin-4 was higher than that of ^{18}F-FBEM-Cys^{0}-exendin-4[80]. Based on the above results, other Cys^{40}-exendin-4 analogs were developed for GLP-1R imaging[81,82].

In vitro receptor competitive binding study confirmed that the nine amino acid sequence at C-terminal of exendin-4 was not key for the biological activity or binding to the receptor. Meanwhile, serine is almost same as cysteine except for the difference in hydroxy and sulfhydryl group. Thus, replacing Ser^{39} with Cys^{39} could

provide a unique site for attachment of a radiolabeling thiol-reactive group (such as ^{18}F-FBEM) and may have less impact on the binding affinity of the peptide to the receptor[83]. Xu *et al*[83] synthesized a novel ^{18}F-labeled exendin-4 analog, ^{18}F-FBEM–Cys^{39}-exendin-4. The tracer showed specific binding to GLP-1R and had better tumor to background radioactivity ratio and lower abdominal backgrounds than those of ^{18}F-FBEM-Cys^{40}-exendin-4[83]. It suggested that ^{18}F-FBEM–Cys^{39}-exendin-4 may be a potential probe for insulinomas imaging[83].

Despite the encouraging results, the tedious radiosynthesis would hinder the tracer to widespread use. Recently, a one-step simple procedure for preparing ^{18}F-labeled peptides *via* chelating ^{18}FAl with NOTA has been reported[84]. Xu *et al*[84] conjugated Cys^{39}-exendin-4 with NOTA-MAL and obtained NOTA-MAL-Cys^{39}-exendin-4. The compound was simply radiolabeled with ^{18}FAl complex by one step in 30 min[85]. ^{18}FAl-NOTA-MAL-Cys^{39}-exendin-4 shows favorable characteristics for insulinoma imaging in mice bearing INS-1 tumor and may be translated to clinical studies[85].

THERAPY WITH RADIOPHARMACEUTICALS

Recently, only few patients have resectable disease. High-dose external radiation to the pancreas may damage the surrounding organs. The intratumoral administration of radiopharmaceuticals delivers the maximum amount of radioactivity to the tumor with limiting side effects[86-88].

During the past several decades, implantation of radioactive isotopes for the treatment has been used. Some basic research indicated that ^{125}I seed with continuous low dose rate irradiation may be beneficial to PC[86-88]. Zhongmin *et al*[89] implanted ^{125}I seeds into PC under CT guidance in thirty-one patients with inoperable PC. It was found that overall responding rate was greater than 60% and median survival time was about 10 mo[89]. The efficacy of intraoperative ultrasound-guided implantation of ^{125}I seeds was also assessed for the treatment of unresectable PC by Wang *et al*[90]. Most of the patients achieved favorable pain relief. These studies revealed that ^{125}I seeds implantation was benefit for the treatment of PC patients[90].

Phosphorus 32 is another ideal unsealed therapeutic radionuclide. Colloid ^{32}P has been applied for the treatment of intracavitary malignancies[91-93]. Preclinical study showed that ^{32}P-chromic phosphate colloid (^{32}P-CP) through intratumoral injection mainly accumulated in the BXPC-3 human tumor and retained for a long time[94]. The safety and efficacy of the therapy to PC was also confirmed[94].

Poly (L-lactic acid) (PLLA) has been widely used as a drug delivery system due to excellent biocompatibility and biodegradability[95-99]. ^{32}P-CP-PLLA microparticle was successfully prepared and used for brachytherapy in several tumor models[95-99]. Yang *et al*[100] evaluated its biodistribution, bioelimination, and therapeutic effect in mice bearing BxPC-3 human PC. Results showed that ^{32}P-CP-PLLA was mostly remained at the tumor (> 95% ID) and almost no radioactivity excretion was observed in urine and feces. As compared, some radioactivity (over 5% ID) of ^{32}P-CP colloid was found in the normal organs[100]. Meanwhile, the tumor volumes was significantly decreased after treatment with ^{32}P-CP-PLLA microparticle[100]. It showed that ^{32}P-CP-PLLA microparticle might be benefit for the management of PC[100].

CONCLUSION

Radiopharmaceuticals are favorable diagnostic and therapy facility for PC. The development of new tracers may be beneficial to personalized management of the disease.

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REVIEW

Molecularly targeted therapy for advanced hepatocellular carcinoma - a drug development crisis?

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Abstract

Hepatocellular carcinoma is the fastest growing cause of cancer related death globally. Sorafenib, a multi-targeted kinase inhibitor, is the only drug proven to improve outcomes in patients with advanced disease offering modest survival benefit. Although comprehensive genomic mapping has improved understanding of the genetic aberrations in hepatocellular cancer (HCC), this knowledge has not yet impacted clinical care. The last few years have seen the failure of several first and second line phase Ⅲ clinical trials of novel molecularly targeted therapies, warranting a change in the way new therapies are investigated in HCC. Potential reasons for these failures include clinical and molecular heterogeneity, trial design and a lack of biomarkers. This review discusses the current crisis in HCC drug development and how we should learn from recent trial failures to develop a more effective personalised treatment paradigm for patients with HCC.

Key words: Hepatocellular carcinoma; Molecular targets; Genomics; Sorafenib; Tyrosine kinase inhibitors

Core tip: This review discusses the current drug therapy landscape for advanced hepatocellular carcinoma, in particular the reasons for failure of several clinical trials of molecularly targeted therapy and future directions of research to address these problems.

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INTRODUCTION

Hepatocellular cancer (HCC) is the sixth most prevalent cancer worldwide and accounts for over 745000 deaths a year[1]. Despite the implementation of screening programs for high-risk individuals, the majority of patients present with incurable disease. Median overall survival for advanced disease remains poor at less than 12 mo and there is an urgent need for more effective treatments[2]. Global epidemiological patterns vary depending on the prevalence of risk factor. Incidence rates are highest in East Asia in areas where hepatitis B and C are endemic[3]. However, improved management of early viral hepatitis in Japan has seen a reduction in new HCC cases[4]. By contrast the upward trends of HCV, obesity and metabolic syndrome in North America and Europe contribute to HCC being the fastest growing cause of cancer related mortality in these regions[5]. Resection, radiofrequency or microwave ablation, and liver transplantation comprise the mainstay of treatment for early disease offering the only chance of cure, but only one third of patients present with disease suitable for these treatments[6]. Loco-regional therapy with trans-arterial chemoembolization (TACE) can lead to sustained disease control for intermediate stage HCC[7,8]. Sorafenib, a multi-targeted tyrosine kinase inhibitor (TKI), remains the only systemic therapy that is effective in advanced disease offering marginal survival benefit without significant improvement in cancer related symptoms or quality of life[2]. After many years of disappointing results with chemotherapy, sorafenib was thought to herald a new era in HCC treatment with great optimism for molecularly targeted therapies. Disappointingly, several negative first and second line phase Ⅲ clinical trials ensued. However, the combination of recent extensive genomic studies and biomarker based clinical trials, provide hope for the development of a more personalised treatment paradigm. This review discusses the current concepts and management of advanced HCC with a particular focus on the failure of molecular targeted therapy beyond sorafenib and outlines how this should be addressed.

Current therapy for advanced disease

Despite only marginal benefits with chemotherapy reported in single arm studies, lack of alternative treatments meant its use was routine prior to the advent of sorafenib. Challenges with toxicities (especially in patients with underlying liver disease) led to chemotherapy being reserved for patients with good performance status and preserved hepatic function. Single agents such as doxorubicin, cisplatin and fluorouracil offer response rates of 10%[9-11]. This increases to 20% with combination regimens, none of which impact survival[9,12]. The recently reported EACH trial, a phase Ⅲ study conducted in China, Taiwan, Korea and Thailand randomly assigned 371 patients with advanced disease to receive either combined oxaliplatin and fluorouracil/leucovorin (FOLFOX4) or doxorubicin[13]. The trial failed to demonstrate a significant survival difference between each arm, although a trend towards improved outcomes with FOLFOX4 was noted (median overall survival was 6.4 mo for FOLFOX4 and 4.97 mo for doxorubicin; P = 0.7; HR = 0.8; 95%CI: 0.63-1.02).

The search for more efficacious treatments eventually led to two large randomised phase Ⅲ trials that reported a significant survival benefit with sorafenib in close succession. The first, conducted in a European, Australian and American population, demonstrated a median overall survival (OS) of 10.7 mo for patients treated with sorafenib (400 mg BD) compared with 7.9 mo for placebo (HR = 0.69; 95%CI: 0.55-0.87; P < 0.001)[2]. The latter, conducted in the Asian-Pacific region reported that patients treated with sorafenib led to a median overall survival of 6.5 mo compared with 4.2 mo (HR = 0.68; 95%CI: 0.50-0.93; P = 0.014)[14]. The survival advantages in both trials were modest and neither study established any improvement in cancer symptoms or quality of life. Yet this benefit was sufficient for sorafenib to become the new standard of care for patients with advanced disease. Data extracted from the prospectively maintained GIDEON database (Global Investigation of Therapeutic Decisions in Hepatocellular Carcinoma and of its Treatment with Sorafenib) showed that in 3202 patients treated with HCC, adverse events were comparable between patients with Child-Pugh A and Child-Pugh B cirrhosis[15]. Yet the frequency of serious adverse events was higher in the Child-Pugh B group (60.4% for Child-Pugh B and 36.0% for Child-Pugh A) and median overall survival was shorter 5.2 mo (4.6-6.3) for Child-Pugh B and 13.6 mo (12.8-14.7) for Child-Pugh A (Table 1).

Four separate phase Ⅲ trials exploring different multi-targeted TKIs have now failed to show superior outcomes to sorafenib. HCCs are vascular tumours and both VEGF and angiopoietin-2 (Ang2) were independent prognostic markers during the SHARP trial and have been associated with tumour growth and metastatic spread[16]. The success of sorafenib was thought to be predominantly related to its anti-angiogenic properties and subsequent studies aimed to identify more potent anti-angiogenic drugs. Sunitinib, a multi-kinase inhibitor targeting VEGFR, PDGFR, c-KIT and FLT-3 has been approved for use in gastro-intestinal stromal tumours and renal cell carcinomas and was more potent that sorafenib in preclinical models[17,18]. Phase Ⅱ studies showed modest benefit in HCC at best although did highlight potential biomarkers such as interleukin-6, stromal-derived factor1alpha and soluble c-KIT, as changes in tumour vascular permeability and circulating inflammatory molecules were associated with poorer outcome[19-21]. Adverse events in these phase Ⅱ studies were concerning with liver related toxicities including encephalopathy and hepato-renal syndrome and 5%-10%

Table 1 First line trials with molecular targeted therapies in advanced hepatocellular cancer

Trial	Drugs	Design	*n*	Median survival	HR	*P* value	Ref.
ASIA-PACIFIC	Sorafenib *vs* placebo	Superiority	150	6.5	0.68	0.01	[14]
			76	4.2			
SHARP	Sorafenib *vs* placebo	Superiority	229	10.7	0.69	0.001	[2]
			303	7.9			
SUNITINIB	Sunitinib *vs* sorafenib	Superiority	530	7.9	1.3	0.001	[22]
			544	10.2			
BRISK-FL	Brivanib *vs* sorafenib	Non-inferiority	577	9.5	1.06	0.31	[28]
			578	9.9			
LIGHT	Linifanib *vs* sorafenib	Non-inferiority	514	9.1	1.04	0.52	[24]
			521	9.8			
SEARCH	Sorafenib/erlotinib *vs* sorafenib/placebo	Superiority	362	9.5	0.92	0.48	[29]
			358	8.5			

of patients died from treatment related causes. The daily dose of 50 mg that is routinely used in other tumour types was deemed too high for patients with HCC where it precipitated liver toxicities including portal hypertension, encephalopathy, oesophageal variceal bleeding, ascites and thrombocytopenia. A subsequent head-to-head phase Ⅲ study of 1074 patients randomised to either sunitinib or sorafenib patients terminated early due to both futility and safety concerns[22]. The most frequent grade 3/4 adverse events in the sunitinib group were thrombocytopenia (29.7%) and neutropenia (25.7%) and in the sorafenib group were hand-foot syndrome (21.2%). Overall survival was also significantly lower in the sunitinib arm (7.9 mo *vs* 10.2 mo $P = 0.0014$). Temporary treatment discontinuation was more frequent with sunitinib (76.6% *vs* 58.7%). The failure of sunitinib was likely related to a combination of inadequate dosing, toxicities and trial design, and highlights the need for caution in over-interpretation of phase Ⅱ data and decision to move to Phase Ⅲ trials.

Pre-clinical studies identified linifanib as a more potent dual vascular epidermal growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) inhibitor than sorafenib (IC_{50} = 25 nmol for linifanib and IC_{50} = 57 nmol sorafenib) and VEGFR (IC_{50} = 8 nmol for linifanib and IC_{50} = 90 nmmol for sorafenib)[23]. A single arm phase Ⅱ trial in the first line setting resulted in a median overall survival of 9.7 mo (10.4 mo in patients with Child-Pugh-A status), which led to a non-inferiority phase Ⅲ trial with sorafenib[24]. The study of 1035 patients failed to reach its end-point with an overall survival of 9.1 mo for linifanib and 9.8 mo for sorafenib (HR = 1.04; 95%CI: 0.89-1.22; $P = 0.001$)[25]. Toxicities of hypertension and hepatic toxicities including encephalopathy were also higher in the linifanib arm.

A single arm first line phase Ⅱ study of 55 patients treated with brivanib, an ATP competitive inhibitor of several kinases including VEGFR2 (IC_{50} = 25 nmol), FGFR-1 (148 nm) and VEGFR1 (380 nmol), resulted in a median overall survival of 10.0 mo[26,27]. Phase Ⅱ studies confirmed that brivanib was well tolerated and one patient had a completed response, three had a partial response and twenty-two had stable disease. Yet BRISK-FL, the subsequent phase Ⅲ direct comparison trial of brivanib and sorafenib, failed to establish a significant survival benefit (9.5 mo for brivanib *vs* 9.9 mo for sorafenib; HR = 1.06; $P = 0.31$)[28]. Due to the trial design, in order to demonstrate non-inferiority, brivanib needed to produce a hazard ratio between 1 and 1.08, which it narrowly failed to reach. The BRISK-FL trial highlighted the difficulties in extracting comprehensive survival data from non-randomised phase Ⅱ trials. Grade 3/4 toxicities for sorafenib and brivanib were hyponatraemia (9% and 23% respectively), elevated liver enzymes (17% and 14%), fatigue (7% and 15%) and hand-foot reaction (15% and 2%). Even if this trial had met its end-point of non-inferiority, the significant toxicity and economic profiles were not more favourable than sorafenib, and thus would have been of little meaningful clinical benefit.

Erlotinib, an epidermal growth factor receptor (EGFR) TKI was tested in a first-line phase Ⅲ trial in combination with sorafenib compared to placebo/sorafenib in a study of 720 patients with advanced disease[29]. The combination had not previously been tested in phase Ⅱ trials, with two single arm phase Ⅱ studies demonstrating modest disease control[29-31]. The combined treatment did not improve overall survival (9.5 mo compared with 8.5 mo for sorafenib alone HR = 0.92; $P = 0.2$). Toxicities in the combination arm were also higher resulting in a reduced median treatment duration that may have contributed to its diminished efficacy. This trial demonstrates both the danger of proceeding to large-scale phase Ⅲ trials without a clear signal of efficacy from earlier phase studies and the difficulties in combining therapies for HCC (especially for drugs that have overlapping toxicities). Robust HCC-specific phase Ⅰ/Ⅱ studies are needed to identify optimal dosing of combination regimens (Table 2).

FGF has been pursued as a potential target in HCC and recent data suggests the FGF signalling pathway may play a key role in the development of resistance to anti-VEGF therapies by activating alternative proangiogenic signalling pathways[32]. Forty-six patients who had not responded to prior anti-angiogenic therapies were treated with brivanib in a single arm phase Ⅱ study[33]. The results were promising with a median overall survival of 9.7 mo. A subsequent phase Ⅲ trial that was conducted in parallel to the BRISK-FL trial compared brivanib with placebo

Table 2 Second line trials with molecular targeted therapies in advanced hepatocellular cancer

Trial	Drugs	Design	*n*	Median survival	HR	*P* value
BRISK-PS	Brivanib *vs* placebo	Superiority	263	9.4	0.89	0.33
			132	8.2		
EVOLVE-1	Everolimus *vs* placebo	Superiority	362	7.6	1.05	0.68
			184	7.3		
REACH	Ramucirumab *vs* placebo	Superiority	277	9.2	0.87	0.14
			276	7.6		

as second line treatment failed to meet its end point[34]. Patients treated with brivanib had a median overall survival of 9.7 mo compared with 8.2 mo in the placebo arm ($P = 0.3$). Yet significant improvements were seen in the secondary end points of overall response rate (10% for brivanib *vs* 2% for placebo $P = 0.003$), disease control rate (61% *vs* 40% $P \leqslant 0.001$) and alpha-feto protein reduction in 74% of patients with elevated baseline levels (> 50% reduction seen in 54% *vs* 7%). These indicate that brivanib has anti-tumour activity despite the negative primary outcome. Furthermore, despite stratification the placebo cohort had fewer patients with macro-vessel invasion and a numerically lower median AFP level. The unexpectedly long survival of patients in the placebo cohort has been cited as one of the reasons for treatment failure. As expected, there were also higher rates of treatment discontinuation and elective patient withdrawal from the brivanib arm, which may have reduced efficacy in this group.

Mammalian target of rapamycin (mTOR) is upregulated in many solid tumours including HCC and appears to have a critical role in pathogenesis[35,36]. A second line study with the mTOR inhibitor everolimus, offered no survival advantage over placebo (7.6 mo for everolimus *vs* 7.3 mo; HR = 1.05; $P = 0.68$)[37]. Ramucirumab is a fully human monoclonal antibody against vascular endothelial growth factor receptor 2 (VEGFR2), which also failed to improve survival compared with placebo (median overall survival for ramucirumab was 9.2 mo compared with 7.6 mo; HR = 0.86, $P = 0.13$) in the REACH trial[38]. However, a pre-planned sub-group analysis revealed that in patients with elevated baseline alpha-feto protein (AFP) of more than 400 ng/mL, ramucirumab extended both overall and progression free survival. Grade 3 toxicities that occurred more frequently in the ramucirumab arm included hypertension (12% compared with 4%) and fatigue (5% compared with 2%), but its toxicity profile is otherwise favourable compared to the multi-targeted TKIs. Due to this data, a phase Ⅲ trial with second line ramucirumab in a select population with AFP > 400 ng/mL is ongoing.

REASONS FOR THE FAILURE OF PHASE Ⅲ TRIALS

Clinical and molecular heterogeneity

So far all phase Ⅲ trials have unexpectedly failed to reach their end-points. There are several reasons for this. In the majority of patients with HCC, the cancer arises predominantly as a consequence of liver injury secondary to a variety of causes. It is clear that underlying liver pathology affects both outcome and treatment response, suggesting trials need to be stratified according to aetiology as well as Child-Pugh status, histological grade and stage[39]. Whilst patients with hepatitis B had longer overall survival and shorter time to progression following treatment with sorafenib in the SHARP trial, these results may have been confounded by the imbalance in numbers between patients with hepatitis B and C[2]. Without prior stratification, it is difficult to analyse the survival between sub-groups, highlighting the need for careful trial design.

Limited understanding of oncogenic drivers mean all recent negative phase Ⅲ trials were for "all comers", yet there is marked molecular heterogeneity amongst HCC tumours. Extensive genomic studies have revealed multiple genetic aberrations with more than 30 somatic mutations per tumour[40,41]. The challenge lies in distinguishing which are oncogenic drivers and which are bystander passenger mutations. Once drivers are identified, trials can be tailored to pertinent pathways. However, several studies have challenged the idea that single biopsies can represent the mutational landscape of the whole cancer. With highly mutated tumours such as HCC, the key is finding the so-called "trunk" mutations that exist in all tumour sites[42]. Even if a driver is found, inhibiting pathways may induce resistant mutations. Whilst "liquid" biopsies evaluating circulating DNA are under evaluation, further research is needed to validate these techniques before their use in the clinical setting[43]. One of the barriers to drug development is that many previous HCC trials did not mandate a tissue diagnosis, relying on clinical criteria alone. Several studies have now highlighted histological changes following treatment with loco-regional therapy such as TACE. In a prospective analysis of 80 nodules found in explant livers following transplantation for HCC, 14 cases of mixed hepatocholangiocellular tumours were found in patients who had received TACE whilst none were seen in the treatment-naive group, implying differentiation into a cholangiocellular phenotype for some patients[44]. Furthermore, the lack of histology arguably impedes both predictive and prognostic biomarker development. For example, a phase Ⅱ trial with the selective non ATP competitive c-MET inhibitor tivantinib, did not offer a survival advantage in patients with advanced HCC but a post study sub-group analysis revealed that the overall survival was longer in patients

with high baseline expression of c-MET (overall survival was 7.2 mo for tivantinib and 3.8 mo for placebo HR = 0.38, P = 0.01)[45]. A phase Ⅲ trial for patients with tumours over-expressing c-MET in the second line setting is on going (NCT01755767). Therefore, several agents that have failed in phase Ⅲ trials may still be efficacious in sub-groups of patients, emphasising the urgent need for tissue collection and more sophisticated trial designs that accommodate molecular stratification.

Underlying liver cirrhosis

Another challenge when treating patients with HCC is the presence of underlying liver cirrhosis. Historically, clinical trials were reserved for patients with good hepatic reserve so that competing liver morbidity does not overshadow outcomes from malignancy. Yet even in patients with preserved baseline hepatic function, reaching the optimal maximum tolerated dose in patients can be limited by hepatotoxicity. Treatment duration in these trials may have been insufficient to elicit a response. Liver dysfunction and co-existing cirrhosis may affect drug metabolism and due to the consequent changes in the pharmacokinetic and pharmacodynamics profiles of drugs, there is now a trend to conduct HCC-specific phase trials rather than extrapolate results from "all-comer" phase 1 studies conducted in patients with normal or near normal liver function.

There are no approved therapies in patients who progress on sorafenib and who retain well preserved liver function and good performance status. Many centres use cytotoxic chemotherapy (usually with FOLFOX due to results of the EACH trial) despite the lack of clear evidence supporting its use. Due to the lack of effective second-line therapy, patients are encouraged to enter clinical trials of novel agents. By definition, patients suitable for second line trials are more likely to have less aggressive disease than the wider HCC population in whom performance status often deteriorates rapidly on progression and is associated with decompensation of liver function. In a number of the recent second-line phase Ⅲ trials comparing novel therapies to placebo, there has been unexpected prolonged survival in the placebo cohort, potentially diminishing the survival differences between groups. Although the trend for overall survival favoured brivanib in the second line BRISK-PS trial, the results were non-significant suggesting the study was not sufficiently powered to detect benefits with brivanib against a placebo controlled population in whom survival was unexpectedly long[34].

Novel direct-acting antivirals (DAA) that target HCV-encoded proteins necessary for viral replication, can offer patients with hepatitis C sustained virological responses (SVR). The increasing use of these novel agents are expected to have a future impact on the incidence of HCV related HCC. Yet the presence of advanced fibrosis will continue to pose a risk for oncogenesis, even in the absence of a detectable viral load, and screening high risk individuals is still required[46]. The development of molecular predictive biomarkers could help identify patients that require ongoing surveillance. Furthermore, biomarker based stratification could be used to enrich HCC chemoprevention trials[47].

Response evaluation

Finally, response criteria in trials must be chosen carefully. Traditional endpoints such as tumour shrinkage relate to chemotherapy treatments and may not be applicable when assessing the benefits of targeted treatments, which can be cytostatic rather than cytoreductive[48]. Drugs that have been deemed failures in phase Ⅲ studies may have therapeutic activity in HCC, but insufficient potency to improve conventional end-points in phase Ⅲ trials[49]. Furthermore liver disease can elicit an inflammatory response, which can be mistaken for progression resulting in premature cessation of treatment. Thus the use of traditional imaging has been highlighted as insufficient in assessing response in HCC whereby functional imaging provides more useful information. RECIST criteria that is routinely used to measure disease response in many solid tumours, has been recognised as insensitive in HCC. In the SHARP trial, despite an improvement in overall survival, only 2% of patients treated with sorafenib underwent a response by RECIST criteria. The RECIST response criteria were amended to incorporate tumour necrosis induced by treatment. The modified RECIST (mRECIST) measures arterially enhancing lesions that are more representative of residual viable tumour[50,51]. Large multi-centre clinical trials in patients with HCC pose unique challenges and future study designs must accommodate these in order to exploit the true potential of novel agents in this disease[52,53].

THE GENETIC BACKGROUND OF HCC

In malignancies such as melanoma, key driver mutations have now been identified, leading to the use of effective targeted therapy that directly translates to improved patient survival[54]. Despite the presence of more than 40 somatic mutations, there does not appear to be solitary frequent genetic defects in the majority of HCC tumours[40,41,55,56]. Polyclonality has been noted in patients with HCC reflecting a complex genetic landscape. The recently proposed concept of "trunk *vs* branch" heterogeneity can be applied to HCC, whereby key mutations that drive tumorigenesis exist in both primary and secondary lesions (trunk) and need to be distinguished from those that are only present in a minority of tissue (branch)[42]. The question remains as to whether the vast number of genetic alterations in HCC reflect multiple "trunk" mutations that would each require inhibition, or if the majority are mere passenger alterations that do not need treating. Recent advances in high throughput sequencing have uncovered several mechanisms of genetic changes, including somatic mutations, copy number alterations, HBV integration and somatic changes of retrotransposons[55,57]. Whole genome sequencing of 88 primary HCC tumours with

matched adjacent liver tissue revealed the predominant oncogenic mutation was beta catenin (15.9%) which is mutually exclusive with the most frequently mutated tumour suppressor gene Tp53 (35.2%) echoing results from previous genomic studies[41,55,58,59]. Further mutations have been found in ARID1 and 2 (both of which regulate chromatin remodelling pathways) and rare mutations in RPS6KA3 which codes for RSK2 (a serine threonine kinase of the MAPK pathway)[60]. A larger study of 503 HCC liver genomes revealed 30 driver genes implicating 11 core pathways in tumorigenesis. Recurrent focal amplifications were seen in 25% of cases, including telomerase reverse-transcriptase (TERT) and CCND1-FGF19. Key oncogenic pathways included TP53-RB, Wnt and mTOR-PIK3CA[61]. Frequently altered in HCC, somatic TERT mutations have also been found in pre-cancerous cirrhotic nodules and hepatic adenomas, suggesting they play a pivotal role in malignant transformation. Sequencing of the promoter region of tissue taken from 305 HCCs revealed recurrent TERT mutations in 179 samples (59%) at two common mutually exclusive hot spots[62]. Yet despite a greater understanding of the role of TERT in HCC, its potential as a druggable target remains unknown. A small early phase Ⅱ study of a telomerase derived peptide, GV1001, failed to elicit any responses, although the trial was not enriched for TERT mutated tumours[63].

