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EDITORIAL

Interaction between diet and genetics in patients with inflammatory bowel disease

Daniéla Oliveira Magro, Ligia Yukie Sassaki, Júlio Maria Fonseca Chebli

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Abstract

In this editorial, we comment on the article by Marangoni *et al*, published in the recent issue of the *World Journal of Gastroenterology* 2023; 29: 5618-5629, about "Diet as an epigenetic factor in inflammatory bowel disease". The authors emphasized the role of diet, especially the interaction with genetics, in promoting the inflammatory process in inflammatory bowel disease (IBD) patients, focusing on DNA methylation, histone modifications, and the influence of microRNAs. In this editorial, we explore the interaction between genetics, gut microbiota, and diet, in an only way. Furthermore, we provided dietary recommendations for patients with IBD. The Western diet, characterized by a low fiber content and deficiency the micronutrients, impacts short-chain fatty acids production and may be related to the pathogenesis of IBD. On the other hand, the consumption of the Mediterranean diet and dietary fibers are associated with reduced risk of IBD flares, particularly in Crohn's disease (CD) patients. According to the dietary guidance from the International Organization for the Study of Inflammatory Bowel Diseases (IOIBD), the regular consumption of fruits and vegetables while reducing the consumption of saturated, trans, dairy fat, additives, processed foods rich in maltodextrins, and artificial sweeteners containing sucralose or saccharine is recommended to CD patients. For patients with ulcerative colitis, the IOIBD recommends the increased intake of natural sources of omega-3 fatty acids and follows the same restrictive recommendations aimed at CD patients, with the possible inclusion of red meats. In conclusion, IBD is a complex and heterogeneous disease, and future studies are needed to elucidate the influence of

epigenetics on diet and microbiota in IBD patients.

Key Words: Diet; Genetics; MicroRNAs; Gastrointestinal microbiome; Inflammatory bowel diseases; Crohn's disease

Core Tip: Diet-related issues are one of the main concerns that inflammatory bowel disease (IBD) patients bring to their clinicians and dietitians and are known to place a substantial burden on patients' quality of life. In this article, we discuss the interaction between diet and genetic factors such as microRNAs and the importance of diet in IBD patients. Furthermore, we provide dietary recommendations for patients during IBD flare as well as healthy nutritional guidelines to be followed during disease remission based on unprocessed or minimally processed foods.

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INTRODUCTION

In this editorial, we comment on the article by Marangoni *et al*[1], published in the recent issue of the *World Journal of Gastroenterology* about "Diet as an epigenetic factor in inflammatory bowel disease". In this review, the authors discuss the role of epigenetics in the pathogenesis of inflammatory bowel disease (IBD) and its modifications through diet as a mechanism for modulating the intestinal microbiota and attenuating the inflammatory process.

The term epigenetic was studied in the middle of the 19th century by biologist British Conrad Hall Waddington[2], to describe the interaction between genes and environment that allows the emergence of phenotypes. The first publication about epigenetics and IBD was in 1996[3]. In patients with IBD, the most studied modifications have been DNA-methylation and noncoding RNA that may be induced by smoking and diet[4,5]. Interestingly, this DNA-methylation is a process that is dependent on cofactors dietary such as substrates and nutrients (folate, vitamins B12, D, and others)[2] and is associated with inflammation, microbiota composition, and microRNAs (miRNAs) which can affect IBD by interfering with T cells differentiation[6]. Marangoni *et al*[1] provided an elegant review of the role of DNA methylation in IBD and its consequences to the inflammatory process.

In the recent review, Natasha and Zilbauer[2] claim that genetic changes may account for modest percentages of IBD, while epigenetic factors could potentially have contributed to the increase in the incidence of disease in recent decades and could be a key role of environmental factors in IBD pathogenesis.

It is worth highlighting that factors determining the degree of cellular epigenetic changes include the type of environmental factors and duration[2]. Some authors consider three critical periods during which the environment may favor the onset of the IBD: In utero, or the early postnatal phase (during gut microbiota colonization), and just before the disease onset[2,7]. Some data suggest that epigenomic reprogramming happens in response to maternal diet modifications, an excess of prenatal micronutrient supplementation (folate, methionine, betaine, and vitamin B12), maternal infection during prenatal, that increase interleukin-6 cytokine and induce epigenetic changes in fetal intestinal, and maternal smoking[7]. These factors could impact the development of IBD in infants.

Therefore, several nutrients present in the diet influence epigenetic modifications. In this way, phenotypic characteristics could be altered through changes in lifestyle and the environment in which the individual lives[3]. The Western diet, characterized by a low fiber content and deficiency of the micronutrients[5], impacts the short-chain fatty acids (SCFAs) levels and can induce epigenetic changes related to IBD, with the decrease in miR-143/145a, miR-148a, and miR-152 in colonocytes[7].

The inhibition of SCFA production (acetate, butyrate, and propionate) due to the low-fiber diet appears to play a critical role in the epigenetic control of the inflammation[5]. SCFA are essential for epithelial cell homeostasis and can epigenetically regulate the immune response and induce intracellular signaling pathways through the activation of G-protein coupled receptors[8].

The high-fat diet or diet rich in n-6 linoleic acid, particularly arachidonic acid, or high sugar diet have a proinflammatory activity that can change the miRNA profile of the visceral adipose exosomes or DNA methylation respectively, resulting in gut microbiota dysbiosis, dysregulating gut immune homeostasis, and increasing the risk of inflammation[5,7]. In the same way, the chronic alcohol consumption increases miR-122 and miR155 expression in the intestine and decreases occludin expression, leading to increased intestinal permeability[7].

Of note, the microbiome may induce epigenetic changes both in the intestinal epithelium and in immune cells[7]. Species bacteria such as *Faecalibacterium Prausnitzii, Roseburia* (Phylum Firmicutes), and Bacteroides genera, have anti-inflammatory action and are reduced in IBD while Proteobacteria (*Enterobacteriaceae and Bilophila*) are increased[8]. The bacteria commensal concerns the bioavailability of methyl groups through their production of folate and affects the host DNA methylation[7]. Many studies support that diet may change genome expression and induce host epigenetic

modification stably changing DNA structure[3].

Notably, lipopolysaccharides, a major component of bacteria Gram-negative[9], also play an important role in the epigenetics of IBD, as it has pro-inflammatory activity, increasing inflammatory cytokines[7] and the intestinal permeability[8].

Furthermore, the interaction between miRNA and gut microbiota in IBD patients has been emphasized by recent studies[10,11]. However, the exact mechanisms through which miRNAs are involved in IBD or dysbiosis are still unexplored. It is hypothesized that miRNAs could act as physiological regulators of the inflammatory process more than it participate in the inflammatory pathogenesis[12]. Intestinal miRNAs can interact with host microbiota and alter the growth and composition of bacterial gene expression[12]. On the other hand, gut microbiota can conversely regulate the expression of miRNA[13], altering the host status and predisposing to diseases. Some examples are the influence of gut microbiota on fecal miRNAs let-7, and miR-148, which target Enterobacteriaceae and Proteobacteria, respectively, and miR-21, which increases the abundance of IBD-related Bacteroidetes phylum and reduces the abundance of protective Firmicutes and Clostridia phylum[13]. In conclusion, the dysregulation of miRNAs could cause microbiota changes, leading to intestinal epithelial dysfunction, and immune hyperactivation[14]. Despite the recent findings, the complex relationship between intestinal microbiota and miRNAs in IBD deserves more attention.

In addition to miRNA, the role of circular RNA (circRNAs) in IBD has also been studied. CircRNAs are noncoding RNAs with covalently closed loop structures[15]. CircRNAs are involved in various diseases such as metabolic disorders [16], cardiovascular diseases[17], cancer[18] and IBD[19], showing their potential role as biomarkers for diagnosis, prognosis, or even as therapeutic targets for IBD. CircRNAs act together with miRNA in various inflammatory process, acting as miRNA sponges and altering their expression, and interacting with proteins[19]. Changes in the expression of circRNAs can impair the intestinal epithelial barrier and intestinal epithelium homeostasis[19], as demonstrated that depletion of circPan3 in human inhibited the renewal of intestinal stem cells, leading to the inhibition of epithelium regeneration[20]. A list of circRNAs has been associated with IBC, both Crohn's disease (CD) or ulcerative colitis (UC), all related to the intestinal epithelium and inflammatory process, and some are overexpressed (Circ_0001187; CircRNA_103765; CircRNA_102610; CircRNA_103516; CircRNA_102685; CircAtp9b; CircRNA_004662; CircSMAD4; CircKcnt2; CircZbtb20) while others are under expressed (CircHECTD1; CircHIPK3; CircGMCL1; Circ_CCND1; CircCDKN2BAS1; Circ_0007919; Circ_0001021)[19]. Despite this, circRNAs are not well characterized, and more in-depth studies are necessary to elucidate its role and applications in clinical practice for IBD patients.

Despite the need for greater elucidation on the role of the interaction between genetics and microbiota in the inflammatory activity of patients with IBD, it is worth highlighting that nutritional therapy is a safe and non-invasive treatment for IBD, by altering the gut microbiota and increases the production of SCFA, which appears to play a critical role in the epigenetic control of the inflammatory response[5].

DIETARY TIPS FOR PATIENTS WITH IBD

Diet-related issues are one of the main concerns that IBD patients bring to their clinicians and are known to place a substantial burden on patients' quality of life.

Every clinician who focuses their practice on IBD patient management is faced with situations where the patient with IBD requests recommendations on what types of foods he/she should avoid or consume since it is not uncommon for the patient to believe and report that certain foods seem to exacerbate his disease. It is important to emphasize that dietary manipulations must be tempered in these settings since there are some risks of restrictive diets in this nutritionally challenged population. Indeed, malnutrition in the IBD population already is high. In addition, many of these patients already have wrong beliefs about diet and adopt a series of food or food group restrictions such as a gluten-free diet, and paleo and vegan diets, which increases the risk of malnutrition. Thus, it's always important to work with a dietitian skilled in IBD management.

While it is well accepted that diet is one of the main modulators of the gut microbiota, thought to play a crucial and causative role in IBD, currently, there are no widely accepted evidence-based dietary approaches for managing patients with IBD[21-23]. In certain clinical contexts, some dietary tips are beneficial and important to emphasize. For example, a Mediterranean diet rich in a variety of fresh fruits and vegetables, monounsaturated fats, complex carbohydrates, and lean proteins, and low in ultra processed foods, added sugar, and salt[23] is recommended for all patients with IBD, a low-residue or fiber diet (avoiding, especially leafy green vegetables, nuts, seeds, beans, and kernels) for CD patients with symptomatic strictures to avoid bowel obstruction, a low FODMAP diet for patients with functional gut symptoms in association with quiescent IBD, or a low-fat diet for bile acid diarrhea after ileocecal resection, and an increased intake of fluids and calcium and reduced intake of oxalate-rich foods for those patients with kidney stones[22,23].

In the meantime, what dietary recommendations could a clinician caring for patients with IBD offer to their patients in daily clinical practice?

Despite the time-honored axiom "you are what you eat", no specific diet or nutritional intervention has been shown to prevent or treat IBD, except for the use of exclusive enteral nutrition as induction therapy for pediatric CD and the Crohn disease exclusion diet (CDED) for adults CD[5,24]. Regardless of this fact, some diet strategies help control symptoms. Based on experience in clinical practice in IBD, some dietary strategies for managing symptoms during flares can be recommended (Table 1).

Conversely, a wide range of recent studies have evaluated the relationship between ultra-processed food consumption (UPF) and IBD pathogenesis and have systematically shown a strong association between higher levels of consumption of UPFs and an increased risk of being newly diagnosed with CD[25,26]. UPF components, such as emulsifiers, thickeners,

Table 1 Tips for dietary adjustments during flares of inflammatory bowel disease[5,9,22,25-27,29]

No.	Tips
1	Avoid foods that may exacerbate diarrhea such as raw vegetables, fresh fruits with peel, prunes, spicy foods, fried or greasy foods, concentrated sweets, and caffeinated beverages
2	Avoid ultra-processed food
3	Prefer smaller, more frequent meals that are better tolerated and can increase calories and nutrient intake
4	Try to incorporate into your feed that constitutes the nutritional basis of the Mediterranean diet
5	Follow a lactose-free diet because it is not uncommon to develop transient lactose deficiency during flares
6	Avoid alcoholic drinks
7	Consider using nutritional supplements if solid foods are not well tolerated during the flare or your appetite is much reduced
8	Consider the use of EEN or CDED (PEN + modified oral diet) for Crohn disease according to the patient's tolerances

CDED: Crohn's disease exclusion diet; PEN: Partial enteral nutrition; EEN: Exclusive enteral nutrition.

salt, artificial sweeteners, phosphate, and food colorants (titanium dioxide, Azo dyes) can negatively affect the intestinal barrier, inducing dysbiosis, affecting the mucus layer, increasing the permeability of the intestinal epithelium, or directly interacting with the immune system[25,27].

Additionally, cumulative evidence suggests that a Mediterranean diet and a specific carbohydrate diet may help induce clinical remission in patients with CD, although this issue is still debated[23,28,29]. Indeed, in a recent randomized trial, researchers compared the consumption of a Mediterranean-style diet to the consumption of the specific carbohydrate diet for 12 wk in adult patients with CD who presented mild to moderate activity[30]. After 6 wk of following these 2 different diets, researchers found similar rates of symptomatic remission (46.5% *vs* 43.5% for the specific carbohydrate diet and Mediterranean diet, respectively; $P = 0.77$). Similarly, a reduction in fecal calprotectin levels was achieved in 34.8% with the specific carbohydrate diet and in 30.8% with the Mediterranean diet ($P = 0.83$). While the specific carbohydrate diet has some evidence that it can be beneficial for patients with CD, this trial was not able to show that it was better than the Mediterranean diet. For the practicing clinician, this finding has fundamental importance: The Mediterranean-style diet is less complex for patients to adopt in their busy lives compared to the specific carbohydrate diet. Moreover, this diet has the potential to bring several health benefits, including cardiovascular health outside of its favorable effects on CD patients[31].

Notably, maintenance dietary strategies in IBD lack evidence, except for the Mediterranean diet and consumption of dietary fibers which are associated with reduced risk of IBD flares, particularly CD[32]. Interestingly, some preclinical data suggest that a Westernized diet rich in saturated fat, refined carbohydrates, proteins from meat, processed foods and food additives influence the abundance, colonization, and phenotypic behavior of *Escherichia coli* in the gut, which may in turn initiate or contribute to gut inflammation. Conversely, the Mediterranean diet and specific dietary fibers may decrease *Escherichia coli* colonization and protect from invasion and adherence and consequently intestinal inflammation [33]. Moreover, from an epidemiological point of view, a Westernized diet that includes low consumption of fiber, fruit, and vegetables, has been shown to have pro-inflammatory effects and has been associated with a wide range of immune-mediated conditions, including a higher prevalence of IBD[34]. Regrettably, the Mediterranean diet has an important limiting factor, that is, its high cost, which can make access difficult in low-income countries.

Hopefully, future studies will be able to better determine the putative interrelationship between the consumption of specific food products, or their constituents, intestinal microbiome, and epigenetic mechanisms in IBD to target these factors in managing IBD. While we await the results of these studies, at present, some dietary tips that can be easily adopted by IBD patients in their busy daily lives can be appropriate, including consuming a well-balanced diet consisting mainly of fresh ingredients such as fruits, vegetables, legumes, whole grains, lean protein, olive oil, fish, limited red meat, and low-fat and nonfat dairy products, while avoiding processed meats, UPFs, food additives and emulsifiers[35-37]. Endorsing these dietary tips recent dietary guidance from the International Organization for the Study of Inflammatory Bowel Diseases (IOIBD) based on the best available evidence to date recommends that CD patients engage in regular consumption of fruits and vegetables while reducing the consumption of saturated, trans, dairy fat, additives, processed dairy or foods rich in maltodextrins, artificial sweeteners containing sucralose or saccharine, and processed food containing nanoparticles[37]. For patients with UC, the IOIBD recommends the increased intake of natural sources of omega-3 fatty acids (for instance, from oil olive, wild salmon, and other natural sources, not from supplements). Likewise, the types of food that patients with UC should avoid are similar to CD with the possible inclusion of red meats [37]. Ultimately, well-designed randomized controlled clinical trials are required before evidence-based dietary recommendations for IBD management can be made. It is also plausible that in the future personalized dietary strategies for each patient could be implemented based on better knowledge of the interaction between nutrients, gut microbiome and metabolome, and individual genetics.

ADVANTAGES AND LIMITATIONS OF EPIGENOME STUDIES

Epigenome-wide association studies brought advantages such as (1) finding novel methylation sites associated with disease; (2) evaluating the environmental impact of genetic regulation; and (3) explaining part of the heritability missed out by genome-wide association analysis. On the other hand, there are still gaps such as (1) expansion of sample size and ethnic diversity; (2) existence of the heterogeneity of sample material; and (3) causal inference of the identified epigenetic is challenging[6].

The complexity and heterogeneity of IBD make it a challenge because it is an evolving disease, and one treatment will not suit all patients. It is believed that precision medicine is the future for the treatment of IBD and among the studies and databases, epigenetics is included. Future perspectives are needed to elucidate the influence of epigenetics on diet and microbiota in IBD patients.

CONCLUSION

Diet is thought to play a role in the pathogenesis of IBD and may contribute to triggering IBD flares. In particular, some dietary components may interact with gut microbiota and genetics to trigger or perpetuate intestinal inflammation. Patients with IBD often requests recommendations on what types of foods he/she should avoid or consume, since it is not uncommon for the patient to associate their diet with symptoms. Briefly, cumulative evidence strongly suggests that higher levels of consumption of UPFs increase the risk of CD. A diet low in UPFs could encourage induced remission or control of symptoms in patients with IBD. Conversely, a healthier or Mediterranean-style diet is likely to be protective for CD development. From a therapeutic point of view, the specific carbohydrate diet, CDED, or a Mediterranean-style diet may be beneficial for the treatment of patients with CD who have mild to moderate symptoms. A diet low in red and processed meat may reduce the risk of flares in UC. In addition, a low FODMAP diet is beneficial for patients with functional gut symptoms in association with quiescent IBD. For patients with IBD in remission, the consumption of the Mediterranean diet and dietary fibers as adjunctive therapies may be recommended to reduce the risk of IBD flares, particularly in CD patients. For patients with UC, the increased intake of natural sources of omega-3 fatty acids and the following of restrictive recommendations aimed at CD patients may be useful in reducing UC flare-ups. All patients with IBD should be monitored for malnutrition, vitamin D, and iron deficiency and, in some situations, for vitamin B12 deficiency.

FOOTNOTES

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EDITORIAL

Pediatric stricturing Crohn's disease

Alessandro Boscarelli, Matteo Bramuzzo

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Abstract

Crohn's disease (CD) is a chronic inflammatory disease of the digestive tract. The incidence of pediatric CD is increasing and is currently 2.5–11.4 per 100000 worldwide. Notably, approximately 25% of children with CD develop stricturing CD (SCD) that requires intervention. Symptomatic stricturing diseases refractory to pharmacological management frequently require non-pharmacological interventions. Non-pharmacological therapeutic strategies include endoscopic balloon dilatation, stricturoplasty, and surgical resection of the strictured segment. However, strictures tend to recur postoperatively regardless of treatment modality. The lifetime risk of surgery in patients with childhood SCD remains at 50%–90%. Thus, new and emerging strategies, advanced diagnostic tools, and minimally invasive approaches are under investigation to improve the outcomes and overall quality of life of pediatric patients with SCD.

Key Words: Stricturing; Crohn's disease; Pediatrics; Insights; Future perspectives

Core Tip: Crohn's disease (CD) is a chronic inflammatory disease of the digestive tract, and approximately one out of four children develop stricturing CD (SCD) requiring intervention. Since strictures tend to recur postoperatively regardless of treatment modality and the estimated lifetime risk of surgery in patients with childhood SCD remains high, new emerging strategies may help to improve the outcomes and overall quality of life of patients with SCD.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder that progressively damages the bowel and causes remarkable morbidity, disability, and reduced quality of life. The etiopathogenesis of CD is still being investigated. However, its underlying pathology is assumed to result from a dysfunctional interaction between the human immune system and intestinal commensal microbiota[1,2]. The overall incidence of CD is continuously increasing. A recent systematic review of population-based studies reported the highest prevalence in Germany (322 per 100000) and Canada (319 per 100000). The incidence of pediatric CD ranges from 2.5–11.4 per 100000 worldwide[3,4]. Common symptoms of CD in children include fever, abdominal pain, bloody or mucopurulent chronic diarrhea, anemia, poor growth, and signs of intestinal obstruction. Other symptoms include perianal anomalies, such as abscesses or fistulas, and extraintestinal manifestations like arthritis, erythema nodosum, and uveitis. Notably, despite available treatment strategies for reducing the progressive inflammatory process of the disease, approximately 25% of children with CD develop stricturing CD (SCD) that requires intervention[2,4,5]. Approximately 10% of patients present with complicated disease at diagnosis. In particular, duodenal SCD is a rare but serious complication, affecting nearly 1% of patients at diagnosis, with an annual incidence of 0.05 per 100000. Conversely, the most frequent CD location is the terminal ileum, which is the part of the bowel most often affected by complications[6,7]. Interestingly, Sato *et al*[8] conducted a retrospective, single-center study of a cohort of 520 patients with initial CD attacks and a mean age at diagnosis of approximately 25 years; they concluded that stenosis or fistula appeared in about half of the patients after 5 years. Moreover, in patients with upper gastrointestinal disease or small intestinal lesions at the time of diagnosis, the cumulative rate of initial surgery was seemingly higher[8].