HCC can be classified into two distinct sub-groups based on genetic aberrations[64-67]. The proliferative subclass is characterised by activation of RAS, mTOR and IGF signalling and has been associated with poor outcomes. This group can be further divided into those with Wnt/transforming growth factor (TGF)-β activation and the progenitor cell group that have higher progenitor cell, epithelial cell adhesion molecules and type 1 cytoskeletal 19 markers. By comparison, the non-proliferative group is more heterogeneous with less shared mutations. The Wnt/beta catenin and JAK/STAT signalling pathways are the most frequently affected pathways, with alterations in as many as 50%-62.5% and 45% of cases respectively[66,68,69]. Several distinct protein-altering JAK1 mutations have been identified, the majority of which affect the kinase domain[55,70]. HCC development is often attributed to chronic inflammation triggered by both viral infection and cell necrosis and the JAK/STAT pathway has been identified as a promoter of carcinogenesis in a sub-set of HCC *via* cytokine-induced JAK/STAT pathway activation[55,71].

Copy number analyses using array based comparative genomic hybridization (aCGH) have revealed recurrent amplifications in genes for p53, Wnt signalling, proliferation pathways with recurrent deletions of genes involved in the immune response, chromatin remodelling and NF-kβ pathways[72,73]. Furthermore, the DNA virus hepatitis B (HBV), a leading cause of HCC, integrates into the host genome affecting gene expression. Deep sequencing of HCC samples on a background HBV found direct genetic disruption, aberrations of viral promoter-driven transcription, viral-human transcription and copy number changes confirming theories that alternate aetiologies lead to distinct genetic alterations[74,75]. Whole exome sequencing of 243 liver tumours revealed mutational signatures that appeared to correlate with specific risk factors for HCC development including CTNNB1 (alcohol) and TP53(HBV)[76]. In addition, different mutations were associated with varying clinical outcomes. Early stage disease harboured TERT promoter mutations whereas FGF, CCDN1, TP53 were associated with more aggressive pathology.

Conclusions from these extensive genetic studies have highlighted not only the heterogeneity of HCC tumours but also the significant differences in key oncogenic drivers of HCC compared with many other solid malignancies. In breast, colorectal and lung for example, MAPK and PI3K as well as EGFR activated pathways dominate progression in distinct cohorts[77-79]. However, for HCC Wnt/β-catenin and JAK/STAT pathways have consistently been identified as responsible for key oncogenic signalling. These differences are likely to explain the failures of therapies in HCC that have provided benefit in other malignancies. Comprehensive genetic mapping will undoubtedly aid drug development for HCC but a major challenge is that the majority of pathways found remain "undruggable" and interacting protein kinases must be targeted instead (Figure 1). A selection of key pathways and novel agents recently or currently under investigation are discussed below.

EMERGING TARGETS IN DRUG DEVELOPMENT

MEK inhibition

The RAF/MEK/ERK pathway plays a pivotal role in several cellular process including proliferation, apoptosis and migration[80,81]. Although RAS and RAF mutations are uncommon in HCC, there is evidence that this pathway is activated in the majority of HCC tumours. Selumetinib, a potent selective MEK 1/2 inhibitor, was assessed in a single arm phase 2 trial in 19 patients who had not received prior systemic therapy. There were no responses and time to progression was short (8 wk). The trial was subsequently terminated at the interim analysis[82]. Examination of pre and post treatment tissue revealed that four out of five patients achieved significant inhibition of phospho-ERK1/2 in tumours suggesting the failure of selumetinib was not due to lack of target inhibition. A small study assessing in combination with sorafenib resulted in three partial responses and six with stable disease. Whilst these numbers were small and therefore difficult to interpret, it suggests that perhaps this combination should be assessed further[83]. A phase Ⅱ study assessing the efficacy and safety of combination inhibition using sorafenib and the MEK inhibitor refametinib, resulted in a median time to progression of 122 d and median OS of 290 d[84]. Toxicities however were significant with rash, diarrhoea, elevated liver enzymes and vomiting and the majority of patients required dose reductions. Interestingly the best responders harboured a RAS mutation and a proof of concept phase Ⅱ trial using this combination for

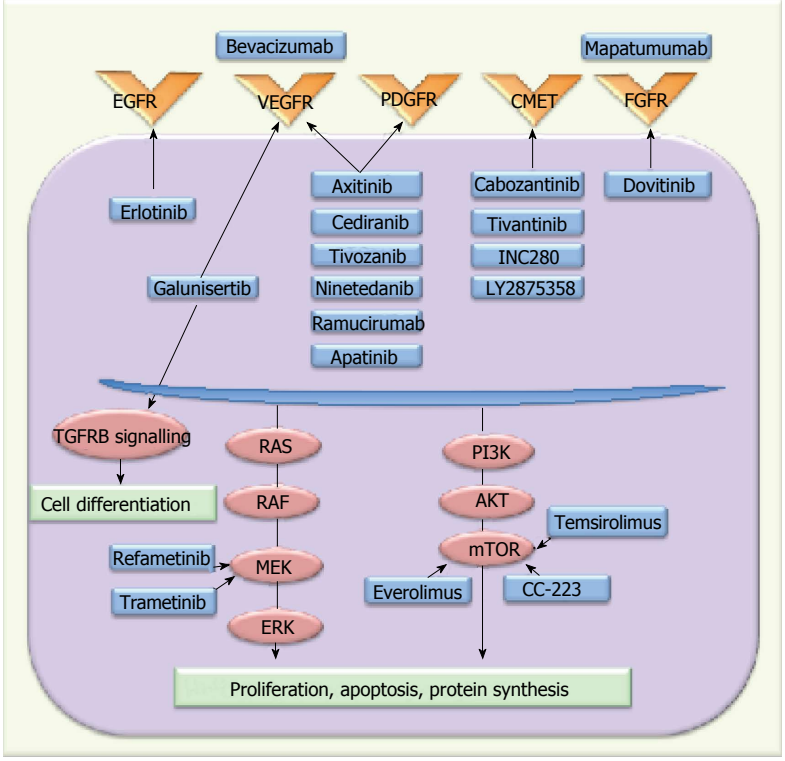

Figure 1 Novel compounds under investigation and their predominant targets. EGFR: Epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet derived growth factor receptor; FGFR: Fibroblast growth factor receptor; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; mTOR: Mammalian target of rapamycin.

patients with RAS mutations is on going (NCT01915602). Crucially, this study is one of the first attempts to select a specific cohort of HCC patients based on molecular genotype utilising cfDNA to detect mutations in RAS. The study raises a number of important issues regarding feasibility and cost given the incidence of RAS mutation is approximately 3%-5%, requiring a large cohort of patients to be prescreened to identify the small group with aberrant genotype (Table 3).

Anti-angiogenic therapy

HCC is a hyper vascular tumour enriched with high levels of angiogenesis due to the presence of growth factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF)[85]. A meta-analysis assessing the prognostic value of VEGF expression confirmed that tissue and serum VEGF levels seemed to predict poor disease free and overall survival[86]. Biomarker data from the SHARP trial also demonstrated that VEGF and angiopoietin-2 [(Ang2) a further critical molecule in angiogenesis] were independent prognostic markers but not predictive of response[16]. Sorafenib has anti-angiogenic properties and its success fuelled the search for more potent, selective anti-angiogenics. Yet several negative clinical trials have questioned the emphasis on VEGF inhibition in HCC, supporting theories that multiple mechanisms may be in play. As discussed the VEGF inhibitors, sunitinib, linifanib and brivanib failed to prove non-inferiority compared with sorafenib. Some commentators have therefore argued that an antiangiogenic monotherapy "ceiling" has been reached, and combination strategies will be required to extend survival beyond this[87]. Trials of sorafenib in combination with other antiangiogenic therapy (bevacizumab), chemotherapy (doxorubicin or FOLFOX) or other molecularly targeted therapy (*e.g.*, everolimus and temsirolimus) are on-going. In order to ensure optimal results with these agents, the development of predictive biomarkers is needed to select patients who are most likely to benefit.

HGF/c-MET pathway

In vitro studies suggest that c-Met may play a role in proliferation, angiogenesis and metastatic spread in HCC and the hepatocyte growth factor (HGF)-cMET axis is therefore an attractive target. Whilst HGF expression in HCC tumours is low compared with surrounding liver tissue, over-expression of cMET has been observed in nearly a quarter of HCC cases and there is some evidence to suggest c-MET expression is a poor prognostic marker[88-90]. Biomarker data from the SHARP trial revealed that HGF levels correlated with tumour size[16]. There is also evidence of an interaction between c-MET and both EGFR and VEGF[91]. Preliminary data from c-MET inhibition with cabozantinib is promising and as previously discussed a phase Ⅲ trial with tivantinib in patients with high levels of MET expression is on going[92].

FGFR inhibition

Fibroblast growth factors are trans membrane receptor kinases that signal downstream pathways including the RAS-RAF-MAPK. FGF3/4 is expressed in normal tissue including benign hepatocytes[93]. Gene array studies and Immunohistochemical expression assays have shown overexpression of FGF3 and FGF4 in HCC tumours that mediate proliferation, cell death and alpha

Table 3 Novel agents currently under evaluation in clinical trials

Drug	Phase	Target	Enriched population	Trial identifier	Study location
Tivantinib	Ⅲ	MET/tubulin	High MET expression	NCT01755767	North America, Europe
Axitinib	Ⅱ	VEGFR/c-KIT/PDGFR	No	NCT01334112	North America
Tivozanib	Ⅰ/Ⅱ	VEGFR	No	NCT01835223	North America
Nintedanib	Ⅰ/Ⅱ	VEGFR/FGFR/PDGFR	No	NCT00987935	Asia
Ramucirumab	Ⅲ	VEGFR2	AFP > 400	NCT02435433	North America, Asia, Europe
Apatinib	Ⅲ	VEGFR2	No	NCT02329860	Asia
Cabozantinib	Ⅲ	MET	No	NCT01908426	North America, Asia, Europe
INC280	Ⅱ	MET	MET aberration	NCT01737827	Asia
LY2875358	Ⅰ/Ⅱ	MET/VEGFR	No	NCT01287546	North America
Refametinib	Ⅱ	MEK	RAS mutations	NCT01915602	North America, Asia, Europe
Trametinib	Ⅰ/Ⅱ	MEK1/2	No	NCT02292173	North America
Dovitinib	Ⅱ	VEGFR, FGFR	No	NCT01232296	Asia
Temsirolimus	Ⅰ, Ⅱ	mTOR	No	NCT01687673	North America
Cc-223	Ⅰ, Ⅱ	mTOR	No	NCT01177397	North America, Europe
Galunisertib	Ⅱ	TGFRβ	No	NCT02423343	North America
Mapatumumab	Ⅰ/Ⅱ	TRAIL-R1	No	NCT01258608	North America, Europe
Nivolumab	Ⅰ	PD1	No	NCT01658878	North America, Europe, Asia
Lenvatinib	Ⅲ	VEGF	No	NCT01761266	North America, Europe, Asia
Enzalutamide	Ⅱ	Androgen receptors	No	NCT02528643	TBC
OMP-54F28	Ⅰ	Wnt signalling	No	NCT02069145	North America

feto protein (AFP) levels[94]. Brivanib, in addition to its anti-angiogenic properties as discussed above, is an ATP competitive inhibitor of FGF1-3. Although it failed to improve survival in the first and second line setting, further multi-kinase inhibitors that also target FGFR are currently underway. The lack of response to brivanib may be partly explained by its use in an unspecified population and biomarkers may aid selection of patients likely to respond to inhibition. Lenvatinib, an oral multi-targeted tyrosine kinase inhibitor of VEGFR-1, FGFDR1-4, PDGFRβ, RET and KIT is currently under evaluation in a non-inferiority study with sorafenib following a phase Ⅱ trial which resulted in a median time to progression of 12.8 mo (95%CI: 7.23-14.7) and median OS of 18.7 mo NCT01761266[95]. The REFLECT phase Ⅲ trial comparing sorafenib to lenvatinib has recently been completed. This trial has attempted to learn the lessons from the previous high profile failures described in this article by utilising stricter criteria for trial entry, excluding poor prognosis groups such as those patients with greater than 50% liver involvement, bile duct invasion, or main branch portal venous infiltration.

Dovitinib, an FGFR, VEGFR and PDGFR TKI demonstrated efficacy in xenograft mouse models and is currently under investigation in a phase Ⅱ trial[96,97]. FGF19, located on chromosome 11q13, a region amplified in 10%-15% of HCC tumours, is a potential predictive biomarker for FGF inhibitors and FGF19 targeted antibodies are under investigation in *in vitro* models[97]. *In vivo* studies with murine models suggest that dual targeting with FGFR and mTOR inhibition impaired tumour growth unlike treatment with the FGFR inhibitor alone providing support for combination trials[98].

TGF-β signalling

TGF-β signalling plays a role in the micro-tumour environment promoting epithelial-mesenchymal transition (EMT), dysplastic nodule formation and subsequent HCC development[99-101]. Patients with higher levels of TGF-β signalling are associated with larger less differentiated tumours with higher levels of AFP[102]. It remains unclear whether TGF-β plays a role in a sub-group of patients, or in the carcinogenesis of all HCCs due to its dual role in tumour suppression in normal tissue and tumour promotion in HCC. TGF-β inhibitors modulate EMT leading to reduced tumour growth in pre-clinical models. Galunisertib, a selective TGF-β TKI is currently under investigation in a phase Ⅱ trial (NCT02178358).

Immunotherapy

Recent years have seen a resurgence in the use of immunotherapy, led partly by the success of anti-CTL4 antibodies in solid tumours such as melanoma and more recently antibodies targeting the programmed death (PD) receptor and its ligand[103,104]. Immunotherapy works by enhancing anti-tumour response, an important mechanism in HCC as the surrounding micro-tumour environment is rich in immune cells. Tremelimumab, a fully human IgG2 monoclonal anti-CTL4 antibody was assessed in a phase Ⅱ study of 24 patients with HCC on a background of HCV. The drug had a good safety profile and a partial response of 17.6% and disease control rate of 76.4%. Time to progression was 6.48 m (95%CI: 3.95-9.14). Changes were also seen in the predominant variants of HCV as well as a reduction in viral loads. These early reports are promising and suggest that immunotherapy may have the dual benefit of treating both HCC and underlying viral hepatitis. Anti-programmed death ligand 1 (PDL1) inhibitors are checkpoint inhibitors that block T cell activation when bound by PD ligands 1 and 2. Patients with tumours that over-express PD-L1 are associated with a poorer prognosis. In a recently reported phase Ⅰ/Ⅱ dose

escalation study, patients received 0.1 to 10.0 mg/kg of the anti-PDL1 agent nivolumab intravenously for up to 2 years. 2 patients had a complete response (CR) and a further 7 patients had a partial response (PR)[105]. The overall survival rate at 6 mo was 72%. Although these results are from a very small early phase trial, they are highly encouraging and a number of trials using checkpoint inhibitors are now planned in both first and second line settings.

CONCLUSION

The era of personalised medicine and treatment stratification has yet to impact clinical practice of HCC and the failure of several clinical trials has been disappointing. Nevertheless our understanding of this unique disease has improved significantly with the benefit of genomic sequencing and biomarker data from clinical trials. Proof of concept studies such as the ongoing phase Ⅱ trial with refametinib for RAS mutated cancers and tivantinib for c-MET positive tumours are a step forward in designing adequate trials to maximise potential benefit of novel agents in pre-determined sub groups. Molecular testing, improved clinical trial design and the development of predictive biomarkers should finally see an improvement in survival for this global disease.

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REVIEW

Neoadjuvant radiotherapeutic strategies in pancreatic cancer

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Abstract

This review summarizes the current status of neoadjuvant radiation approaches in the treatment of pancreatic cancer, including a description of modern radiation techniques, and an overview on the literature regarding neoadjuvant radio- or radiochemotherapeutic strategies both for resectable and irresectable pancreatic cancer. Neoadjuvant chemoradiation for locally-advanced, primarily non- or borderline resectable pancreas cancer results in secondary resectability in a substantial proportion of patients with consecutively markedly improved overall prognosis and should be considered as possible alternative in pretreatment multidisciplinary evaluations. In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have been observed with neoadjuvant radio- or radiochemotherapy in several phase Ⅰ/Ⅱ trials, which justify further evaluation of this strategy. Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials in order to improve comparability of the current results with other treatment modalities. This should include the evaluation of optimal sequencing with newer and more potent systemic induction therapy approaches. Advances in patient selection based on new molecular markers might be of crucial interest in this context. Finally modern external beam radiation techniques (intensity-modulated radiation therapy, image-guided radiation therapy and stereotactic body radiation therapy), new radiation qualities (protons, heavy ions) or combinations with alternative boosting techniques widen the therapeutic window and contribute to the reduction of toxicity.

Key words: Pancreatic cancer; Neoadjuvant; Radiation therapy; Review; Radiation techniques

Core tip: This review summarizes the current status of neoadjuvant radiation approaches for pancreatic cancer. Neoadjuvant chemoradiation for locally-advanced cases results in secondary resectability in a substantial proportion of patients with consecutively improved overall prognosis. In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have

been observed in several phase Ⅰ/Ⅱ trials. Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials and included evaluation of optimal sequencing with chemotherapy and patient selection based on molecular markers. Modern radiation techniques widen the therapeutic window and contribute to the reduction of toxicity.

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INTRODUCTION

Multimodal treatment of patients with pancreatic cancer remains one of the largest challenges in gastrointestinal oncology. Surgery is the cornerstone of curative intent treatment[1], however only 10%-20% of the patients are deemed resectable at presentation while 30%-40% already suffer from locally-advanced, irresectable disease and the remaining group shows distant metastases[2]. Given a median survival of approximately 24 mo and a 5-year overall survival rate of roughly 10%-20% even in the most favourable group with primarily resectable, locally confined disease, pancreatic cancer remains a disease with one of the most dismal prognosis in oncology[3].

While neoadjuvant strategies are already part of the standard approaches in most other gastrointestinal tumors (*e.g.,* rectal cancer, esophageal cancer)[4,5], surgery followed by adjuvant treatment still represents the standard of care for resectable pancreatic cancer. Adjuvant chemotherapy seems the preferred approach in Europe based on the Conko-001 trial[6], while adjuvant chemoradiation is frequently used in the US based on the GITSG trial[7] and several non-randomized single-center studies with excellent results[8-10]. In primarily non-resectable locoregionally confined tumors, mainly definitive-palliative strategies have been used so far, which either consist of systemic therapy alone, combined chemoradiation or various combinations of both[11-13]. However, the mentioned strategies show limited success in terms of overall prognosis. On the other hand, the high rates of microscopically incomplete resections[14] with consecutively significant local recurrence rates[15] and the high frequency of locoregionally confined but primarily non-resectable tumors in combination with the clear advantages of neoadjuvant treatment strategies shown in other gastrointestinal tumor diseases despite clearly more favourable resectability, may form a strong rationale for the use of neoadjuvant strategies both for locally-advanced non-resectable as well as for primarily resectable pancreatic cancer patients.

Such strategies have different aims and include different possible advantages dependent on the resectability of the primary lesion: (1) in primarily non-resectable locoregionally confined pancreatic cancer, the main aim of neoadjuvant chemoradiation consists of tumor shrinkage including a drawback of the tumor from the major vessels to achieve secondary resectability; and (2) in primarily resectable cases, the main aim consists in enhanced local control either by increased probability of microscopic complete resection (R0) due to tumor shrinkage or by sterilization of microscopic tumor remnants in case of a microscopically incomplete (R1) resection. Substantial potential benefits further exist independent of the resection margin: (1) neoadjuvant treatment allows a local response evaluation which may reflect the overall disease prognosis; (2) neoadjuvant chemoradiation usually requires several weeks, which enables a stratification of patients with response or stable disease *vs* patients with rapid systemic progress. This may allow a potential omission of major surgery in those patients who are unlikely to benefit. The radiation therapy component thereby prevents patients without rapid systemic progression from a worsened overall prognosis due to local progression caused by locally insufficient effects of systemic therapy alone; (3) efficacy of radiation is enhanced in the neoadjuvant setting in comparison to postoperative radiotherapy because of the increased oxygenation of the untreated tissue; (4) the probability that additional therapy must be cancelled due to postoperative complications is reduced; and (5) target volume definition is simplified, resulting in smaller safety margins with consecutively lower dose to organs at risk and reduced toxicities.

Due to the complexity of the disease and the different aims in distinct stages, a variety of neoadjuvant concepts exist. They include chemotherapy, radiation therapy, chemoradiation or combinations like induction-chemotherapy followed by chemoradiation. This review focuses mainly on neoadjuvant radiotherapeutic strategies (radiation alone, radiation with concurrent chemotherapy) and advances in radiation technique rather than neoadjuvant concepts using chemotherapy alone or induction chemotherapy.

NEOADJUVANT RADIOTHERAPEUTIC TECHNIQUES AND CONCEPTS

3D-conformal radiation therapy

3D-conformal radiation therapy has been the standard radiation technique for the treatment of pancreatic cancer for the last two decades. This technique includes three-dimensional treatment planning based on computed tomography as first step. In the neoadjuvant setting, the target volume includes the primary tumor and the regional lymph nodes with a safety margin for daily repositioning error and tumor motion. If and to what extent the regional lymph node areas have to be included into the target volume is indeed part of an ongoing discussion. Multiple radiation fields are arranged in a way to ensure sufficient coverage of the target volume with best possible sparing of organs at risk at the same time (so called forward treatment

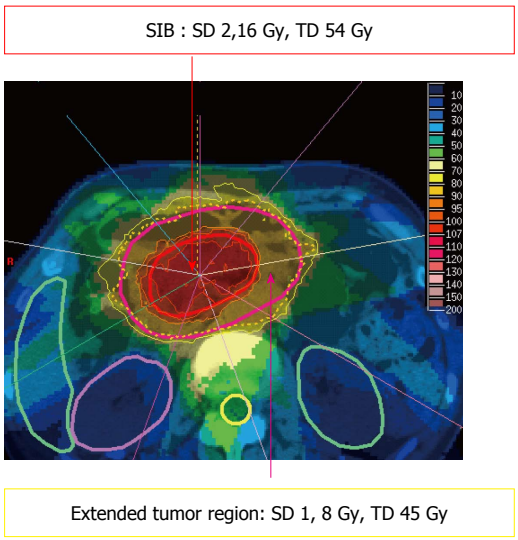

Figure 1 Example for a 9-beam intensity modulated radiation therapy treatment plan in a pancreatic cancer patient with simultaneously integrated boost. SD: Single dose; TD: Total dose in 25 fractions; SIB: Simultaneously integrated boost.

planning) with small bowel and kidneys representing the main dose-limiting structures. Usually total doses of 45-54 Gy are applied in conventional fractionation (5-6 wk overall treatment time) in combination with simultaneous 5-FU or gemcitabine based chemotherapy.

Intensity-modulated radiation therapy

Treatment of irregularly shaped target volumes directly adjacent to radiation sensitive organs at risk can generally be improved by the use of so called "complex" photon irradiation techniques like intensity-modulated radiation therapy (IMRT). In contrast to 3D-conformal therapy, IMRT allows the delivery of different doses to certain segments of the same radiation field, creating a so called "fluence matrix" for every beam. By addition of multiple segments within several beams, superior coverage of irregularly shaped target volumes can be achieved. At the same time, steep dose gradients are possible, allowing improved sparing of directly adjacent organs at risk remain possible. Treatment planning is called "inverse" planning, because in contrast to 3D-conformal therapy the field geometry is usually not directly adjusted by the planner. Instead, doses are prescribed to the target volume(s) and to the outlined organs at risk (so called "dose constraints") with a prespecified arrangement of beams. The treatment plan is then generated by an iterative computer-aided process by adjusting the fluence matrix and/or the constraints. This technique further allows the treatment of regions inside the target volume (for example gross tumor) with a higher dose, and other regions (for example elective nodal areas) with a lower dose within the same fraction. This enables dose escalation in certain areas within an unchanged number of fractions (so called "SIB = simultaneously integrated boost", Figure 1).

Numerous dosimetric studies showed clear advantages for intensity-modulated techniques compared to 3D-conformal treatments. In particular, lower doses in small bowel and liver could be achieved[16] and the possibility of a dose escalation up to 65 Gy was suggested[17]. Further on, several clinical studies have clearly confirmed, that these dosimetric advantages translate into reductions of acute and late side effects[18].

Image-guided radiation therapy

In general, several sources of uncertainty must be addressed in external beam radiation therapy regarding the coverage of target volumes with the prescribed dose. Intrafractional motion is mainly caused by respiration. On the other hand interfractional variations are the result of a combination of different factors. One major source is the displacement of the target by different filling of adjacent distensible structures like stomach or small bowel. Another source is the so-called "set-up error", *i.e.,* the uncertainty due to variation in daily positioning of the patient. All these variations must be compensated by safety margins. However, if directly adjacent organs at risk are present, every increase of safety margins consequently leads to increased side effects, which builds up the rationale for image-guided radiation therapy (IGRT). In doing so, three-dimensional datasets in treatment position are generated with imaging devices directly mounted on the linear accelerators (so called "on-board imaging"). These allow a comparison of the actual situation with the one during treatment planning and a real time correction of the position prior to irradiation. The increased precision of treatment application consequently allows a reduction of the safety margin. Several analyses have shown, that the safety margins needed to compensate for set-up error in the upper abdominal region can be reduced from 10 to 5 mm if IGRT is used[19]. In a tumor with 5 cm diameter, this margin reduction would lead to a 30% decrease of irradiated volume[18] with a significant dose reduction in small bowel, kidneys and liver[20].

Intrafractional respiratory motion differs from patient to patient, but can reach several centimeters[21]. Different strategies have been used to reduce the safety margins needed to account for such large variations. First, the individual respiratory motion can be measured for example by 4-dimensional computed tomography and allow definition of individualized anisotropic margins. This strategy can result in a mean reduction of the target volume by one third compared to the use conventional margins[20]. Some modern linear accelerators also allow gating, *i.e.,* on board detection of tumor motion and application of radiation only at distinct positions of the tumor in its motion cycle. Another technique supported by some accelerators is a continuous adjustment of the beams to the particular tumor position (so-called "tracking"). Especially for these methods, the implantation of fiducials into the tumor may further increase the precision of dose application[22].

Adaptive radiation therapy strategies

In contrast to the mentioned techniques, adaptive

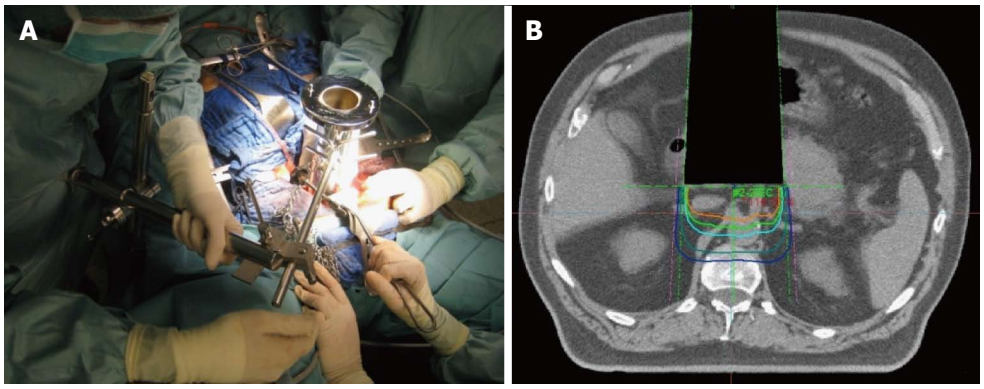

Figure 2 Intraoperative electron radiation therapy. A: Placement of the applicator after resection; B: Schematic dose distribution.

strategies use regular imaging to adapt the radiation treatment plan semi-automatically to anatomical changes during the radiation therapy series, for example due to tumor shrinkage. Although the models for routine use of these techniques are currently still under development, theoretical studies suggest marked reductions in dose to several organs at risk, for example duodenum[23].

Intraoperative radiation therapy

Although modern radiation techniques allow an improved sparing of surrounding organs at risk, dose-limitations in external beam therapy still exist, mainly due to directly adjacent structures with low radiation tolerance. Intraoperative electron radiation therapy (IOERT) offers an elegant possibility to overcome these dose-limitations after neoadjuvant radio (chemo)therapy. Due to its unique opportunity to guide a high single dose directly to the tumor bed or residual tumor during surgery, while adjacent organs at risk can be manually removed, IOERT can effectively prevents adjacent organs at risk from radiation exposure. Further advantages of IOERT in comparison to an external beam boost include at least theoretically smaller field sizes (because safety margins for daily positioning errors can be omitted) and the higher biological effectiveness of a single dose compared to the same amount of fractionated radiation therapy[24-29]. If typical dose concepts for the combination are used (10-15 Gy IOERT + 45-54 Gy EBRT), total doses can be reached which are biologically equivalent to 70-90 Gy of conventionally fractionated external beam radiation therapy without markedly increased toxicity[24-29]. Practically, an applicator of appropriate size is placed inside the abdominal cavity under visual control to cover the tumor bed/residual tumor after performed/attempted resection and manual removal of adjacent structures at risk. After moving and adjusting the patient below the accelerator, the irradiation itself is performed inside the operation theater, lasting about 1-2 min. Adequate depth coverage is achieved by appropriate selection of the electron energy (Figure 2). Unfortunately, this technique is only available at a limited number of centers so far (although numbers are heavily increasing in the recent years). Efficacy of IOERT remains difficult to assess, since many series report only single-center experiences covering large observation periods. Regarding primarily resectable pancreatic cancer, several Italian series reported significantly decreased local recurrence rates with the addition of IOERT[30,31]. Reni *et al*[32] confirmed these results in a larger comparison *vs* surgery alone. Beside an increased local control rate, they also found a significantly improved median overall survival in the subgroup of patients with early stages without increased perioperative morbidity. A multi-institutional series from Japan described a local recurrence rate of only 15% in 210 patients after gross complete resection with IOERT[33] and a European pooled analysis reported a very encouraging median overall survival of 30 mo for the combination of neoadjuvant chemoradiation, surgery and IOERT in a series of 270 patients[34]. Regarding primarily unresectable pancreatic cancer, IOERT can be used for dose escalation after neoadjuvant chemoradiation both in case of achieved secondary resectability as well as in case of further irresectability, resulting not only in improved local control but in the achievement of durable pain control in 75%-90% of the patients[35]. The Mayo group reported a series of 115 patients with unresectable pancreatic cancer and found that the addition of IOERT during explorative laparotomy after neoadjuvant irradiation resulted in a significantly increased 1-year local control rate (48% *vs* 82%)[36]. Shibamoto *et al*[37] compared EBRT, EBRT + IOERT and IOERT alone in a cohort of 150 patients and described an improved survival for the combination in the subgroup of patients with an initial CA 19-9 < 1000. The MGH group reported a median overall survival of 12 mo for the combination of EBRT and IOERT in their series of 194 patients with irresectable pancreatic cancer[38]. If the combined local treatment was further enhanced by a systemic treatment component, several series consistently reported median overall survival times of 16-18 mo with 2-year local control rates around 70%[39,40]. Even in patients with isolated local pancreatic cancer recurrences, the combination of EBRT, surgery and IOERT resulted in high local control and encouraging overall survival rates[26].

In summary, IOERT provides an elegant possibility to escalate dose allowing total doses which could not be achieved by EBRT alone even with the use of the most sophisticated techniques. In resectable pancreatic

cancer, IOERT seems clearly to improve local control, while its influence on overall survival cannot be finally assessed at present. In primarily irresectable pancreatic cancer, it can be suggested based on large single-center experiences, that especially the combination of EBRT, IOERT and chemotherapy achieves improved quality of life due to durable pain control, high local control rates and encouraging overall survival rates compared to other treatment approaches, although no phase Ⅲ data currently exists to confirm these results.

Stereotactic body (ablative) radiation therapy

Stereotactic radiation therapy was primarily developed to treat small intracranial tumor lesions (for example brain metastases) and was successfully used in these situations for several decades. Initially it was defined as a treatment with a high single dose, which was guided precisely to the target using a stereotactic frame as external coordinate system for target localisation and very rigid patient fixation systems to reduce safety margins to a minimum (also known as radiosurgery). After expansion of the technique to extracranial sites and introduction of image guided radiation therapy, the definition of stereotactic body radiation therapy (SBRT) has widened. Today it summarizes different methods, which consistently apply so called "ablative" doses (biologically equivalent doses far beyond those achievable by conventional fractionation) in one to few fractions with optimal precision aiming at durable control of macroscopic tumor lesions. This technique has for example emerged as the standard of care in medically inoperable patients with early stage non-small cell lung cancer[41]. Currently it is used also in pancreatic cancer, although due to the lower contrast to the surrounding tissue (compared with lung cancer) implantation of fiducials is usually needed to achieve a safe detection of tumor position and motion with simple imaging modalities. Fiducial based approaches can additionally be combined with motion compensating radiation techniques (gating or tracking) to reduce safety margins to a minimum.

The present clinical experience for SBRT in pancreatic cancer is based mainly on small series of patients with irresectable locally-advanced pancreatic cancer[42]. Although very different dose schemes (1 × 15-25 Gy, 3 × 8-15 Gy, 5 × 6.5 Gy) have been used[42], these series consistently report very high local control rates of 80%-100% with partially very encouraging overall survival especially if combined with sequential chemotherapy[43-45]. However, the therapeutic window of this technique is narrow and therefore dose to directly adjacent organs at risk (like duodenum) must be strictly limited to avoid major complications[46], as shown by the range of gastrointestinal grade 3 complications reaching from 14% to 79% in the major series, and depending mainly on target volume size and dose to the duodenum[45,47]. Adaptive dose prescriptions depending for example on the distance between tumor and duodenum seem to be beneficial[44].

Recently, SBRT has also been introduced into neoadjuvant treatment approaches. One series describes the use of SBRT in 73 patients of whom 56 were deemed borderline resectable[48]. Treatment consisted of 3 cycles induction-chemotherapy followed by SBRT which guided 35-50 Gy to the vessel-approaching tumor parts and 25-30 Gy to the remaining tumor parts in 5 fractions. Seventy-seven percent of the borderline resectable patients responded and were surgically explored. Resection was possible in 56% of the patients (97% R0), showing a significantly improved survival. Severe gastrointestinal toxicity (grade 3) was observed only in 5% of the patients[48].

In summary, SBRT yields high local control rates, which seem so be superior to the results of conventionally fractionated RT. However, SBRT in the upper abdomen remains a demanding technique with a narrow therapeutic window and has been so far investigated mainly in irresectable pancreatic cancer. Nevertheless, it seems to be a promising approach also in the neoadjuvant setting especially if combined with systemic therapy.

Particle therapy

A least theoretically, more advantages could be exploited by the use of radiation qualities like protons or heavy ions. In contrast to photons, particle beams deposit most of the dose in a narrow range of tissue depth depending on the beam energy. This so-called "Bragg-peak" can be used to focus the dose very precisely to the target volume, while adjacent tissues can be safely spared (Figure 3). Especially heavy ions further show an enhanced biological effectiveness, because they generate a different pattern of DNA-damage in the tumor cells which is less easily repaired by cellular DNA-repair mechanisms in comparison to damages set by photon therapy. Some drawbacks remain in the upper abdomen due to difficulties to account for bowel gas movement during treatment planning. These can lead to large dosimetric uncertainties compared to photons[49]. Nevertheless several encouraging preliminary results have been reported by several centers. For example, the MGH group showed a very low severe gastrointestinal toxicity rate of 4% during chemoradiation in a phase Ⅰ/Ⅱ trial, where neoadjuvant proton radiotherapy with 5 × 5 Gy combined with simultaneous capecitabine and followed by resection and adjuvant gemcitabine was evaluated in primarily resectable pancreatic cancer. With a median follow-up of 38 mo, they reported a local recurrence rate of 16% and a median overall survival of 17 mo[50]. Investigators from Chiba (Japan) launched a phase Ⅰ trial including 26 patients with resectable pancreatic cancer, treated with increasing doses of 30-36.8 Gy in 8 fractions with carbon ions of whom 81% proceeded to surgery. They reported a local control rate of 100% with 1- and 5-year survival rates of 89% and 52% in resected patients[51]. Irresectable pancreatic cancer patients were also included into a dose escalation trial with doses of 38.4-52.8 Gy in 12 fractions at the same center resulting in 81% local control and 60% overall survival after one year[52].

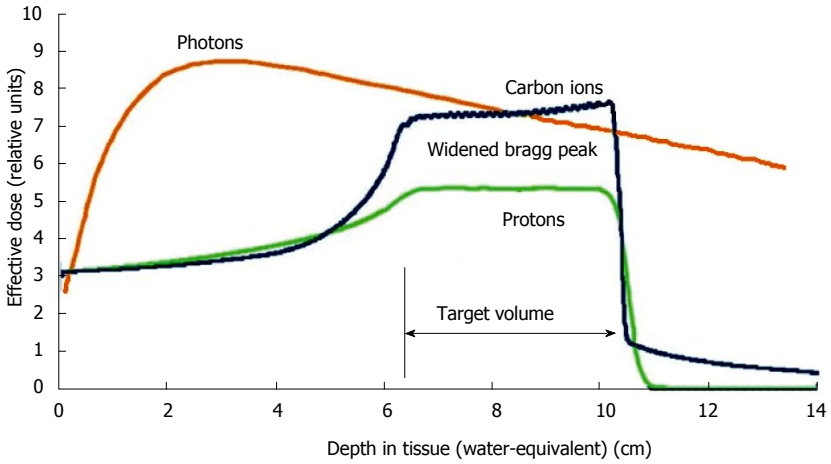

Figure 3 Schematic comparison of the depth dose curves of photons and particles. Lower dose distribution in the radiation path before and after the target with particles by exploiting the Bragg-peak.

In summary, particle radiation therapy seems to be a promising modality with regard to high local control rates with low toxicity. However, the current knowledge is based only on a few studies with low patient numbers and short follow-up and has therefore to be regarded as preliminary. Due to the known uncertainties in dose calculation because of bowel gas movement, patients with pancreatic cancer should be treated only in prospective studies at experienced centers.

Current status of neoadjuvant radio (chemo) therapy in locally-advanced primarily non-resectable pancreatic cancer

The interpretation of the literature regarding the optimal therapy for locally-advanced pancreatic cancer is difficult for different reasons. First of all, very different treatment strategies exist, ranging from aggressive approaches with curative intent such as multimodal neoadjuvant treatment including regular surgical exploration to purely palliative systemic treatment approaches, with all conceivable steps in between. Further on, even if only series using very similar neoadjuvant approaches aiming at secondary resectability are assessed, they differ extensively in terms of patient selection. The distinction between resectable and irresectable lesions is flawed by a certain subjectivity, which clearly correlates with the experience of surgeon and center. Even if a lesion is deemed primarily irresectable, different sub-terms are in use. In most of the US literature, patients are sub-divided in borderline-resectable and unresectable depending on the extent of vessel involvement, while this differentiation is not commonly used in most parts of Europe and Asia. This results in the inclusion of very different advanced lesions in neoadjuvant approaches compromising reasonable comparisons. The primary aim of neoadjuvant approaches in patients with locally-advanced irresectable pancreatic cancer is the induction of tumor shrinkage and thereby the achievement of secondary resectability *per se* and the increase of the rate of microscopic negative (R0) resections. Secondary aims are response evaluation for further treatment stratification and improvement of quality of life by prevention of local symptoms in case of persistent irresectability. The impact of neoadjuvant radio(chemo)therapy has been evaluated in numerous retrospective and prospective studies. These show a wide range of results and therefore seem less reliable when taken individually[53]. However, Gillen *et al*[2] described some fundamental findings in an impressive metaanalysis including 111 studies with 4400 patients. They included trials evaluating primarily resectable and primarily irresectable patients but analyzed them separately. In the group deemed primarily resectable they found a final resection rate of 74% after neoadjuvant therapy which is very similar to the rate reported for surgery alone. In the group primarily deemed irresectable, they observed a final resection rate of 33% after neoadjuvant (mainly radiochemo-) therapy. Radiological response assessment after neoadjuvant therapy described complete and partial remission in 4% and 29% with a 21% progression rate. These rates were not different between resectable and irresectable patients. However, the most important finding was a median survival of 21 mo and a 2-year overall survival rate of 50% in the group of patients, who reached secondary resectability after neoadjuvant treatment. This equals the result in the primarily resectable group (median survival 24 mo, 2-year overall survival 47%), while patients in whom resection was not achieved showed a significantly worse overall survival (median 10 mo), independent of their initial resectability status. Morganti *et al*[53] performed another metaanalysis including 13 trials with 510 patients, which were deemed irresectable and had received neoadjuvant chemoradiation with at least 45 Gy. Interestingly, they reported similar results: final resection rate was 27% with 88% being complete (R0). Median survival after secondary resection (24 mo) was significantly improved in comparison to persistent irresectability (10 mo)[53]. One of the largest single center analyses from Heidelberg again showed similar results[54]. In 257 patients treated with neoadjuvant (mainly radiochemo-) therapy and surgical exploration, secondary gross total resection rate was 40% with a median survival of 25 mo after R0-resection[54]. Postoperative morbidity and mortality does not seem to be increased after neoadjuvant chemoradiation compared to surgery alone[55,56]. In summary, neoadjuvant radiochemotherapy for patients with primarily irresectable, locally-advanced pancreatic

cancer results in secondary resectability in a substantial portion (30%-40%) of the patients, accompanied by a significantly improved prognosis in this subgroup. The median survival time (median approximately 24 mo) is similar to patients with primary resection and adjuvant chemotherapy. Even if secondary resectability is not achieved, the results after neoadjuvant chemoradiation are found at the upper end of the range reported for chemotherapy alone in the current literature, including the advantage of improved quality of life due to durable prevention of local complications by tumor progression.

Current status of neoadjuvant radio (chemo) therapy in primarily resectable pancreatic cancer

The rationale for neoadjuvant treatment approaches in resectable pancreatic cancer is based on several findings. First of all, locoregional progression is at least a component of disease progression in 50%-75% as shown by pattern of recurrence analyses after resection alone[57]. Even with the use of adjuvant chemotherapy, several studies reported local recurrence rates of 30%-60%. This suggests that eradication of locally persistent tumor cells by chemotherapy alone is not safely ensured[6,58]. As locoregional recurrences often result in local complications, the aim of achieving adequate local control seems justified with regard to quality of life.

Neoadjuvant strategies have replaced or at least supplemented sole adjuvant approaches as the standard of care in many other resectable gastrointestinal tumors, for example rectal cancer or esophageal cancer[5,59]. Unfortunately, no randomized data comparing neoadjuvant and adjuvant approaches in resectable pancreatic cancer have been published so far, although neoadjuvant approaches are investigated increasingly because of their potential benefits. Benefits include an improved local control rate for example due to an increased R0-resection rate, early initiation of at least a systemic therapy component to control potentially existing distant micrometastases, a simplified access to additional therapies and of course an optimal patient selection by exclusion of patients with early distant failure. The ongoing development will be illustrated exemplary by the work of the MDACC group, which designed and performed a number of consecutive phase Ⅱ trials over nearly 2 decades. They started with conventionally-fractioned radiation therapy combined with 5-FU[60], went on with a shortened radiochemotherapy with additional IOERT[61] and ended up with paclitaxel[62] and finally gemcitabine-based preoperative chemoradiation[63]. The last concept was evaluated in a phase Ⅱ trial with 86 patients, who received a shortened radiation therapy (10 × 3 Gy in 2 wk) in combination with weekly gemcitabine 400 mg/m^2 over 7 wk. Resection was finally achieved in 74% of the patients. The median overall survival for the entire cohort was 23 mo with a 5-year survival rate of 27%. Resectable patients had a significantly improved median survival of 34 mo compared to 7 mo in irresectable patients. The same was true for the 5-year survival rate (36% *vs* 0%). The local control rate in resected patients was 89%[63]. The authors concluded, that neoadjuvant chemoradiation allows a good selection of patients, who probably will not profit from major surgery. They recommended further investigation of neoadjuvant gemcitabine-based chemoradiation in resectable patients based on the very encouraging overall survival results[63], especially because a parallel trial by the same group with additional induction chemotherapy showed no further benefit[64]. The consistency regarding definition of resectability, surgical treatment and histological examination further suggests a good comparability of the results in the different MDACC trials[65]. Gemcitabine-based radiochemotherapy resulted in improved response rates, improved R0-resection rates and longer median survival in comparison to combinations with 5-FU or paclitaxel[65]. A pooled analysis of the MDACC studies including 240 patients treated with surgery after neoadjuvant therapy finally revealed a median disease-free survival of 15 mo and a median overall survival of 34 mo[66]. The potential benefit of neoadjuvant radiation therapy for resectable pancreatic cancer is further supported by a SEER-analysis on more than 3800 patients, which described a significant improved median survival of 24 mo after neoadjuvant radiation therapy compared to 17 mo with adjuvant and 12 mo without radiation therapy[67].

In summary, neoadjuvant radio- or radiochemotherapy offers several potential benefits compared to adjuvant strategies, although no randomized data are currently available to support this assumption. Nevertheless, neoadjuvant radio(chemo)-therapy has shown outstanding results in terms of response, local control and overall survival at least in phase Ⅱ trials. These results clearly justify further investigation of neoadjuvant radiation therapy approaches. In this context, further shortening of neoadjuvant radiation therapy schemes might be beneficial, as currently investigated in several prospective trials evaluating modern photon or proton techniques[28,50].

Future directions

As mentioned earlier, this article focuses on radiotherapeutic strategies including radiotherapy alone or combined with concurrent chemotherapy in the neoadjuvant setting. Within such approaches, chemotherapy is used mainly as a radiation sensitizer rather than as systemic treatment resulting in low doses and usually single drug treatment to keep combined toxicity acceptable. However, recently new chemotherapy agents and combinations like Gemcitabine/nab-Paclitaxel[68] or FOLFIRINOX[69] have been successfully introduced into the treatment of metastatic pancreatic cancer and resulted in improved response and overall survival. Therefore it seems reasonable to use these schemes also in the neoadjuvant setting either to target possible distant micrometastases as early as possible in patients with resectable disease or to induce tumor shrinkage in irresectable patients to achieve secondary resectability. Due to the increased toxicity profile of these potent combinations, concurrent application of radiation does not seem possible even with the most sophisticated radiation techniques. Therefore sequential

applications for example induction chemotherapy with FOLFIRINOX followed by chemoradiation with 5-FU or gemcitabine seem to be very promising and are currently under investigation (for example in the german CONKO 007 study), with some groups already showing very encouraging preliminary results[70]. Therefore additional aims for future radiation research in pancreatic cancer should include the evaluation of optimal sequencing of systemic and radiotherapeutic approaches as well as the identification of biomarkers to predict the pattern of disease progression in the individual patient.

Biomarker for stratification

One of the main challenges in the treatment of pancreatic cancer remains the insufficient possibilities for an early prediction of disease progression. This compromises a reasonable stratification of patients in terms of treatment combinations. Established and new biomarkers could be helpful. This will be illustrated exemplary in the following with CA 19-9 serving as example for an established and SMAD for a new marker. Several groups established an association between increased pretreatment CA 19-9 levels and an unfavourable outcome[71], with very high values indicating an already disseminated disease. Kim *et al*[72] for example showed stage-dependent median CA 19-9 levels between 40 and 748 U/mL in stage IA-ⅢⅠ compared to a median CA 19-9 level of 3239 U/mL in stage Ⅳ. However, two major disadvantages limit the value of pretreatment CA 19-9 levels for prediction of disease prognosis: 5%-10% of patients with pancreatic cancer show negative CA 19-9 levels due to a defect in the gen coding for Lewis enzyme[73] and CA 19-9 levels can be heavily influenced by other factors, for example cholestasis. Therefore increasing interest has been paid to new markers like SMAD4. The SMAD family of proteins plays a role in TGF-beta signaling, which is heavily involved in the regulation of cell proliferation, differentiation and apoptosis[74]. SMAD4 has been recently suggested as the most important candidate in regard to pancreatic cancer because it has been linked not only with tumor development but also with the pattern of disease progression[75]. In this context, the presence of intact SMAD4 seems to be associated with a rather locally-destructive growth, while loss of SMAD4 correlates with early distant metastasis[76]. These findings were supported by a trial performed by Crane *et al*[77], which found that 73% of the patients with intact SMAD4 showed locoregional progression while 74% of the patients with inactive SMAD4 developed distant failure. In summary, although current knowledge about biomarkers seems premature in regard to treatment stratification, this might be an encouraging opportunity to allow an improved allocation of patients to locally-aggressive *vs* systemic treatment approaches to strengthen personalized medicine also for pancreatic cancer in the future.

SUMMARY

In the absence of randomized data, published studies show consistently that neoadjuvant chemoradiation for locally-advanced, primarily non- or borderline resectable pancreas cancer results in secondary resectability in a substantial proportion of patients with consecutively markedly improved overall prognosis in this subgroup. Even if the goal of secondary resectability is not reached, radiation therapy may contribute to improved quality of life by the prevention of local complications. Neoadjuvant chemoradiation should therefore be considered as possible alternative in multidisciplinary pretreatment evaluations.

In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have been observed with neoadjuvant radio- or radiochemotherapy in several phase Ⅰ/Ⅱ trials. These undoubtly justify further evaluation of this strategy. In this context, further shortening of the radiation therapy series to allow a simplified integration into multimodal concepts is evaluated in ongoing trials.

Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials in order to improve comparability of the current results with other treatment modalities. This should include the evaluation of optimal sequencing with newer and more potent systemic induction therapy approaches. Advances in patient selection based on new molecular markers might be of crucial interest in this context.

Finally modern external beam radiation techniques (IMRT, IGRT, SBRT), new radiation qualities (protons, heavy ions) or combinations with alternative boosting techniques (IOERT) widen the therapeutic window and contribute to the reduction of toxicity by improving normal tissue sparing and/or increasing efficacy by dose escalation or enhanced biological effectiveness. These techniques offer innovative treatment strategies, which should be further evaluated in prospective controlled trials.

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REVIEW

Ubiquitin proteasome system research in gastrointestinal cancer

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Abstract

The ubiquitin proteasome system (UPS) is important for the degradation of proteins in eukaryotic cells. It is involved in nearly every cellular process and plays an important role in maintaining body homeostasis. An increasing body of evidence has linked alterations in the UPS to gastrointestinal malignancies, including esophageal, gastric and colorectal cancers. Here, we summarize the current literature detailing the involvement of the UPS in gastrointestinal cancer, highlighting its role in tumor occurrence and development, providing information for therapeutic targets research and anti-gastrointestinal tumor drug design.

Key words: Ubiquitin proteasome system; Gastrointestinal cancer

Core tip: The ubiquitin proteasome system (UPS) is involved in almost every cellular process, playing an important role in maintaining body homeostasis. Increasing evidence indicates that alterations in the UPS are correlated with gastrointestinal malignancies. Here, we review current information describing UPS members involved in gastrointestinal cancer, providing a resource for further study and clinical application.

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INTRODUCTION

The ubiquitin proteasome system (UPS) is important for the degradation of proteins in eukaryotic cells. Approximately 80%-90% of intracellular proteins involved in every cellular function are degraded though the UPS[1]. Compared with the lysosomal system, the UPS is highly selective. It can regulate the proteins that it degrades *via* the ubiquitination-proteasome-deubiquitination mechanism to maintain homeostasis in the body. When regulatory proteins are stabilized by a decrease in degradation or are lost due to accelerated degradation, an imbalance is generated and diseases such as cancer occur.

Gastrointestinal cancer is a cancer of different organs of the digestive system, the most frequently occurring cancers of which are esophageal, gastric and colorectal. Despite improvements in diagnostic and therapeutic methods, gastrointestinal cancers remain a significant threat to patients[2]. Recently, growing evidence has indicated that the UPS is linked to the development of gastrointestinal cancer. In this review, we discuss the members of the UPS thought to be involved in gastrointestinal cancer and highlight their roles in tumor occurrence and development.

The UPS is an important pathway for intracellular protein degradation

In cells, there are two main systems utilized for protein degradation: The autophagy-lysosome system and the UPS[3,4]. The lysosomal pathway degrades extracellular proteins imported into the cell by endocytosis or pinocytosis, while the UPS controls the degradation of intracellular proteins[5,6].

The UPS is composed of ubiquitin, ubiquitination enzymes, deubiquitination enzymes (DUBs), and proteasomes.