INSIGHTS ON SCD DIAGNOSIS

Endoscopy is the gold standard for diagnosing and monitoring inflammatory bowel diseases in children. However, it is less desirable for pediatric than for adult patients because of its invasiveness, the need for sedation and bowel preparation, and additional procedural challenges. In addition, while irradiation should be limited in pediatric patients during follow-up of a chronic disease such as CD, suspicion of acute SCD remains an indication for abdominopelvic computed tomography[9,10]. Intestinal ultrasound (IUS) is an imaging tool that has recently been shown to have comparable accuracy to magnetic resonance enterography when evaluating transmural inflammation of the entire bowel. Advantages of IUS include being well-tolerated, non-radiating, and less expensive. Furthermore, IUS showed high sensitivity in detecting small bowel CD, particularly active ileal inflammation[11]. The International Bowel Ultrasound Group's Pediatric Committee proposed the first pediatric IUS monitoring algorithm to better assess and characterize complications such as SCD. Following endoscopy and trans-abdominal IUS, magnetic resonance enterography should be considered to establish disease extension and activity, leaving small bowel capsule endoscopy for selected cases in which clinical suspicion remains high[9,11].

Recently, Ungaro *et al*[12] identified panels of blood biomarkers, including the proteins C-C motif chemokine ligands 3 and C-C motif chemokine ligands 4 and cluster of differentiation 40 selected by random survival forest modeling, that appear to predict the development of complications. These biomarkers may assist with risk stratification at the time of diagnosis of CD in pediatric patients[12]. Further studies are needed to better investigate the capacity of these biomarkers to predict SCD.

ADVANCES IN SCD MANAGEMENT

A recent population-based study by Ley *et al*[13] evaluated the impact of current therapeutic strategies on long-term outcomes in a cohort of 1007 patients with CD recognized before the age of 17 years over 26 years. They concluded that the increased use of immunosuppressants and anti-tumor necrosis factor (TNF) antibodies decreased the likelihood of bowel resection and SCD within 5 years after diagnosis, leading to a reduction in surgical interventions. Anti-TNF therapy has been shown to have good short-term success but a modest long-term response in patients with SCD[13,14]. Moreover, a recent study suggested that early anti-TNF exposure may reduce disease progression, while body mass index was directly associated with an increased likelihood of surgery[15].

Notably, a retrospective analysis of a cohort of 57 children in 2022 highlighted that female gender, stricturing and/or penetrating disease, and perianal disease at diagnosis were independent risk factors for surgical intervention. In addition, Spencer *et al*[16,17] reported a recurrence rate of 46% within a pediatric CD cohort of 78 patients who had undergone ileocolic resection.

As stated above, endoscopy is a cornerstone for diagnosing and following up with children with CD, and video capsule endoscopy is considered a valuable adjunctive and alternative tool for managing these patients. Recent data suggest that endoscopic balloon dilatation is an emerging safe and effective alternative that should be considered in

selected cases. However, its use is limited by the need for dedicated centers and expert endoscopists[18-20].

CONCLUSION

Advanced interventional techniques, such as endoscopic stricturotomy and stricturoplasty or endoscopic stenting with self-expandable metal stents, are feasible and effective in treating SCD in adults. However, post-procedural complications and long-term follow-up have been poorly investigated, and data on indications, descriptions, and results in children with SCD are scarce[18-21]. Further studies are required to evaluate the application of these emerging techniques in pediatric patients. The concomitant advent of robotic technologies will likely influence this process of treatment evolution.

FOOTNOTES

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EDITORIAL

Gut microbiota and female health

Meng-Yao Wang, Li-Xuan Sang, Si-Yu Sun

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Abstract

The gut microbiota is recognized as an endocrine organ with the capacity to influence distant organs and associated biological pathways. Recent advancements underscore the critical role of gut microbial homeostasis in female health; with dysbiosis potentially leading to diseases among women such as polycystic ovarian syndrome, endometriosis, breast cancer, cervical cancer, and ovarian cancer *etc.* Despite this, there has been limited discussion on the underlying mechanisms. This editorial explores the three potential mechanisms through which gut microbiota dysbiosis may impact the development of diseases among women, namely, the immune system, the gut microbiota-estrogen axis, and the metabolite pathway. We focused on approaches for treating diseases in women by addressing gut microbiota imbalances through probiotics, prebiotics supplementation, and fecal microbiota transplantation (FMT). Future studies should focus on determining the molecular mechanisms underlying associations between dysbiosis of gut microbiota and female diseases to realize precision medicine, with FMT emerging as a promising intervention.

Key Words: Gut microbiota; Female health; Estrogen; Polycystic ovarian syndrome; Endometriosis

Core Tip: Maintaining intestinal microbial homeostasis is essential for human health. Dysbiosis of gut microbiota has been demonstrated in patients with polycystic ovarian syndrome, endometriosis, breast cancer, cervical cancer, and ovarian cancer, disordered gut microbiota may affect the occurrence and development of these diseases through the immune system, estrogen, or metabolite pathways. In the future, maintaining gut microbiota homeostasis may be a promising treatment.

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INTRODUCTION

The human body contains trillions of microbes, rapidly diversifying after birth. Recent developments in genome sequencing, transcriptome analysis, and metabolomics have enabled researchers to explore the microbiota in more detail, particularly their functions. The gut microbiota plays a pivotal role in nutrient transformation and absorption, maintaining vital interactions with multiple tissues and organs, an indispensable factor for human health. The primary bacteria found in the gut are *Firmicutes* and *Bacteroidetes*, accounting for 90% of the flora in the gut[1], other bacterial constituents include *Actinobacteria* and *Proteobacteria*.

While a universally healthy gut microbiota remains undefined, dysbiosis has been associated with diseases ranging from irritable bowel syndrome to cancer. Previous studies have reported sex differences in the distribution of gut microbiota and disease prevalence[2], of which, the female gut microbiota emerges as a compelling area for investigation. Marano *et al*[3] underscore the strategic role of gut microbiota in crucial life stages for women, from childhood through adolescence, fertile age to pregnancy-partum, and up to menopause. This editorial aimed to discuss the potential mechanisms and therapeutic targets associated with the impact of gut microbiota dysbiosis on female diseases.

POSSIBLE MECHANISMS OF GUT MICROBIOTA DYSBIOSIS AFFECTING FEMALE HEALTH

Immune system

The gut microbiota can both promote and inhibit inflammatory response by influencing inflammatory factors, thus affecting the onset and progression of female diseases. In a study by Xu *et al*[4], ovarian cancer cells transplanted into mice with gut microbiota dysbiosis demonstrated increased xenograft tumor sizes. This dysbiosis stimulated macrophages, resulting in increased circulating levels of interleukin (IL)-6 and tumor necrosis factor-α, thus inducing the progression of ovarian cancer epithelial-mesenchymal transition[4]. One study examined the gut microbiota in patients with preeclampsia, revealing that *Akkermansia muciniphila* significantly suppressed inflammation and alleviated preeclamptic symptoms in rats by promoting autophagy and M2 polarization of macrophages in the placental bed[5]. Dysbiotic shifts in the gut microbiota are associated with increased gut permeability, leading to increased translocation of bacterial endotoxins, primarily lipopolysaccharide (LPS)[6]. The activation of the innate immune system through toll-like receptor 4 by LPS increases the expression of proinflammatory cytokines *via* nuclear factor κB translocation[7].

Gut microbiota - estrogen axis

In premenopausal women, ovaries use cholesterol derived from saturated fats for estrogen synthesis. Following menopause, adipose tissue, adrenal glands, and other organs convert circulating androgens into estrogens through the aromatase enzyme[8,9]. As shown in Figure 1, circulating estrogens undergo conjugation in the liver through glucuronidation or sulfonation, facilitating their excretion in bile, urine, and stool. The gut microbiota significantly influences estrogen levels by secreting β-glucuronidase (GUS), an enzyme that converts conjugated estrogen into deconjugated estrogen in the gastrointestinal tract. This transformation allows it to bind to estrogen receptors, initiating downstream signaling and physiological effects[10,11]. In the lower female reproductive tract, estrogen regulates the microenvironment by increasing epithelial thickness, glycogen levels, mucus secretion, and indirectly lowering vaginal pH through the promotion of *Lactobacilli* abundance and lactic acid production[12]. Additionally, estrogen can modify gut epithelial barrier integrity[13].

Decreased GUS activity may lead to reduced deconjugation of estrogen, resulting in decreased circulating estrogen levels and contributing to pathologies such as obesity and polycystic ovarian syndrome (PCOS). In contrast, increased GUS activity can elevate estrogen levels, leading to conditions such as endometriosis and cancer[14,15]. Endogenous estrogen is a major factor in the development of postmenopausal breast cancer. Certain bacterial genera and species in the human gut, including *Bacteroides, Escherichia*, and *Lactobacillus*, contain genes encoding GUS[16]. In a study on mice with letrozole-induced PCOS, serum estradiol levels positively correlated with the abundance of *Bifidobacterium* and *Bacteroides* , while negatively correlating with the abundance of *Prevotella*[17]. The investigation by Shin *et al*[18] on 26 healthy women revealed that those in the high-estradiol group harbored a more diverse gut microbiota compared to the low- and medium-estradiol groups. The drop in serum estradiol concentration was attributed to the relative overabundance of *Slackia* and *Butyricimonas*[18]. Another study found a significant and positive association between non-ovarian urine

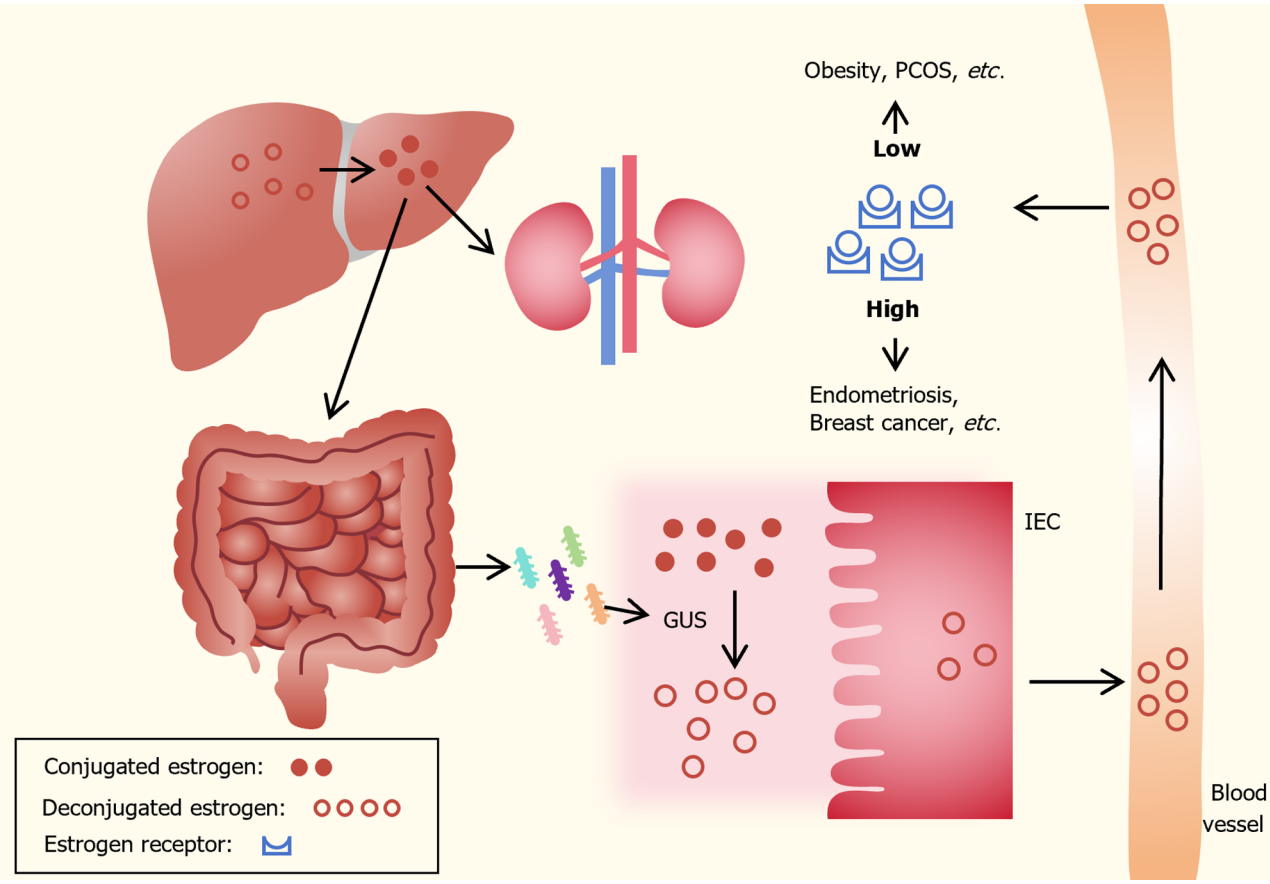

Figure 1 Effects of gut microbiota on estrogen metabolism. Estrogens are primarily produced in the ovaries, adrenal glands, and adipose tissue and circulate in the bloodstream and first undergo metabolism in the liver, where estrogens are conjugated. Conjugated estrogens are eliminated from the body by metabolic conversion to water-soluble molecules, which are excreted in urine or bile into the gut. The gut microbiota significantly influences estrogen levels by secreting β-glucuronidase (GUS), an enzyme that converts conjugated estrogen into deconjugated estrogen in the gastrointestinal tract. This transformation allows it to bind to estrogen receptors, initiating downstream signaling and physiological effects. Decreased GUS activity may lead to reduced deconjugation of estrogen, resulting in decreased circulating estrogen levels and contributing to pathologies such as obesity and polycystic ovarian syndrome. In contrast, increased GUS activity can elevate estrogen levels, leading to conditions such as endometriosis and cancer. IEC: Intestinal epithelial cell; GUS: β-glucuronidase; PCOS: Polycystic ovarian syndrome.

estrogen levels and *Clostridia* taxa in the *Firmicutes* (including *non-Clostridiales* and three genera in the family *Ruminococcaceae*)[19].

Metabolite pathway

Through the breakdown of organic matter, gut microbiota produces metabolites such as short-chain fatty acids (SCFA) and bile acids. SCFAs, including acetic acid, propionic acid, and butyric acid, provide energy for colon cells, regulate the intestinal barrier, and influence the inflammatory response[20-23]. *Firmicutes* predominantly synthesize butyrate[24], while *Bacteroides* are major producers of acetate and propionate[25]. Butyric acid has been shown to regulate progesterone and estradiol secretion through the cAMP signaling pathway in porcine granulosa cells[26]. The study by Liu *et al*[27] demonstrated that supplementing with butyrate can alleviate nonalcoholic fatty liver disease in ovariectomized mice. One study found that SCFAs have anti-inflammatory properties mediated through the G protein-coupled receptor pathway and histone acetylase[28]. Notably, SCAFs may have anti-cancer properties in cervical cancer through the activation of free fatty acid receptor 2[29].

Bile acid plays an important role in maintaining intestinal homeostasis, regulating lipid and carbohydrate metabolism, and influencing immune function. The study by Qi *et al*[30] reported that elevated levels of *Bacteroides vulgatus* were observed in the gut microbiota of individuals with PCOS, and this elevation was accompanied by reduced levels of glycodeoxycholic acid and tauroursodeoxycholic acid. The study found that glycodeoxycholic acid induced intestinal group 3 innate lymphoid cell IL-22 secretion through GATA binding protein 3. IL-22, in turn, improved the PCOS phenotype[30]. In an *in vitro* experiment, it was demonstrated that urolithin A reduces the Rac1 and PAK1 activity, leading to a decrease in actin polymerization and consequently reducing cell migration in human endometrial carcinoma cells[31], urolithin A may offer new avenues for the development of novel cancer therapeutics.

CHANGES OF GUT MICROBIOTA IN PATIENTS

PCOS

PCOS is a prevalent endocrine disorder in women, characterized by symptoms such as anovulation, obesity, insulin resistance, and hyperandrogenism. Tremellen and Pearce[32] highlighted that disturbances in the gut microbiota resulting from a poor diet can lead to increased gut mucosal permeability. Subsequently, this allows LPS from Gram-negative colonic bacteria to enter the systemic circulation. The subsequent activation of the immune system interferes with insulin receptor function, elevating serum insulin levels, consequently contributing to increased androgen production by the ovaries and disruption of normal follicle development[32]. A study involving 18 obese patients with PCOS and 15 obese women without PCOS revealed that the richness and diversity of gut microbiota were lower in the obese PCOS group compared to the control group, *Lachnoclostridium*, *Fusobacterium*, *Coprococcus_2*, and *Tyzzerela 4* were identified as the characteristic genera in obese patients with PCOS[33]. Additionally, mice transplanted with stool from women with PCOS exhibited fewer pups compared to mice transplanted with stool from healthy controls[30]. Modulating gut microbiota could hold significant value for the treatment of PCOS[34,35].

Endometriosis

Endometriosis is a chronic inflammatory disease characterized by the presence of endometrial tissue (glands and stroma) outside the uterus, significantly impacting the quality of life of women of childbearing age[36]. Although endometriosis is known to be estrogen-dependent, studies have revealed that the growth of ectopic lesions persists even in ovariectomized animals. This suggests that in addition to ovarian steroids, the innate immune system of the pelvic environment can also regulate the growth of ectopic lesions in endometriosis[37]. Notably, gut microbiota dysbiosis has been associated with the occurrence and development of endometriosis. A study involving 14 women with histologically confirmed stage 3/4 endometriosis and 14 healthy controls demonstrated significant decreases in the genera *Sneathia*, *Barnesella*, and *Gardnerella* in stool samples of the endometriosis group[38]. Two patients in the endometriosis group exhibited higher levels of *Escherichia/Shigella* in stool, and subsequent follow-up revealed severe bowel involvement by endometriosis in these patients[38]. Moreover, a study using antibiotic-induced microbiota-depleted (MD) mice to investigate endometriosis progression demonstrated that MD mice exhibited reduced endometriotic lesion growth, and the transplantation of gut microbiota through oral gavage of feces from mice with endometriosis caused the endometriotic lesion growth[39]. Thus, these findings underscore the close relationship between the occurrence and development of endometriosis and gut microbiota.

Cancer

Dysbiosis of microbiota can impact the occurrence and progression of tumors by regulating host immune response and inflammatory pathways. Breast cancer is one of the most prevalent malignant tumors among women worldwide and is closely linked to estrogen levels. The gut microbiota plays a role in deconjugating estrogens through the bacterial secretion of GUS, enabling estrogens to bind to estrogen receptors. Subsequently, the activation of estrogen receptors increases the number of G0/G1 cells entering the cell cycle, promoting cell proliferation, which is particularly well-defined in breast cancer[40]. The study conducted by Bobin-Dubigeon *et al*[41] demonstrated a reduction in gut microbiota diversity, a relative enrichment in *Firmicutes*, and a depletion in *Bacteroidetes* among patients with breast cancer as compared to those of healthy women. In another controlled study, patients with breast cancer exhibited a lower abundance of some microbial taxa, including *Bacteroidetes phylum*, *Firmicutes phylum*, *Verrucomicrobia phylum*, *Clostridium genus*, *Shigella genus*, *Bifidobacterium genus*, *Akkermansia muciniphila*, *Clostridium perfringens*, *Escherichia coli*, *Bacteroides uniformis*, *Clostridium hathewayi*, and *Faecalibacterium prausnitzii*[42].

Cervical cancer ranks as the fourth most common cancer in terms of morbidity and mortality, primarily attributed to human papillomavirus infection. In a study involving 42 patients with cervical cancer, gut microbiota 16S rDNA analysis revealed differences in both α and β diversity between the patient group and the control group[43]. The patient group exhibited a higher abundance of *Prevotella*, *Porphyromonas*, and *Dialister*, while the control group showed a higher abundance of *Bacteroides*, *Alistipes*, and members of the *Lachnospiracea* family[43]. Additionally, Chang *et al*[44] conducted an enrichment analysis of gut microbiota from patients with cervical cancer patients and healthy controls and found that the functions of the differentially expressed genes in the two groups, primarily associated with REDOX reactions, biosynthesis of other secondary metabolites, and amino acid transport and metabolism.

Ovarian cancer, with the highest mortality among female genital tract malignancies, is often diagnosed at advanced stages with metastasis. Endoscopic ultrasound is a non-invasive and accurate method for the early diagnosis of early carcinoma in the upper gastrointestinal tract and liver diseases[45,46]. Additionally, this tool is effective in detecting ovarian cancer infiltration of surrounding organs[47]. Xu *et al*[4] demonstrated *in vitro* that gut microbiota dysbiosis can promote the growth of ovarian cancer cells and induce epithelial-mesenchymal transition. This underscores the need for further exploration of the role of gut microbiota in the occurrence and development of various tumors. Table 1 summarizes the list of studies that highlighted the gut microbiota changes in patients with PCOS, endometriosis, breast cancer, cervical cancer, and ovarian cancer.

THERAPY

Probiotics have emerged as a modulator of gut microbiota. Recently, probiotics have been successfully used in the regulation of disrupted gut microbiota and the improvement of diseases such as gestational diabetes mellitus (GDM) and

Table 1 Gut microbiota alterations in polycystic ovarian syndrome endometriosis, breast cancer, cervical cancer, and ovarian cancer of the human studies

Diseases	Changes in gut microbiota (human studies)
PCOS	Increase: *Lachnoclostridium*, *Fusobacterium*, *Coprococcus_2*, and *Tyzzerela 4*[33]; *Bacteroides*, *Escherichia/Shigella*, and *Streptococcus*[48]
	Decrease: *Tenericutes* and *Firmicutes/Bacteroides ratio*[33]; *Akkermansia* and *Ruminococcaceae*[48]
Endometriosis	Increase: *Bacteroides*, *Parabacteroides Oscillospira*, and *Coprococccus*[49]
	Decrease: *Paraprevotella* and *Lachnospira*[49]; *Clostridia Clostridiales*, *Lachnospiraceae Ruminococcus*, *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae Ruminococcus*[50]
Breast cancer	Increase: *Firmicutes*, *Clostridium cluster IV*, and *cluster XIVa*[41]
	Decrease: *Bacteroidetes*[41]; *Bacteroidetes phylum*, and *Verrucomicrobia phylum*[42]
Cervical cancer	Increase: *Prevotella*, *Porphyromonas*, and *Dialister*[43]; *Succinivibrio*, *Ruminococcus*, *Morganella*, *Shewanella*, and *Proteus*[51]
	Decrease: *Bacteroides*, *Alistipes*, and *Lachnospiracea*[43]; *Phascolarctobacterium* and *Halomonas*[51]
Ovarian cancer	Increase: *Proteobacteria*[52]
	Decrease: *Actinobacteria*, *Bifidobacterium*, and *Coprococcus*[52]

PCOS: Polycystic ovarian syndrome.

endometriosis. In a prospective study involving 256 pregnant women randomized to receive probiotics or a placebo during the first trimester, the probiotic intervention resulted in a reduced incidence of GDM[53]. Additionally, oral administration of lactic acid bacteria was found to alleviate endometriosis-related pain[54]. In a study by He *et al*[34], a PCOS-induced rat model treated through letrozole treatment showed that a 4-wk strain intervention, particularly with *Lactobacillus plantarum* HL2, was protective against PCOS-like pathological changes in the ovaries. Prebiotics are defined as a nondigestible food ingredient that selectively stimulates the growth and/or activity of specific bacteria in the colon; thus, improving host health[55]. Prebiotics improve the balance of gut microbiota and produce various beneficial effects on the human host, such as improving insulin resistance[56] and regulating intestinal immunity[57].