Ubiquitin is a highly conserved 76 amino acid protein, wild-expressed in eukaryotic cells[7]. During the ubiquitination process, multiple ubiquitin proteins can be covalently attached to a target protein by ubiquitination enzymes[8]. These ubiquitination enzymes include ubiquitin activating enzyme (E1), ubiquitin carrier protein (E2), and ubiquitin protein ligase (E3). Initially, E1 activates ubiquitin in an ATP-dependent manner, forming a thioester linkage between the carboxy-terminal glycine residue of ubiquitin and a cysteine in the active site of the E1 enzyme. Activated ubiquitin is then transferred from an E1 enzyme to a cysteine residue of an E2 enzyme. E3 then catalyzes the final step of the ubiquitination process by transferring ubiquitin to lysine residues of targeted proteins, forming a polyubiquitin chain that earmarks the targeted proteins[8-10]. Humans possess two E1 enzymes (UBA1 and UBA6), several dozen E2 enzymes, and several hundred E3 enzymes[11-13]. The specificity of the E3 enzymes determines the specific recognition of target proteins, providing selectivity in which proteins are targeted to the proteasome for degradation[10,14,15].

Upon ubiquitination, targeted protein is degraded by the 26S proteasome in an ATP-dependent manner[16,17]. The 26S proteasome is a complex consisting of a proteolytic core particle (20S proteasome) that is capped at both ends by 19S regulatory particles (19S regulatory complex). The 20S proteasome is a barrel-shaped complex comprised of four stacked rings and contains multiple catalytic centers in the chamber. The 19S proteasome recognizes a polyubiquitinated protein, unfolds it, liberates it from the polyubiquitin chain, and translocates the protein into the proteasome chamber for degradation. The ubiquitin molecules are recycled, and the peptides generated are used for antigen presentation or are degraded into amino acids that are recycled for new protein synthesis[9,10,17].

DUBs are a cluster of enzymes that oppose the action of the E3 ligases by cleaving the isopeptide bonds between lysine residues of targeted proteins and the C-terminal glycine of ubiquitin. They play important roles in maintaining the balance of the UPS. Analysis of the human genome has indicated the presence of - 100 functional DUBs[18-20].

Tumorigenesis consists of several steps, including self-sufficiency in growth signals, insensitivity to growth inhibitor signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis[21-24]. The UPS is an important regulator of protein degradation that is involved in every cellular function, including cell proliferation, apoptosis, migration and invasion. Thus, deregulation of this system may lead to tumorigenesis.

The UPS in esophageal cancer

Esophageal cancer (EC) is the eighth most common type of cancer, and ranks as the sixth leading cause of cancer-related mortality worldwide. The incidence of EC varies internationally, with the highest rates found in Eastern Asia and in Eastern and Southern Africa[2,25].

Several E2 enzymes, including ubiquitin-conjugating enzyme H10 (UBCH10, also known as UBE2C), ubiquitin-conjugating enzyme E2L3 (UBE2L3, also known as UBCH7), E2-EPF ubiquitin carrier protein (UCP), and ubiquitin-conjugating enzyme E2D3 (UBE2D3) have been shown to be involved in the development of EC. UBCH10 is expressed in cancerous and dysplastic esophageal lesions, but not in normal tissue. Its expression is positively correlated with lymph nodes metastasis (LNM), TNM classification, and clinical stages, and negatively correlated with relapse-free survival period. Down-regulation of UBCH10 can inhibit cell proliferation and induce the sensibility to the treatment of MG-262[26,27]. Knockdown of UBE2L3 expression can reduce the anoikis resistance of EC cells[28]. Higher level of UCP has been linked to a greater tumor burden, poor response to neoadjuvant therapy, and worse overall survival for EC patients. Furthermore, UCP down-regulation can inhibit the proliferation, migration and invasion of EC cells, probably through the VHL/HIF-1α-TGF-β1 pathway[29]. UBE2D3 expression is significantly lower in EC tissues and is correlated with histological grade, N stage, and

recurrence, suggesting that it acts as a tumor suppressor in EC. UBE2D3 may be involved the hTERT signal pathway, which can promote the development of invasive esophageal squamous cell cancer by interacting with the epidermal growth factor receptor and p53[30,31].

Numerous E3 enzymes participate in the development of EC, some of which promotes tumor development. C-terminal Hsp-interacting protein (CHIP) exhibits a higher expression level in metastatic lymph nodes and is positively correlated with a poor survival rate in stage Ⅲ EC patients[32]. F-box protein 31 (FBXO31) is an ubiquitin ligase whose cytoplasmic expression is concordant with the nuclear expression of cyclin D1. In EC tissues, higher FBXO31 expression level is significantly correlated with depth of tumor invasion, clinical stage, and poorer prognosis[33]. p53-associated cellular protein-testes derived (PACT) is highly up-regulated in EC. Experimental studies have revealed that knockdown of PACT significantly attenuates the p53-Hdm2 interaction, reduces p53 polyubiquitination, and enhances p53 accumulation, leading to both apoptosis and cell growth retardation[34]. SMAD specific E3 ubiquitin protein ligase 2 (Smurf2) targets TGF pathway-restricted Smad2. In EC, high expression of Smurf2 is correlated with depth of invasion, LNM, and poor survival rate. High expression of Smurf2 can down-regulate Smad2, which in turn regulates the TGF signaling pathway[35]. S-phase kinase-interacting protein 2 (Skp2) can interact with the S-phase kinase Cdk2/cyclinA and is involved in the ubiquitin-dependent degradation of p27. Skp2 expression is closely correlated with TNM stage. High expression of Skp2 is associated with poor overall survival in resectable EC. Further study reveals that knockdown of Skp2 inhibits cell migration and invasion and sensitizes cancer cells to anoikis, at least in part through the phosphoinositidyl 3-kinase-Akt pathway[36,37]. Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is overexpressed in EC tissues. Its expression is correlated with the T-stage and N-stage as well as with differentiation. Down-regulation of UHRF1 can enhance the radio-sensitivity of TE-1 cells by altering cell cycle progression, increasing apoptosis and decreasing DNA damage repair capacity[38].

Some E3 enzymes act as tumor suppressors in EC. F-box protein 4 (FBX4) is a ubiquitin ligase that directs the ubiquitylation of cyclin D1. Increased FBX4 function can enhance the normal activity of EC cells, and 14-3-3ε is involved in regulating its function[39]. F-box and leucine-rich repeat protein 19 (FBXL19) functions as an antagonist of Rac3 by regulating its stability, and it also regulates the TGFβ1-induced down-regulation of E-cadherin. Over-expression of FBXL19 attenuates TGFβ1-induced E-cadherin down-regulation and the elongation phenotype of EC cells[40,41]. F-box and WD repeat domain containing 7 (FBXW7, E3 ubiquitin protein ligase) can induce the degradation of positive cell cycle regulators, such as Myc, cyclin E and Jun. In EC tissues, a decrease in FBXW7 copy number regulates FBXW7 mRNA expression, and reduced expression of FBXW7 is an independent prognostic factor in EC[42].

Besides, there are still some E3 enzymes play controversial roles in EC. MDM2 (MDM2 proto-oncogene) is a key negative regulator of P53, but its expression state and clinicopathological parameters in esophageal squamous cell carcinoma are controversial. This may be due to a lack of sufficient case numbers in each study or the use of different methods to detect MDM2 expression. Meta-analysis suggests that MDM2 acts as a potent marker of early primary tumor stages but poses a high risk of regional LNM in EC. Notably, the MDM2 309GG genotype may be associated with an increased risk of EC among Asians[43,44].

DBUs, including ubiquitin specific peptidase 7 (USP7), ubiquitin specific protease-9X (USP9X), Ubiquitin-specific protease 22 (USP22), ubiquitin carboxyl-terminal hydrolase 37 (UCH37), and ubiquitin carboxyl-terminal hydrolase1 (UCHL1) are correlated with EC development. USP7 can deubiquitylate p53 and protect it from proteasome-mediated degradation. EC cells can be protected against metformin-induced growth inhibition by siRNA against USP7[45]. Up-regulation of USP9X in EC tissues plays an important role in the formation and progression of precancerous lesions, and its increased expression is significantly correlated with poorer survival rate in EC patients[46]. High expression of the USP22 protein is significantly associated with tumor progression, relapse and poor prognosis[47]. The expression of UCH37 is higher in EC tissues and is associated with outcome and recurrence. UCHL1 is silenced by promoter region hypermethylation in EC, and the restoration of its expression suppresses EC cell colony formation[48,49].

The UPS in gastric cancer

Gastric cancer (GC) is the second most common cause of cancer-related death, and its incidence rates are highest in Eastern Asia[2].

The E2 enzyme UBCH10 is known to be involved in GC. UBCH10 is expressed at higher levels in primary stomach tumors compared with corresponding normal tissues[50].

The oncogenic E3 ubiquitin ligases involved in GC are discussed in detail below. Autocrine motility factor receptor (AMFR) expression is significantly increased in GC tissues and is associated with invasion depth and LNM. Its expression is correlated with poor overall survival and an increased risk of recurrence in GC cases. AMFR is thought to participate in the EMT pathway because its expression is negatively correlated with E-cadherin expression and is positively correlated with N-cadherin[51]. Cullin 1 (CUL1) overexpression is significantly correlated with GC TNM stage, depth of invasion, LNM, worse overall survival rate, and 3-year survival rate in GC patients. Experimental studies have demonstrated that CUL1 knockdown inhibits cell growth by up-regulating p27 expression and decreases cell adhesion by suppressing the expression of Src family kinases and focal adhesion kinase[52]. MDM2 protein level is significantly up-regulated in GC and is significantly correlated with clinicopathologic

characteristics and a shorter overall survival of GC patients. Similar to its role in EC, the MDM2 309GG genotype may be significantly associated with an increased risk of GC[43,53]. Makorin ring finger protein 1 (MKRN1) can simultaneously induce p53 and p21 ubiquitination as well as proteasome-dependent degradation. In GC cells, MKRN1 could affect gastric tumorigenesis by repressing cellular senescence and tumor-suppressive effects through the down-regulation of p14ARF in either a p53-dependent or -independent manner[54]. RING box protein-1 (RBX1) exhibits a higher expression level in GC tissues, and silencing it significantly inhibits the proliferation of GC cells *in vitro*[55].

E3 ubiquitin ligases with tumor suppressor activity in GC are discussed in detail below. checkpoint with forkhead and ring finger domains (CHFR) is reported to promote the ubiquitination and degradation of oncogenic proteins such as Aurora A and polo-like kinase 1[56]. It is frequently down-regulated in GC as a result of CHFR promoter methylation, suggesting that it acts as a tumor suppressor in GC. Methylation of the CHFR promoter is correlated with tumor differentiation. CHFR methylation is significantly higher in poorly differentiated GC samples[57]. Moreover, CHFR promoter methylation is a sensitive marker of the effect of docetaxel in GC patients[58]. CHIP expression is significantly lower in GC tissues. CHIP down-regulation is correlated with LNM and tumor differentiation. Further study has demonstrated that CHIP down-regulation results in increased angiogenesis and contributes to GC progression and a poor prognosis, probably through the NF-κB signaling pathway[59-61]. FBXO31 expression is dramatically decreased in GC tissue and is significantly associated with tumor size, infiltration, clinical grade and patient prognosis. *In vitro*, FBXO31 overexpression significantly decreases colony formation, induces a G1-phase arrest, and inhibits the expression of CyclinD1 in GC cells. *In vivo*, ectopic expression of FBXO31 dramatically inhibits xenograft tumor growth in nude mice[62]. FBXW7 mRNA expression in GC samples is markedly decreased, and its deregulation is associated with the presence of LNM and GC stage Ⅲ-Ⅳ, as well as poor prognosis. Reduced FBXW7 expression is associated with MYC overexpression and a more invasive phenotype in GC cells[63]. Neural precursor cell expressed, developmentally down-regulated 4-like (NEDD4L) is strongly related to the invasion and metastasis of GC. Tumors lacking NEDD4L expression exhibit a greater extent of LNM, lymphatic invasion, and venous invasion, and present poor clinical outcomes for GC patients[64]. RNF180 (Ring finger protein 180) acts as a tumor suppressor in GC, and the methylated CpG site count of the RNF180 DNA promoter is highly associated with patient survival[65]. Zinc and ring finger 3 (ZNRF3) is down-regulated in gastric adenocarcinoma tissues. There is also a correlation between the down-regulation of ZNRF3 and poor tissue differentiation. Further study has revealed that ZNRF3 inhibits GC cell growth and promotes cell apoptosis by affecting the Wnt/beta-catenin/TCF signaling pathway[66].

E3 ubiquitin ligases with controversial functions in GC are discussed in detail below. Cbl proto-oncogene B (CBLB) is highly expressed in GC tissue with EGFR, and their expression levels have been linked to the invasion and development of GC. However, some studies have revealed that CBLB represses IGF-I-induced EMT, likely by targeting IGF-IR for degradation and further inhibiting the Akt/ERK-miR-200c-ZEB2 axis in GC cells[67,68]. Constitutive photomorphogenic 1 (COP1, also known as RFWD2) has been shown to regulate c-Jun and p53. One study found that COP1 mRNA was significantly decreased in GC tissues, and knockdown of COP1 in GC cells promoted cell proliferation and the expression of MMP1, MMP7 and MMP10[69]. However, another study showed that COP1 overexpression was associated with poor prognosis in primary GC[70]. Neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) is a regulator of PTEN and plays a complicated role in GC. One study revealed that overexpression of NEDD4 was tightly associated with TNM stage and a lower GC survival rate. And knockdown of NEDD4 dramatically inhibited GC cell migration and invasion[71]. However, another study demonstrated that NEDD4 increased in intestinal metaplasia compared to normal gastric mucosa and decreased in gastric carcinoma compared to dysplasia[72].

The DUBs involved in GC are discussed in detail below. UCHL1 is frequently methylated in primary GCs and has been found to be more frequently methylated in diffuse-type GCs than in intestinal-type GCs. Moreover, UCHL1 is involved in galangin-induced apoptosis in human GC cells[49,73]. Ubiquitin-specific protease 10 (USP10) is expressed at lower levels in GC tissues and cells compared to their wild-type equivalents. A lack of USP10 expression results in a marked propensity toward gastric wall invasion, LNM, highly malignant biological behavior, and poor survival[74].

The UPS in colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. The highest incidence rates are in Australia and New Zealand, Europe, and Northern America.

The E2 enzymes related to CRC are discussed in detail below. UBCH10 is highly expressed in CRC. The depletion of UBCH10 hinders tumorigenesis both *in vitro* and *in vivo*, probably by regulating the expression of cell cycle proteins such as cyclin A and cyclin B1. Furthermore, n-acetyl-leu-leu-norleucinal (ALLN) treatment is more effective in tumors with lower UBCH10 expression[75]. Ubiquitin-conjugating enzyme E2Q family member 2 (UBE2Q2) expression is increased in 65.11% colorectal carcinoma tissues compared with their corresponding normal tissues[76]. Ubiquitin-conjugating enzyme E2I (UBE2I) RNAi suppresses the 3D growth of KRAS mutant CRC cells *in vitro* and attenuates tumor growth *in vivo*[77].

The oncogenic E3 ubiquitin ligases in CRC are discussed in detail below. F-box and leucine-rich repeat

protein 20 (FBXL20) is overexpressed in human colorectal adenocarcinoma. Moreover, the inhibition of FBXL20 expression can effectively suppress cell proliferation and promote apoptosis in CRC cells, while the overexpression of FBXL20 promotes the invasive ability of CRC cells, possibly by inducing the degradation of SET and E-cadherin through caspase activation[78,79]. HECT, UBA and WWE domain containing 1 (HUWE1) is required for the growth of CRC cells in culture and in orthotopic xenograft models. HUWE1 shRNA suppresses the clonogenic growth of CRC cells, and small molecule inhibitors of HUWE1 can inhibit MYC-dependent transactivation in CRC cells but not in stem and normal colon epithelial cells[80]. UHRF1 expression is up-regulated in approximately two-thirds of CRC specimens and is particularly expressed in right compared with left hemicolon cancer. High UHRF1 expression tends to be associated with the depth of invasion and with E2F-1 expression. Knockdown of UHRF1 suppresses cellular growth in colon cancer cell lines[81-83]. ubiquitin-like with PHD and ring finger domains 2 (UHRF2) is up-regulated at both the transcriptional and translational levels in tumor tissues. Overexpression of UHRF2 is highly linked to clinical stage, depth of invasion, nodal involvement, tumor histologic grade and the presence of metastases. Patients with UHRF2-positive tumors have a much lower disease-free survival and overall survival[84]. Skp2-siRNA effectively inhibits proliferation, increases the level of apoptosis, and induces G0/G1 phase arrest of colon cancer cells, along with increasing p27 and p16 protein levels. Tumorigenicity experiments show that the inhibition of Skp2 significantly increases the survival Skp2 is associated with a poor therapeutic response and adverse outcomes in rectal cancer patients treated with neoadjuvant chemoradiotherapy[87].

E3 ubiquitin ligases exhibiting tumor suppressor activity in CRC are discussed in detail below. CHIP is down-regulated, predominantly in the late stages of CRC, and the CHIP promoter is hypermethylated in CRC specimens. Overexpression of CHIP results in impaired tumor growth in nude mice and decreased migration and invasion abilities of tumor cells. Further study reveals that CHIP negatively regulates NF-κB signaling by promoting the ubiquitination and degradation of p65. The suppressive effect of CHIP leads to decreases in the expression of NF-κB-targeted oncogenes, including Cyclin D1, c-Myc, MMP-2, VEGF and IL-8[88]. Neural precursor cell expressed, developmentally down-regulated 4-like (NEDD4L) mRNA is significantly down-regulated in all CRCs. NEDD4L protein is significantly decreased in CRC compared to adjacent normal mucosa. Moreover, NEDD4L inhibits canonical Wnt signaling at or below the level of β-catenin *in vitro*[89]. Neuregulin receptor degradation protein-1 (NRDP1) is significantly decreased in CRC tissues. Knockdown of NRDP1 enhances the proliferation of CRC cells, while the overexpression of NRDP1 inhibits the proliferation of CRC cells. Further analysis shows that NRDP1 may induce the degradation of its target ErbB3 to inhibit the activation of both the ERK/MAPK and PI3K/Akt pathways in CRC cells, which seems to affect cell proliferation *via* the nuclear retention of a major cell-cycle inhibitor, p27. In addition, NRPD1 inhibits the expression of MMP7, which is required for cell invasion[90,91]. Ring finger protein 43 (RNF43) can negatively regulate Wnt signaling, and its gene mutations in this gene has been found in over 18% of colorectal adenocarcinomas. Truncating mutations of RNF43 are more prevalent in microsatellite-unstable tumors and show mutual exclusivity with inactivating APC mutations in colorectal adenocarcinomas. These results indicate that RNF43 is one of the most commonly mutated genes in CRC[92].

FBXW7 plays a controversial role in CRC. On one hand, FBXW7 mRNA expression is significantly lower in tumor tissues, an expression pattern correlated with poorer prognosis. *In vitro*, FBXW7-specific siRNA enhances the expression of c-MYC and cyclin E and promotes cell proliferation[93]. Moreover, studies have found that the FBXW7 mutation is correlated with colorectal tumorigenesis[94]. On the other hand, a large-scale study has revealed that there is no strong association between patient prognosis and FBXW7 mutation[95].

The DUBs involved in CRC are discussed in detail below. OTU deubiquitinase, ubiquitin aldehyde binding 1 (OTUB1) is overexpressed in CRC tissues, and its expression level is associated with metastasis. A high OTUB1 expression level is also associated with poor survival, and OTUB1 serves as an independent prognostic factor in multivariate analysis. Further study has revealed that OTUB1 promotes the metastasis of CRC cell lines *in vitro* and *in vivo* by regulating EMT[96]. Ubiquitin specific peptidase 11 (USP11) overexpression is frequently observed in CRC tissues and is correlated with poor survival. CRC cell lines expressing high levels of USP11 exhibit strong resistance to Smac mimetic-induced cIAP2 degradation. Furthermore, USP11 down-regulation sensitizes these cells to apoptosis induced by TRAIL and BV6, and suppresses tumor growth in a xenograft model[97]. Ubiquitin-specific protease 22 (USP22) expression is significantly higher in primary CRCs than in the paired non-cancerous tissues at both the mRNA and protein levels. Higher USP22 expression is significantly associated with shorter periods of disease-specific survival and shorter disease-free survival. In addition, USP22 expression is significantly correlated with BMI-1, c-Myc and cyclin D2 and is a novel regulator of the SIRT1-STAT3 signaling pathway[98-100]. Ubiquitin-specific protease 28 (USP28) deletion results the fewer intestinal tumors of the murine model in CRC. And in established tumors, USP28 deletion reduces tumor size and dramatically increases lifespan[101]. Ubiquitin-specific protease 33 (USP33) expression is down-regulated in CRC samples, and a reduced USP33 mRNA level is correlated with increased tumor grade, LNM and poor patient survival. USP33 acts as a tumor suppressor in CRC by mediating the inhibitory function of Slit-Robo signaling on CRC cell migration[102]. USP44

Table 1 The roles of ubiquitin proteasome system members in gastrointestinal cancer

Enzyme	Esophageal cancer			Gastric cancer			Colorectal cancer		
	P[1]	S[2]	C[3]	P[1]	S[2]	C[3]	P[1]	S[2]	C[3]
E2 enzyme	UBCH10 UBE2L3 UCP	UBE2D3		UBCH10			UBCH10 UBE2Q2 UBE2I		
E3 enzyme	CHIP FBXO31 PACT Smurf2 Skp2 UHRF1	FBX4 FBXL19 FBXW7	MDM2	AMFR CUL1 MDM2 MKRN1 RBX1	CHFR CHIP FBXO31 FBXW7 NEDD4L RNF180 ZNRF3	CBLB COP1 NEDD4	FBXL20 HUWE1 UHRF1 UHRF2 Skp2	CHIP NEDD4L NRDP1 RNF43	FBXW7
DUBs	UCH37 USP9X USP22	UCHL1 USP7			UCHL1 USP10		OTUB1 USP11 USP22 USP28	USP33 USP44	UCHL1

[1]Tumor promoter role; [2]Tumor suppressor role; [3]Controversial role. AMFR: Autocrine motility factor receptor; CBLB: Cbl proto-oncogene B; CHFR: Checkpoint with forkhead and ring finger domains; CHIP: C-terminal Hsp-interacting protein; COP1: Constitutive photomorphogenic 1; CUL1: Cullin 1; DUB: Deubiquitination enzymes; FBXO31: F-box protein 31; FBX4: F-box protein 4; FBXL: F-box and leucine-rich repeat protein; FBXW7: F-box and WD repeat domain containing 7; HUWE1: HECT, UBA and WWE domain containing 1; MDM2: MDM2 proto-oncogene; MKRN1: Makorin ring finger protein 1; NEDD4: Neural precursor cell expressed, developmentally down-regulated 4; NEDD4L: Neural precursor cell expressed, developmentally down-regulated 4-like; NRDP1: Neuregulin receptor degradation protein-1; OTUB1: OTU deubiquitinase, ubiquitin aldehyde binding 1; PACT: p53-associated cellular protein-testes derived; RBX1: RING box protein-1; RNF: Ring finger protein; Smurf2: SMAD specific E3 ubiquitin protein ligase 2; Skp2: S-phase kinase-interacting protein 2; UBCH10: Ubiquitin-conjugating enzyme H10; UBE2L3: Ubiquitin-conjugating enzyme E2L3; UBE2D3: Ubiquitin-conjugating enzyme E2D3; UCP: E2-EPF ubiquitin carrier protein; UHRF: Ubiquitin-like with PHD and ring finger domains; UCH37: Ubiquitin carboxyl-terminal hydrolase 37; USP: Ubiquitin specific protease; UCHL1: Ubiquitin carboxyl-terminal hydrolase1; UBE2Q2: Ubiquitin-conjugating enzyme E2Q family member 2; UBE2I: Ubiquitin-conjugating enzyme E2I; ZNRF3: Zinc and ring finger 3.

is hypermethylated in all CRC cell lines and in most colorectal adenomas, but rarely in normal mucosa samples[103]. UCHL1 plays a controversial role in CRC. Some investigations have shown that UCHL1 is more frequently methylated in CRC tissues than in normal colorectal tissues, whereas other studies have indicated that high UCHL1 expression is related to colorectal tumor progression, invasion, LNM, and poor clinical outcome[104].

CONCLUSION

The UPS plays an essential role in controlling every cellular process and in maintaining the homeostasis of the body. In this review, we discussed the members of the UPS known to be involved in gastrointestinal cancer. Among the UPS, the dysregulation of the enzymes E2, E3 and DUBs play the most prominent role in tumorigenesis and development. As shown in Table 1, some enzymes may be just involved in one type cancer, while others may be involved in two or three types. Moreover, a single enzyme may play different roles in different cancers, as is the case for CHIP and UCHL1. This suggests that these enzymes may exhibit tissue specificity or may function through different mechanisms in different situation. Further study is necessary to better understand the biological function of the UPS and for the development of new therapeutic targets and anti-tumor drugs.

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ORIGINAL ARTICLE

Case Control Study

Risk factors for the development of colorectal carcinoma: A case control study from South India

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Data sharing statement: Technical appendix, statistical code and dataset available with the corresponding author at drvikramkate@gmail.com.

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Abstract

AIM: To study the association of colorectal carcinoma (CRC) with diet, smoking, alcohol, physical activity, body mass index, family history and diabetes.

METHODS: All consecutive patients with CRC confirmed by histopathology diagnosis were included. Age (± 5 years) and gender matched controls were selected among the patients admitted in surgery ward for various conditions without any co-existing malignancy. Food frequency questionnaire (FFQ) was developed and validated after pretesting by investigator trained in data collection techniques. Cases and controls were interviewed ensuring privacy, in similar interview setting, with same duration of time for both cases and controls without any leading question. Biological variables like family history of CRC in first degree relatives, history of diabetes mellitus; behavioral factors like tobacco use both smoking and smokeless form, alcohol consumption and physical activity were recorded. Dietary details were recorded using a FFQ consisting 29 food items with seven categories. Analysis was done using appropriate statistical methods.

RESULTS: Ninety-four histopathologically confirmed cases of CRC and equal number of age and gender

matched controls treated over a period of two years were studied. Age distribution, mean age, male to female ratio, education level and socioeconomic status were similar in cases and controls. Intake of food items was categorized into tertile due to skewed distribution of subjects as per recommended cut off for consumption of food item. On univariate analysis red meat [OR = 7.4 (2.935-18.732)], egg [OR = 5.1 (2.26-11.36)], fish, fried food and oil consumption were found to be risk factors for CRC. On multivariate analysis red meat consumption of more than 2-3 times a month (OR = 5.4; 95%CI: 1.55-19.05) and egg consumption of more than 2-3 times a week (OR = 3.67; 95%CI: 1.23-9.35) were found to be independent risk factors for the development of CRC.