The technology of fecal microbiota transplantation (FMT) has gradually matured and found applications in various complex intestinal diseases. However, there has been limited research on the use of FMT for the treatment of gynecological diseases. In a study, rats with PCOS were observed to have lower levels of *Lactobacillus* and *Clostridium*, and higher levels of *Prevotella* compared to control rats[35]. Following treatment with FMT and *Lactobacillus* from healthy rats, the abnormal estrous cycle improved, and androgen biosynthesis decreased in all rats in the FMT group and 75% of the rats in the *Lactobacillus* group. Moreover, ovarian morphology normalized, and the composition of the recovered gut microbiota in the FMT and *Lactobacillus*-treated groups demonstrated an increase in *Lactobacillus* and *Clostridium* and a decrease in *Prevotella*[35]. Huang *et al*[2] performed ovariectomy on 12-wk-old mice and subsequent follow-up revealed vaginal atrophy and disrupted intestinal microbial balance at 4 wk post-operation. Subsequent transplantation of gut microbiota from normal female mice to ovariectomized mice resulted in enhanced proliferation of vaginal epithelium and significant alleviation of epithelial atrophy. The abundance of bacteria positively influencing vaginal epithelial regeneration (*Proteobacteria*, *Verrucomicrobia*, *Akkermansia*) increased as observed in the study. Therefore, further research on FMT could offer a new alternative treatment for gynecological diseases.

CONCLUSION

The impact of gut microbiota on the body's immune system and hormonal balance is significant, dysbiosis of gut microbiota has been associated with the promotion of common gynecological diseases, such as PCOS, endometriosis, and malignant tumors. Conversely, these diseases can further disrupt the balance of gut microbiota. Interventions targeting the imbalanced gut microbiota, including the use of probiotics, prebiotics, and FMT, have shown promising results in animal experimental models. This approach offers a new perspective on the treatment of gynecological diseases, although further clinical studies are necessary to validate these findings. In the future, exploring the possibility of "matching" FMT donors and recipients or using bioengineering to synthesize bacterial solutions for precise disease treatment is worth further exploration.

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EDITORIAL

Multiparametric ultrasound as a new concept of assessment of liver tissue damage

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Abstract

Liver disease accounts for approximately 2 million deaths per year worldwide. All chronic liver diseases (CLDs), whether of toxic, genetic, autoimmune, or infectious origin, undergo typical histological changes in the structure of the tissue. These changes may include the accumulation of extracellular matrix material, fats, triglycerides, or tissue scarring. Noninvasive methods for diagnosing CLD, such as conventional B-mode ultrasound (US), play a significant role in diagnosis. Doppler US, when coupled with B-mode US, can be helpful in evaluating the hemodynamics of hepatic vessels and detecting US findings associated with hepatic decompensation. US elastography can assess liver stiffness, serving as a surrogate marker for liver fibrosis. It is important to note that interpreting these values should not rely solely on a histological classification. Contrast-enhanced US (CEUS) provides valuable information on tissue perfusion and enables excellent differentiation between benign and malignant focal liver lesions. Clinical evaluation, the etiology of liver disease, and the patient current comorbidities all influence the interpretation of liver stiffness measurements. These measurements are most clinically relevant when interpreted as a probability of compensated advanced CLD. B-mode US offers a subjective estimation of fatty infiltration and has limited sensitivity for mild steatosis. The controlled attenuation parameter requires a dedicated device, and cutoff values are not clearly defined. Quantitative US parameters for liver fat estimation include the attenuation coefficient, backscatter coefficient, and speed of sound. These parameters offer the advantage of providing fat quantification alongside B-mode evaluation and other US parameters. Multiparametric US (MPUS) of the liver introduces a new concept for complete noninvasive diagnosis. It encourages examiners to utilize the latest features of an US machine, including conventional B-mode, liver stiffness evaluation, fat quantification, dispersion imaging, Doppler US, and CEUS for focal liver lesion characterization. This comprehensive approach allows for

diagnosis in a single examination, providing clinicians worldwide with a broader perspective and becoming a cornerstone in their diagnostic arsenal. MPUS, in the hands of skilled clinicians, becomes an invaluable predictive tool for diagnosing, staging, and monitoring CLD.

Key Words: Multiparametric ultrasound; Ultrasound-based elastography; Liver stiffness; Noninvasive diagnostic test for chronic liver disease; Liver steatosis assessment; Portal hypertension evaluation

Core Tip: Multiparametric ultrasound (MPUS) of the liver introduces a new concept for complete liver evaluation. It encourages examiners to utilize the latest features of an ultrasound (US) machine, including conventional B-mode, liver stiffness evaluation, fat quantification, dispersion imaging, Doppler US, and contrast-enhanced US for focal liver lesion characterization. MPUS, in the hands of skilled clinicians, becomes an invaluable predictive tool for diagnosing, staging, and monitoring chronic liver disease.

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INTRODUCTION

Chronic liver disease (CLD) poses a global health challenge, contributing to approximately two million deaths annually worldwide[1]. The nature of these diseases, arising from diverse etiologies, present a complex array of structural and functional abnormalities. The assessment of liver tissue damage is a critical aspect of managing various liver diseases. Historically, liver tissue damage assessment relied heavily on invasive methods such as liver biopsy. Histological changes in liver tissue are characteristic of CLDs, encompassing toxic (alcoholic), genetic, autoimmune, and infectious etiologies [2]. Accumulation of extracellular matrix material, fats, triglycerides, or tissue scarring are common manifestations. The gold standard for evaluating CLDs is a liver biopsy. This is because examining the histologic specimen not only helps with fibrosis staging but also provides additional information about necroinflammation and other pathological changes. Offering direct insights into histopathological changes, it is an invasive procedure carrying potential complications and limitations such as sampling errors and interobserver variability. This underscores the necessity for noninvasive alternatives[3-5].

Traditional B-mode ultrasound (US) has been a cornerstone in diagnosing liver diseases, providing valuable insights into structural abnormalities[6]. Recent developments have expanded the diagnostic capabilities of US. Doppler US, when combined with B-mode imaging, offers a nuanced evaluation of hepatic vessel hemodynamics and identifies findings associated with hepatic decompensation. Contrast-enhanced US (CEUS) enhances tissue perfusion assessment, facilitating the differentiation between benign and malignant liver lesions[7]. US-based elastography, measuring liver stiffness, emerges as a pivotal tool for assessing liver fibrosis. However, its interpretation must consider clinical evaluation, the etiology of the liver disease, and the patient's comorbidities. These measurements prove most clinically relevant when viewed as a probability of compensated advanced CLD (cACLD)[8]. Accurate diagnosis of liver disease is essential for effective management and timely intervention. Multiparametric US (MPUS) addresses this challenge by combining multiple imaging parameters to offer a detailed and nuanced assessment of liver health. The advent of MPUS marks a paradigm shift in liver disease diagnosis. By integrating various US features such as B-mode, liver stiffness, fat quantification, dispersion imaging, Doppler US, and CEUS, clinicians gain a comprehensive diagnostic perspective in a single examination. MPUS, when wielded by skilled clinicians, becomes an invaluable predictive tool for diagnosing, staging, and monitoring CLDs. The ability to provide a broader perspective enhances diagnostic accuracy, empowering clinicians worldwide with efficient diagnostic tools. The evolution of noninvasive methods, particularly MPUS, has revolutionized the landscape of liver disease diagnosis.

COMPONENTS OF MPUS

Traditional B-mode US remains a fundamental component, providing a structural overview of liver tissue. However, its limitations in detecting mild steatosis emphasize the need for a more comprehensive approach. When coupled with B-mode imaging, Doppler US enhances the evaluation of hepatic vessel hemodynamics. This addition aids in identifying early signs of hepatic decompensation, contributing to a more thorough diagnostic picture. Vascular thrombosis can be diagnosed very simply with standard US and with Doppler evaluation. CEUS provides valuable information on tissue perfusion, enabling accurate differentiation between benign and malignant focal liver lesions. The enhanced imaging

capabilities contribute significantly to the diagnostic accuracy of MPUS. US-based elastography serves as a surrogate marker for liver fibrosis. However, the interpretation of these measurements requires a holistic consideration of clinical evaluation, the underlying etiology, confounding factors, and the patient comorbidities. The limitations of B-mode US in estimating fatty infiltration underscore the need for comprehensive approaches. The controlled attenuation parameter, though requiring a dedicated device, contributes valuable insights. Quantitative US parameters like attenuation coefficient, backscatter coefficient, and speed of sound offer a holistic evaluation of liver fat, complementing B-mode assessments. Interpreting results from noninvasive methods requires a nuanced understanding of the underlying liver disease, patient comorbidities, and the specific modality used. A comprehensive clinical evaluation is essential for accurate diagnosis. The availability of advanced diagnostic technologies varies globally, impacting the accessibility of these noninvasive methods. Efforts to enhance accessibility and reduce disparities are crucial for widespread adoption. Standardizing the interpretation of results and establishing cutoff values for different modalities remain ongoing challenges. Consistent guidelines are necessary to ensure uniformity in assessments across healthcare settings. Standardization efforts are essential to enhance reliability and comparability. The field of liver tissue damage assessment is rapidly evolving. Future advancements may involve the integration of artificial intelligence for enhanced diagnostic accuracy, the development of novel serum biomarkers, and the refinement of existing technologies to address current limitations. Introduction of these new modules of evaluation (stiffness, fatty quantification) to a middle-class US machine is essential for the future accessibility of these new developments of the method.

BEYOND FIBROSIS: THE COMPREHENSIVE ROLE OF ELASTOGRAPHY IN ASSESSING LIVER TISSUE HEALTH

Liver fibrosis, a key feature of CLDs caused by various factors, can progress to liver cirrhosis along with its associated complications[3]. Evaluating the presence and extent of liver fibrosis is crucial in managing CLD patients as it can anticipate the prognosis and potentially impact treatment decision. Initially developed to estimate liver fibrosis by measuring tissue stiffness, elastography has transcended its original purpose. Elastography, once primarily associated with fibrosis assessment, has evolved into a versatile method offering insights into various aspects of liver tissue health (Figure 1).

Now, many experts explore the expanding role of elastography beyond fibrosis evaluation, highlighting its diverse applications in assessing the dynamic nature of liver tissues[8]. A model centered on applications of elastography beyond fibrosis offers several options including: (1) Liver steatosis assessment. Elastography has shown promise in quantifying liver steatosis, providing a noninvasive means to evaluate fat content. Identifying and quantifying fat infiltration contributes to a more comprehensive understanding of liver health; (2) inflammation detection. The dynamic nature of elastography allows for the detection of inflammatory changes within liver tissues. By assessing tissue stiffness alterations, elastography aids in identifying inflammation, a crucial factor in the progression of various liver diseases; (3) portal hypertension evaluation. Elastography provides valuable insights into portal hypertension by assessing liver stiffness. Monitoring changes in stiffness aids in understanding the impact of portal hypertension on liver tissues and guides appropriate interventions; and (4) monitoring treatment response. Elastography serves as a tool for monitoring responses to therapeutic interventions. Whether assessing the effectiveness of anti-inflammatory treatments or tracking changes in liver stiffness post-treatment, elastography offers real-time feedback on treatment outcomes (Figure 1).

However, there are some confounding factors that can increase the liver stiffness. These confounding factors can contribute to a false increase in liver stiffness. Cholestasis refers to the impaired flow of bile, leading to the accumulation of bile acids and other substances within the liver. The accumulation of bile acids and other components in liver tissue may lead to inflammation and fibrosis. Elastography measurements in cholestatic conditions may indicate increased liver stiffness, reflecting the fibrotic changes associated with chronic cholestasis.

Hepatic congestion, often seen in conditions such as congestive heart failure, can impact liver stiffness as well. Congestion in the liver causes increased pressure within the hepatic vasculature. This elevated pressure can affect the mechanical properties of liver tissue, leading to changes in stiffness. Elastography may detect increased liver stiffness in cases of hepatic congestion, indicating the mechanical alterations caused by elevated intrahepatic pressure (Figure 1). Assessing the severity of cholestasis, the degree of congestion, and other contributing factors is essential for accurate diagnosis and appropriate clinical management. However, the interpretation should be conducted in the broader clinical context, considering the underlying causes and potential coexisting factors influencing liver health.

Various techniques, such as shear wave elastography (SWE) and strain elastography, have demonstrated their efficacy in assessment of the mechanical properties of liver tissues. Various SWE techniques evaluate the speed of shear waves produced through mechanically induced stress. US SWE methods encompass vibration-controlled transient elastography (VCTE) and techniques based on acoustic radiation force impulse (ARFI). In VCTE, shear waves result from vibration controlled at the body surface, while in ARFI-based techniques, the waves stem from the push-pulse of a focused US beam. ARFI-based techniques comprise point SWE (pSWE), assessing stiffness in a specific and constant region, and two-dimensional SWE (2D-SWE), measuring stiffness across a broader area, accompanied by a color-coded parametric map of stiffness. The results of US SWE techniques are typically presented in meters per second (m/s), representing shear wave velocity. Alternatively, they can be converted to Young's modulus in kilopascals (kPa), although this conversion relies on assumptions that may not always be accurate[9].

Regular monitoring of liver stiffness can aid in assessing disease progression and the effectiveness of interventions in managing these conditions. It is crucial to interpret liver stiffness values in the context of the patient's clinical history, including the underlying cause of cholestasis or congestion. The ongoing evolution of elastography suggests a promising

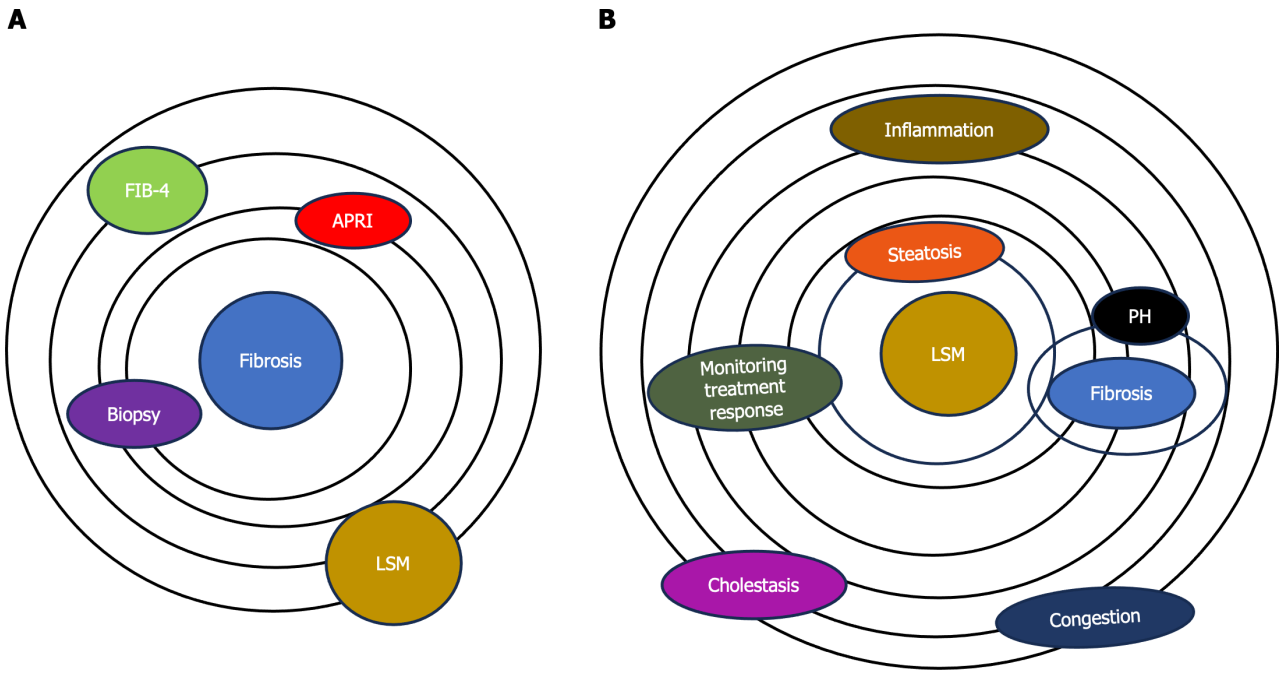

Figure 1 Role of elastography in assessing liver tissue health. A: Model of assessment of fibrosis. Elastography, once primarily associated with fibrosis assessment (biopsy, different noninvasive scores like fibrosis index based on 4 factors, aspartate aminotransferase-to-platelet index, *etc*); B: Model of assessment of liver stiffness. Now, elastography has evolved into a versatile method offering assessment of the mechanical properties and dynamic nature of liver tissues such as the quantification of liver steatosis by providing a noninvasive means for evaluation of fat content and the detection of inflammatory changes within liver tissues. Elastography may provide valuable insights into portal hypertension and monitor responses to therapeutic interventions. There are some confounding factors (cholestasis and heart congestion) that can contribute to increasing the liver stiffness. LSM: Liver stiffness measurement; FIB-4: Fibrosis index based on 4 factors; APRI: Aminotransferase-to-platelet index; PH: Portal hypertension.

future in liver tissue assessment. Advances in technology and research may lead to further refinements, increased standardization, and expanded applications, solidifying elastography as a cornerstone in liver health diagnostics. Elastography has transcended its initial role in fibrosis assessment, emerging as a powerful tool for comprehensive liver tissue evaluation. From steatosis to inflammation and portal hypertension, the diverse applications of elastography offer a nuanced understanding of liver health. As technology and standardization efforts progress, elastography is poised to play an increasingly central role in noninvasive liver assessments, shaping the future of liver disease diagnosis and management.

LIVER STIFFNESS MEASUREMENT IS USED TO STRATIFY THE SEVERITY OF LIVER DISEASE

The acronym advanced CLD (ACLD) is employed for individuals in the advanced stages of CLD and serves as an alternative to the term "cirrhosis," which is based on histology[10,11]. This designation is intended to encompass a wide range of patients, including those with significant liver fibrosis (bridging fibrosis) as observed in histology and those with compensated cirrhosis[12].

Many studies and meta-analyses proposed different cutoff values for liver stiffness evaluation with VCTE and in connection with different etiologies. In the Baveno VI and Baveno VII consensus[13] “rule of 5” was accepted. This is a very simple modality of stiffness value classification where < 5 kPa means normal liver, less than 10 kPa excludes cACLD, more than 15 kPa assumes cACLD, and more than 25 kPa assumes clinically significant portal hypertension (CSPH). This rule in daily practice can be used for a lot of purposes, like assessment of fibrosis and determining cACLD or CSPH (if liver stiffness is more than 25 kPa, the upper endoscopy can be avoided). Using the VCTE system in a patient and starting with the controlled attenuation parameter, we can stratify severity of steatosis and significant fibrosis can be determined in a very short time. It is important to note that while VCTE provides valuable information about liver stiffness, the interpretation should always be performed in conjunction with other clinical assessments, including medical history, laboratory tests, and potentially additional imaging studies and excluding confounding factors (including fasting, elevated aminotransferases, obstructive cholestasis, or right heart failure). As a prognostic tool, adopting the rule of 5 with cutoff values of liver stiffness measurement (LSM) using VCTE (10-15-20-25 kPa) is suggested. This approach enables a rapid estimation of the risk of decompensation and liver-related deaths, irrespective of the etiology of ACLD (Figure 2).

ARFI methods (pSWE and 2D-SWE) are implemented in a US system and can be used for standard US evaluation, Doppler examination, fatty quantification, stiffness measurement, and lesion discovery (focal liver lesion). Immediately, a CEUS examination can be performed. Then finally, this evaluation a MPUS method.