CONCLUSION: Egg and red meat consumption found to be independent risk factors for CRC. Smoking, alcohol, physical activity and family history were not associated with increased risk.

Key words: Dietary factors; Smoking; Rectal cancer; Red meat; Colorectal malignancy

Core tip: In this hospital based case control study, egg consumption of 2-3 times a week and red meat consumption of 2-3 times a month were found to be independent risk factors for the development of colorectal carcinoma. On the other hand smoking, alcohol, physical activity, diabetes and family history were not associated with an increased risk. There was no conclusive evidence to suggest that fruits and vegetable consumption has protective effect on colorectal carcinoma. Since red meat and egg had an increased risk, the community needs to be educated to reduce the consumption of red meat such as mutton and egg.

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INTRODUCTION

Colorectal cancer (CRC) is one amongst the leading cause of cancer related morbidity and mortality. CRC share 10% of the total cancers worldwide and accounts for 8% of all cancer related mortality; caused 608000 deaths worldwide[1,2]. In India data from population based cancer registry at Bangalore, Chennai and Delhi showed significantly increased incidence of CRC from 1982-2006[3].

Epidemiological studies have estimated that up to 70%-80% of CRCs could be ascribed to dietary, environmental and lifestyle factors; suggesting majority of the risk factors are modifiable[4]. It has been demonstrated that diet significantly influences the risk of developing CRC, and up to 70% reduction in the cancer burden can be achieved by changing the food habits[5]. Many epidemiological studies across the globe have tried to evaluate the role of dietary and life style factors in the development of CRC, however a fair share of controversies exist among the observations[6]. Majority of the studies that investigated the role of high vegetable and fruit diet failed to prove any significant reduction in the incidence of CRC.

For a long time, it was believed that low meat intake and high fiber vegetarian diet by Indian population is the reason for the low incidence of CRC in India. It was found that only two studies have been reported in literature from India regarding factors associated with CRC[7,8]. Identifying the factors associated with decreased CRC incidence among Indian population may help in the prevention of CRC. Hence an attempt was made to study these factors through a case control study. The objective of the study was to find the association of CRC with life style variables (diet, smoking, alcohol, physical activity) and Biological Variables [body mass index (BMI), family history of CRC in 1st -degree relatives, history of diabetes mellitus].

MATERIALS AND METHODS

The study was conducted in Department of Preventive and Social Medicine in collaboration with department of Surgery in a tertiary care referral and research institute of India. This study was conducted from period of two years. This study was approved by the Institute Ethics Committee. The nature, methodology of the study was explained to the patient and informed consent was obtained. All the information collected was kept confidential and patient was given full freedom to withdraw from the study at any point during the study. All provisions of the Declaration of Helsinki were followed in this study.

All consecutive patients with confirmed histopathology diagnosis were included. Histopathology was done either pre-operatively or postoperatively. Diagnosis of CRC was confirmed by per-rectal sigmoidoscopic or endoscopic biopsy. In case where resection for colorectal malignancy was done as an emergency surgical procedure, the diagnosis was confirmed post operatively. CRC patients with co-existing malignancy were excluded. Age (± 5 years) and gender matched controls were selected among the patients admitted in Surgery ward for various conditions like inguinal hernia, varicose veins, necrotizing fasciitis and diabetic foot.

Patients with co-existing malignancies, familial adenomatous polyposis and patients admitted with any abdominal disorders were excluded from the study. Controls were selected within one week after selecting the case. When more than one control was eligible then

control was selected by simple random methods using lots. During initial phase of the study, food frequency questionnaire (FFQ) was developed and face validation was carried out by circulating among the faculty who were involved in the study. Pre-testing was done among 10 patients admitted in the surgery ward by investigator trained in data collection techniques. It helped to estimate the average time taken for questionnaire administration, examination and to check for comprehensibility of participants to the questions.

After pre-testing of questionnaire necessary modifications were carried out. After obtaining informed consent cases and controls were interviewed ensuring privacy, in similar interview setting, with same duration of time for both cases and controls without any leading question. Average time taken for each interview was around 45 min. Anthropometric measurements was taken at the end of the interview. Pre-tested questionnaire which elicited information on demographic parameters like name, age, gender; Social variables like education, occupation, income, presenting complaints; biological variables like family history of CRC in first degree relatives, history of diabetes mellitus; behavioral factors like tobacco use both smoking and smokeless form, alcohol consumption and physical activity.

The alcohol consumption among study participants was measured and classified as per the World Health Organization STEPwise approach to surveillance of non-communicable diseases. The STEPS questionnaires used for the study are available in the internet from: http://www.who.int/ncd_surviellance/en/steps_framework_dec03.pdf. The alcohol consumption pattern of drinkers (amount, type and frequency) was noted and converted in terms of average alcohol consumed in grams per day. These were further classified as abstainers (who never consumed alcohol in past 12 mo), grade 1 (< 39.9 g/d), grade 2 (40-59.9 g/d) and grade 3 (> 60 g/d). The physical activity was measured using international physical activity questionnaire-short version. Metabolic equivalent (MET) levels for walking, moderate and vigorous intensity activities were taken as 3.3, 4.0 and 8.0. The activities were measured separately (MET level × minutes of activity/day × days per week) and expressed as total MET min/wk. Based on the total scores, study participants were categorized in to low (< 600 MET min/wk), moderate (600-3000 MET min/wk) and high (> 3000 MET min/wk) level of physical activity.

Dietary details were recorded using a FFQ consisting 29 food items with seven categories (never or hardly ever, once a month, 2-3 times a month, once a week, 2-3 times a week, 4-6 times a week, once a day or more) for egg, chicken, mutton, beef, pork, fruits, vegetables, fried foods, type of oil, type of food, tea, coffee; anthropometric measurements including weight, height, hip circumference, waist circumference also were recorded.

Sample size was calculated using *n* Master software 2.0 for matched case control study, taking exposure in controls for non-vegetarian food as 58% and OR 3.38 at 95%CI, 80% power the minimum sample size was 93[9].

Table 1 Socio demographic details of study population, *n* (%)

Variable	Cases	Controls
Age (yr)		
< 40	9 (9.6)	7 (7.4)
40-49	21 (22.3)	18 (19.1)
50-59	28 (29.8)	29 (30.9)
60-69	30 (31.9)	32 (34)
⩾ 70	6 (6.4)	8 (8.5)
Educational status		
Never attended school	37 (39.4)	33 (35.1)
1-4	23 (24.5)	34 (36.2)
5-7	15 (16)	10 (10.6)
8-10	14 (14.9)	9 (9.6)
11-12	1 (1.1)	6 (6.4)
Graduation	4 (4.3)	2 (2.1)
Occupation		
Non worker	23 (24.5)	19 (20.2)
Skill Ⅰ	44 (46.8)	59 (62.8)
Skill Ⅱ	25 (26.6)	16 (17)
Skill Ⅲ	2 (2.1)	0
PCI in indian rupees/mo		
Class Ⅰ > 4400	1 (1.1)	0
Class Ⅱ 2200-4399	1 (1.1)	2 (2.1)
Class Ⅲ 1320-2199	5 (5.3)	6 (6.4)
Class Ⅳ 660-1319	34 (36.2)	38 (40.4)
Class Ⅴ < 660	53 (56.4)	48 (51.1)

PCI: Per Capita Income (after adjusting for Consumer Price Index of 2011).

Analysis was done using SPSS version 20[10]. Socio-demographic details and frequency of food intake were expressed in proportions. Univariate analysis for categorical variables (diet, smoking, alcohol, physical activity, BMI, history of diabetes, family history) were done using χ^2 test. Seven frequencies of food item intake were categorized into tertile. Tertile1 corresponds to lowest frequency of intake and tertile 3 corresponds to highest frequency of intake. OR was calculated for highest tertile of intake relative to lowest tertile by logistic regression. Factors having *P* value < 0.05 in univariate analysis were included as parameter for multivariate analysis using logistic regression. Results of multivariate analysis were given as OR with 95%CI. All *P* values were two tailed and significant when values were less than 0.05.

RESULTS

A total of 94 cases and controls were included in the study. The mean age group of cases and controls were 54.1 ± 11.5 years and 55 ± 11.8 years respectively. Age distribution of cases and controls were in the range of 17-78 years. There was almost equal distribution of males and females 48.9% and 51.1% respectively among the study subjects (Table 1). Around 39.4% cases and 35.1% of controls never attended school. In both cases and controls more than 50% of them belonged to class Ⅴ socio economic status.

The distribution of subjects as per recommended cut off for consumption of food item was much skewed

Table 2 Frequency of food intake among cases and controls, *n* (%)

Food item		Never or hardly ever	Once a month	2-3 times/mo	Once a week	2-3 times/wk	4-6 times/wk	Once a day	Total
Egg	Case	7 (7.4)	17 (18.1)	6 (6.4)	28 (29.8)	21 (22.3)	7 (7.4)	8 (8.5)	94
	Control	8 (8.5)	36 (38.3)	10 (10.6)	27 (28.7)	8 (8.5)	-	5 (5.3)	94
Chicken	Case	13 (13.8)	31 (33)	9 (9.6)	36 (38.3)	5 (5.3)	-	-	94
	Control	12 (12.8)	45 (47.9)	14 (14.9)	19 (20.2)	4 (4.3)	-	-	94
Mutton	Case	23 (24.5)	40 (42.6)	4 (4.3)	25 (26.6)	1 (1.1)	1 (1.1)	-	94
	Control	44 (46.8)	42 (44.7)	3 (3.2)	4 (4.3)	1 (1.1)	-	-	94
Fish	Case	26 (27.7)	49 (52.1)	2 (2.1)	6 (6.4)	10 (10.6)	1 (1.1)	-	94
	Control	27 (28.7)	61 (64.9)	1 (1.1)	2 (2.1)	1 (1.1)	2 (2.1)	-	94
Beef	Case	68 (72.3)	1 (1.1)	1 (1.1)	18 (19.1)	6 (6.4)	-	-	94
	Control	81 (86.2)	6 (6.4)	-	7 (7.4)	-	-	-	94
Pork	Case	81 (86.2)	9 (9.6)	1 (1.1)	2 (2.1)	1 (1.1)	-	-	94
	Control	87 (92.6)	5 (5.3)	-	2 (2.1)	-	-	-	94
Fried foods	Case	3 (3.2)	32 (34.0)	6 (6.4)	35 (37.2)	18 (19.1)	-	-	94
	Control	5 (5.3)	45 (47.9)	14 (14.9)	28 (29.8)	2 (2.1)	-	-	94
Fruits	Case	32 (34.0)	37 (39.4)	7 (7.4)	6 (6.4)	5 (5.3)	3 (3.2)	4 (4.3)	94
	Control	36 (38.3)	23 (24.5)	14 (14.9)	13 (13.8)	3 (3.2)	-	5 (5.3)	94
Vegetables	Case	-	-	-	-	13 (13.8)	7 (7.4)	74 (78.7)	94
	Control	-	-	-	-	2 (2.1)	8 (8.5)	84 (89.4)	94
Coffee	Case	81 (86.2)		-	-	-	-	13 (13.8)	94
	Control	87 (92.6)	-	-	-	-	-	7 (7.4)	94
Tea	Case	20 (21.2)		-	-	-	-	74 (78.7)	94
	Control	11 (11.7)	-	-	-	-	-	83 (88.3)	94

Table 3 Colorectal carcinoma risk associated with individual dietary item

Food item			Adjusted OR (CI)	*P* value
Egg	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	1.6 (0.85-3.33)	0.133
	Tertile 3	> 2-3 times a week	5.1 (2.26-11.36)	0.001
Chicken	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	0.6 (0.25-1.51)	0.297
	Tertile 3	Once a week	1.6 (0.64-4.19)	0.297
Mutton	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	1.8 (0.93-3.45)	0.070
	Tertile 3	More than 2-3 times a month	7.4 (2.93-3.45)	0.001
Fish	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	0.8 (0.44-1.60)	0.588
	Tertile 3	More than 2-3 times a month	3.2 (1.13-9.53)	0.028
Beef	Tertile 1	-	-	-
	Tertile 2	Never or hardly ever	1	
	Tertile 3	More than once a month	2.3 (0.13-4.99)	0.237
Fruits	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	1.8 (0.89-3.64)	0.099
	Tertile 3	More than 2-3 times a month	0.8 (0.39-1.68)	0.540
Vegetables	Tertile 1	2-3 times a week	1	
	Tertile 2	Once a day	0.4 (0.19-1.00)	0.050
	Tertile 3	-	-	-
Fried foods	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	0.61 (0.21-1.74)	0.350
	Tertile 3	2-3 times a month	2.52 (1.35-4.70)	0.004

as shown in (Table 2). Among cases 22.3% consumed egg 2-3 times a week compared to only 8.5% among the controls. In cases about one-fourth 24.5% never or hardly ever consumed mutton compared to 46.8% in controls. Beef consumption was reported to be low among both cases and controls, 72.3% of cases and 86.2% of controls never or hardly ever consumed beef. Similarly more than 80% of cases and controls never or hardly ever consumed pork. Majority of cases 78.7% and controls 89.4% consumed vegetables once a day.

As distribution of subjects as per recommended cut off for consumption of food item was much skewed, intake of food items was categorized into tertile. The frequency cut-off into tertile is not same for all the food items. For certain food items (beef, pork, vegetables, tea, coffee) ranking into tertile was not possible due to its skewed distribution. Univariate logistic regression analysis was done considering these tertile groups as shown in (Table 3). It was observed that consumption of egg for more than 2-3 times a week increases the risk of getting CRC by five times [OR = 5.1 (2.26-11.36)] compared to those who never or hardly consume egg. Mutton consumption of more than 2-3 times a month increases the risk of CRC by 7 times [OR = 7.4 (2.935-18.732)] compared to those never or hardly consumes mutton. Consuming fish and fried foods more than 2-3 times a month increases the risk for CRC. Coffee consumption was not significantly associated with CRC [OR = 1.95 (0.76-5.43)]. Similarly Tea consumption also did not show any significant association with CRC in the present study [OR = 0.49 (0.22-1.70)].

Compared to never smokers, subjects who smoked < 10 pack years, 10-20 pack years and > 20 pack years were not at increased risk for CRC. Alcohol consumption of < 39.9 g/d, 40-59.9 g/d and > 60 g/d was not associated with increased risk for CRC compared to non-users. High (3000 METs/wk) and moderate (600-3000 METs/wk) level of physical activity was not protective for CRC. BMI greater than 25 is not associated with CRC risk. History of diabetes was not significantly

Table 4 Association of variables with colorectal carcinoma, *n* (%)

Variable	Cases	Controls	OR (CI)
Type of oil			
Refined	29 (30.9)	22 (23.4)	1
Groundnut	15 (16)	42 (44.7)	0.271 (0.12-0.61)
Palm	50 (53.2)	30 (31.9)	1.264 (0.62-2.59)
Type of food			
Moderate spicy	73 (77.7)	82 (87.2)	1
Very spicy	21 (22.3)	12 (12.8)	1.97 (0.91-4.28)
Smoking status			
Non-smoker	74 (78.7)	75 (79.7)	1
< 10 pack years	3 (3.19)	5 (5.31)	0.60 (0.14-2.63)
10-20 pack years	5 (5.31)	10 (10.6)	0.50 (0.16-1.55)
> 20 pack years	12 (12.8)	4 (4.3)	3.04 (0.93-9.85)
Alcohol use			
Non users	68 (72.3)	74 (78.7)	1
Grade Ⅰ (< 39.9 g/d)	19 (20.2)	10 (10.6)	2.06 (0.89- 4.75)
Grade Ⅱ (40-59.9 g/d)	4 (4.2)	6 (6.3)	0.72 (0.19-2.68)
Grade Ⅲ (> 60 g/d)	3 (3.2)	4 (4.2)	0.81 (0.17-3.78)
Physical activity (METs/wk)			
Low (< 600)	18 (19.1)	24 (25.5)	1
Moderate (600-3000)	51 (54.3)	44 (46.8)	1.54 (0.74-3.21)
High (> 3000)	25 (26.6)	26 (27.7)	1.28 (0.56-2.91)
BMI (kg/m^2)			
< 18.5 (underweight)	19 (20.2)	10 (10.6)	1
18.5-22.99 (normal)	47 (50)	57 (60.6)	0.43 (0.18-1.02)
23-24.99 (over weight)	14 (14.9)	17 (18.1)	0.43 (0.15-1.22)
⩾ 25 (obese)	14 (14.9)	10 (10.6)	0.73 (0.24-2.24)
Diabetes mellitus			
No	73 (53.3)	67 (46.7)	1
yes	21 (41.2)	30 (58.8)	1.62 (0.85-3.12)

METs/wk: Metabolic equivalents minutes per week; BMI: Body mass index.

Table 5 Factors independently associated with colorectal carcinoma

Food item			Adjusted OR (CI)	*P* value
Mutton	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	2.62 (0.08-6.33)	0.137
	Tertile 3	> 2-3 times a month	5.41 (1.55-19.05)	0.008
Egg	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	1.54 (0.63-3.70)	0.340
	Tertile 3	> 2-3 times a week	3.67 (1.23-9.35)	0.013
Fried foods	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	0.76 (0.22-2.54)	0.655
	Tertile 3	> 2-3 times a month	2.03 (0.95-4.43)	0.060
Fish	Tertile 1	Never or hardly ever	1	
	Tertile 2	Once a month	0.02 (0.08-0.58)	0.195
	Tertile 3	> 2-3 times a month	0.39 (0.09-1.62)	0.237
Type of oil	Refined	NA	1	
	Ground nut	NA	0.4 (0.15-1.00)	0.068
	Palm	NA	1.6 (0.75-4.04)	0.240

associated with CRC risk (Table 4). Multivariate logistic regression results (Table 5) for those factors found to be statistically significant in univariate analysis (mutton, egg, fish, fried foods and type of oil) showed egg and mutton as independent risk factor.

DISCUSSION

Though population based cancer registries showed a statistically significant increase in the incidence of CRC in India from 1982-2006, very few studies have been done in India to document the association of modifiable risk factors with CRC. The present study attempted to identify the modifiable risk factors so that appropriate preventive measures can be planned. Red meat consumption more than 2-3 times a month found to be an independent risk factor in multivariate regression analysis and increased the odds of developing CRC by 5.41 (1.55-19.05) times compared to those never or hardly consume. This was similar to study by Nayak *et al*[7] which reported beef consumption more than once a week has increased risk compared to those who do not consume beef [OR = 4.25 (2.02-8.94)]. A study from Uruguay[9] reported a positive association between CRC and high intake of red meat with OR = 3.38 (2.37-6.20). Similarly Singh *et al*[11] reported red meat intake more than once a week increased the risk compared to non-consumers [RR = 1.90 (1.16-3.11)].

In the western studies red meat consumption included beef, pork and mutton. However, in present study population due to cultural practices and beliefs beef and pork consumption were minimal. Subjects who consumed egg more than 2-3 times a week had 3.6 (1.23-9.35) times higher risk compared to those who never or hardly ever consume egg. This was similar to the study[12] which reported consumption of egg more than 2-3 times/wk is associated with increased risk of CRC compared to those who never or hardly consume egg [OR = 2.95 (1.75-5)]. In the present study fish consumption more than 2-3 times a month is associated with increased risk for CRC in univariate analysis. In contrast Nayak *et al*[7] from Kerala showed 20% decreased risk of CRC with consumption of fish with every meal [OR = 0.32 (0.13-0.98)]. European study reported fish consumption more than 80 g/d was inversely associated with CRC compared to those consuming < 10 g/d [OR = 0.69 (0.54-0.88)][13]. Discrepancy between present study finding and other studies could be due to difference in type of fish consumed, amount of fish consumed, method of cooking and method of preservation.

Fruits and vegetable consumption was not found to be protective for CRC, similar to the findings reported in studies from Western countries[14-16]. Frequent intake of fried food a proxy variable for high fat intake was associated with CRC [OR = 2.52 (1.35-4.70)] in univariate analysis but it was not an independent risk factor. In contrast studies reported consuming deep fried foods more than once a month was not associated with increased risk[17,18]. Coffee consumption was not significantly associated with CRC [OR = 1.95 (0.76-5.43)]. A meta-analysis by Je *et al*[19] in 2008 showed no significant association between coffee consumption and colorectal cancer [RR = 0.91 (0.81-1.02)], nevertheless; studies have also shown protective effect of coffee in the development of CRC. Kato *et al*[20] in Japan found daily coffee consumption

had protective effect on both colon and rectal carcinoma compared with the non drinkers with RR = 0.43 (0.25-0.73) and RR = 0.53 (0.27-1.03), respectively. Reasons for varying results across studies are due to difference in type of coffee, serving size, brewing method and also cutoffs for high and low exposure categories varies between studies. Tea consumption did not show any significant association with CRC in the present study [OR = 0.49 (0.22-1.70)]. Similar findings were found by Nayak *et al*[7] where highest quartile of tea consumption has not shown any risk difference compared to lowest quartile with OR = 1.03 (0.62-1.71). In 2005, Michels *et al*[14] from United States reported that tea consumption of more than 5 cups per day was not significantly associated with CRC [HR = 1.01 (0.83-1.22)].

Smoking and alcohol use was not associated with CRC in contrast to increased risk reported in few studies[21-25]. As smoking and alcohol were considered as undesirable behavior in community people tend to under report the use due to social desirability bias[26]. This could be the reason for no association in the present study. High level of physical activity was not associated with decreased risk for CRC compared to low level of physical activity as reported in other studies[27,28]. BMI was not significantly associated with CRC; in contrast studies reported high BMI increased risk for CRC[29,30]. This could be due to underlying limitation of hospital based case-control study, where cases are ill and admitted to the hospital in late stage of disease. By the time patients seek medical attention they would have lost considerable amount of weight. The weight recorded at the time of admission may not find the true association.

Selection of appropriate controls is crucial to establish the true association between exposure (diet, smoking, alcohol, physical activity) and outcome (CRC). Selection of controls remains a major concern when designing a case-control study due to the issues involved in the internal validity and cost. Scientifically there is scope for introducing bias (selection bias and information bias) while selecting hospital based controls[31]. However there is several advantage of selecting hospital controls such as feasibility, cost, travel time and better recall among hospital controls. Validation studies conducted by Li *et al*[32], González *et al*[33], Inoue *et al*[34] showed that hospital based controls elicit similar information to community controls in assessment of dietary risk factors. Hospital controls are preferred in a hospital based case-control study in view of the issues of practicability. It also reduces the cost involved in the travel and decreases the time taken for face-to-face interviews at field. It has also been demonstrated that the capacity to recall and report the exposures are better in those who are actively seeking health care advise than the members randomly selected from the population[35].

Since it measures long term, average and habitual dietary intake; FFQ as a mean of dietary assessment have been found appropriate in many nutritional and epidemiological studies[36]. FFQ captures pattern of food consumption over a period of time ranging from months to years. Pandey *et al*[37] from India reported FFQ had good correlation (0.8) with 5 d diet record and was reproducible. The quantity of food consumed is considered an important factor in estimating the dietary intake of an individual; however, the frequency rather than the serving size has been found to be a better contributor to the variance in the intake of most foods.

Primary limitation of the study was dietary items were not quantified. Though efforts were taken to minimize the recall bias, change in dietary pattern of cases after development of symptoms might have led to biased reporting of their diet.

In conclusion, this hospital based case control study showed egg consumption of 2-3 times a week and mutton consumption 2-3 times a month as independent risk factor. On other hand smoking, alcohol, physical activity, history of diabetes and family history were not associated with increased risk for CRC and no conclusive evidence to suggest fruits and vegetable consumption as protective factor. Cohort study is required to assess the risk associated with commonly consumed dietary items in a given population.

As it was found that persons consuming red meat (mutton) had an increased risk of developing CRC (OR = 5.4), the community needs to be educated to reduce the consumption of red meat such as mutton, so that they can minimize their risk for developing CRC. Similarly, egg consumption was found to increase the odds of developing CRC (OR = 3.6), people especially adults need to be advised to reduce the egg consumption.

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COMMENTS

Background

Epidemiological studies have shown that significant proportion of colorectal cancer (CRC) incidence could be ascribed to dietary, environmental and lifestyle factors; suggesting majority of the risk factors are modifiable. Regional variation in the dietary and social habit could play a vital role in the causation of CRC and may be responsible for the geographical variations in the occurrence of CRC.

Research frontiers

For a long time, it was believed that low meat intake and high fiber vegetarian diet by Indian population is the reason for the low incidence of CRC in India. Only two studies have been reported in literature from India regarding factors associated with CRC and more research studies are require to evaluate or to confirm the risk factors. Identifying the factors associated with decreased CRC incidence among Indian population may help in the primary prevention of CRC across the globe.

Innovations and breakthrough

This study found that red meat consumption of more than 2-3 times a month egg consumption of more than 2-3 times a week are independent risk factors for the development of CRC. Contrary to common belief the study showed no association between CRC and smoking, alcohol consumption, physical activity,

body mass index or diabetes. Consumption of coffee or tea were also not associated with CRC.

Applications

As it was found that persons consuming red meat (mutton) had an increased risk of developing CRC (OR = 5.4), the community needs to be educated to reduce the consumption of red meat such as mutton, so that they can minimize their risk for developing CRC. Similarly, egg consumption was found to increase the odds of developing CRC (OR = 3.6), people especially adults need to be advised to reduce the egg consumption.

Peer-review

This is a good case control study taken a very relevant and significant problem to be studied. The article evaluated important life style and dietary factors for the possible relationship with colorectal cancer in South Indian population. The study showed significant association between red meat and egg consumption and certainly gives better insight and understandings about the other risk factors including smoking, alcohol consumption, body mass index, diabetes and physical activity, *etc.*

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ORIGINAL ARTICLE

Retrospective Cohort Study

Correlation of *Helicobacter pylori* and interleukin-8 mRNA expression in high risk gastric cancer population prediction

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Abstract

AIM: To evaluate (1) the association of the *Helicobacter pylori* (*H. pylori*) test and interleukin-8 (*IL-8*) mRNA expression alone and the severity of gastric cancer (GC); (2) the association of both tests were added to patients' characteristics to identifli Thai suspected patients of gastric cancer who would receive the most benefit; and (3) diagnostic value of levels of *IL-8* mRNA expression for gastric cancer.