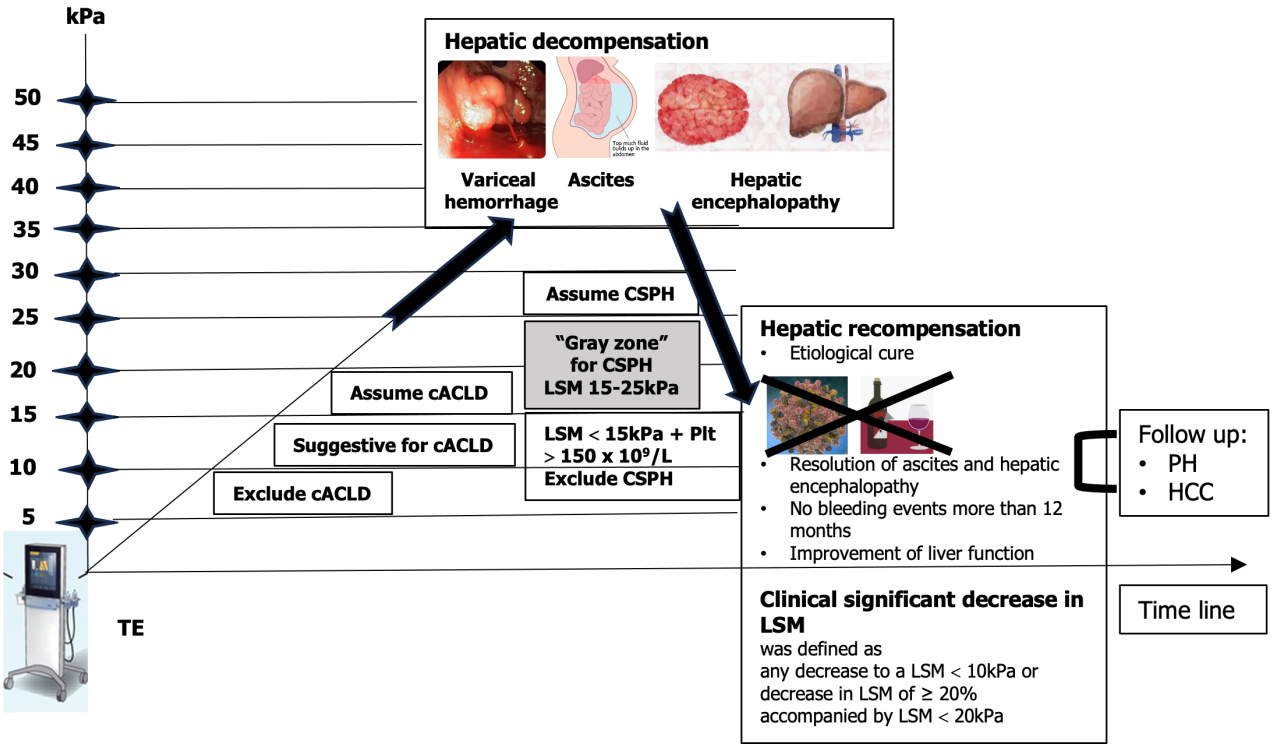

Figure 2 Use of noninvasive tests according to the rule of 5 to determine compensated advanced chronic liver disease and clinically significant portal hypertension. Dynamic use of noninvasive tests for assessment of hepatic decompensation or recompensation. Patients having a liver stiffness measurement (LSM) < 10 kPa rules out compensated advanced chronic liver disease (cACLD) in the absence of other clinical/imaging signs. LSM values between 10 kPa and 15 kPa are suggestive of cACLD, and LSM ≤ 15 kPa plus platelets ≥ 150 × 10^9/L rule out clinically significant portal hypertension (CSPH) in the majority of etiologies. LSM measured by transient elastography (TE) > 15 kPa are considered as a high likelihood of cACLD in all etiologies. Patients with intermediate values of LSM between 15 kPa and 25 kPa are in a "gray zone" of CSPH. The best cutoff to determine the presence of CSPH was an LSM ≥ 25 kPa (specificity and positive predictive value > 90%) in alcoholic liver disease, chronic hepatitis B, chronic hepatitis C, and non-obese patients with nonalcoholic steatohepatitis. Hepatic recompensation includes all of the following criteria: Suppression or removal of the underlying etiology of cirrhosis; Resolution of ascites and hepatic encephalopathy after discontinuation of diuretics and prophylactic therapies; Absence of variceal bleeding for 12 months; Sustained improvement of biochemical liver function, assessed by serum albumin, bilirubin, and international normalized ratio[13]. LSM: Liver stiffness measurement; HCC: Hepatocellular carcinoma; PH: Portal hypertension; TE: Transient elastography.

For many years, every company proposed their own cutoff values. Then in practice it was quite difficult to use these values. In 2020 a proposed algorithm, the "Rule of 4" for interpretation of liver stiffness (5-9-13-17 kPa), was presented [14]. In this system, it is quite easy to use the cutoffs for ARFI methods. If the values are < 5 kPa, the liver is normal, and below 9 kPa rules out cACLD. Values between 9-13 kPa are suggestive for cACLD and more than 13 kPa suggests the presence of cACLD. Values > 17 kPa are suggestive for CSPH (Figure 3). Concerning the practical value of SWE methods for liver stiffness evaluation, many published papers show the good results of these methods. There are meta-analyses and prospective studies (with most using liver biopsy as the gold standard). All these studies show that the area under the receiver operating characteristic curve of the methods increases with the severity of fibrosis, with more than 90% for liver cirrhosis[15-18].

Conventionally, cirrhosis progression was seen as a one-way street, transitioning inevitably from a compensated to a decompensated stage[19]. Yet, a growing body of evidence suggests that effective treatment or the elimination of the underlying liver disease etiology not only decelerates disease advancement but can even result in disease regression. The outlook is more optimistic than we once thought! The evolution in how we perceive things led to the development of the idea of hepatic recompensation[13]. This involves a significant improvement in hepatic function, along with a reduction in functional and structural factors like hepatic inflammation, fibrosis, and portal hypertension, all stemming from the successful treatment of the underlying cause. It emphasizes the encouraging potential for positive changes in liver health.

Numerous studies have investigated the significance of LSM in predicting liver-related events in individuals with liver diseases. However, a majority of these studies rely on one-time assessments. Precision in determining the long-term risk of liver complications based on a single LSM remains challenging. This is due to the fact that patients may encounter various situations over time, such as alterations in alcohol consumption, the emergence of metabolic disturbances, resolution of the underlying etiologic factor, or the introduction of new contributing factors, all of which can impact their prognosis. Repeated LSM offer an enhanced understanding of the liver disease's natural progression, potentially enabling personalized treatment decisions when integrated into clinical decision-making. However, certain aspects still require further exploration. Determining the optimal frequency of LSMs and the intervals between them must be established and proven to be cost-effective. Changes in LSM over time can be regarded as a dynamic prognostic biomarker. Repeated LSM holds the potential to refine predictions and individualize treatment strategies in clinical practice.

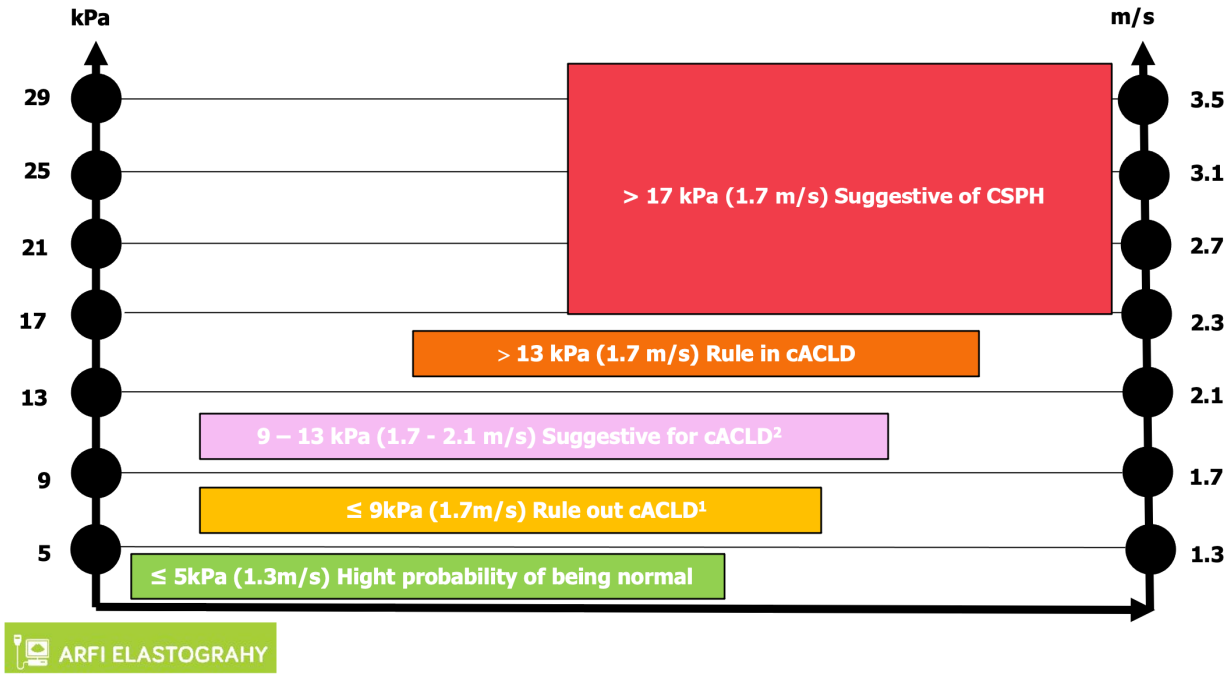

Figure 3 Interpretation of liver stiffness value with acoustic radiation force impulse techniques. Based on some published studies, the consensus panel Baveno VII proposed a vendor-neutral "rule of 4" (5, 9, 13, and 17 kPa) for the acoustic radiation force impulse techniques for viral etiologies and nonalcoholic fatty liver disease, liver stiffness of 5 kPa (1.3 m/sec) or less has a high probability of being normal. Values greater than 13 kPa (2.1 m/sec) are highly suggestive of compensated advanced chronic liver disease (cACLD). There is a probability of clinically significant portal hypertension with liver stiffness values greater than 17 kPa (2.4 m/sec), but additional patient testing may be required. In some patients with nonalcoholic fatty liver disease, the cutoff values for cACLD may be lower and follow-up or additional testing in those with values between 7 kPa and 9 kPa is recommended[10]. For other causes such as alcoholic hepatitis, primary biliary cirrhosis, Wilson's disease, autoimmune hepatitis, sclerosing cholangitis, and drug-induced liver disease, there is insufficient data to make a conclusion. [1]Liver stiffness less than 9 kPa (1.7 m/sec), in the absence of other known clinical signs, rules out compensated advanced chronic liver disease (cACLD). [2]Values between 9 kPa (1.7 m/sec) and 13 kPa (2.1 m/sec) are suggestive of compensated advanced chronic liver disease but may need further testing for confirmation. cACLD: Compensated advanced chronic liver disease; CSPH: Clinically significant portal hypertension.

NONINVASIVE TESTS TO GUIDE CLINICAL DECISION MAKING

Prognostic biomarkers quantify the likelihood of clinical events, disease recurrence, or disease progression. As transitioning from a compensated to decompensated state is the single most important factor affecting survival in patients with cirrhosis, prediction of decompensation is a major prognostic target[20]. An LSM by transient elastography (TE) is the best validated prognostic marker for determining liver-related morbidity and mortality in patients with compensated liver disease. A study of 3028 patients with mixed etiologies found a cumulative incidence of decompensation of 3.7% after 5 years for patients with TE values < 15 kPa, increasing to 19% for patients with baseline TE values ≥ 25 kPa[21]. Other elastography techniques such as pSWE, 2D-SWE, and magnetic resonance elastography also exhibit comparable accuracy as prognostic markers of decompensation and mortality, but variation in published cutoffs and heterogeneity attributable to equipment from different manufacturers limit their generalizability. It is important to switch off assessment of fibrosis to evaluation of clinically important ACLD.

CONCLUSION

The assessment of liver tissue damage has witnessed a transformative shift from invasive to noninvasive methods, providing safer alternatives for patients. The continuous refinement of noninvasive diagnostic methods, particularly the MPUS approach, signifies a crucial stride in managing CLDs. As this technology becomes more accessible and its applications expand, it promises to reshape clinical practices, offering a holistic and efficient means of diagnosing, staging, and monitoring liver diseases on a global scale. Addressing current challenges and embracing emerging technologies will pave the way for more effective management and personalized treatment strategies for patients with liver diseases.

FOOTNOTES

Author contributions: Peltec A and Sporea I provided significant intellectual contributions to this paper, including to the writing and editing of the manuscript, illustrations for the figures, and review of the literature; Peltec A designed the overall concept and outline of the manuscript; Sporea I contributed to the discussion and design of the manuscript.

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EDITORIAL

Advancements in medical treatment for pancreatic neuroendocrine tumors: A beacon of hope

Somdatta Giri, Jayaprakash Sahoo

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Abstract

This editorial highlights the remarkable advancements in medical treatment strategies for pancreatic neuroendocrine tumors (pan-NETs), emphasizing tailored approaches for specific subtypes. Cytoreductive surgery and somatostatin analogs (SSAs) play pivotal roles in managing tumors, while palliative options such as molecular targeted therapy, peptide receptor radionuclide therapy, and chemotherapy are reserved for SSA-refractory patients. Gastrinomas, insulinomas, glucagonomas, carcinoid tumors and VIPomas necessitate distinct therapeutic strategies. Understanding the genetic basis of pan-NETs and exploring immunotherapies could lead to promising avenues for future research. This review underscores the evolving landscape of pan-NET treatment, offering renewed hope and improved outcomes for patients facing this complex disease.

Key Words: Pancreatic neuroendocrine tumor; Medical management; Somatostatin analog; Immunotherapy; Everolimus

Core Tip: The evolving landscape of pancreatic neuroendocrine tumor treatment showcases tailored approaches based on tumor subtype. Cytoreductive surgery and somatostatin analogs are pivotal, whereas peptide receptor radionuclide therapy and molecular targeted agents are offering hope for refractory cases. Understanding genetic markers and exploring immunotherapies open promising avenues for future research.

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INTRODUCTION

Pancreatic neuroendocrine tumors (pan-NETs) present unique challenges in the field of oncology. These tumors account for 1%-2% of all pancreatic cancers and are mostly sporadic. Sometimes, pan-NETs are associated with various syndromes, such as multiple endocrine neoplasia 1 (MEN1), von Hippel-Lindau disease, tuberous sclerosis or neurofibromatosis. Often, these tumors are diagnosed at an advanced stage in the course of disease with metastasis to multiple organs, making curative surgery less successful. The role of medical therapy is as follows.

Pan-NETs

Most patients with advanced pan-NETs (50%-75%) have nonfunctioning tumors and do not have any associated hormonal syndrome. Somatostatin analogs (SSAs) provide a valuable palliative option by reducing hormonal secretion as well as the tumor burden. The European Neuroendocrine Tumor Society, North American Neuroendocrine Society, and National Comprehensive Cancer Network suggest the initiation of SSA in patients with unresectable, asymptomatic, well-differentiated pan-NETs and a high tumor burden, as SSAs have been shown to improve survival in the PROMID[1] and CLARINET trials[2]. Treatment of highly symptomatic patients may be initiated with short-acting octreotide with rapid transition to a long-acting formulation (LAR) and subsequent titration of the dose to optimize symptom control while the LAR formulation is starting to take effect (approximately 10-14 d). The depot preparation, *e.g.*, octreotide LAR, has largely eliminated the need for daily octreotide injections. Patients may use additional short-acting octreotide for breakthrough symptoms while doses are being titrated. Lanreotide can be administered once monthly *via* deep subcutaneous injection and appears to have efficacy similar to that of octreotide.

Notably, molecular targeted therapies, peptide receptor radionuclide therapy (PRRT), and chemotherapy are typically reserved for patients refractory to SSAs[3]. According to recent European Society of Medical Oncology guidelines, the mammalian target of rapamycin (mTOR) inhibitor everolimus is recommended for the treatment of G1/G2 pan-NETs[4]. A good treatment effect of everolimus was also recorded irrespective of the volume of liver metastasis in the RADIANT-4 trial[5]. Everolimus in combination with SSAs in advanced and metastatic pan-NETs demonstrated greater benefit than everolimus monotherapy in the RADIANT-1 and RADIANT-3 trials. In contrast to these data, the combination of everolimus with pasireotide did not prove to exert any further benefit. Everolimus should be carefully co-administered with glucocorticoids since, in the RADIANT-3 cohort, their combination resulted in a fatal episode of acute respiratory distress, as everolimus can cause immunosuppression. The progression-free survival (PFS) associated with the combination of everolimus and metformin was better than that associated with everolimus alone[6]. The mechanisms affected by metformin include a reduction in blood glucose, insulin, and insulin like growth factor-1 levels; inhibition of mitochondrial oxidation; activation of AMP-activated protein kinase; and antibacterial cell autonomy *via* mTOR inhibition as well as oncogenic effects.

PRRT has emerged as a beacon of hope for patients with metastatic but low-grade pan-NETs where curative surgery is not possible. This targeted therapy utilizes radionuclides to deliver systemic treatment. In the NETTER-1 trial, patients who received ^{177}Lu-DOTATATE with SSA experienced a 54.4% increase in estimated PFS at 20 months *vs* SSA alone[7].

On the other hand, neuroendocrine carcinoma (NEC) is genetically more similar to pancreatic cancer than to G1/G2 neuroendocrine tumors (NETs). Here comes the role of chemotherapy. Capecitabine plays synergistic role with temozolomide, perhaps by downregulating the DNA repair enzyme methylguanine methyltransferase. Although combined therapy is more effective for treating pan-NET, overall survival in patients with NEC is not rewarding (22 *vs* 4.6 months) [8]. In NEC patients, cisplatin-based therapy with etoposide or irinotecan remains the standard first-line chemotherapy option[9]. Distinguished clinical trials related to the management of pan-NETs are summarized in Table 1.

Gastrinomas

In gastrinomas, where surgical intervention might pose significant risks, medical therapy with high-dose proton pump inhibitor therapy (PPI) with or without SSAs can effectively control symptoms and tumor growth. Patients without imageable pancreatic tumors in MEN1 exhibit promising survival rates, *i.e.*, 5-year survival rates of approximately 90% and a 10-year survival rate of 54% in patients with disseminated distant metastasis, underscoring the importance of tailored approaches[10]. Long-acting PPIs such as omeprazole 60 mg a day or an equivalent dose of other PPIs once or twice a day are durable and effective in patients with sporadic gastrinoma without evidence of tachyphylaxis. The goal of PPI therapy is to restrict basal acid output to < 10 mEq/h during the hour before the next dose. Patients with MEN1-related gastrinoma may require a daily dose of 80-120 mg of omeprazole. Effective treatment of hypercalcemia in MEN1 patients is of paramount importance because it reduces gastric acid hypersecretion. It is advised to continue PPI therapy for at least 3-6 months after resection of the tumor due to the continued risk of gastroesophageal reflux disease complications caused by the increased parietal cell mass. Vigilance is required for potential side effects of long-term PPI therapy, such as vitamin B12 deficiency, hypomagnesemia and the risk of bone fracture. The role of SSA in PPI refractory patients was well documented in a retrospective study of 12 patients with gastrinoma[11]. In this study, all but one patient achieved complete clinical control with octreotide or lanreotide.

Table 1 Clinical trials related to medical therapy in pancreatic neuroendocrine tumours

Trial	Population	Study design	Drug	NET	Outcome
PROMID	85	RCT	Octreotide LAR	Metastatic Midgut NET	PFS 14.3 *vs* 6 m
CLARINET	204	RCT	Lanreotide	G1, G2 non-functioning NET	32% increase in PFS at 24 m
RADIANT 4	302	RCT	Everolimus	Non-functioning NET	PFS 11 *vs* 4 m; 52% reduction of death
Kurita *et al*[6]	100	Retrospective	Everolimus	Pan-NET	PFS is longer with G1, G2 than G3, NEC; Metformin increases PFS
NETTER 1	229	RCT	Lu DOTATATE with Octreotide LAR	Metastatic Midgut NET	54.4% increase in PFS at 20 m
Rogowski *et al* [8]	32	retrospective	capecitabine and temozolomide	G3 NET	PFS 15.3 m in G3 NET, 3.3 m in NEC
Morizane *et al* [9]	170	RCT	etoposide and cisplatin *vs* irinotecan and cisplatin	NEC	PFS 5.6 months in EP *vs* 5.1 months in IP
Bernard *et al* [13]	12	Retrospective	Everolimus	Malignant insulinoma	Hypoglycemia resolve in 11/12. Median recurrence at 6.5 m
TELECAST	76	RCT	Telotristat ethyl	Carcinoid syndrome	Sustained reductions in u5-HIAA and diarrhea

NET: Neuroendocrine tumor; RCT: Randomized control trial; PFS: Progression-free survival; NEC: Neuroendocrine carcinoma; LAR: Long-acting release.

Insulinomas

Insulinomas present a unique challenge, as patients develop hypoglycemia. Diazoxide (50-600 mg daily) is the primary medical therapy, and in refractory cases, glucocorticoids, verapamil, and diphenylhydantoin may be considered. SSAs can reduce insulin levels and are pivotal for the antiproliferative control of well-differentiated tumors. Since there is usually low expression of somatostatin receptor type (SSTR) 2 in benign insulinomas, SSA therapy may result in paradoxical worsening of hypoglycemia, as it also decreases glucagon secretion. In contrast, advanced insulinomas usually express SSTR2 and SSTR5. The pan-somatostatin receptor agonist pasireotide is a promising option for patients with refractory hypoglycemia, especially for those with metastatic insulinoma[12]. Akt/mTOR pathway is abnormally activated in NETs. Everolimus has both antineoplastic and antisecretory effects as shown in Figure 1 and has been successfully tested for refractory insulinoma[13]. However, exhaustion of its antineoplastic effect is observed after 2 years due to downregulation of mediators involved in the mTOR pathway instead of a true resistance[14]. Therefore, rechallenge with everolimus can be considered since this phenomenon appears to be transient.

Glucagonomas

For glucagonomas, SSAs are considered first-line treatments, as they have shown remarkable efficacy in controlling hormonal symptoms. In advanced cases, management must include SSAs to improve patient performance status, enabling surgical options to be considered[14]. Amino acid infusion and zinc therapy have shown promise in improving skin lesions associated with necrolytic migratory erythema.

VIPoma

VIPoma can cause severe and life-threatening diarrhea. Supportive care with intravenous fluids and electrolytes is crucial. SSAs have shown substantial promise in improving VIPoma-associated symptoms. Chemotherapy and sunitinib improved diarrhea in 10 out of 12 patients in a French study. Hypercalcemia associated with VIPoma is caused by the secretion of parathyroid hormone-related peptide and responds well to both zoledronate and denosumab[15].

Carcinoid syndrome

Management of crisis due to carcinoid syndrome is a medical emergency that requires the infusion of octreotide along with serotonin antagonists such as ondansetron, cyproheptadine, and ranitidine. A bolus of octreotide 100-500 μg should be immediately administered, followed by a maintenance dose of 50-100 μg/h (maximum 500 μg/h). Sympathomimetics can precipitate hormonal release by the tumor, paradoxically leading to distributive shock, and should be used cautiously. However, if a patient needs to be on sympathomimetics, the selective alpha1-agonist phenylephrine and vasopressin are the preferred vasopressors in this context.

For the prophylaxis for carcinoid crisis, SSAs can be started before surgery or before PRRT. It is advised to keep SSA-free period before the start of PRRT as short as possible (8-24 h), with safe reintroduction of SSA 1 h after the infusion of ^{177}Lu-DOTATATE in order to prevent functioning symptoms deterioration due to release of hormone following PRRT. Antidiarrheal agents such as loperamide are also useful. However, refractory diarrhea responds well to telotristat ethyl, a tryptophan hydroxylase inhibitor[16]. Patients with niacin deficiency or pellagra should be started on nicotinamide. A

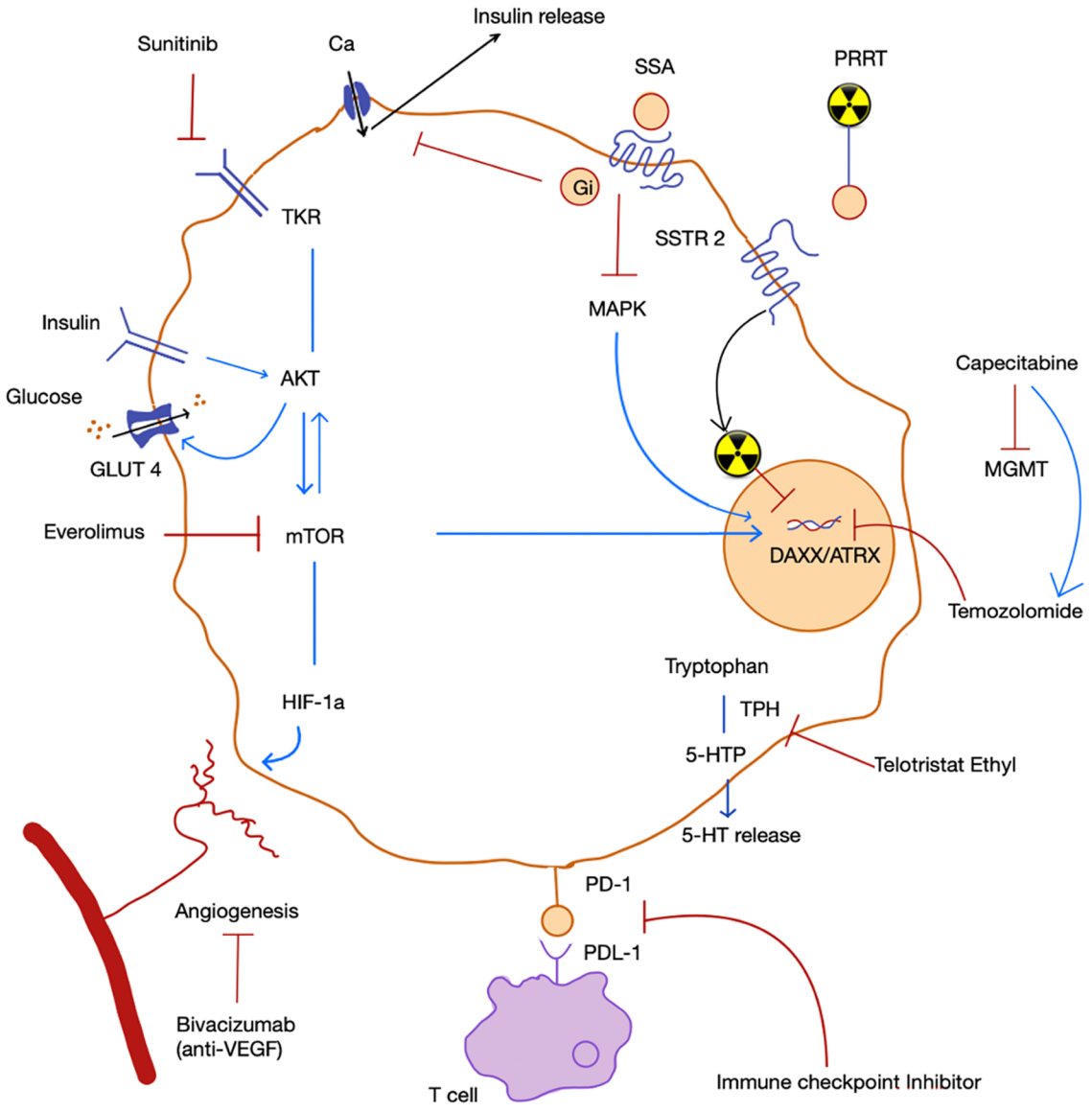

Figure 1 Mechanism of action various medical therapeutics. Everolimus by inhibiting mammalian target of rapamycin pathway can decrease cell proliferation as well as insulin action by supressing AKT. Somatostatin analog can supress hormone release by inhibiting Calcium channel and decrease proliferation by supressing Mitogen activated protein kinase. Temozolomide being an alkylating agent cause DNA damage. Capecitabine augment its activity by reducing methylguanine-DNA methyltransferase which is a DNA repair enzyme. Red lines are used to show inhibition and blue lines are used to show stimulation. GLUT 4: Glucose transporter 4, HIF-1a: Hypoxia inducible factor 1 alpha; 5-HTTP: 5 hydroxytryptophan; 5-HT: 5 hydroxy tryptamines; MAPK: Mitogen activated protein kinase; MGMT: Methylguanine-DNA methyltransferase; mTOR: Mammalian target of rapamycin; PD-1: Programmed cell death-1; TKR: Tyrosine kinase receptor; TPH: Tryptophan hydroxylase; VEGF: Vascular endothelial growth factor; SSA: Somatostatin analog.

simplified flow diagram showing approach to medical therapy in pan-NETs is depicted in Figure 2.

Future medical therapy in pan-NETs

Elucidating the genetic underpinnings of different pan-NET subtypes opens new avenues for tailored therapies. Mutations in genes such as *DAXX/ATRX*, *EPHB4*, *ROS1*, and *KMT2A* provide potential targets for future research[17]. Compared to NETs of extra-pancreatic origin, pan-NETs have higher expression levels of programmed cell death protein 1 (PD-1) and more tumor-infiltrating lymphocytes[18]. Immunotherapies, particularly PD-1 inhibitors, in combination with anti-vascular endothelial growth factor (bevacizumab) have shown potential in controlling pan-NETs.

CONCLUSION

The landscape of pan-NET treatment has evolved significantly, offering patients a more tailored and effective approach. From surgical interventions to targeted therapies and immunomodulatory agents, advancements in medical treatment for pan-NETs represent a beacon of hope for those facing this challenging disease. As research continues to uncover the intricacies of this condition, we can look forward to even more promising developments in the future.

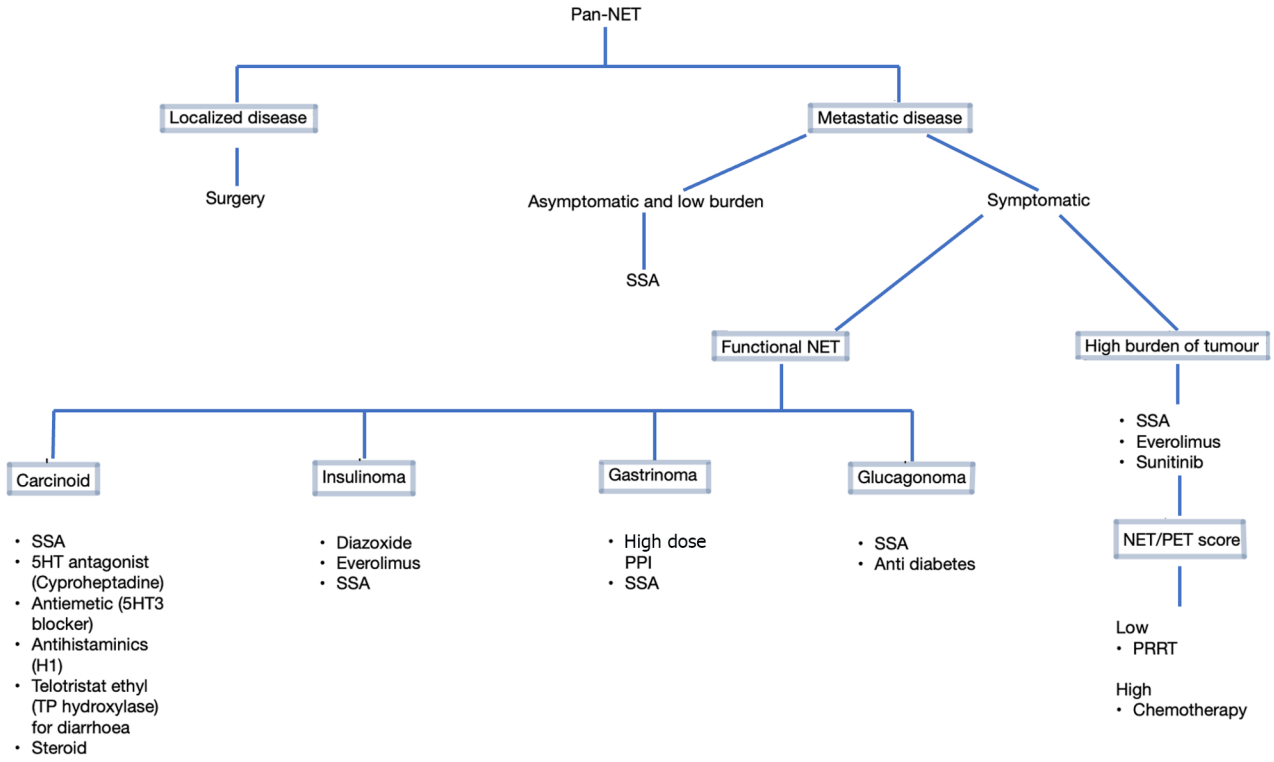

Figure 2 Approach to medical therapy in pancreatic neuroendocrine tumors. SSA: Somatostatin analog; PPI: Proton pump inhibitor; Pan-NET: Pancreatic neuroendocrine tumor; PRRT: Peptide receptor radionuclide therapy.

FOOTNOTES

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OPINION REVIEW

New direction for surgery: Super minimally invasive surgery

En-Qiang Linghu

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Abstract

The top goal of modern medicine is treating disease without destroying organ structures and making patients as healthy as they were before their sickness. Minimally invasive surgery (MIS) has dominated the surgical realm because of its lesser invasiveness. However, changes in anatomical structures of the body and reconstruction of internal organs or different organs are common after traditional surgery or MIS, decreasing the quality of life of patients post-operation. Thus, I propose a new treatment mode, super MIS (SMIS), which is defined as "curing a disease or lesion which used to be treated by MIS while preserving the integrity of the organs". In this study, I describe the origin, definition, operative channels, advantages, and future perspectives of SMIS.

Key Words: Super minimally invasive surgery; Minimally invasive surgery; Treatment mode; Traditional Surgery; New direction for surgery

Core Tip: The top goal of modern medicine is treating diseases without destroying organ structures and making patients as healthy as they were before their sickness. Minimally invasive surgery (MIS) has predominated among surgeries, but it fails to avoid some downsides of traditional surgery, as it still changes anatomical structures and leads to reconstruction of internal organs or different organs. In this study, I describe a new treatment mode, super MIS, which is defined as "curing the disease while preserving the integrity of the organs".

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INTRODUCTION

With the development of new equipment and the growing experiences of experts, surgery has become less invasive. Minimally invasive surgery (MIS) has dominated the surgical realm for years, but, no matter how developed MIS is, the treatment mode is still ultimately the same. Classic surgical operations are based on organ resection. This breaks the anatomical structures of the body to some degree. Reconstruction of internal organs or different organs is common after traditional surgery and MIS. The reconstruction changes the programmed body functions, which might lead to dysfunction of organ(s) or discomfort in the patients. Although the disease has been cured, the quality of life (QoL) of patients has decreased. Such treatment methods may be required for advanced cancers, but they can be too radical for early cancers or benign lesions.

The human body continues to evolve to adapt to its environment. The current physiological and anatomical structure should be most suitable for the modern environment. Each organ of the human body plays its own unique roles and they interact with each other. The operating mechanism of the body is complex, and little is known about it. Scientists have failed to explain the mystery of the human body comprehensively, and even the functions of individual organs have not been fully explained. For example, the appendix, which used to be regarded as useless, is now known to regulate immune functions. Treatments such as biomaterial implantation and organ transplantation aim to restore the original structure of the human body as much as possible and simulate its natural condition. The top goal of modern medicine (or treatment modes) is treating the disease without destroying the organ structure and making patients as healthy as they were before their sickness.

Therefore, a new treatment mode, named "super MIS (SMIS)", was first proposed by Linghu[1] in 2016. This new mode is defined as "curing the diseases that used to be treated by MIS while preserving the integrity of the organs". Organ resection is inevitable during MIS and traditional surgery. Organ resection and reconstruction are not involved in SMIS, making the procedures less invasive and promoting a better QoL[2,3]. SMIS includes not only endoscopic surgery, but also some surgical operations. Therapies for diseases are advancing over time and there are always more expected operative methods than existing ones.

MAIN CONTENT

SMIS is mainly operated through four operative channels. The first channel is the natural lumen, through which the surgery can be called natural orifice translumenal endoscopic surgery (NOTES). The following treatment methods are examples of SMIS through the natural orifice: Endoscopic submucosal dissection (ESD), endoscopic mucosal resection, endoscopic submucosal excavation, endoscopic full-thickness resection[4], and so on. The second one is the submucosal tunnel. A tunnel is made between mucosal and muscularis propria layers. This kind of SMIS is also called the digestive endoscopic tunnel technique (DETT) for gastrointestinal lesions[5]. The following treatment methods are classical examples of SMIS through the submucosal channel: Endoscopic submucosal tunnel dissection (ESTD), submucosal tunnel endoscopic resection, and peroral endoscopic myotomy. The third tunnel is the transmural channel, exemplified by video-assisted thoracoscopic enucleation which only resects lesions through the skin or cholangioscopy-assisted extraction of choledocholithiasis without endoscopic sphincterotomy[6,7]. The last one is multi-cavity channels. This type of SMIS includes the cooperation of different treatment methods, such as laparoscopy- or thoracoscopy-assisted endoscopic surgery.

Taking digestive diseases as an example, SMIS can be mainly divided into two types, resection and drainage. Resection can be applied to most of the benign lesions and early malignant lesions which need surgical removal while drainage is mainly applicable to benign lesions. Benign lesions, such as gastrointestinal stromal tumors, lipomas, leiomyomas, and polyps, can be resected by SMIS while malignant lesions, such as early gastrointestinal tumors and neuroendocrine tumors, could also be resected by SMIS without breaking the integrity of their located organs. For example, SMIS has been used to resect a large tumor at the cardia without breaking the integrity of the organ (Figure 1). Drainage is mainly used to treat pancreatic pseudocysts, pancreatic walled-off necrosis, gallbladder stones, biliary tract stones, suppurative cholangitis, and suppurative appendicitis. The applications of SMIS are restricted by surgical instruments and the experience of the operators. Not all benign lesions can be treated by SMIS. Lesions with a large size or rich blood supply are not indicated for SMIS. However, I believe that SMIS will have a broader scope for benign diseases in the near future. With more and more malignant lesions being detected in their early stage, more malignant lesions will also be treatable by SMIS.

The different treatment modes between SMIS and MIS/traditional surgery can lead to distinct prognoses. Taking early cancer located on the gastric cardia or near the gastric cardia as an example, ESD or ESTD was used to resect the tumor, leaving the cardial structures free from damage (Video 1). Both MIS and traditional surgery would resect not only the tumor but also the cardia, and even all or some of the stomach and some of the esophagus. The esophagus and stomach were connected to reconstruct an artificial gastroesophageal conjunction (Video 2). The gastroesophageal anastomosis failed to play the role of low esophageal sphincter, which has anti-reflux effects. Losing the cardia made patients suffer from abnormal gastrointestinal dynamics and reflux. These patients must sit up in bed to avoid the symptoms of heartburn. As compared to patients after MIS and traditional surgery, those after SMIS lived as healthy as they did without cardial cancer.

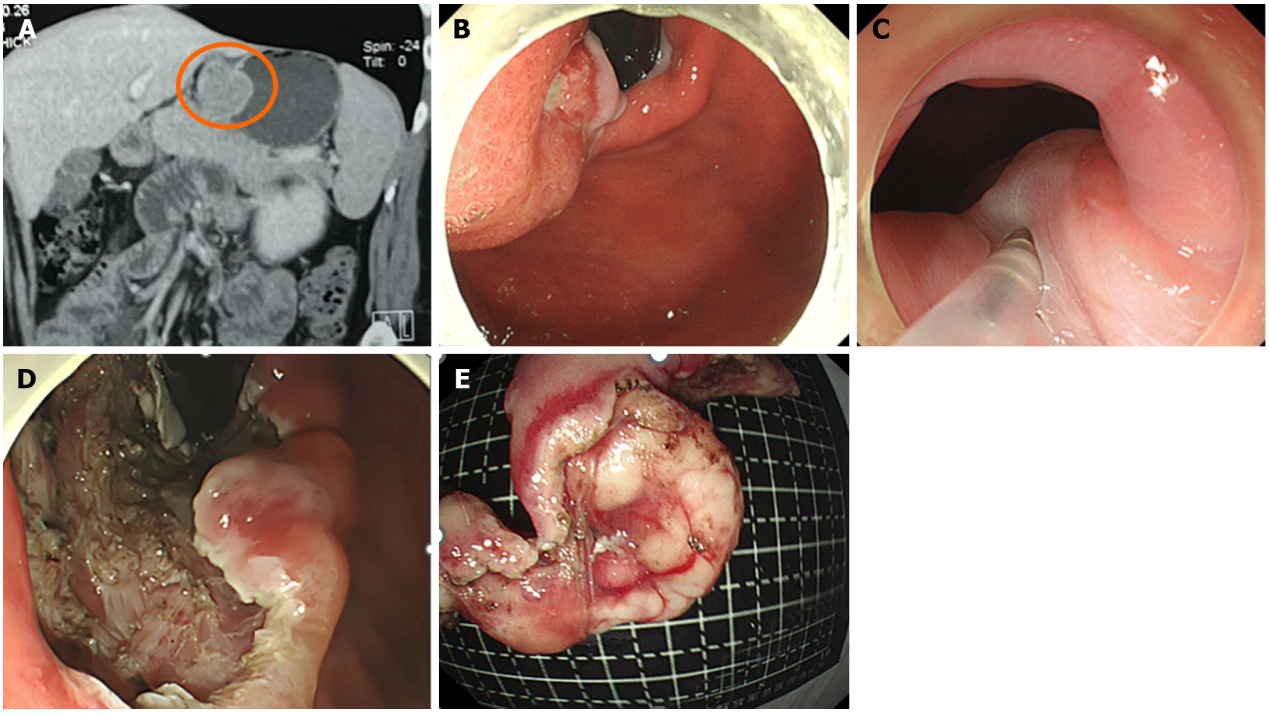

Figure 1 Resection of a large tumor at the cardia without breaking the integrity of the organ by super minimally invasive surgery. A: Huge tumor at the cardia found on CT scanning; B: Super minimally invasive surgery (SMIS) for the giant tumor; C: SMIS procedure; D: Postoperative wound of SMIS; E: The large tumor sample cut by SMIS.

CONCLUSION

I expect SMIS to be widely used in the near future. I hope that it can point to the new direction of surgery and be applicable to more than digestive diseases. I believe that all diseases could eventually be treated without changing any anatomic structure. SMIS could be regarded as a goal for the treatment of diseases that will be widely used for diseases of many systems.

FOOTNOTES

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REVIEW

Liquid biopsy for gastric cancer: Techniques, applications, and future directions

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Abstract

After the study of circulating tumor cells in blood through liquid biopsy (LB), this technique has evolved to encompass the analysis of multiple materials originating from the tumor, such as nucleic acids, extracellular vesicles, tumor-educated platelets, and other metabolites. Additionally, research has extended to include the examination of samples other than blood or plasma, such as saliva, gastric juice, urine, or stool. LB techniques are diverse, intricate, and variable. They must be highly sensitive, and pre-analytical, patient, and tumor-related factors significantly influence the detection threshold, diagnostic method selection, and potential results. Consequently, the implementation of LB in clinical practice still faces several challenges. The potential applications of LB range from early cancer detection to guiding targeted therapy or immunotherapy in both early and advanced cancer cases, monitoring treatment response, early identification of relapses, or assessing patient risk. On the other hand, gastric cancer (GC) is a disease often diagnosed at advanced stages. Despite recent advances in molecular understanding, the currently available treatment options have not substantially improved the prognosis for many of these patients. The application of LB in GC could be highly valuable as a non-invasive method for early diagnosis and for enhancing the management and outcomes of these patients. In this comprehensive review, from a pathologist's perspective, we provide an overview of the main options available in LB, delve into the fundamental principles of the most studied techniques, explore the potential utility of LB application in the context of GC, and address the obstacles that need to be overcome in the future to make this innovative technique a game-changer in cancer diagnosis and treatment within clinical practice.

Key Words: Liquid biopsy; Gastric cancer; Circulating tumor cells; Cell-free DNA; Circulating tumor DNA; Molecular

Core Tip: Liquid biopsy (LB) has the potential to revolutionize cancer diagnostics, offering reduced invasiveness and improved understanding of tumor heterogeneity. Going beyond examining circulating tumor cells and cell-free DNA in blood, LB studies now explore a variety of structures and non-blood samples. Despite advancements in LB for tumor detection, prognostic assessment and treatment guidance, challenges remain, including complex and expensive techniques, lack of standardization, and suboptimal scientific evidence. In the context of gastric cancer, LB represents a promising approach, especially in advanced stages. This review navigates through LB intricacies, emphasizing its benefits while urging for future improvements to achieve clinical impact.