METHODS: A cross-sectional analytical study was completed with 220 patients with 86 GC patients who underwent endoscopy with gastric surgery divided into non-metastasis and metastasis groups, and 134 patients with benign lesions who underwent endoscopic examination, at the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital between 2006 and 2010. Of 220 patients, 86 cases of diagnosed gastric adenocarcinoma were in an advanced stage and 134 cases were non-cancer patients.

RESULTS: The *IL-8* mRNA expression showed predominant association with advanced GC when compared to *H. pylori* infection alone [OR (95%CI); 0.86 (0.49-1.53) *vs* 5.44 (3.08-9.62)] when including the patients' characteristics the highest of the area under the receiver operating characteristic curves (AuROC) of the model were males older than 40 years of age [AuROC (95%CI); 0.81 (0.75-0.86)]. However, preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression to predict the severity of GC cases found an increasing suboptimal trend from the likelihood ratio of positive to differentiate the severity in the GC group. The *IL-8* mRNA expression showed a predominant association with GC when compared to *H. pylori* infection, especially in males older than 40 years of age who may benefit most from this test.

CONCLUSION: The future research of *IL-8* mRNA expression to predict severity in the gastric cancer group should be warranted.

Key words: *Helicobacter pylori*; Interleukin-8 mRNA

expression; Gastric cancer

Core tip: The author reviewed updated basic research studies regarding linkages of inflammatory cytokine genetic expression level and gastric cancer (GC) risk prediction in the Thai population. The review focused on *interleukin-8* (*IL-8*) mRNA expression and *Helicobacter pylori* (*H. pylori*) infection in which are found an increasing risk for GC and aggressive histologic types. We performed the epidemiologic data in-depth analysis, and make the various cut off points to discern which level of *IL-8* mRNA expression has remarkable predictive risk value in comparison with *H. pylori* infection to predict GC occurrence.

Chongruksut W, Limpakan (Yamada) S, Chakrabandhu B, Ruengorn C, Nanta S. Correlation of *Helicobacter pylori* and interleukin-8 mRNA expression in high risk gastric cancer population prediction. *World J Gastrointest Oncol* 2016; 8(2): 215-221 Available from: URL: http://www.wjgnet.com/1948-5204/full/v8/i2/215.htm DOI: http://dx.doi.org/10.4251/wjgo.v8.i2.215

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers and continues to remain a major public health problem in the world, with a varying prevalence from 11% to 56% in different areas. The prevalence of GC in the United States was an estimated 74035 people[1] and estimated 934000 cases, 56% of new cases from Eastern Asia, 41% from China, and 11% from Japan[2]. In Thailand, the incidence rate of GC was 4.1:100000 for males and 2:100000 for females, especially in the northern part of Thailand which has a higher GC incidence rate with 6.6:100000 in males, and 4.5:100000 in females[3].

GC is one of the most common causes of death from cancer worldwide, and most of the cases occur in developing countries[4]. A gender difference of mortality of GC was reported; 14.3 per 100000 in men and 6.9 per 100000 in women worldwide[5], as well as geographic countries; 20% mortality rate in Western countries *vs* up to 60% in Asia[6].

Early diagnosis is crucial because of the possibility of early metastasis to other organs such as, liver, pancreas, omentum, esophagus, bile ducts, and lymph nodes[7]. If GC was detected at an early stage, the five year survival was approximately 90%[8]. Thus, in developing countries, early detection is most needed. The standard method or diagnosing GC is through the upper digestive endoscopy combined with biopsy and histopathological evaluation of the biopsy samples[4]. This method has a high diagnostic accuracy of 95% to 99%[9].

Helicobacter pylori (H. pylori) infection is widely regarded as the most important risk factor in the development of GC[6] with from 0.5% to 2.0% developing gastric adenocarcinoma[10]. A meta-analysis of 34 cohort and case-control studied patients found that *H. pylori* carried a relative risk of GC of 3.02 (95%CI: 1.92-4.74) in high risk settings (China, Japan and Korea) and 2.56 (95%CI: 1.99-3.29) in low risk settings (Western Europe, Australia, and the United States)[11]. Epidemiologic data indicates that GC occurs more frequently in populations with higher rates of *H. pylori* infection, and the World Health Organization has classified this bacterium as a Class 1 carcinogen for GC[6]. *H. pylori* infection was important in the process of tissue remodeling, angiogenesis, tumor invasion and metastasis[12] and induces a number of genes in host cells that are potential determinants of inflammation, angiogenesis, and metastasis including *interleukin-8 (IL-8)* gene expression[13]. However, it remains unclear how *H. pylori* infection activates specific transcription factors and induces gene expression. Yamada *et al*[14] indicated that the *H. pylori* infection in Thai GC patients was reported by combined histopathology and *H. pylori* IgG antibody test with 77.1% and 97.4% of sensitivity and specificity, respectively.

Moreover, *IL-8* mRNA expression is one of the factors that were possible influences which affect GC[15], Yamada *et al*[14,16] reported that GCs were detected in more than 80% of Thai patients with high levels of *IL-8* mRNA expression, while *H. pylori* infection and *IL-8* mRNA expression were relative risks for Thai GC, therefore *IL-8* mRNA expression may be a useful diagnostic and prognostic risk marker for GC. Similarly, Macrì *et al*[17] indicated that the level of serum *IL-8* mRNA expression may act as marker of GC. The high expression of *IL-8* mRNA expression was directly demonstrated with a poor prognostic histologic type in GC[14,16].

Although, the association of *H. pylori* infection and *IL-8* mRNA expression and GC were demonstrated from several studies, most studies did not evaluate the association of these biomarkers and the GC severity, *i.e.*, no cancer, non-metastasis, and metastasis stage. There might be an increasing possibility of GC by gradient of *IL-8* mRNA expression. Moreover, some studies showed an association with independent factors such as advanced age, sex, and alcohol drinking. Therefore, this present study aimed to evaluate (1) the association of *H. pylori* test and *IL-8* mRNA expression alone and the severity of GC; (2) the association of both tests added to patients' characteristics to identify Thai suspected patient risk of GC who will receive the greatest benefit for follow up endoscopy; and (3) diagnostic value of four different levels of *IL-8* mRNA expression for GC cases.

MATERIALS AND METHODS

A cross-sectional analytical study was conducted in patients over 18 years of age. Eighty-six patients who underwent endoscopy were diagnosed with GC, and 134 patients who underwent endoscopic examination were diagnosed as non-GC, at the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital between 2006 and 2010. All patients were comprehensively examined

by a gastrointestinal pathologist for *H. pylori* infection and combined histopathological diagnostic results. The outcomes of the study were divided into non-GC, and GC. In GC patients, those who were categorized in cancer Stages Ⅰ, Ⅱ, Ⅲ and Ⅳ were in the GC group.

Tissue samples were taken by endoscopy with tissue *IL-8* mRNA expression conducted by real time relative quantitation polymerase chain reaction. Additionally, baseline characteristics; gender, age, alcohol drinking, smoking, stages of cancer, histological pathology were obtained by a physician and nurse, using a case record form. All enrolled patients were examined by endoscopy with a pathology result for *H. pylori* infection and received biopsy of tissues with *IL-8* mRNA expression. This study excluded all patients without results of pathology or tissue *IL-8* mRNA expression. The present study was approved by the Institutional Review Boards of the Faculty of Medicine, Chiang Mai University.

Statistical analysis

The demographic data were analyzed using χ^2 to test between groups, and the test for trend was used to test for proportion. An ordinal logistic regression, both univariable and multi-variable models, were performed to determine association of *H. pylori* and *IL-8* mRNA expression and severity of GC with or without patients' characteristics presented with crude and adjusted odds ratio with 95%CI. The area under the receiver operating characteristic curves (AuROC) was calculated and compared using a standard method. *IL-8* mRNA expression level was divided into four different cut-off points, and AuROC was compared to select the best cut-off point. Performance of each *IL-8* mRNA expression cut-off point was then evaluated for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LHR+), and negative likelihood ratio (LHR-) in only GC cases. A statistical significance level or alpha of 0.05 was selected for Type Ⅰ error.

RESULTS

Of the 220 patients enrolled in this study, 86 cases were diagnosed with GC and underwent endoscopy with gastric surgery, and 134 non-cancer patients underwent endoscopic examination. Among those diagnosed with a non-GC, 45 cases were normal, 46 had benign lesions (polyps, erosion, mild superficial gastritis), and 42 cases had chronic active gastritis. When categorized by staging, 41 cases (47.67%), and 34 cases (39.53%) were in Stage ⅢB, and Stage Ⅳ, respectively.

Two groups of patients were therefore assigned by order of severity to non-cancer, and advanced GC. Patients' characteristics found statistically significant when testing for trend were sex, age, and smoking status. The majority of GC patients were male, aged ⩾ 40 years, and had a history of smoking (Table 1).

According to *H. pylori* active infection status and pathology, no statistical differences were found among the groups. While, *IL-8* mRNA expression had the highest level in the metastatic GC group (median 325) and non-cancer group (median 19.72), respectively. An *IL-8* mRNA expression was transformed to $\log_{10}$ and divided into five cut-off points. The higher the cut-off point, the higher proportion of the severity of GC was demonstrated (Table 2).

The value of *H. pylori*, and *IL-8* mRNA expression as biomarkers, alone or combined with patients' characteristics, were determined. The results showed the predominant performance of *IL-8* mRNA expression over *H. pylori* pathology and serum IgG results (Model 1 *vs* Model 2). *H. pylori* pathology results in accordance with significant demographic characteristics, *i.e.,* sex and age showed lower performance compared to *IL-8* mRNA expression alone (Model 3 *vs* Model 2). Adding *IL-8* mRNA expression in a model with sex and age, the AuROC of probability to predict severity occurrence of GC was increased (Model 4 *vs* Model 2). However, adding *H. pylori* pathology in the last model did not enhance a predictability of GC severity in the last model (Model 5 *vs* Model 4). Therefore, *IL-8* mRNA expression is useful to differentiate severity of GC especially when combined with sex and age (Table 3). The *IL-8* mRNA expression used was the best cut-off point of two to predict the severity of GC; AuROC of cut-off-point one through four was 0.64, 0.71, 0.60, and 0.53, respectively (data not shown).

We further analyzed the prediction ability of the model containing *IL-8* mRNA expression across age group and sex. The likelihood positive ratio of all models

Table 1 Characteristics of gastric cancer and non-gastric cancer patients, *n* (%)

Characteristics	Gastric cancer *n* = 86	Non-gastric cancer *n* = 134	*P* value
Sex			< 0.001
Male	52 (60.47)	41 (30.60)	
Female	34 (39.53)	93 (69.40)	
Age			0.003
⩾ 40	5 (5.81)	28 (20.90)	
< 40	81 (94.19)	106 (79.10)	
Mean ± SD	56 ± 11.29	48.5 ± 11.21	
Alcohol drinking,	36 (41.87)	51 (38.06)	0.679
Smoking	24 (27.91)	12 (8.96)	< 0.001
Diseases			
Normal	0	45 (33.83)	
Benign lesion	0	46 (34.59)	
Chronic active gastritis	0	42 (31.58)	
Gastric cancer	86 (100)	0	
Stage			
Ⅰa	-	-	
Ⅰb	-	-	
Ⅱa	1 (1.16)	-	
Ⅱb	2 (2.23)	-	
Ⅲa	8 (9.30)	-	
Ⅲb	41 (47.67)	-	
Ⅳ	34 (39.53)	-	
Histological grade			
Poorly differentiated	25 (29.76)	-	
Signet ring cell	36 (42.82)	-	
Moderate differentiated	16 (19.05)	-	
Well differentiated	7 (8.33)	-	

Table 2 Serum interleukin-8 mRNA expression and *Helicobacter pylori* infection status detection results in gastric cancer and non-gastric cancer patients, *n* (%)

Variables	Gastric cancer *n* = 86	Non-gastric cancer *n* = 134	*P* value
H. pylori pathology			0.267
Negative	32 (37.21)	60 (44.78)	
Positive	54 (62.79)	74 (55.22)	
H. pylori infection status			0.121
Negative	20 (24.4)	37 (36.0)	
Positive	62 (75.6)	96 (64.0)	
IL-8 raw RQ			< 0.001
< 100	33 (38.37)	105 (78.36)	
⩾ 100	53 (61.63)	29 (21.64)	
Median (IRQ)	325 (2326.37)	19.72 (105.74)	
IL-8 Log 10			< 0.001
Min-0.99	5 (6.49)	36 (34.95)	
1.00-1.99	16.36 (22.08)	38 (36.90)	
2.00-2.99	28 (36.36)	13 (12.62)	
3.00-3.99	20 (25.97)	12 (11.65)	
⩾ 4.00	7 (9.09)	4 (3.88)	

H. pylori: *Helicobacter pylori*.

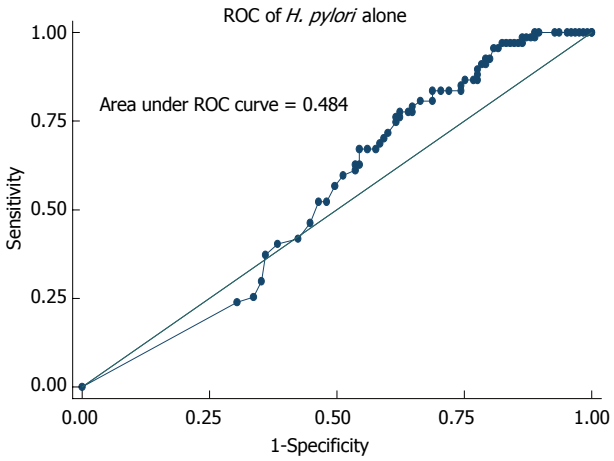

Figure 1 Area under the receiver operating characteristic curve of *Helicobacter pylori* in prediction of gastric cancer. *H. pylori*: *Helicobacter pylori*; ROC: Receiver operating characteristic curves.

was statistically significant. The largest yield of the LHR+ was the model with all variables. Apparently, *IL-8* mRNA express has the highest yield of the LHR+ in males who are older than 40 years old compared to the younger age or in the female group (LHR+ 14.54 *vs* 3.38) due to acceptable LHR+ (more than 5.0, theoretical suggested LHR+). Therefore, *IL-8* mRNA expression may be most useful in the Thai male with the age older than 40 years (Table 4).

Because the trend of prediction in the severity of GC of *IL-8* mRNA expression was observed, diagnostic indices were determined only in 86 GC patients who were categorized by metastatic status although it was still localized. Under four different cut-off points of *IL-8* mRNA expression, the sensitivity was highest in the *IL-8* mRNA expression Level one (96.8%) and continuously declined to 12.9% in the cut-off point of Level four. In the

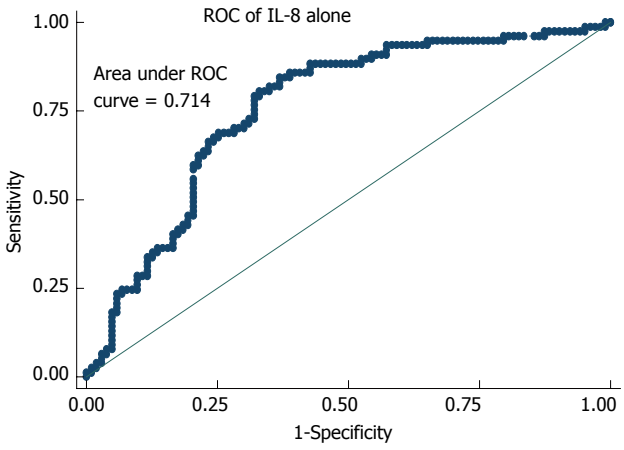

Figure 2 Area under the receiver operating characteristic curve of interleukin-8 mRNA Expression in prediction of gastric cancer. IL-8: Interleukin-8; ROC: Receiver operating characteristic curves.

opposite, specificity for GC metastasis increased from 8.7% in *IL-8* mRNA expression level one to 93.5% in Level four. The LHR+ increased from 1.06 to 1.98 of the Level one to Level four (Table 5). The AuROC of all of the cut-off points were not statistically significant (*P*-value of difference = 0.832) with less than a 60% range in all groups. There might have been a lack of ample sample size so the *IL-8* mRNA expression level could differentiate severity in the diagnosed GC group (Table 5).

The AuROC of *H. pylori* alone and AuROC of *IL-8* mRNA expression alone in prediction of GC occurrence is shown in Figures 1 and 2. Comparable AuROC of *IL-8* mRNA expression in an adjusted model by sex, age, and *H.pylori*, and additional AuROC of both *IL-8* mRNA expression and *H.pylori* infection in prediction of GC are shown in Figure 3.

DISCUSSION

In this study, performances of *H. pylori* infection and *IL-8* mRNA expression were determined as to whether there was an association with GC which was divided into two groups: Non-GC, and GC. The *IL-8* mRNA expression showed a predominant association with GC when compared to *H. pylori* infection, especially in males older than 40 years of age. In addition, there was a trend of the probability of GC with increasing levels of *IL-8* mRNA expression. Further, preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression to predict severity of GC cases was performed. However, we found an increasing suboptimal trend from the likelihood ratio of positive (less than five times) which may be due to the small sample size to differentiate severity in GC groups.

GC has a high incidence rate in the northern part of Thailand. Epidemiological studies have shown that *H. pylori* is associated closely with the development of GC and it is widely regarded as the most important modifiable risk factor for GC[18]. However, when categorizing GC

Table 3 Logistic models and the area of the receiver operating characteristic curves comparing *Helicobacter pylori* pathology results and interleukin-8 mRNA expression with/without demographic characteristics

Variable	Model 1 Crude OR (95%CI)	Model 2 Crude OR (95%CI)	Model 3 Adjusted OR (95%CI)	Model 4 Adjusted OR (95%CI)	Model 5 Adjusted OR (95%CI)
Sex	-	-	2.99 (1.72-5.20)	3.31 (1.84-5.94)	3.29 (1.83-5.93)
Age group	-	-	3.79 (1.38-10.41)	4.25 (1.47-12.34)	4.19 (1.44-12.20)
H. pylori	0.86 (0.49-1.53)	-	0.94 (0.52-1.71)	-	0.91 (0.48-1.70)
IL-8 mRNA expression ⩾ 2	-	5.44 (3.08-9.62)	-	6.05 (3.32-11.02)	6.07 (3.33-11.04)
AuROC	0.48 (0.42-0.54)[a]	0.71 (0.64-0.77)[a]	0.70 (0.63-0.76)	0.80 (0.75-0.86)[b]	0.81 (0.75-0.86)[b]

[a]*P* value difference of AuROC = 0.532; [b]*P* value difference of AuROC < 0.001; *H. pylori*: *Helicobacter pylori*; AuROC: Area under the receiver operating characteristic curves.

Table 4 Likelihood ratio of positive of models including interleukin-8 mRNA expression, sex, and age group

Variable	LHR+ (95%CI)	*P* value
Interleukin-8 mRNA expression alone	2.90 (2.02-4.16)	< 0.001
Interleukin-8 mRNA expression + age > 40 or male	3.38 (2.26-5.04)	< 0.001
Interleukin-8 mRNA expression + age > 40 + male	14.54 (4.56-46.36)	< 0.001

Table 5 Diagnostic values of each interleukin-8 mRNA expression cut-off point, 95%CI and gastric cancer diagnosis only gastric cancer cases (*n* = 86)

IL-8 level cut-off point	Sensitivity	Specificity	PPV	NPV	LHR+	LHR-	AuROC
1	96.8 (92.8-100.0)	8.7 (2.4-15.0)	41.7 (30.6-52.7)	80.0 (71.1-88.9)	1.06 (0.95-1.18)	0.37 (0.04-3.16)	0.53 (0.48-0.58)
2	64.7 (54.6-74.8)	38.4 (28.2-48.7)	40.7 (30.4-51.1)	62.5 (52.3-72.7)	1.05 (0.76-1.46)	1.09 (0.62-1.93)	0.58 (0.41-0.62)
3	41.9 (30.9-53.0)	69.6 (59.3-79.8)	48.2 (37.0-59.3)	64.0 (53.3-74.7)	1.38 (0.75-2.52)	1.20 (0.84-1.71)	0.56 (0.45-0.67)
4	12.9 (5.4-20.4)	93.5 (88.0-99.0)	57.1 (46.1-68.2)	61.4 (50.6-72.3)	1.98 (0.48-8.23)	1.07 (0.92-1.25)	0.53 (0.46-0.60)

P value of all AuROC = 0.832. PPV: Positive predictive value; NPV: Negative predictive value; LHR+: Positive likelihood ratio; LHR-: Negative likelihood ratio.

by severity, *H. pylori* lost its association in our findings. Unlike, *IL-8* mRNA expression, the results from our study found the superiority of prediction of GC severity over *H. pylori* infection. *IL-8* mRNA expression is one of factors that possibly affects GC[15]. Thai people in the advanced stage of GC showed that gastric mucosal tissue *IL-8* mRNA expression has a higher level value and percentage of poorer differentiated cell type more than in favorable histology or differentiated cell type[14]. This finding was consistent with the results of Yamada *et al*[14,16] which showed that a high level of *IL-8* mRNA expression was detected more than 80% in Thai advanced GC patients, of cases and they demonstrated that gastric mucosal *IL-8* mRNA expression was a relative risk for Thai GC. Thus *IL-8* mRNA expression may also be a useful diagnostic risk marker for GC. It is possible to use *IL-8* mRNA expression as a good indicator for advanced GC or aggressive types of cancer treatment selection especially in poor prognostic cell type.

Moreover, this study demonstrated the AuROC of *IL-8* mRNA expression when comparing gender with age found that males more than 40 years of age predicted the severity of GC with LHR+ 14.5 times. This may explain recent indications that men have a higher incidence rate and may have a poorer prognosis than women[19]. The increased incidence rate of males could be due to the difference in the lifestyles and habits from females; such as smoking and alcohol consumption[20]. A previous study on sex differences in GC incidence based on the study of etiological hypothesis indicated that the predominance of GC in men was a global phenomenon, and was related to a 10 to 15 year delay in the appearance and onset of GC of intestinal subtype in women compared with men[21,22]. Our data showed that *IL-8* mRNA expression may be a helpful tool to identify advanced risk of GC in Thai patients especially males with an age older than 40 years.

We further investigated diagnostic performances

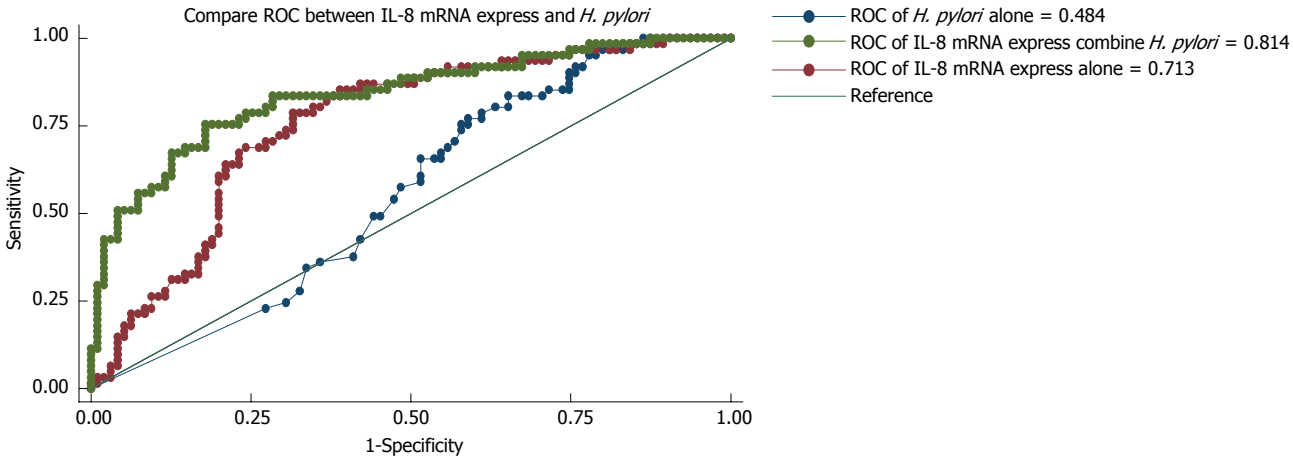

Figure 3 Comparable area under the receiver operating characteristic curve between interleukin-8 mRNA expression and *Helicobacter pylori* in prediction of gastric cancer. *H. pylori*: *Helicobacter pylori*; IL-8: Interleukin-8; ROC: Receiver operating characteristic curves.

of *IL-8* mRNA expression for advanced stages of GC. Although there was no statistical significance among the four cut-off points of *IL-8* mRNA expression of the AuROC, and the LHR+ values were lower than five times, there was a trend of increasing predictability of GC severity and prognosis. Future study is warranted to prove the predictive values of *IL-8* mRNA expression in GC patients with a larger clinical sample size. The limitation of this study was its retrospective nature and as a result some important available data could have been omitted due to a lack of medical records.

The *IL-8* mRNA expression showed predominant association with GC when compared to *H. pylori* infection, especially in males with age older than 40 years who may be benefit the most from this test. The preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression showed a suboptimal trend to differentiate severity in the GC group.

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COMMENTS

Background

The association of *Helicobacter pylori* (*H. pylori*) infection and interleukin-8 (*IL-8*) mRNA expression and gastric cancer (GC) were demonstrated from several studies. However, there was a lack of evidence of the association of these biomarkers and GC severity.

Research frontiers

The association of *H. pylori* test and *IL-8* mRNA expression alone and the severity of GC; (1) the association of both tests added to patients' characteristics to identify Thai suspected patient risk of GC who will receive the greatest benefit for follow-up endoscopy; and (2) diagnostic value of four different levels of *IL-8* mRNA expression for GC cases.

Innovations and breakthroughs

The *IL-8* mRNA expression showed predominant association with GC when compared to *H. pylori* infection, especially in males with age older than 40 years who may be benefit the most from this test. The preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression showed a suboptimal trend to differentiate severity in the GC group.

Peer-review

It might be interesting to analysis *IL-8* expression level in different stage and different histological grade, in order to demonstrate whether *IL-8* is more sensitive than *H. pylori* in different stage and grade GC.

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Evaluation of antibody-dependent cell-mediated cytotoxicity activity and cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients

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Author contributions: Lo Nigro C, Ricci V and Merlano MC designed the study; Ricci V, Miraglio E and Granetto C enrolled the patients and collected clinical data; Vivenza D, Monteverde M and Tonissi F performed ADCC assays and genotyping analyses; Strola G performed all the cytofluorimetric studies; Fortunato M selected patient's tumoral samples; Vivenza D and Lucio F performed the statistical analysis; Lo Nigro C, Ricci V and Merlano MC critically visioned the results and were equally involved in the writing of the paper with Vivenza D.