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INTRODUCTION

Over the past few decades, remarkable technical and molecular advancements in the field of biological sciences, particularly in cancer research, have given rise to innovative techniques that improve conventional approaches. Liquid biopsy (LB) has emerged as a groundbreaking method for detecting and analyzing specific molecules and cells in bodily fluids. While the majority of LB studies have focused on circulating tumor cells (CTCs) or circulating tumor DNA (ctDNA) in peripheral blood samples, other possibilities, such as the analysis of exosomes or tumor-educated platelets (TEPs), or the utilization of non-blood samples, have come to the forefront. The primary objectives of LB in cancer encompass early tumor diagnosis, treatment monitoring, timely detection of relapses, prognosis determination, and the identification of therapeutic biomarkers in both early and advanced cancer cases. In contrast to conventional biopsy, LB holds the advantages of being a minimally invasive technique that can capture the spatial and temporal tumor heterogeneity, by reflecting various cell clones present in the body at a given moment. However, LB techniques must be highly sensitive, and pre-analytic factors, patient and tumor features significantly impact the detection threshold, diagnostic methodology, and potential outcomes. Additionally, LB techniques are complex, expensive, and currently lack standardization. Consequently, the integration of LB into clinical practice faces several challenges.

On another note, gastric cancer (GC) is a disease with a poor prognosis, particularly in Western countries. Significant differences exist between Western and Asian patients, with GC being more prevalent in Asian countries. Geographic variability has been also observed in clinical, histological, prognostic, molecular and treatment response factors. While some high-incidence countries like Japan and Korea have implemented screening strategies that improve patient prognosis, these techniques are not considered to be cost-effective in Western countries, leading to the detection of most cases at late stages. Furthermore, recent advances in molecular pathology and targeted therapies have not significantly influenced the prognosis of GC patients. Therefore, there is a need to facilitate early diagnosis, improve patient selection for various treatments, and identify new therapeutically relevant biomarkers. In this scenario, the application of LB holds great potential for GC. Previous studies have assessed the utility of analyzing CTCs, ctDNA, and other molecules in peripheral blood samples from GC patients in different contexts. The potential value of using non-blood samples, such as gastric juice or urine, is also under investigation.

In this comprehensive review, from a pathologist's perspective, we provide an overview of the main options available in LB. We delve into the fundamental principles of the most studied techniques, explore the potential utility of LB in GC, and address the obstacles that need to be overcome in the future to make this innovative technique a game-changer in cancer diagnosis and treatment within clinical practice.

DEFINITION

LB is a term commonly used to refer to the detection and analysis of circulating DNA or cells in peripheral blood. However, it encompasses laboratory examinations of various bodily fluids, and includes the detection of diverse tumor-associated structures[1] (Figure 1).

Consequently, LB can be applicable to fluids such as blood, serum, plasma, urine, sputum, or cerebrospinal fluid. In addition, it enables the identification of CTCs, cell-free DNA (cfDNA), ctDNA, cell-free RNA (cfRNA), long non-coding RNA (lncRNA), microRNA (miRNA), extracellular vesicles, TEPs, proteins, or metabolites[2].

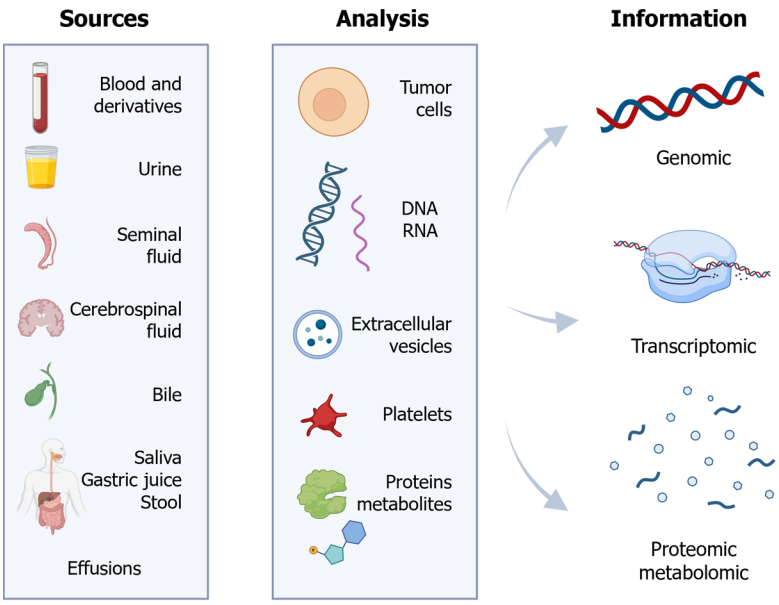

Figure 1 Key specimens, analyzable structures, and information obtained through liquid biopsy techniques. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

HISTORICAL OVERVIEW

Exploring the theoretical foundations of LB reveals its historical roots in ancient Greece, where the belief in the diagnostic potential of bodily fluids was prevalent[3]. Notably, in the 5th century B.C., Hippocrates formulated the humoral theory, linking the onset of disease to imbalances among the four humors of the body[4].

Moving closer to the present, the subspecialty of cytology emerged in the 19th century in the field of pathology. Cytology involves the analysis of fluids for diagnostic purposes, as opposed to the use of "solid" tissue fragments in conventional histology. Exfoliative and aspiration cytology are the two primary branches[5] (Figure 2).

The evolution of cytology in pathology parallels advances in cancer knowledge: originally a tool for morphological diagnosis under an optical microscope, it has become instrumental in the morphological-molecular diagnosis[6]. Despite some overlap between cytology, when employed for current molecular diagnostic purposes, and LB, key distinctions arise. Notably, blood, plasma, or serum samples are not typical subjects of cytological studies. Moreover, cytology enables the correlation of findings from complementary techniques (molecular, immunohistochemical, or histochemical) with the microscopic morphology of the cells present in the sample. Additionally, detection procedures in LB should exhibit heightened sensitivity, owing to the scarcity of target molecules or cells in LB samples.

Narrowing the focus to the field of LB, its foundational contributions can be attributed to Dr. Thomas Ashworth in 1869. In that year, a study discovered the existence of CTCs in the plasma of a patient with metastatic cancer[7]. Subsequent observations highlighted the prognostic value of CTCs in oncology, their presence in early-stage tumors, and their potential utility in guiding patient prognosis or treatment[8]. The first commercial approval for CTC utilization in clinical practice materialized in 2004, with the United States Food and Drug Administration (FDA) endorsing the CellSearch® platform for isolating and enumerating CTCs in metastatic breast cancer patients. This approval extended in 2007 and 2008 to encompass patients with colorectal and prostate cancer[9]. While the CellSearch® system remained the sole entity granted for capturing CTCs until 2022, other technologies, such as the Parsortix® PC1 system, have recently garnered approval[10].

The analysis of nucleic acids in blood was addressed later, beginning with cfDNA. The first detection of this molecule in plasma occurred in 1948[11]. Decades later, it was observed that the amount of cfDNA is higher in cancer patients, and it was confirmed that these molecules could originate from tumor cells. In the 1990s, a significant stride was made as mutations began to be discerned using cfDNA extracted from blood and other samples of patients with solid and hematological tumors, followed by the performance of methylation studies[12]. Clinical determination of genetic alterations in cfDNA became possible in 2005, but it was not until 2014 and 2016 that cfDNA-based mutational tests secured approval from two pivotal international regulatory bodies: European Medicines Agency (EMA) and FDA. In 2014, the EMA authorized the identification of EGFR mutations in cfDNA extracted from blood samples of lung carcinoma patients, with the aim of guiding gefitinib treatment when no tissue sample was available[13]. In 2016, the FDA approved the detection of specific EGFR mutations in blood samples to facilitate patient selection for erlotinib treatment[14]. Simultaneously, in 2016, the use of cfDNA was approved to detect the EGFR T790M mutation and guide treatment with osimertinib in lung cancer patients[15].

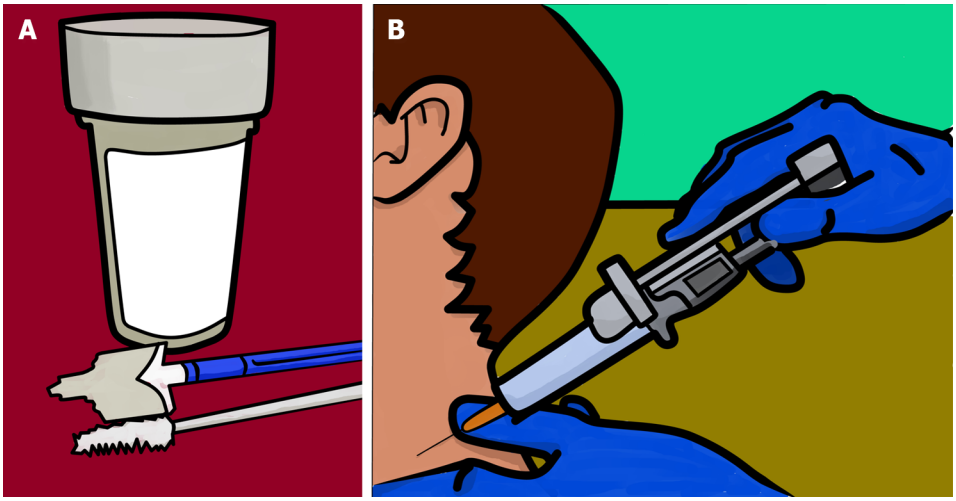

Figure 2 Main branches of cytology. A: Exfoliative cytology; the study of cells shed from body surfaces, collected either spontaneously or mechanically; B: Aspiration cytology; analysis of fluids obtained *via* fine-needle aspiration, from both superficial and deep tissues, with or without image-guided control. Image created by Tony Punk (contacto@tonypunk.es).

ADVANTAGES AND DISADVANTAGES OF LB

Advantages

LB offers notable advantages, including: (1) The non/minimal invasiveness of the technique; (2) the ability to investigate both the spatial and temporal heterogeneity of tumors; and (3) the possibility of conducting serial studies[16]. Considering the molecular divergence between primary tumors and various metastatic sites, it is advisable to study the profile of each of them[17]. This approach proves particularly valuable in patients with already diagnosed primary tumors and metastatic lesions in challenging locations, in order to tailor specific treatments for each cell subclone and enhance the likelihood of eradicating all tumor cells in the body.

Disadvantages

However, LB is not without its drawbacks. A significant limitation arises from the substantial methodological variability observed across different studies, hindering the standardization of LB procedures and the comparison of research results. Further exploration of these disadvantages is provided in the section addressing "future challenges".

TYPES OF DETERMINATION AND TECHNICAL FOUNDATIONS

As previously mentioned, LB can analyze CTCs, circulating nucleic acids, extracellular vesicles, TEPs, proteins, or metabolites[2]. Among these, the most studied structures are CTCs and cfDNA.

CTCs

Metastases are the leading cause of therapeutic failure and cancer-related deaths. The metastatic process involves the steps depicted in Figure 3[18,19].

CTCs are tumor cells that detach from the primary tumor and circulate in the blood. They generally constitute a very small percentage of blood-circulating cells, which are predominantly leukocytes[20]. Remarkably, CTCs are considered as rare as one in a billion circulating cells[21]. Furthermore, only a small percentage of these cells will survive and give rise to metastases, underscoring the crucial interaction between the blood and metastatic niche microenvironment and CTCs [22].

Blood circulation and interaction with immune cells: CTCs circulate in the peripheral blood both individually and in clusters, and can interact with various immune cells. This interaction may protect CTCs from the aggressive blood microenvironment and promote their survival, proliferation, migratory capacity, and metastatic potential[23].

Interaction with neutrophils: Neutrophils are the most abundant leukocytes in the blood. The CTC-neutrophil interaction can support tumor progression, either through direct neutrophil-CTC interaction (via adhesion molecules) or indirectly, through the release of certain substances or the formation of structures by neutrophils, including neutrophil extracellular traps (NETs)[24,25].

Interaction with macrophages: *In vitro* studies have shown that the interaction between CTCs and circulating monocytes in the blood can promote their differentiation into macrophages, the secretion of various mediators for leukocyte recruitment, and the migration and invasiveness of CTCs[26]. Moreover, this interaction may enhance the intravasation of CTCs and epithelial-mesenchymal transition (EMT), boosting tumor heterogeneity and improving the metastatic potential of CTCs[27].

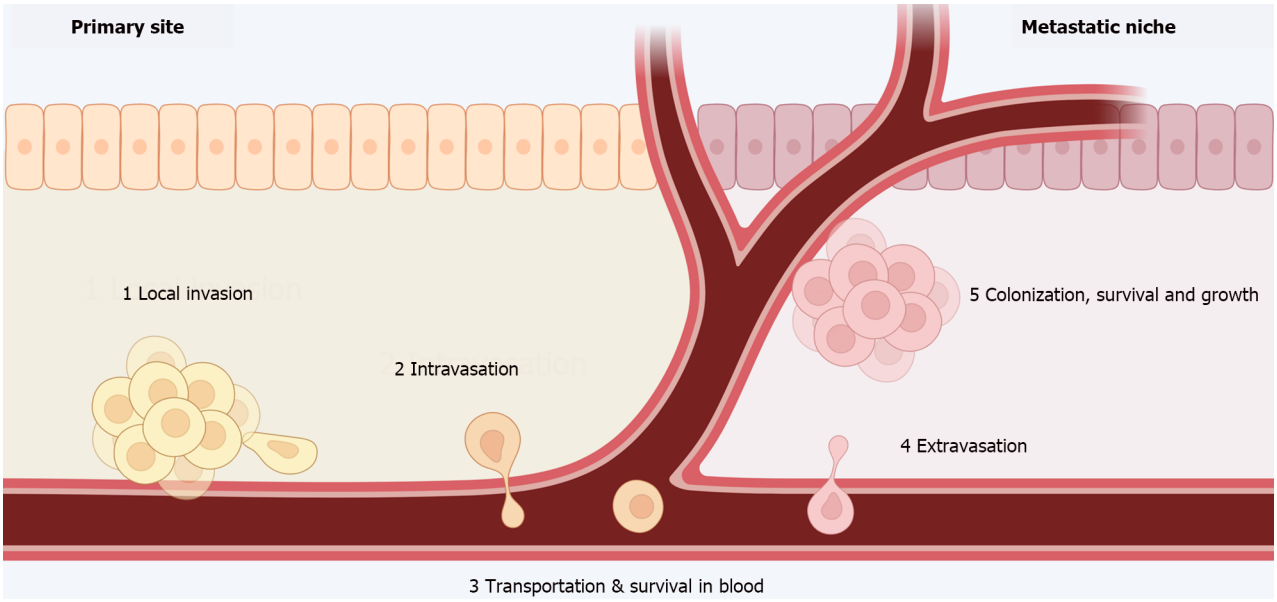

Figure 3 Metastatic process. (1) Local invasion of tumor cells into adjacent tissues; (2) intravasation; (3) transportation and survival through the blood as circulating tumor cells; (4) extravasation; and (5) colonization, survival, and growth in distant organs until detectable metastases develop. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

Interaction with platelets: Activated circulating platelets promote the survival of CTCs and their colonization and growth in secondary sites[28]. CTC-platelet aggregates appear to protect CTCs from mechanical stress and NK cells[29]. Additionally, CTC-platelet interactions can promote vascular permeability and CTC adhesion to endothelial cells, facilitating tumor extravasation to metastatic locations[30].

Interaction with myeloid-derived suppressor cells (MDSCs): MDSCs have immunosuppressive and metastasis-promoting capabilities. The MDSC-CTC interaction seems to help CTCs evade T-cell-mediated responses and promote their proliferation[31].

Interaction with cancer-associated fibroblasts (CAFs): Preliminary studies indicate that CTCs can transport CAFs to metastatic locations, and CAFs could protect CTCs from mechanical stress and promote their survival, invasion, and EMT [32,33].

Isolation and detection: As mentioned earlier, CTCs are extremely rare, occurring at a frequency of approximately 1 in a billion peripheral blood cells. This poses a significant technological challenge for their isolation. Advances in detection techniques, as reflected in recent studies, are anticipated to continue progressing[9,23]. Due to their scarcity, enrichment strategies become essential for separating CTCs from blood cells, enabling the acquisition of a CTC-enriched fraction for CTC purification or downstream analyses.

For the isolation and detection of CTCs, various methods have traditionally been employed, including physical methods based on density, deformability, or cell size, magnetic techniques, or immune-based methods[34] (Figure 4). In addition to antibody-based strategies, other techniques such as fluorescence in situ hybridization, cytometric methods, or nucleic acid-based detection (RT-qPCR) can be utilized for CTC detection. Many systems combine these approaches to directly isolate and detect CTCs. These methods can also be categorized as "negative" if they remove immune cells to isolate CTCs or "positive" if they actively select CTCs[35].

It is crucial to note that while the immune positive approach is commonly used, it comes with limitations. The primary challenge lies in the potential loss of CTCs, as a significant number may lose EpCAM expression, due to the EMT phenomenon, the acquisition of stemness features or a histologic type-dependent decrease in expression[36-39]. To overcome this limitation, antigen-based negative methods can be employed, including antibodies to identify blood cells such as CD45. Moreover, novel platforms utilizing microfluidics and/or nanomaterials have emerged[40]. Examples include CTC-iChip, which combines leukocyte marking with antibodies and cell selection based on size, enhancing CTC purity and viability[41]. The main challenges for these new systems lie in their long analysis time and high cost, prompting efforts to automate the process and to reduce costs in subsequent studies[42-45].

Main applications of the study of CTCs: Initially, CTC isolation platforms were used to detect the presence or absence of CTCs in blood, count them, and correlate these counts with patient prognosis or treatment response. For instance, in the years 2004, 2007 and 2008, the CellSearch® system was approved for determining the prognosis of metastatic breast, colorectal and prostate cancer patients, respectively[9] (Figure 5). The identification and enumeration of CTCs in peripheral blood was associated with decreased progression-free and overall survival (OS) in various studies[46,47].

Subsequently, technological advances enabled the study of the genome, transcriptome, and proteome of CTCs. In 2022, the FDA approved the use of the Parsortix® PC1 platform for isolating CTCs in patients with metastatic breast cancer, allowing downstream analysis using validated molecular techniques[10] (Figure 6). This approval specifically pertained to CTC enrichment, and it did not include the identification and enumeration of CTCs with prognostic or patient

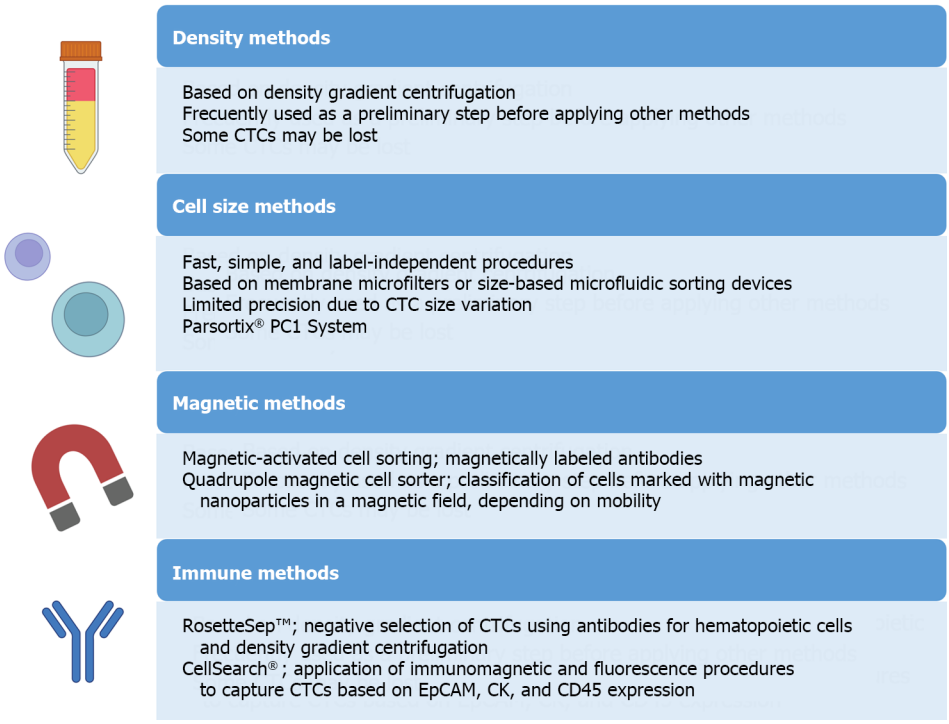

Figure 4 Principal methods developed for the isolation and detection of circulating tumor cells. CTCs: Circulating tumor cells. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

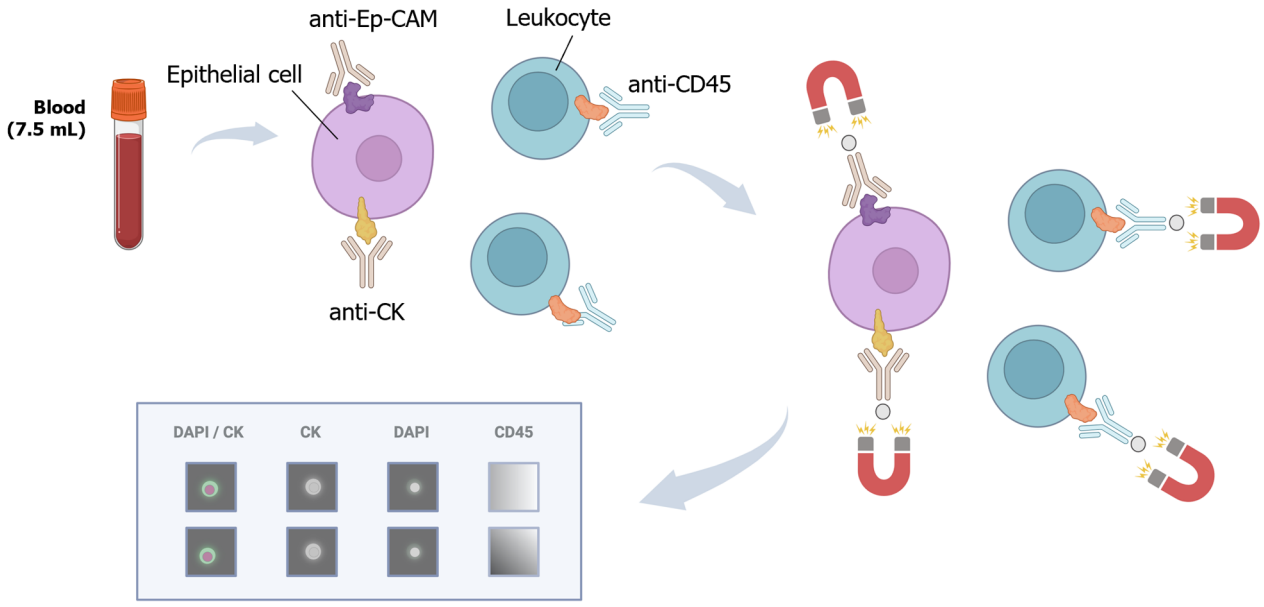

Figure 5 CellSearch® platform. The system analyzes a 7.5 mL blood sample using a ferrofluidic capture reagent, immunofluorescent reagents, and a magnetic field. The ferrofluid reagent comprises a magnetic core surrounded by a polymer layer coated with EpCAM antibodies to capture circulating tumor cells. Fluorescent reagents (CK-PE, DAPI, and anti-CD45) are added, and the sample is loaded into a cartridge within a strong magnetic field. The platform scans the surface of the cartridge, acquires images, and presents them in a gallery format for final classification. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

management purposes. Characterizing and analyzing isolated CTCs can be useful for diagnosis, prognosis determination, treatment response, patient management, and for identifying clinically relevant CTC subtypes, as seen in previous studies including patients with lung, breast, colorectal, hepatocellular, prostate cancer or melanoma[48-57]. Interestingly, differences have been observed between the genomic, transcriptomic, and proteomic profiles of the primary tumor and CTCs[58].

Advantages and disadvantages of CTCs: Studying CTCs within the context of LB presents several advantages over investigating other cells or molecules[23]. Firstly, as CTCs are considered pre-metastatic populations, delving into their

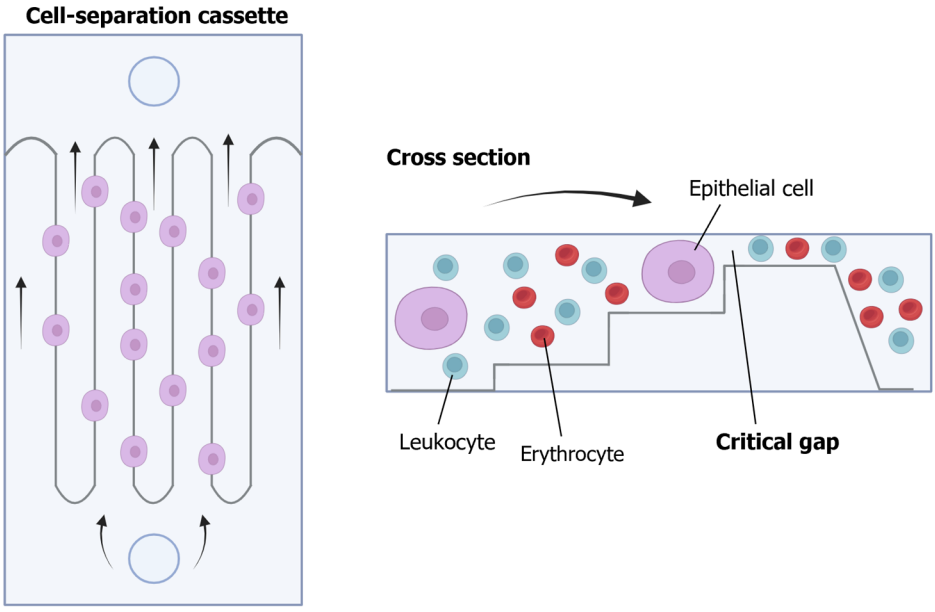

Figure 6 Parsortix® PC1 system. This platform is a physical method for detecting circulating tumor cells from a blood sample, suitable for subsequent user-validated downstream analyses. It incorporates single-use cell separation cassettes, allowing blood to pass through until reaching a critical gap where circulating tumor cells are captured. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

study has the potential to significantly enhance our comprehension of the cancer life cycle and the development of metastases. Secondly, CTCs provide an opportunity for cell cultures or can be implanted in mice to establish tumor models, thereby greatly facilitating the personalization of treatment[59].

However, the challenges in analyzing CTCs in blood lie in their scarcity, the absence of entirely sensitive and specific markers for their detection, the difficulty in obtaining viable cells for culture, and the lack of standardization, in addition to high associated costs and large processing times[60,61].

Addressing the first obstacle necessitates the development and enhancement of highly precise technologies for both isolating and detecting CTCs and subsequently analyzing them. As for the second challenge, rescuing the inadequate isolation of CTCs by EpCAM-based technologies is feasible by incorporating both epithelial and mesenchymal cancer markers, or by using marker-independent detection methods[62,63]. The utilization of mesenchymal markers alongside epithelial markers offers the potential to identify distinct CTC populations. Despite the drawback of including circulating mesenchymal cells associated with the tumor as CTCs, some studies suggest that these cells also correlate to patient prognosis and enhance the metastatic potential of CTCs[64]. Concerning the third challenge, most platforms currently in use fail to yield cells suitable for culturing or mouse models due to processes that compromise their viability, primarily during cell labeling and immobilization. Certain technologies, however, enable the retrieval of cells with enhanced viability for subsequent studies[41]. As for the fourth challenge, it is foreseeable that these techniques will evolve, become more automated, and undergo standardization in the coming years. In this process, digital pathology could play an essential role[61].

cfDNA/ctDNA

cfDNA consists of double-stranded DNA fragments found in bodily fluids, originating from both normal and pathological cells[65]. cfDNA typically circulates bound to proteins that shield it from degradation, and it primarily arises from cell death phenomena, including necrosis and apoptosis. Other significant sources of cfDNA include NETosis or extracellular vesicles[66-68]. Elevated levels of cfDNA have been observed in patients with benign lesions, inflammatory diseases, or tissue trauma[69]. cfDNA also holds value in the non-invasive prenatal diagnosis of fetal genetic diseases, as a percentage of fetal DNA is present in the peripheral blood of pregnant women[70]. In cancer patients, cfDNA comprises a mixture of DNA from normal cells and, theoretically, DNA from different tumor subclones and locations, forming ctDNA[71]. Moreover, the presence of cell-free mitochondrial DNA circulating as small fragments has been identified, correlating with the severity of physical or psychological trauma, cancer detection and burden, as well as the occurrence and severity of other diseases[72,73].

Origin and characteristics: According to previous studies, the majority of circulating cfDNA fragments in blood are 150-180 base pairs long, aligning with the DNA content of a nucleosome[74]. The relationship between fragment length and the presence or absence of cancer is contradictory. In various cancer types, both an increase and a decrease in cfDNA integrity have been observed. Generally, it is accepted that ctDNA is more fragmented than cfDNA. Some studies have shown that ctDNA is longer in tumors with a high necrotic rate and shorter in cases with increased apoptosis[75,76].

The percentage of cfDNA is typically low but varies among patients. This percentage is higher in cancer patients, yet, in most cases, it remains below 100 ng/mL. Additionally, in most cancer patients the majority of cfDNA comes from non-

tumor cells, including both blood cells and infiltrating non-tumor cells[77]. The reported range of ctDNA relative to cfDNA varies between < 0.5% to 95.0%, being lower in early stages (around 1%) and higher in advanced stages (up to 40%)[78-80]. This percentage varies not only with disease burden but also with features such as proliferation and apoptosis rates, necrosis, inflammation, tumor microenvironment, patient and treatment-related factors.

Finally, cfDNA undergoes rapid turnover, resulting in a very short half-life. Pioneering studies in pregnant women observed that fetal cfDNA in maternal blood has a half-life of approximately 15 min post-partum, becoming undetectable after 2 h[81]. Currently, cfDNA is considered to undergo a two-phase clearance, with a rapid phase within the first 10 min to the first hour and a slow phase with a half-life of 13 h. However, in cancer patients, the half-life of ctDNA seems to be less than 2 h[82].

Main applications of ctDNA analyses: As observed with CTCs, ctDNA has multiple potential clinical applications, including screening, characterizing early disease, detecting molecular residual disease (MRD), predicting relapses, genotyping advanced cancer, early assessment of treatment efficacy, monitoring response, and identifying mechanisms of resistance to therapy.

To date, the FDA has approved several ctDNA-based companion diagnostic assays for the safe and effective use of various targeted therapies, mainly in metastatic tumors[83]. These indications include PCR-based techniques for detecting EGFR or PIK3CA alterations in NSCLC and breast cancer, respectively, as well as the use of next-generation sequencing (NGS) platforms in various cancers, such as metastatic breast, prostate, lung, or ovarian carcinomas. However, a key limitation of currently FDA-approved ctDNA-based assays is that a proportion of patients with mutation-positive tumors in tissue-based testing may not be detected[84].

Ongoing studies aim to validate the analysis of ctDNA in early-stage patients and other clinical contexts, exploring additional genetic alterations and applications beyond specific drug treatments in metastatic patients. For example, in early stages, the detection of MRD can prove valuable in identifying patients at risk of relapse and tailoring treatment accordingly. Across different tumor types, the use of ctDNA has shown high positive predictive value with an acceptable negative predictive value[85,86]. Interestingly, serial ctDNA determinations can enhance the sensitivity of MRD detection. Additionally, relapse can be detected in LB before it becomes visible in other clinical or radiological tests. In the metastatic context, ctDNA determination can be useful for monitoring tumor evolution, assessing treatment resistance, determining patient management, or indicating other treatments, such as immunotherapy[87-89].

Advantages and disadvantages: As advantages over CTCs, ctDNA is more abundant, making its study less technologically complex and potentially more sensitive and specific[90]. CTCs reflect more of the metastasis-initiating cells, while ctDNA represents more of the tumor burden. Thus, ctDNA carries the evolutionary information of both primary and metastatic tumors as cancer cells dynamically evolve in response to intermittent drug treatment. Moreover, in recent years, there has been progress in ctDNA analysis, transitioning from mostly discrepant results to having validated tools that can be implemented in clinical practice.

However, the drawbacks of ctDNA analysis should be considered. Firstly, there is currently no platform that serves all potential applications of ctDNA, as technologies vary depending on whether they are used to detect cancer in early stages, monitor patients in advanced stages, detect genetic alterations for treatment resistance, or other purposes[84]. Pre-analytical variables such as tumor-related factors, patient characteristics, treatment history, collection tube, storage conditions, and processing methods significantly impact results[91]. Secondly, despite the higher abundance of ctDNA compared to CTCs, false negatives may occur due to concentration variations and technique-related issues. Thirdly, false positives have also been detected with cfDNA studies, as normal cells can present somatic alterations and clonal expansion[92]. Finally, the clotting process during serum preparation induces cell lysis, so ctDNA analysis might be hampered by increased levels of high-molecular cfDNA when using serum instead of plasma. Due to this, plasma has been suggested as the better specimen type for ctDNA analysis[93].

Other molecules or structures to analyze in LB

While ctDNA and CTCs dominate LB research, several other structures in body fluids hold potential clinical value, albeit with challenges due to limited evidence and procedural standardization.

cfRNA: cfRNA reaches the blood mainly through passive release due to apoptosis and necrosis phenomena, active secretion by microvesicles, and secretion with nucleoproteins or protein-RNA complexes. It is usually found in association with extracellular vesicles and lipoprotein complexes that prevent its degradation and can be secreted by both normal and pathological cells[94]. Free RNA is unstable and degrades within seconds of incubation, requiring these vehicles for stabilization[66].

Beyond serving as a prognostic marker or aiding in early cancer and relapse diagnosis, cfRNA allows the analysis of the expression signature of a tumor. This is particularly valuable for identifying biomarkers, gene expression patterns, understanding intercellular communication, the tumor immune environment, and determining the tissue of origin and/or subtype of a tumor through specific markers[95]. However, challenges include the low quantity of cfRNA in blood, its susceptibility to degradation, and the complexity of its extraction.

Clinical implementation of cfRNA has seen fewer trials compared to ctDNA, with limited available evidence. Notably, the FDA approved one platform in 2012, the PROGENSA PCA3 assay, which detects PCA3 RNA in urine and is useful for determining the risk of prostate cancer[96,97].

Extracellular vesicles: These structures are secreted by all living cells and can be detected in any fluid. They are heterogeneous, represent their cells of origin, and contain associated molecules (proteins, nucleic acids, lipids, and metabolites) relevant for diverse applications in LB[98].

EVs can be classified as exosomes if they come from the intracellular endosomal system, or ectosomes/microvesicles formed directly from the plasma membrane[99]. Additional categories include "apoptotic bodies", "large oncosomes", and "migrasomes"[100]. On the other hand, EVs can also be classified as "small EVs" if they are 200 nm or less and "large EVs" if they are larger[101]. In recent years, exomers, small non-membranous particles of 50 nm or less, have also been described, which seem to be related to the regulation of metabolic pathways[102].

The main function of EVs is intercellular communication, with their specific function varying depending on the type and state of the originating cell[100]. In cancer, EVs seem to promote metastasis, angiogenesis, immune evasion, adaptation, and resistance to treatment[103]. Interestingly, they not only serve for communication with nearby cells but also seem to reach distant cells.

For their isolation and detection, techniques similar to those used for CTCs have been developed[104,105]. For their characterization, transmission electron microscopy can be used, or quantification and characterization can be done with methods such as dynamic light scattering technique or nanoparticle tracking analysis[105]. After their isolation, the molecules present in EVs can be studied with lipid, proteomic, transcriptomic, or genomic analysis[98].

The study of EVs holds clinical potential in detecting biomarkers for cancer diagnostics, treatment monitoring, predicting disease progression, and assessing therapy resistance. However, only three platforms for EV analysis have been commercialized: ExoDx™ Lung, ExoDx™ Prostate IntelliScore, and MedOncAlyzer™ 170[98,104,106] (Figure 7).

The study of EVs has the advantage of encompassing multiple types of molecules, enabling its use in multi-analyte platforms to detect various alterations. In addition, EVs are more abundant than CTCs in all body fluids and more stable than cfDNA due to the protection of their lipid membranes, so EV-based techniques could be more sensitive than these other two approaches. However, EVs are very heterogeneous structures, and there are no detailed characterization techniques available. They are highly variable depending on the physiological or pathological process, and the available evidence is generally scarce.

TEPs: Platelets are anucleated blood cells derived from megakaryocytes that, when activated, form aggregates and secrete a considerable amount of substances into the environment. Traditionally recognized for their role in hemostasis, thrombosis, and wound healing processes, platelets have also been observed to play roles as mediators in cancer development, particularly in the onset of metastasis[107]. TEPs alter their function depending on signals produced by tumor cells, developing a distinctive phenotype and releasing substances that promote cell growth and survival, neoangiogenesis, immune escape, and extracellular matrix remodeling, thereby facilitating tumor progression, migration, and spread[108].

Concerning their potential clinical value, the analysis of TEPs has shown utility in cancer detection, monitoring treatment response, and studying RNA profiles[109].

MAIN TYPES OF SAMPLES

While blood and its derivatives, such as plasma or serum, are the most commonly used samples, studies on LB can be conducted using various other sample types[110,111]. Depending on the tumor type and/or its anatomical location, these alternative fluids may offer greater sensitivity and specificity compared to blood sources. This is due to their increased contact with the tumor, resulting in a higher concentration of tumor-derived molecules like ctDNA. Additionally, these alternative samples can complement plasma studies, and some can be self-collected by the patient. However, their clinical implementation is complex due to the need to standardize pre-analytical factors, the lack of commercially available kits, and the requirement for invasive procedures to obtain certain samples, such as effusions or cerebrospinal fluid. This complexity may limit their serial use for monitoring purposes. Table 1 summarizes the main applications, advantages, and limitations of non-blood samples, according to the review by Tivey *et al*[112] and recent research articles[113-115].

GASTRIC CANCER

Overview

GC ranks as the fifth most common cancer worldwide, with over one million new cases diagnosed in 2020[116]. It exhibits significant geographical variability, being more prevalent in Asian countries and some Eastern European nations, while less common in Western regions[117]. Certain countries, such as Japan and Korea, have implemented screening strategies based on imaging techniques that have improved early diagnosis and prognosis for GC patients[118,119]. However, disparities not only exist between Asian and Western countries regarding GC incidence and early detection. Geographical variations have also been observed in clinical, morphological and molecular features, prognosis, and risk factors for GC [120-122]. In terms of patient outcomes, GC is an aggressive tumor ranking as the third leading cause of cancer death globally. In Western countries, it is often diagnosed at advanced stages, presenting 5-year survival rates of 20%-30%[123, 124].

In recent years, significant advances in technical tools have enhanced the understanding of the molecular features and immune environment of tumors, and their potential prognostic and therapeutic roles. This knowledge has led to an expanded therapeutic arsenal, focusing on immunotherapy and targeted therapies, which generally exhibit better efficacy and fewer side effects than conventional treatments. However, these advancements have not significantly improved the prognosis for a substantial portion of GC patients[125].

Table 1 Main non-blood specimens for liquid biopsy studies

Sample	Tumor	Main features
Cerebrospinal fluid	Primary and metastatic CNS	Helpful due to the frequent metastatic seeding, the difficulty in obtaining tissue biopsies from this location, and the obstruction of the blood-brain barrier
Urine and seminal fluid	Prostate, urothelial, renal	Renal cancer-derived structures may be limited by glomerular filtration; cfDNA concentration and fragment size in seminal fluid seem to differ between healthy and prostate cancer patients
Stool	Colorectal	ctDNA extraction can be complex; ctDNA may be present in low concentrations (mixed with DNA from the bacterial flora)
Pleural or peritoneal fluid	Lung, mesothelioma, peritoneal spread	Determination of predictive biomarkers in lung cancer; detection of minimal residual disease or early involvement of the peritoneum
Saliva	Oral cancers	Monitoring of minimal residual disease and treatment response; low ctDNA concentration, limited fragment size
Bile	Biliary tract	Useful in difficult-to-biopsy cases; invasive procedure
Gastric juice	Gastric	Improvement of diagnostic sensitivity; invasive procedure (endoscopy)

CNS: Central nervous system; ctDNA: Circulating tumor DNA; cfDNA: Cell-free DNA.

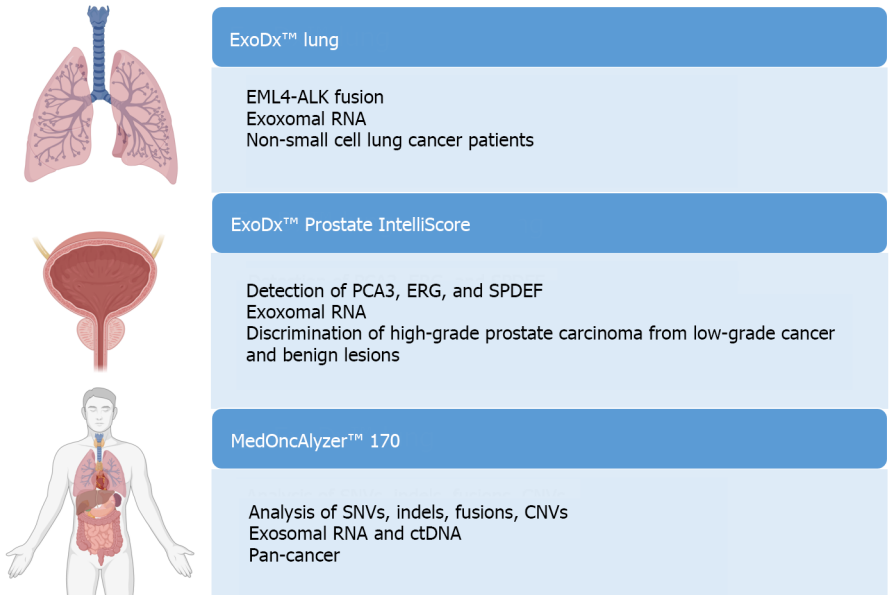

Figure 7 Primary platforms approved for the analysis of extracellular vesicles in liquid biopsy. SNV: Single nucleotide variant; CNV: Copy number variant; ctDNA: Circulating tumor DNA. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

Diagnosis and treatment of GC

GC diagnosis relies on the interpretation of tissue biopsies by a pathologist. Microscopically, it can be classified according to the latest World Health Organization (WHO) system from 2019, into four main types: Tubular, poorly cohesive, papillary, and mucinous[126]. According to the Laurén classification, described in 1965 and still used in clinical practice, GC can be divided into intestinal, diffuse, and mixed types[127]. As observed by our research team in a previous study, supporting findings from other investigators, there are differences in clinical, molecular, and prognostic characteristics between intestinal and diffuse GC[128]. Therefore, studying Laurén subtypes separately could enhance patient stratification both in clinical practice and in clinical trials. Other microscopic findings, such as tumor budding or tumor infiltration by inflammatory cells, may also have prognostic implications or aid in treatment selection[129].

After diagnosis, the only curative treatment available for GC is surgery. In early GC cases, which are scarce in Western countries, endoscopic surgery techniques, such as endoscopic mucosal resection or endoscopic submucosal dissection, can be performed[130]. In other resectable cases, total or subtotal gastrectomy, usually associated with D2 lymphadenectomy, is commonly performed. However, in advanced cases, treatment primarily relies on chemotherapy regimens. The only specifically approved targeted therapies for GC are trastuzumab for cases with amplified HER2 and anti-VEGF/VEGFR drugs[131]. Furthermore, GC is included in the FDA-approved indication for pembrolizumab (anti-PD-L1) for all solid tumors in cases with high microsatellite instability (MSI) or deficiency in the DNA mismatch repair system[132].

Molecular characteristics of GC

A broad spectrum of molecular alterations has been identified in GC, owing to the wealth of information generated by new genomic and transcriptomic studies[133]. These alterations can be categorized into isolated changes, disregulation of pathways involved in GC pathogenesis, gene expression profiles, and molecular classifications integrating available molecular information[134-136].