Conflict-of-interest statement: The authors declare no conflicts of interest regarding this manuscript.

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Abstract

AIM: To investigate the prognostic role of invariant natural killer T (iNKT) cells and antibody-dependent cell-mediated cytotoxicity (ADCC) in wild type *KRAS* metastatic colorectal cancer (mCRC) patients treated with cetuximab.

METHODS: Forty-one *KRAS* wt mCRC patients, treated with cetuximab and irinotecan-based chemotherapy in Ⅱ and Ⅲ lines were analyzed. Genotyping of single nucleotide polymorphism (SNP)s in the *FCGR2A*, *FCGR3A* and in the 3' untranslated regions of *KRAS* and mutational analysis for *KRAS*, *BRAF* and *NRAS* genes was determined either by sequencing or allelic discrimination assays. Enriched NK cells were obtained from lymphoprep-peripheral blood mononuclear cell and iNKT cells were defined by co-expression of CD3, TCRVα24, TCRVβ11. ADCC was evaluated as *ex vivo* NK-dependent activity, measuring lactate dehydrogenase release.

RESULTS: At basal, mCRC patients performing ADCC activity above the median level (71%) showed an improved overall survival (OS) compared to patients with ADCC

below (median 16 *vs* 8 mo; $P = 0.026$). We did not find any significant correlation of iNKT cells with OS ($P = 0.19$), albeit we observed a trend to a longer survival after 10 mo in patients with iNKT above median basal level (0.382 cells/microliter). Correlation of OS and progression-free survival (PFS) with interesting SNPs involved in ADCC ability revealed not to be significant. Patients carrying alleles both with A in FCGR2A and TT in FCGR3A presented a trend of longer PFS (median 9 *vs* 5 mo; $P = 0.064$). Chemotherapy impacted both iNKT cells and ADCC activity. Their prognostic values get lost when we analysed them after 2 and 4 mo of treatment.

CONCLUSION: Our results suggest a link between iNKT cells, basal ADCC activity, genotypes in FCGR2A and FCGR3A, and efficacy of cetuximab in *KRAS* wt mCRC patients.

Key words: Metastatic colorectal cancer; Single nucleotide polymorphism in Fc-γ receptors; Cetuximab; RAS family; Antibody-dependent cell-mediated cytotoxicity; Invariant natural killer T cells

Core tip: A high number of invariant natural killer T (iNKT) cells and a high antibody-dependent cell-mediated cytotoxicity (ADCC) activity, evaluated before therapy, do correlate significantly with a longer overall survival in metastatic colorectal cancer patients treated with irinotecan-based chemotherapy and cetuximab in Ⅱ and Ⅲ lines. Chemotherapy impacted both iNKT cells and ADCC activity. The prognostic value of ADCC above the median basal level, get lost when we analysed those parameters after 2 and 4 mo of treatment. Correlation of overall survival and progression-free survival with interesting single nucleotide polymorphisms reported as involved in ADCC ability, either in the FCGR2A, FCGR3A or in the 3' untranslated regions of *KRAS* gene, revealed not to be significant.

Lo Nigro C, Ricci V, Vivenza D, Monteverde M, Strola G, Lucio F, Tonissi F, Miraglio E, Granetto C, Fortunato M, Merlano MC. Evaluation of antibody-dependent cell-mediated cytotoxicity activity and cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients. *World J Gastrointest Oncol* 2016; 8(2): 222-230 Available from: URL: http://www.wjgnet.com/1948-5204/full/v8/i2/222.htm DOI: http://dx.doi.org/10.4251/wjgo.v8.i2.222

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, accounting for 940000 million new cases annually and nearly 500000 deaths each year. Metastatic colorectal cancer (mCRC) previously untreated patients have demonstrated substantial improvements, with a median overall survival time now reaching more than 24 mo, by the development of systemic chemotherapy, including molecular-targeted therapy[1].

The epidermal growth factor receptor (EGFR) signalling pathway is involved in cell differentiation, proliferation, migration, angiogenesis and apoptosis, all processes dysregulated in cancer cells.

Cetuximab is a chimeric immunoglobulin G1 (IgG1) monoclonal antibody (mAb) which binds EGFR with high affinity and inhibits ligand binding[2].

KRAS activating mutations have been reported in 40% of mCRC showing a negative effect on response to anti-EGFR antibodies[3,4]. Mutations in other downstream effectors of the *EGFR* signalling pathway, such as *BRAF, NRAS* and *PI3*kinase, might also impact the efficacy of monoclonal therapy. Thus, the absence of mutations in *RAS* appears to be a reliable marker for predicting the efficacy of cetuximab which was been restricted to mCRC patients with wild-type *RAS*[5]. Several studies supported the biological activity of cetuximab in advanced CRC. Cetuximab enhances response rate and progression-free survival (PFS) in first-line therapy in combination with Folfiri and Folfox regimen of chemotherapy[6,7]. However, some clinical studies have failed to show a significant correlation between EGFR expression and the response to cetuximab[8]. The proposed working mechanism of cetuximab is thought to include antibody-dependent cell-mediated cytotoxicity (ADCC)[9].

ADCC utilizes the response of innate immune cells to provide antitumor cytotoxicity triggered by the interaction of the Fc portion of the antibody with the Fc receptor on the immune cell. Immunotherapeutics that target natural killer (NK) cells, γδ T cells, macrophages and dendritic cells can, by augmenting the function of the immune response, enhance the antitumor activity of the antibodies[10].

Invariant CD1d-rescricted natural killer T (NKT) cells are T lymphocytes characterized by an invariant T-cell antigen receptor-chain rearrangement that co-express NK cell markers[11].

Molling *et al*[12] in 2007 demonstrated that a severe circulating invariant NKT (iNKT) cell deficiency was related to poor clinical outcome in head and neck squamous cell carcinoma patients, suggesting their critical contribution to antitumor immune responses. Furthermore, screening for iNKT cell levels may be useful for determining which patients can benefit from immunotherapeutic adjuvant therapies aimed at reconstitution of the circulating iNKT cell pool.

Whether ADCC is associated with EGFR expression and/or the mutational status of *RAS* and *BRAF* in CRC remains unclear. Seo *et al*[13] demonstrated that the ADCC activities were significantly associated with the cell surface expression levels of EGFR but not with the mutational status of *KRAS* and *BRAF*.

In this study we aimed to evaluate the prognostic and predictive value of cetuximab-mediated ADCC and circulating iNKT cells levels in mCRC and to analyse their correlation with *EGFR* level, mutational status of

Table 1 Characteristics of 41 patients in II and III line and tumours

	Number of patients	Rates	Median age (range) yr
Gender			
Male (M)	23	56%	67.5 (51-84)
Female (F)	18	44%	64.6 (49-83)
Primary tumour			
Right colon	7	17%	
Left colon	21	51%	
Rectal	13	32%	
Grade			
G1/G2	27	65.8%	
G3	13	31.7%	
NA	1	2.5%	
Metastasis			
Liver only	12	29.3%	
Liver plus other sites	14	34.1%	
Extra-hepatic sites	15	36.6%	
Response			
Responders			
CR	4	9.8%	
PR	12	29.3%	
SD	8	19.5%	
Non-responders			
PD	17	41.4%	
Line of treatment			
II	33	8%	
III	8	2%	

NA: Not available; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

KRAS, *NRAS*, *BRAF,* PFS and overall survival (OS) in a prospective cohort of mCRC patients treated with cetuximab-based therapy.

MATERIALS AND METHODS

Patients and clinical samples

A total of 41 mCRC patients were enrolled in this study from March 2008 to September 2014. Characteristics of the 41 patients are described in Table 1. An informed consent for tissue collection and use for scientific purpose was obtained from each patient enrolled in this study, approved by the local Ethical Committee and carried out in the respect to Helsinki Declaration. Inclusion criteria for mCRC patients were: Suitability for combination therapy including cetuximab with irinotecan-based chemotherapy in second and third lines and *KRAS* wild type (wt) status. Patients were evaluated for PFS, OS and response at the end of treatment with CT scan according to RECIST criteria[2]. Median follow-up was 25 mo (range 10-70).

DNA extraction, genotyping and mutational analyses

Genotyping of rs1801274 (A > G) in the *FCGR2A*, rs 396991 (T > G) in *FCGR3A* and rs61764370 in the 3' untranslated regions (3' UTR) of *KRAS* gene was done on genomic DNA isolated from whole peripheral blood samples using the EZ1 DNA Blood 200 Kit (Qiagen, Germany) according to the manufacturer's instructions. Analyses were determined using the appropriate "allelic discrimination assay" from Life Technologies (Foster city, CA, United States): c_9077561_20 for rs1801274; c_25815666_10 for rs396991 and 1350086 for rs61764370 using the ABIPRISM 7000 Sequence Detection System (Applied Biosystems Foster City, CA, United States).

Mutational analyses for *KRAS* (codons 12-13-59-61-146), *BRAF* (codon 600) and *NRAS* (codons 12-13-59-61-117-146) genes were determined on patients' DNA extracted from Formalin Fixed Paraffin Embedded (FFPE) tumor tissues archived at diagnosis in the Pathology Department of our Institution, by a standard protocol that included proteinase K treatment (EuroClone, Pero, IT).

KRAS and *BRAF* gene analyses were performed by pyrosequencing using PyroMark ID System (Biotage, Uppsala, Sweden), while a Real-Time PCR (OncoSreen NRAS; Relab, Jesi, Italy) was employed for *NRAS* gene using the Rotor-Gene 6000 (Corbett Research, Pty Ltd; Sydney, Australia) according to the manufacturer's protocol.

Antibody-dependent cell-mediated cytotoxicity assay

Twelve milliliter peripheral blood samples were collected at start of therapy for all the 41 patients and ADCC and NK cells were evaluated at basal level. After 2 and 4 mo of treatment a second collection of blood was done in 30 and 23 patients respectively where ADCC and NK cells were longitudinally studied.

Enriched NK cells were obtained from lymphoprep-peripheral blood mononuclear cell pellets using the human NK Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany). NK cells were defined as $CD56^+/CD3^-$; T cells as $CD3^+/CD56^-$ and invariant NKT (iNKT) cells by co-expression of CD3, TCR Vα24, TCR Vβ11.

ADCC was evaluated as *ex vivo* NK-dependent activity with a standard lactate dehydrogenase (LDH) assay (Cytotox 96® non radioactive cytotoxicity assay, Promega, Madison, WI) as set up in our Laboratory[14].

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5 (San Diego, CA, United States) and SPSS version 13 (SPSS, Chicago, IL) programs. The association between ADCC median levels was analyzed using the Fisher's exact test or the Pearson's test when appropriate. OS analyses were based on the time from treatment start to death or last contact in which the survivors were censored. PFS analyses were based on the time from treatment start to first event; patients without an event were censored at their last follow-up. OS was calculated using the Kaplan-Meier method with log-rank test for statistical significance. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Clinical and molecular characteristics of patients

Clinical characteristics of the 41 mCRC patients are

Table 2 *FCGR2A* (rs1801274; A > G), FCGR3A (rs366991; T > G) and single nucleotide polymorphism rs61764370 of KRAS 3' untranslated region genotypes with the correspondent aminoacid change in the 41 metastatic colorectal cancer patients included in this study

Gene	SNP	Genotype	Aminoacid change	Number of subjects (%)
FCGR2A	rs1801274	A/A	H131H	14 (34.1%)
		A/G	H131R	20 (48.8%)
		G/G	R131R	7 (17.1%)
FCGR3A	rs396991	T/T	F158F	14 (34.1%)
		T/G	F158V	21 (51.2%)
		G/G	V158V	6 (14.7%)
KRAS 3' UTR	rs61764370	T/T	---	32 (78%)
		T/G	---	9 (22%)
		G/G	---	0 (0%)

SNP: Single nucleotide polymorphism; UTR: Untranslated region.

detailed in Table 1. Genotyping analyses for single nucleotide polymorphisms are reported in Table 2. All patients were wt for *KRAS* gene. Determination of *NRAS* and *BRAF* mutations failed in 1 out the 41 patients, due to poor quality of DNA obtained from tumoral tissue. In particular we identified 6 mutations in *NRAS* gene (1 mutation in G12x-G13x; 1 in G61R; 1 in Q61K, 1 in Q61L and 2 in A59x-Q61H codons) and 1 mutation in *BRAF* gene (V600E).

Survival analysis according to iNKT cells

iNKT cells evaluated before treatment were analysed to seek correlation with OS and PFS either as number of cells/microliter or as % of T cells, since a low level of circulating iNKT cells has been reported to predict poor clinical outcome in patients with head and neck squamous cell carcinoma[12]. iNKT cells median value at basal determination, before treatment, was 0.382 cells/microliter. We did not find any significant correlation of iNKT cells with OS (P = 0.19), albeit we observed a trend to a longer survival after 10 mo in the population of patients (n = 21) with iNKT above median level (Figure 1).

Survival analysis according to ADCC activity

Median ADCC activity before treatment for all the 41 mCRC patients was 71% (range 10%-99%). Comparison between patients with ADCC above and below median value is reported in Table 3. There were no differences in the clinical characteristics between the two groups, although *EGFR* over-expression was more common in patients with ADCC activity above the median level (P = 0.052; Fisher's exact test). Correlation with OS and PFS was evaluated. Median OS was 12 mo (range 3-37) and PFS was 6 mo (range 3-37). Patients performing ADCC activity above the median level showed an improved OS compared to patients with ADCC activity below this value (median 16 *vs* 8 mo; P = 0.026; Long-rank Mantel-Cox Test) (Figure 2). On the contrary, there was no difference in PFS between patients with ADCC below or above the median level (data not shown). When we

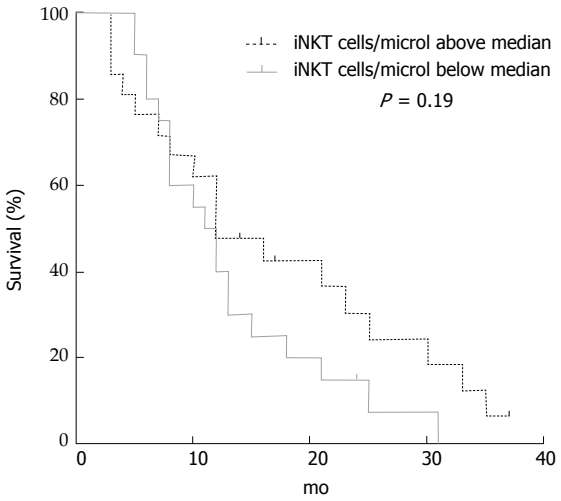

Figure 1 Overall survival in 41 metastatic colorectal cancer treated with cetuximab in II and III lines according to median basal level of invariant natural killer T cells (0.382 cells/microliter). iNKT: Invariant natural killer T.

stratified patients for both iNKT and ADCC activity at basal level, below and above the respective median level, we observed a better OS in patients having both values above the median level compared to all the other combinations (median 23 *vs* 10 mo; P = 0.0075; Long-rank Mantel-Cox Test) (Figure 3).

Survival analysis according to genotypes of FCGR2A, FCGR3A genes and in KRAS 3'UTR

Correlation in terms of OS and PFS with each genotype, either rs1801274 (A > G) in the *FCGR2A*, rs396991 (T > G) in *FCGR3A* or rs61764370 in the 3' UTR of *KRAS* gene reveal not to be significant (data not shown).

Patients carrying alleles both with A in *FCGR2A* (AA/AG genotypes) and TT in *FCGR3A* presented a longer PFS (median 9 *vs* 5 mo; P = 0.064; Long-rank Mantel-Cox Test) in comparison to all the other subgroups (Figure 4), although the difference was not significant.

Survival analysis according to mutational status in RAS family genes

Due to the limited number of patients we were not able to perform OS and PFS analyses according to all-*RAS* gene mutations. Nevertheless we observed that of ADCC activity, as median level, was not affected by the presence of a *NRAS* or a *BRAF* mutation (data not shown).

How the treatment influenced iNKT cells and ADCC activity

Both iNKT cells and ADCC activity were evaluated over time to seek for dynamic changes during treatment and to investigate the impact of therapy on patients' ability to perform ADCC and their clinical outcome. iNKT cells median number decreased from 0.382 cells/microliter at basal, before treatment, to 0.193 after 2 mo and to 0.165 after 4 mo of treatment. Likewise, ADCC activity was longitudinally evaluated up to 2 and 4 mo and its median level fell down from of 71% to 45% at two

Table 3 Comparison of characteristics of 41 patients on the basis of antibody-dependent cell-mediated cytotoxicity activity

	ADCC < 71%	*n* = 20	ADCC > 71%	*n* = 21	*P*
Gender					
Male (M)	11	55%	12	57%	0.9[1]
Female (F)	9	45%	9	43%	
Primary tumour					
Right colon	5	25%	2	10%	0.28[2]
Left colon	8	40%	13	62%	
Rectal	7	35%	6	29%	
Grade					
G1/G2	13	65%	14	67%	0.87[2]
G3	7	35%	6	29%	
NA	0	0%	1	5%	
Metastasis					
Liver only and liver plus other sites	12	60%	14	67%	0.65[1]
Extra-hepatic sites	8	40%	7	33%	
Response					
Responders					
CR	3	15%	1	5%	0.36[2]
PR	4	20%	8	38%	
SD	3	15%	5	24%	
Non-responders					
PD	10	50%	7	33%	
Line of treatment					
Ⅱ	17	85%	17	81%	1[2]
Ⅲ	3	15%	4	19%	
EGFR					
Neg; 1+; 2+	19	95%	14	67%	0.052[2]
3+	1	5%	5	24%	
NA	0	0%	2	10%	

[1]Pearson's Test; [2]Fisher's Exact Test; NA: Not available; Neg: Negative; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; ADCC: Antibody-dependent cell-mediated cytotoxicity; EGFR: Epidermal growth factor receptor.

withdrawal further analyses.

Survival analysis according to variation in ADCC activity during treatment

ADCC determination during treatment lost its prognostic value since there was no difference in OS between patients with ADCC activity above or below the median level after 2 and after 4 mo of treatment (data not shown). Variation of ADCC values was analyzed by stratifying patients on the basis of to the median values at basal level (71%, 41 patients), after 2 mo (45%, 30 patients) and after 4 mo (45%, 23 patients) of treatment. Combination of longitudinal values generated 4 groups of patients: the first included patients showing both ADCC activities above the median level [ADCCbas above/Ⅱ (or Ⅲ) above, where Ⅱ means on blood drawn after 2 mo and Ⅲ after 4 mo], the second group a decrease from a basal above median to a Ⅱ or Ⅲ determination below median [ADCCbas above/Ⅱ (or Ⅲ) below], the third group patients showing instead an increase from below at basal and above at Ⅱ or Ⅲ determination [ADCCbas below/Ⅱ (or Ⅲ) above] and the fourth group patients with both ADCC activities below the median level [ADCCbas below/Ⅱ (or Ⅲ) below].

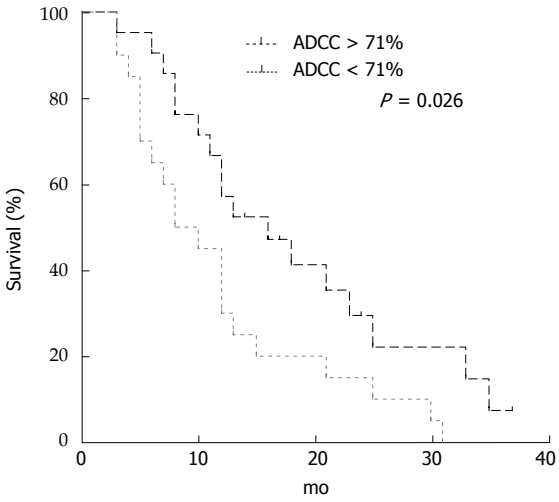

Figure 2 Overall survival in 41 metastatic colorectal cancer treated with cetuximab in II and III lines according to median basal level of antibody-dependent cell-mediated cytotoxicity activity (71%). ADCC: Antibody-dependent cell-mediated cytotoxicity.

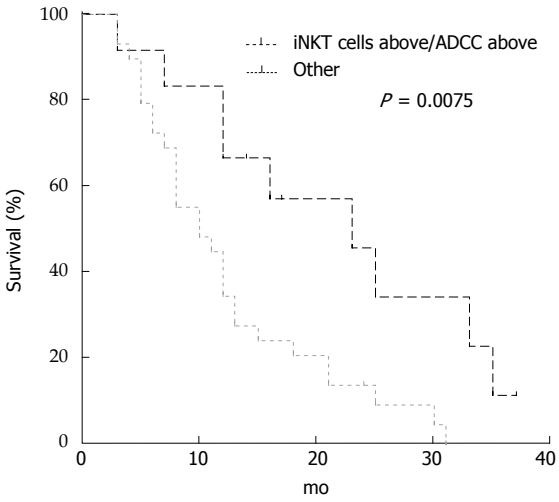

Figure 3 Overall survival in 41 metastatic colorectal cancer patients stratified for both invariant natural killer T and antibody-dependent cell-mediated cytotoxicity activity at basal level, below and above the respective median level. iNKT = 0.382 cells/microliter; ADCC = 71%. iNKT: Invariant natural killer T; ADCC: Antibody-dependent cell-mediated cytotoxicity.

We then analysed correlation with OS in the 4 groups. Patients performing ADCC activity above the median value both at basal level and either after 2 and/or 4 mo presented a trend in longer OS, albeit not significant (Figure 5).

When we focus on patients presenting ADCC values above the median levels in both determinations (basal and after 2 mo) we found that this 9 out of the 30 patients (30%) showed a higher OS compared to other patients (median 21 *vs* 13 mo, *P* = 0.5; Long-rank Mantel-Cox Test). After 4 mo, 8 patients out of 23 (35%) had both values above the median levels of ADCC activity, but their OS was not statistically different from that of the other patients (median 18.5 *vs* 15 mo; *P* = 0.42; Long-rank Mantel-Cox Test) (data not shown).

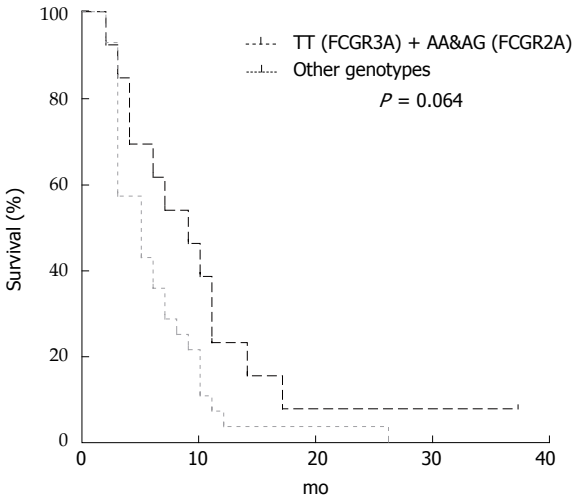

Figure 4 Progression free survival in compound heterozygote patients for single nucleotide polymorphisms rs1801274 in *FCGR2A* and rs396991 in *FCGR3A* genes in the 41 metastatic colorectal cancer patients.

DISCUSSION

Treatment of mCRC requires a multidisciplinary approach and multiple treatment options are nowadays available[1]. Advances in the understanding of tumor biology have led to the development of *EGFR*-targeted therapies as mAbs. In fact, the *EGFR*-signalling pathway regulates important processes involved in cell differentiation, proliferation, migration, angiogenesis and apoptosis, all of which become deregulated in cancer cells. However, the mechanisms that mediate the therapeutic effect of these mAbs are still unclear.

Cetuximab is a chimeric monoclonal antibody that specifically targets *EGFR* with high affinity and prevents the ligand-mediated activation of the *EGFR*-dependent pathway. *KRAS* mutations occur in 35%-45% of mCRC and preclude responsiveness to *EGFR*-targeted therapy with cetuximab or panitumumab. Initial response rates of about 10% were seen with cetuximab monotherapy in patients with heavily pretreated mCRC. A phase Ⅱ BOND study demonstrated the ability of cetuximab to circumvent irinotecan-based chemotherapy resistance[2]. Less than 20% patients displaying wild-type *KRAS* tumors achieve objective response. In fact, it subsequently became clear that tumors without mutations in codon 12 or 13 of the *KRAS* gene responded in 13%-17% of cases, whereas only 0.1%-2% of the *KRAS* mutant tumors did[15].

Alterations in other effectors downstream of the *EGFR* and deregulation of the *PIK3CA/PTEN* pathway have independently been found to give rise to resistance. Moreover, the *PIK3CA* gene is mutated in approximately 20% of CRCs. *BRAF* is the principal downstream effector of *KRAS* and its oncogenic V600E mutation is mutually exclusive with *KRAS* mutations in CRCs[4,16].

It has recently become clear that IgG1 mAb, like cetuximab, may have mechanisms of action other than the selective blockade of tumoral membrane receptors. Among them, the Fc region of the mAb may also trigger

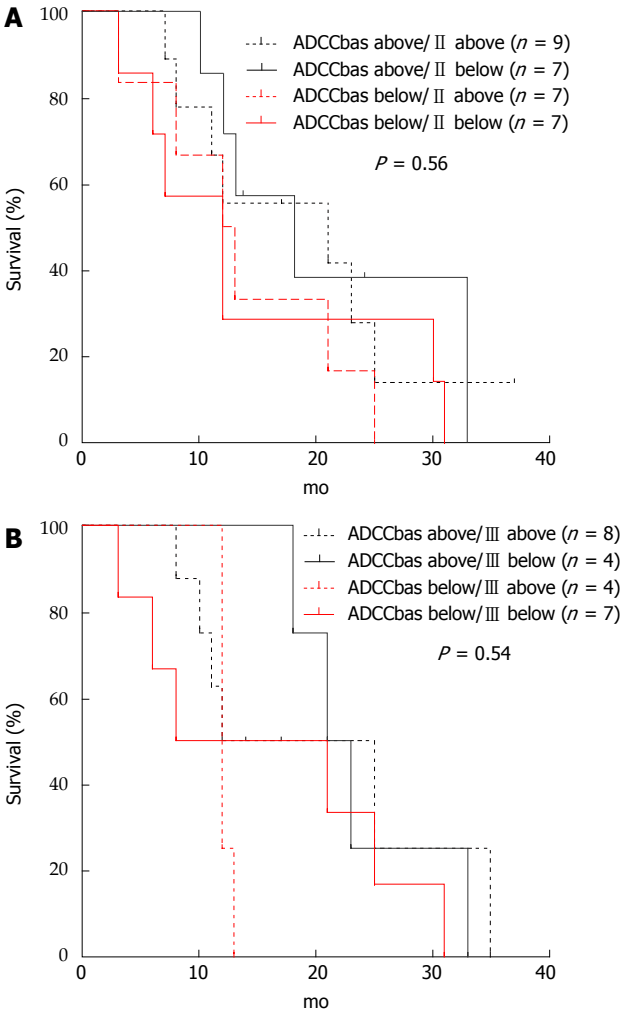

Figure 5 Overall survival in 30 metastatic colorectal cancer patients with a blood drawn after 2 mo (A) and in 23 patients with a blood drawn after 4 mo of treatment (B). Analysis was done in 4 groups of patients: The first included patients showing both ADCC activities above the median level [ADCCbas above/Ⅱ (or Ⅲ) above, where Ⅱ means on blood drawn after 2 mo and Ⅲ after 4 mo], the second group a decrease from a basal above median to a Ⅱ or Ⅲ determination below median [ADCCbas above/Ⅱ (or Ⅲ) below], the third group patients showing instead an increase from below at basal and above at Ⅱ or Ⅲ determination [ADCCbas below/Ⅱ (or Ⅲ) above] and the fourth group patients with both ADCC activities below the median level [ADCCbas below/Ⅱ (or Ⅲ) below]. ADCC: Antibody-dependent cell-mediated cytotoxicity.

ADCC, binding *via* Fv regions the target cell to any of the Fc-γ receptors, *i.e.*, CD16, CD32 and CD64, which are expressed, with different patterns, by cells of the innate immune system, namely monocytes, macrophages, granulocytes and NK. The contribution of the different cell types to the anti-tumor ADCC exerted *in vivo* by anti-*EGFR* mAbs is still debated. In general these cell are thought to play a relevant role controlling tumor growth and in preventing metastatic dissemination in humans[17,18].

In particular, NK cells have been suggested to be the major mediators of the ADCC-dependent therapeutic effect of cetuximab[19]. Moreover, invariant CD1d-restricted NKT cells has been reported to play an allegedly pivotal role in such responses *via* transactivation of immune effector cells. In particular, a severe circulating iNKT cell

deficiency was related to poor clinical outcome in head and neck squamous cell carcinoma patients[12].

Thus, the number of iNKT and the level of ADCC activity exerted by NK cells from tumor patients in the presence of cetuximab might be useful prognostic or predictive parameters for response to treatment. With this in mind, we investigated 41 mCRC patients suitable for combination therapy including cetuximab with irinotecan-based chemotherapy in second and third lines and *KRAS* wild type.

Analyses were carried out at start of therapy for all the 41 patients and ADCC and iNKT cells were evaluated at basal level. After 2 and 4 mo of treatment additional determinations were done in 30 and 23 patients respectively where ADCC and NK cells were longitudinally studied.

Main aim of the project was to study *ex-vivo* the prognostic and predictive value of the number of iNKT cells and the level of cetuximab-mediated ADCC and to analyse their correlation with *EGFR* level, mutational status of *KRAS*, *NRAS*, *BRAF* and PFS and OS in our prospective cohort of mCRCs.

We did not find any significant correlation of iNKT cells at basal level with PFS nor with OS, albeit we observed a trend to a longer survival after 10 mo in the population of patients with iNKT above median level. Instead, patients performing, at basal determination, ADCC activity above the median level showed an improved OS compared to patients with ADCC activity below this value.

Moreover, if we combine iNKT number and ADCC basal level and we stratified patients for both determinations, as below or above the respective median level, we observed a better OS in patients having both values above the median level compared to all the other combinations. Of note, when we analysed the same parameters after 2 and 4 mo of treatment, levels of circulating T, NK, iNTK cells were significantly reduced. On the clinical side, we observed that cancer patients exhibited a lower capacity to perform ADCC as compared to the beginning of therapy; this observation has to be replaced in the global context of an immunosuppressed state of cancer patients and the immunosuppressive effect of chemotherapy and it is consistent with earlier reports[20].

Intriguing, during treatment, neither low level of iNKT nor low ADCC activity did correlate to prognosis. In our study this could be in apparent contrast with what observed by us at the beginning of therapy and also with what reported by others, which is patients with a severe numeric iNKT cell deficiency have a strikingly poor clinical outcome in response to chemo and radiotherapy[12].

On the other hand, we're analysing NK levels and their activity in peripheral blood; we did not have the picture of the functional properties of tumor-infiltrating T and NK cells in patients. A reduced number of NK and iNKT cells in periphery might be "the other side of the coin" and may reflect an increased activity in the tumor infiltrates[21].

The impact of ADCC on the efficacy of cetuximab might also be influenced by the occurrence of polymorphic forms of genes coding receptors for the antibody Fc region. The most relevant polymorphisms regulating Fc:FcR interactions are phenylalanine (F) or valine (V) expression at position 158 of the Fc fragment[22]. In particular, differential response to therapeutic mAbs has been reported to correlate with specific polymorphisms in two of these genes: *FCGR2A* (H131R) and *FCGR3A* (V158F)[23]. However, previous studies exploring the relation between the FCGR polymorphisms and cetuximab efficacy in mCRC have demonstrated conflicting and have been mostly low-powered studies with small sample sizes[24].

More recently a variant allele in a let-7 microRNA complementary site within the 3'UTR of *KRAS* (rs61764370) has been correlated with clinical outcome in mCRC patients receiving cetuximab[25].

In our cohort of mCRCs, correlation in terms of OS and PFS with each genotype, either rs1801274 (A > G) in the *FCGR2A*, rs396991 (T > G) in *FCGR3A* or rs61764370 in the 3' UTR of *KRAS* gene didn't reveal to be significant. Interestingly enough, patients carrying alleles both with A in *FCGR2A* (AA/AG genotypes) and TT in *FCGR3A* presented a longer PFS, although the difference was not significant, probably due to the low number of patients.

For the same reason, we were not able to perform OS and PFS analyses according to all-*RAS* gene mutations. It is well know, in fact, that activating *KRAS* mutations are negative predictors of the response to cetuximab therapy in patients with mCRC, since cetuximab is widely considered to be unable to block the signal initiated by oncogenic *KRAS*[26,27].

Nevertheless we observed that of ADCC activity, as median level, was not affected by the presence of a *NRAS* or a *BRAF* mutation.

Seo *et al*[13] demonstrated cetuximab-mediated ADCC in human CRC cell lines and observed that ADCC activities for the tumor cells were higher in CRC patients with a high expression level of EGFR. Furthermore, the ADCC activity level was significantly associated with EGFR, but not with the *KRAS/BRAF* mutational status.

This has to be considered also in the light of the preclinical studies of nakadate and colleagues, who demonstrated that, in an ADCC assay, perforin-dependent target cell lysis was not affected by the *KRAS* mutation status. On the other hand, perforin-independent ADCC was observed only in CRC cells with wild-type *KRAS*, but not in cells with mutant *KRAS*. Their experiments also revealed that the Fas-Fas ligand (*FasL*) interaction was responsible for the induction of apoptosis and perforin-independent ADCC. Thus, their findings clearly suggested that ADCC is an important mode of action of cetuximab and that *KRAS* mutation impairs the therapeutic effect exerted by cetuximab-mediated ADCC. In our study, regrettably, the limited number of patients precluded any definitive confirmation of this in our clinical setting of mCRC patients[27]. Therefore, all together, our results seem to suggest a link between iNKT cells, basal ADCC

activity, genotypes in *FCGR2A* and *FCGR3A*, and efficacy of cetuximab in *KRAS* wild-type mCRC patients.

The efficacy of monoclonal anti-EGFR antibodies, like cetuximab, has been proven in mCRC patients. It has been established clearly that response to anti-EGFR antibody treatment is only possible in selected patient groups. However, predictive factors for the efficacy of anti-EGFR therapy have still to be completely elucidated. A factor identified in multiple studies as essential for appropriate assessment of eligibility for cetuximab or panitumumab treatment is the absence of *KRAS* gene mutations. EGFR expression on the surface of cancer cells does not seem to have a decisive influence on the efficacy of the therapy. There are ongoing studies assessing the predictive value of the number of copies of the *EGFR* gene, mutations in the *NRAS*, *PI3KCA*, *P53* and *PTEN* genes, concentration of EGFR ligands and polymorphisms in the EGF and EGFR, and the FCGR2A and FCGR3A, genes. In our study, we observed that combining iNKT number and ADCC basal level allowed to identify a group of mCRC patients, having both determinations above the respective median level and a longer OS. This combination looks like the best prognosticator in our population of patients. However, it has not as of yet been examined in large randomized prospective studies and hence should still be better elucidated before using as a basis for mCRC patient eligibility for cetuximab treatment.

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COMMENTS

Background

The efficacy of monoclonal anti-epidermal growth factor receptor (EGFR) antibodies, like cetuximab, has been proven in metastatic colorectal cancer (mCRC) patients. It has been established clearly that response to anti-EGFR antibody treatment is only possible in selected patient groups. However, predictive factors for the efficacy of anti-EGFR therapy have still to be completely elucidated. A factor identified in multiple studies as essential for appropriate assessment of eligibility for cetuximab or panitumumab treatment is the absence of mutation in *RAS* genes. EGFR expression on the surface of cancer cells does not seem to have a decisive influence on the efficacy of the therapy. There are ongoing studies assessing the predictive value of the number of copies of the *EGFR* gene, mutations in the *NRAS, PI3KCA*, *P53* and *PTEN* genes, concentration of EGFR ligands and polymorphisms in the *EGF* and *EGFR*, and the *FCGR2A* and *FCGR3A* genes.

Research frontiers

In this study the authors aim to evaluate the prognostic and predictive value of cetuximab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and circulating invariant natural killer T (iNKT) cells levels in mCRC; the authors shall analyse their correlation with *EGFR* level, mutational status of *KRAS*, *NRAS*, *BRAF*, progression free survival and overall survival in a prospective cohort of mCRC patients treated with cetuximab-based therapy.

Innovations and breakthroughs

The prognostic value of circulating iNKT cell and ADCC basal level reported here adds to previous notions and strengthens the hypothesis that human iNKT cells may also contribute to antitumor responses in cancer patients and to cetuximab efficacy. These results indicate also that the contribution of the indirect action of cetuximab may be relative high, compared with the direct anti-*EGFR* action, toward the clinical therapeutic effect.

Applications

In summary, the authors demonstrated here, in a prospective study, that a low level of circulating iNKT cells and a low ADCC activity before treatment in mCRC patients are significantly associated with poor survival. These data suggest that reconstitution of the iNKT cell pool (*e.g.*, by adoptive transfer of *ex vivo* expanded autologous iNKT cells) provides a promising immunotherapeutic strategy for mCRC. Furthermore, screening for iNKT cells and ADCC levels in peripheral-blood samples might provide a noninvasive, straightforward prognostic parameter and may also be useful for determining which patients can benefit from cetuximab therapy.

Terminology

The antibody-dependent cell-mediated cytotoxicity is a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. It is one of the mechanisms of the adaptive immune response through which antibodies can act to limit and contain tumors; classical antibody-dependent cell-mediated cytotoxicity is mediated by NK cells, which express CD16 which is an Fc receptor. This receptor recognizes, and binds to, the Fc portion of an antibody, such as the IgG1 anti-*EGFR* cetuximab; cetuximab is a recombinant chimeric mAb composed of the variable regions of a murine anti-EGFR antibody and of the constant regions of a human IgG1 kappa immunoglobulin. It is indicated for the treatment of squamous cell carcinoma of the head and neck and of *RAS* wild type mCRC.

Peer-review

This manuscript contributes to shed light to monoclonal therapy response in mCRC patients.

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CASE REPORT

Rectal neuroendocrine tumor with uncommon metastatic spread: A case report and review of literature

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Abstract

Neuroendocrine tumors of the gastrointestinal tract are rare neoplasms. Rectal neuroendocrine tumors consist approximately the 5%-14% of all neuroendocrine neoplasms in Europe. These tumors are diagnosed in relatively young patients, with a mean age at diagnosis of 56 years. Distant metastases from rectal neuroendocrine tumors are not very common. Herein we describe a case of a rectal neuroendocrine tumor which metastasized to the lung, mediastinum and orbit. This case underscores the importance of early identification and optimal management to improve patient's prognosis. Therefore, the clinical significance of this case is the necessity of physicians' awareness and education regarding neuroendocrine tumors' diagnosis and management.

Key words: Rectum; Uncommon metastatic spread; Neuroendocrine tumor; Rectal neuroendocrine tumor; Rectal neuroendocrine neoplasm

Core tip: Rectal neuroendocrine tumors consist approximately 5%-14% of all neuroendocrine neoplasms in Europe. Distant metastases from rectal neuroendocrine tumors are not very common. Herein we describe a case

of a rectal neuroendocrine tumor with an uncommon natural history as well as a review of the literature. The present case underscores the importance of early identification and management of these tumors.

Tsoukalas N, Galanopoulos M, Tolia M, Kiakou M, Nakos G, Papakostidi A, Koumakis G. Rectal neuroendocrine tumor with uncommon metastatic spread: A case report and review of literature. *World J Gastrointest Oncol* 2016; 8(2): 231-234 Available from: URL: http://www.wjgnet.com/1948-5204/full/v8/i2/231.htm DOI: http://dx.doi.org/10.4251/wjgo.v8.i2.231

INTRODUCTION

The gastrointestinal tract has the largest component of neuroendocrine cells. In spite of this, neuroendocrine tumors of the colon and rectum are rare entities, with a reported incidence ranging from 0.3% to 3.9% of all colorectal malignancies[1]. The introduction of more sensitive diagnostic tools (*e.g.*, immunohistochemical stains) and an overall increased awareness among physicians, have largely contributed to the rising incidence of neuroendocrine tumors[2]. Here we describe an interesting case of a rare neuroendocrine neoplasm of the rectum with an uncommon natural history.

CASE REPORT

A 54-year-old man with free medical or family history came to our hospital reporting rectal bleeding in May 2005. Colonoscopy demonstrated a rectal polypoid mass, 15 mm in diameter, located 6 cm from the anus. Biopsies were taken and histopathology evaluation showed an adenocarcinoma which invaded submucosa. An extensive work up with computed tomography (CT) scans was negative for distant metastases but there was an infiltration of pericolic fat. After that, the patient underwent low anterior resection of the rectum and the mesorectum. The histopathological examination of the dissected specimen showed a grade 2 adenocarcinoma with infiltration of pericolic fat and regional lymph nodes (stage C1 Astler-Coller). Adjuvant chemotherapy with 6 cycles of FOLFOX4 was administered without radiotherapy.

Two years later, during the scheduled follow-up, the CT scans revealed a mass in the lower left lobe of the lung, which was surgically resected and the pathology showed a neuroendocrine tumor with well differentiation. The review of both histologic specimens (paraffin tube of rectum and lung specimens, Figures 1 and 2) showed that there were medium to large tumor cells, displaying a trabecular growth pattern with nuclear pleomorphism, hyperchromasia and prominent nucleoli. Tumor cells were often spreading individually infiltrating. No lymphovascular invasion was detectable. There were a few punctate foci of necrosis. The tumor cells invaded perirectal tissues and 2 regional lymph nodes were infiltrated. Pathologic staging was pT3N1M1 and the clinical stage Ⅳ. Moreover, the immunohistochemistry analysis revealed positivity, in both specimens, for CK18, CK20, chromogranin, synaptophysin, CD56 and Ki-67, while CK7 and TTF1 were negative. Synaptophysin and chromogranin showed a diffuse positive staining of the tumor cells. These findings led to the conclusion that the primary tumor was that in the rectum and it was a neuroendocrine neoplasm well differentiated. In particular, Ki-67 was 8%-9% and the tumour was classified as well differentiated neuroendocrine tumor, intermediate grade (G2 NET). At that time patient refused to receive any further treatment.

One year later the planned follow-up showed a mass in the mediastinum. The octreoscan that followed showed increased uptake in the same anatomic region (Figure 3). Subsequently, the patient underwent radiotherapy (44 Gy) for the mass in the mediastinum. Moreover, the patient developed a mass in the left orbit, something that was discovered after a bilateral visual impairment and was treated with stereotactic radiosurgery (Cyber-Knife 18 Gy). Despite the medical advices patient refused to receive any systemic treatment. At the same period of time new lesions in left lung, mediastinum, adrenals and scalp were found. The patient was administered chemotherapy with the regiment Cisplatin 75 mg/m^2 d1 plus Etoposide 100 mg/m^2 d1-d3. Unfortunately, patient died after 4 cycles of chemotherapy due to uncontrolled systemic infection.

DISCUSSION

Rectal neuroendocrine neoplasms are usually small; polypoid lesions located in the mid-rectum, 5 to 10 cm from the anal verge and are submucosal in location, mainly discovered incidentally on routine surveillance endoscopies. If there are any symptoms, they include rectal bleeding, pain (as happened in our case) and change in bowel habits. However, 50% of patients are asymptomatic[3].

They belong to a heterogeneous group of tumours, which all present a common phenotype with immunoreactivity for markers such as chromogranin A and synaptophysin[4,5]. Neuron-specific enolase (NSE) and CD56 are frequently expressed in GEP-NETs, but are not specific. At present, immunohistochemistry for Ki-67 (MIB-1) is mandatory to grade the tumor according to the 2010 World Health Organization (WHO) classification and divides the tumors into NET G1, NET G2 and poorly differentiated neuroendocrine carcinoma (NEC G3)[4].

Prognostic factors for metastases are tumor size, depth of invasion, and lymph node involvement of the rectal NETs. These factors may be assessed by transrectal ultrasound, if feasible, and pelvic MRI. One study revealed that metastases emerged in only 2% of tumors not bigger than 2 cm, which had not infiltrated the muscularis propria, compared to 48% of those infiltrating the muscularis layer[6]. Although neuroendocrine tumours metastasize in 50%-75% of patients with the most common sites being lymph nodes, liver, and bones, metastases to the orbits, as happened in our case, have

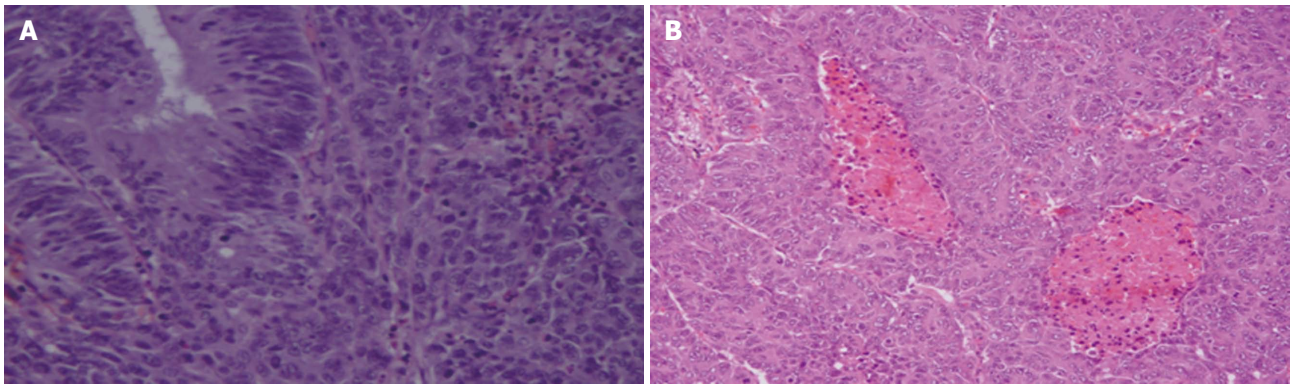

Figure 1 Biopsy of the rectal tumour (A) and lung tumour (B).

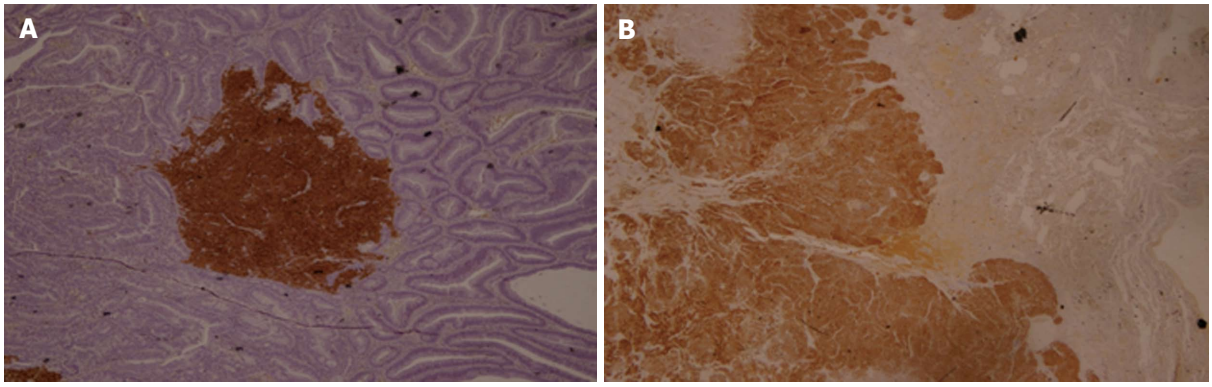

Figure 2 Immunohistochemical positivity for CD56 (rectum) (A) and CD56 (lung) (B).

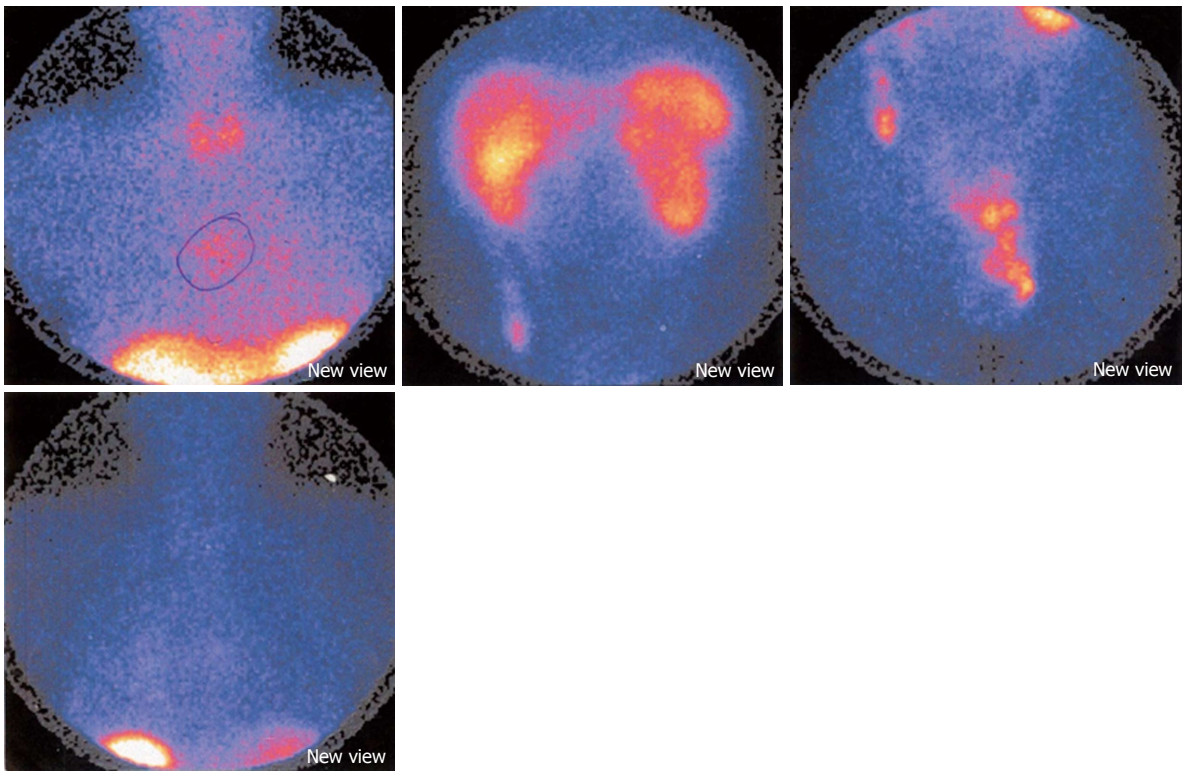

Figure 3 Octreoscan.

only rarely been reported (about 32 cases until 2006) and are believed to occur through hematogeneous

spread[7]. Orbital neuroendocrine tumors tend to arise from the gastrointestinal tract, whereas bronchial neuroendocrine tumors show a propensity to uveal metastasis and typically present with a mass or diplopia while visual failure is unusual[8], characteristics that verified in our case.

Obviously, metastatic disease at diagnosis will suggest a worse prognosis despite the available treatment options. In fact, surgery may have a palliative role to the complications associated with an advanced rectal tumour mass[9]. Adjuvant therapy for well differentiated tumours after surgery is not considered, although an argument exists for applying chemotherapy in non-differentiated tumours with incomplete resection[3]. Well differentiated neuroendocrine tumors is an uncommon indication for systemic chemotherapy[10]. When used for progressive disease, streptozotocin combined with 5-fluorouracil with or without doxorubicin is most often applied even though the response rate is < 25%[4]. The effectiveness of systemic chemo-regimens is optimal in poorly-differentiated tumours and the combination of cisplatin or carboplatin and etoposide have showed satisfactory results[4]. Newer anti-angiogenesis or mTOR inhibitors may be used as well as peptide receptor radionuclide therapy peptide in patients with advanced or metastatic disease[4,11]. Additionally, more chemotherapy regimens such as temozolomide and capecitabine are under clinical investigation for patients with advanced or metastatic neuroendocrine neoplasms[12].

In conclusion, rectal neuroendocrine tumors are rare and cases with distant metastases are even rarer. This case underscores the necessity of physicians' awareness and education regarding neuroendocrine tumors' diagnosis and management.

COMMENTS

Case characteristics

A 54-year-old man with rectal bleeding.

Clinical diagnosis

A rectal polypoid mass, 15 mm in diameter.

Differential diagnosis

Rectal neuroendocrine tumor; Non-neoplastic polyp; Lung neuroendocrine tumor.

Laboratory diagnosis

A well differentiated rectal neuroendocrine tumor (G2 NET) with metastases to left lung, mediastinum and left orbit.

Imaging diagnosis

Computed tomography scans revealed masses in the lower left lobe of the lung, in the mediastinum and in the left orbit.

Pathological diagnosis

The histopathological examination showed a well differentiated rectal G2 NET.

Treatment

Chemotherapy with 6 cycles of FOLFOX4 at the beginning and then regimen Cisplatin 75 mg/m^2 d1 plus etoposide 100 mg/m^2 d1-d3.

Peer-review

This is an interesting case report describing a potentially malignant behavior of a primary neuroendocrine tumor of the rectum.

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