Isolated alterations primarily include mutations and copy number alterations (CNAs), although heterozygosity and gene fusions have also been observed[133]. Mutations in genes such as TP53, BRCA2, cell adhesion genes, or genes encoding proteins in chromatin regulatory complexes or histone regulatory complexes are notable. Affected pathways include the WNT/beta-catenin pathway, alterations in CTNNB1, or different tyrosine kinase receptor pathways (HER, PI3K, *etc.*). Regarding CNAs, amplifications in genes associated with tyrosine kinase receptor pathways, such as HER2, EGFR, MET, KRAS, or FGFR2, are prominent[137,138]. Amplification of PD-L1 and PD-L2, genes related to the cell cycle, and various transcription factors has also been observed. Epigenetic alterations include the inactivation of various tumor suppressor genes by promoter methylation, such as CDH1, MLH1, CDKN2a, or PTEN.

On the other hand, the most important molecular classifications in GC have been published by the Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG)[137,138] (Figures 8 and 9). These systems have been associated with clinical, prognostic and predictive factors. Additionally, other molecular classifications have been published by Shah *et al*[139], Tan *et al*[140], Lei *et al*[141], Wang *et al*[142], Wang *et al*[143], or Furukawa *et al*[144].

Unfortunately, these categorizations have not been translated into clinical practice, primarily due to the need for extensive molecular studies and the variety of alterations included in each molecular group. Our research team and other investigators have managed to formulate stratification systems derived from these classifications, primarily those of TCGA and ACRG, using immunohistochemistry as a surrogate marker[145]. However, validation studies are needed to identify the most sensitive and specific markers and cutoff points for their implementation in clinical practice.

LB IN GC

Given the previously discussed aspects regarding prognosis, therapeutic options, and the molecular landscape of GC, there is a need to optimize patient classification, identify easily implementable prognostic or therapeutic biomarkers, and enhance the understanding of GC tumorigenesis and progression. These efforts aim to improve patient prognosis and treatment cost-effectiveness. In this context, LB emerges as a non-invasive option to enhance theoretical knowledge with potential immediate clinical impact. However, it should be noted that while research on LB applications is more advanced in other tumors, it is still in early stages for GC. The main applications of LB in GC are outlined in Figure 10. Tables 2 and 3 present the key findings from previous studies regarding the use of CTCs, cfDNA/ctDNA, RNA and proteins in the early detection, prognostic determination, and therapy response of GC. These tables summarize and adapt the data presented in the reviews by Zhang *et al*[146], Ma *et al*[147], and Ha *et al*[148].

CTCs in GC

GC detection: Previous studies demonstrated an elevated number of CTCs in GC patients compared to healthy controls, and this count tends to increase with the progression of the disease[149]. Concerning the use of CTCs for GC detection, there is generally a high specificity (> 95%), while sensitivity varies depending on the method, patient factors, and tumor characteristics, mainly GC stage. A 2013 meta-analysis showed a pooled sensitivity and specificity of CTC detection for GC diagnosis of 42% and 99%, respectively[150]. It is worth noting that most studies included in this analysis employed LB techniques based on RT-PCR for detecting CK or carcinoembryonic antigen (CEA) expression[151]. The cutoff point for considering a result as positive has not been standardized, although a previous study found that the limit of 2 CTCs per 7.5 mL of blood presented the highest sensitivity and specificity at 85.3% and 90.3%, respectively[152]. Interestingly, this study included resectable patients, mostly in stage pT1 (54%), and without nodal metastasis (59%). These researchers detected CTCs using a centrifugal microfluidic system with a fluid-assisted separation technique.

Unfortunately, due to the scarcity of CTCs detected with currently available methods, the application of LB for early detection of GC is limited.

Characterization of GC: CTCs in GC also hold potential for assessing prognostic or predictive biomarkers. Biomarkers associated with approved targeted therapies are particularly valuable, as LB enables their non-invasive analysis in GC patients. In cases with metastatic disease, where accessing metastases can be challenging, these biomarkers become especially crucial. CTCs may better represent systemic tumor heterogeneity, and LB techniques serve as a valuable complement to the study of tissue samples from the primary tumor. Additionally, LB allows for serial determinations during the disease course, which can be useful for monitoring genotypic and phenotypic changes in the tumor cell burden of the body. Previous studies have highlighted the utility of evaluating PD-L1 expression or HER2 status in CTCs from GC patients[153,154].

Correlation with clinicopathological data and prognosis: Previous research has established correlations between the number of CTCs and various clinicopathological factors, including Laurén type, histological grade, perineural infiltration, lymphovascular invasion, TNM stage, and cellular proliferation (Ki-67 index). Diffuse cases according to the Laurén classification, tumors with high histological grade, perineural and vascular infiltration, higher proliferation rate, and at more advanced stages are associated with a higher likelihood of detectable CTCs[153,155].

Table 2 Liquid biopsy and early detection of gastric cancer. Summary of the main published studies[146-148]

Role	Structure	Approach	Accuracy	Findings/challenges
Diagnosis	CTCs	Count	S: 42-85%; E: 90%-99%	Low sensitivity; diverse cutoffs
		CD44+	S: 92%; E: 100%	Small sample size
	cfDNA/ctDNA	Methylation	S: 50%-69%; E: 75%-92%	Diverse markers
		Quantification	S: 79%-96%; E: 91%-94%	Diverse methodology
	RNA	MiRNA	S: 67%-99%; E: 66%-95%	Diverse markers
		CircRNA	S: 78%-89%; E: 45%-84%	Diverse markers
		LncRNA	S: 65%-84%; E: 53%-87%	Diverse markers
	EVs RNA	MiRNA	S: 26%-95%; E: 56%-100%	Up or down expression; diverse markers
		CircRNA	S: 41%-87%; E: 68%-98%	Down expression; diverse markers
		LncRNA	S: 70%-88%; E: 60%-98%	Up expression; diverse markers
	Proteins	TTF1-3, CDH17, TFF3, and TXNRD1	S: 62%-96%; E: 57%-83%	Diverse markers; panels

CTC: Circulating tumor cells; S: Sensitivity; E: Specificity; EV: Extracellular vesicle.

Table 3 Liquid biopsy, gastric cancer prognosis and therapy. Summary of the main published studies[146-148]

Role	Structure	Approach	Association	Findings/challenges
Prognosis	CTCs	Count	↓OS/D-RFS	Diverse cutoffs
		EpCAM, CEA, CD44, CD133, TWIST, ploidy, FR, PD-1	↓OS/D-RFS	Diverse methodology
	cfDNA/ctDNA	Quantification	↓OS/D-RFS	Diverse methodology
		Amplification: BRAF, FGFR2, MET; mutation: TP53, ARAF	↓OS	Diverse methodology
		Methylation	↓OS/DFS	Diverse markers
	EVs RNA	MiRNA	↓OS/D-RFS	↑ or ↓ expression; Diverse markers
		CircRNA	↓OS/RFS	↑ or ↓ expression; diverse markers
		LncRNA	↓OS/DFS	↑Expression; diverse markers
Predictive	CTCs	Count	Response to ST	
		HER2, PD-L1 status	Trastuzumab resistance/IT	
	cfDNA/ctDNA	Quantification	Response to ST-CT/anti PD-1	
		Panels Amplification: HER2; mutation: PIK3CA, NF1, HER2, EGFR, TP53, BRCA MSI	Targeted therapy; response to ST; trastuzumab resistance; treatment monitoring; IT	

CTCs: Circulating tumor cells; FR: Folate receptor; OS: Overall survival; D-RFS: Disease free survival/relapse free survival; EV: Extracellular vesicle; ST: Systemic therapy; IT: Immunotherapy; CT: Chemotherapy; MSI: Microsatellite instability.

According to some authors, the CTC count correlates better with GC prognosis than other currently available markers in clinical practice[152]. CTC detection has been variably correlated with the OS and recurrence-free/disease-free survival (RFS/DFS) of GC patients, with cutoff points generally between 1-5 CTCs[156-158]. A recent meta-analysis including 14 retrospective studies and over 1000 patients showed that CTC-positive patients, with a cutoff of 2.8, had significantly lower OS. The relationship between OS and CTCs depended on factors such as the chosen cutoff, the sample size of the study, the type of treatment (mainly resected vs. unresected GC), and the method employed for CTC detection. Furthermore, CTC-positive patients showed higher TNM stage, high histological grade, and more frequent distant metastases[159].

Treatment monitoring: The serial determination of CTCs proves valuable in assessing treatment response in various tumors. In GC, patients with a higher CTC count tend to exhibit a poorer response to chemotherapy and a worse

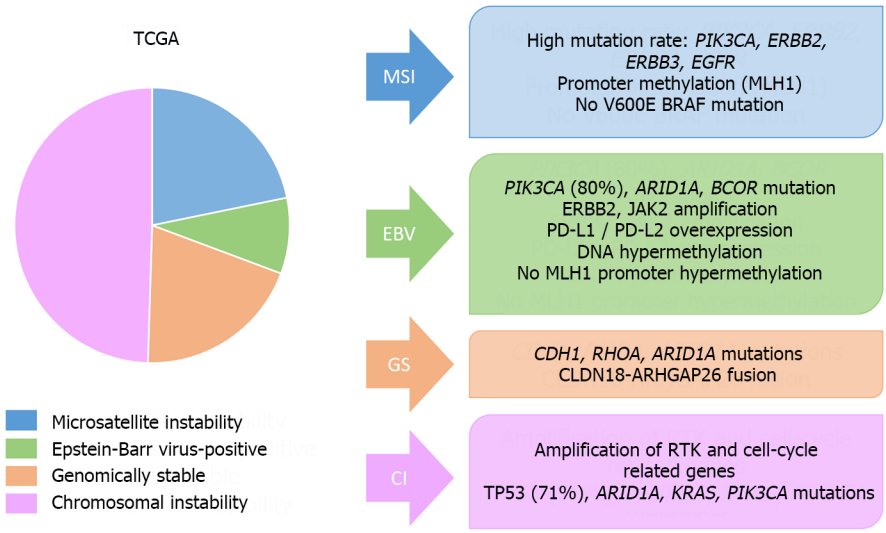

Figure 8 Molecular classifications of gastric cancer. Main molecular features of The Cancer Genome Atlas classification. TCGA: The Cancer Genome Atlas; MSI: Microsatellite instability; EBV: Epstein-Barr virus; GS: Genomically stable; CI: Chromosomal instability.

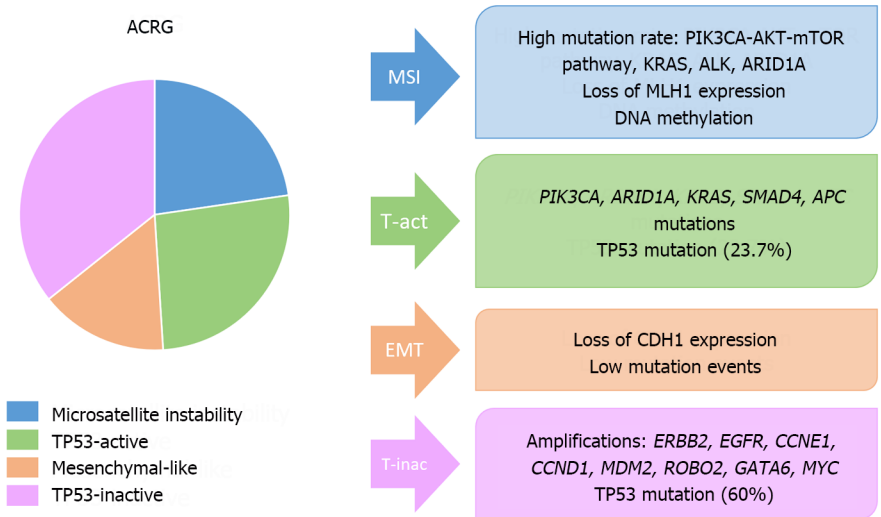

Figure 9 Molecular classifications of gastric cancer. Main molecular features of the Asian Cancer Research Group classification. MSI: Microsatellite instability; ACRG: The Asian Cancer Research Group; EMT: Epithelial-mesenchymal transition.

prognosis compared to those with a lower count[160]. Additionally, the rapid decline of CTCs following treatment initiation is associated with a better prognosis, and the conversion to a higher number of CTCs may be an early indicator of treatment failure.

cfDNA/ ctDNA in GC

Main approved tests: ctDNA analysis stands out as the predominant LB approach for gastrointestinal tumors, with approved indications in GC, particularly for biomarker determination. For instance, ctDNA can be employed to assess HER2 status in GC cases where tissue testing is impractical or urgent treatment decisions are required[84].

GC detection: For the detection of GC, quantitative analysis of ctDNA (total copy number analysis in blood or serum) or qualitative detection, involving the identification of tumor-specific genetic changes, can be utilized (Table 2)[161]. Similar to CTCs, the cfDNA/ctDNA load is found to be higher in GC patients compared to healthy controls or those with preneoplastic lesions, escalating with tumor stage[162].

Overall, ctDNA analysis has demonstrated higher sensitivity than CTCs for GC detection[163]. Notably, the study by Kim *et al*[163] achieved a sensitivity and specificity exceeding 90%. Similarly, Qian *et al*[164] observed that cfDNA concentration was more sensitive than conventional markers like CEA, carbohydrate antigen (CA) 19-9, CA72-4, or CA50 in distinguishing between patients with benign gastric disease or healthy individuals and those with early GC. However, other studies have shown contradictory results. Cabel *et al*[165] observed limited sensitivity of ctDNA testing in resectable patients. A meta-analysis published in 2017 calculated a pooled sensitivity and specificity of the presence of certain

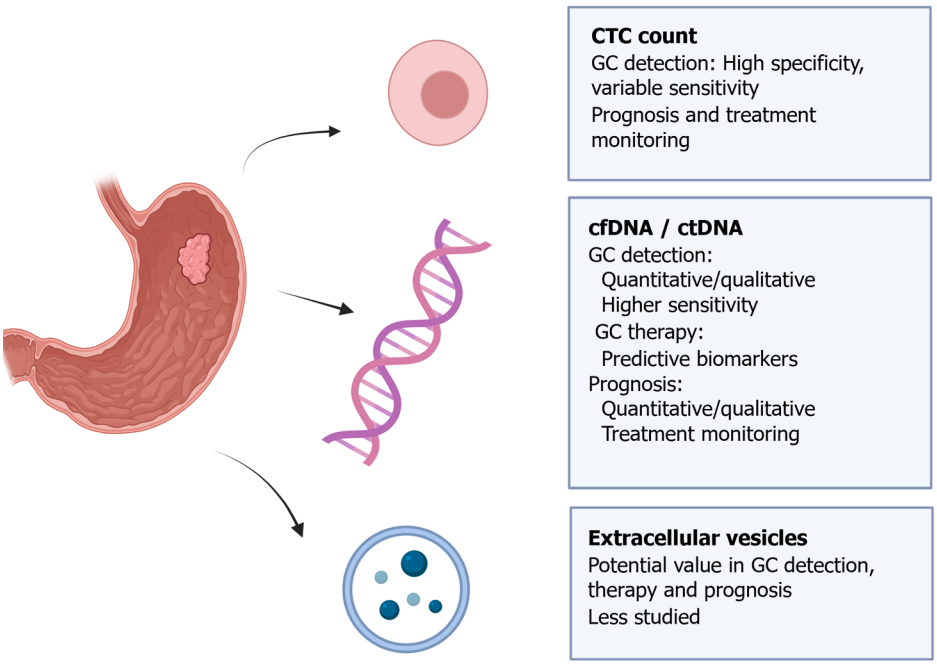

Figure 10 Key applications of analyzing circulating tumor cells, cell-free DNA/circulating tumor DNA, and extracellular vesicles in gastric cancer. CTC: Circulating tumor cell; cfDNA/ctDNA: Cell-free DNA/circulating tumor DNA. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

ctDNA markers of 62% and 95%, respectively[166]. It should be noted that the sensitivity of these determinations, as seen with CTCs, is influenced by factors related to the detection technique and the tumor, particularly its size and extension [167]. In early-stage tumors, the sensitivities observed in previous studies are not yet sufficient to recommend the implementation of these tests in clinical practice[161].

In addition to assessing ctDNA levels, as previously mentioned, a qualitative study of ctDNA can also be conducted. For instance, the analysis of DNA methylation patterns for GC screening, such as the Galleri multicancer detection test, has been explored[168]. However, the sensitivity of this technique is notably influenced by the disease stage, ranging from 16.7% in stage I GC to 100% in stage IV tumors[162].

Characterization of GC: The study of ctDNA proves particularly valuable for determining prognostic or therapeutic biomarkers. ctDNA sequencing has been useful in gastrointestinal tumors, revealing various genetic and epigenetic alterations, such as alterations in EGFR, HER2, TP53, PIK3CA, MSI, or germline mutations of BRCA[169]. Similar to CTCs, ctDNA analysis offers the advantages of non/minimal invasiveness and representation of tumor heterogeneity compared to tissue biopsy, with higher sensitivity than CTCs, although it should be noted that the sensitivity of detecting mutations in ctDNA may also be influenced by the tumor stage.

Regarding the study of HER2 amplification by LB, of clinical importance in GC, a previous study published by Gao *et al*[170] analyzed 70 patients and found a 91.4% correlation between tumor tissues and ctDNA, indicating that LB is a good surrogate for HER2 analysis in GC.

As seen with CTCs, the presence of certain ctDNA markers has been variably associated with various clinicopathological factors, including tumor size, level of infiltration, number of lymph node metastases, presence of distant metastases, or *Helicobacter pylori* infection[166].

Correlation with prognosis: Previous studies have demonstrated that the detection of ctDNA correlates with a worse prognosis, linking it to OS, DFS/RFS, and/or the presence of recurrences or distant metastases[171,172]. The 2017 meta-analysis conducted by Gao, encompassing 16 studies and over 1000 GC patients, further confirmed the relationship between a high level of ctDNA and significantly worse OS and DFS in GC[167]. Subsequently, the meta-analysis by Min *et al*[173] included 34 articles with more than 5000 subjects, revealing a significant association between ctDNA detection in blood and OS, DFS, and PFS, with hazard ratios of 2.74, 3.13, and 3.04, respectively.

Therefore, the study of ctDNA holds promise for detecting MRD and predicting the likelihood of recurrences after treatment. In the study by Yang *et al*[167], it was noted that all patients with detectable ctDNA after surgery experienced recurrences, with ctDNA detected in blood an average of 6 months before recurrence detection by imaging methods. Additionally, the investigation of various genetic or epigenetic alterations in ctDNA can also correlate with patient prognosis. For example, a previous study analyzed CBLB mutations in ctDNA, revealing that patients with these alterations presented significantly shorter OS and DFS[171].

Treatment monitoring: In the realm of treatment monitoring, Zhou *et al*[171] observed that combining the analysis of ctDNA levels with traditional tumor markers and radiological control improved the accuracy of the latter. Kim *et al*[174] studied cancer-specific rearrangements in ctDNA and observed positive ctDNA detection 4 months before detecting a

clinical recurrence. Moreover, previous studies have shown that postoperative ctDNA detection, the persistence of high ctDNA levels, or their increase can be an early indicator of recurrence in patients with resected GC[175,176]. Lastly, ctDNA analysis shows potential for monitoring the response to immunotherapy with anti-PD-1 agents or resistance to trastuzumab in patients with metastatic GC[177,178] (Table 3).

Extracellular vesicles in GC

As mentioned earlier, exosomes hold significant interest because they are composed of multiple structures derived from tumors, enabling studies beyond genomic analysis. Notably, they are more abundant and stable than ctDNA and can be found in non-blood fluids, making them particularly valuable when analyzing non-blood samples[179].

In GC, exosomes seem to play an important role in tumorigenesis and tumor progression, influencing angiogenesis, immune evasion, cell proliferation, invasiveness, and therapy resistance[180-182]. Additionally, exosomes are implicated in the peritoneal dissemination of GC[183].

Concerning their clinical application, several studies have analyzed exosomes and exosomal-derived RNAs and proteins[184-187]. The detection of these molecules has shown diagnostic, prognostic, predictive, or therapeutic potential. In the diagnostic domain, miRNAs, lncRNAs, circRNAs, and proteins such as TRIM 3 have been studied[188]. Generally, miRNAs have been studied in combination (as profiles), while lncRNAs and circRNAs have been investigated individually[179]. For prognostic value, various miRNAs, lncRNAs, circRNAs, and proteins such as TGF-β1, apolipoprotein E, or MET have been analyzed[187,189,190]. Interestingly, the exosomal PD-L1 content has been found to be an independent predictor of OS and negatively correlated with the CD4+ and CD8+ T cell count in a cohort of metastatic GC patients[191].

An intriguing aspect of exosomes, apart from the previously described functions, is their potential role as a drug delivery method in GC[192].

Non-blood samples in GC

A comprehensive systematic review performed by Lopes *et al*[115] compiled over 80 articles related to the use of LB for GC detection utilizing non-blood samples. The majority of articles included Asian patients, and they mainly focused on samples of gastric juice and urine, and to a lesser extent, salivary or stool samples. It should be noted that studies on peritoneal LB for purposes beyond diagnosis were excluded from the review.

Regarding gastric juice, Yamamoto *et al*[193] analyzed the BARHL2 methylation in ctDNA and exosomes, observing differences between GC samples and controls and concluding that this alteration could assist in the early diagnosis of GC, with high sensitivity and specificity. Other researchers have analyzed different types of RNA, proteins, amino acids, microbiota, or metabolites in gastric juice, primarily aiming to detect GC and improve the sensitivity of upper gastrointestinal endoscopy. Proteins such as pepsin A and gastricsin, along with miR-21 and miR-133a, have shown promising discriminatory value[115].

In urine samples, according to the review by Lopes *et al*[115], the markers with the best diagnostic value in isolation were the metabolite glycerol and endothelial lipase. Profiles including combinations of metabolites or amino acids have also been published, showing good diagnostic accuracy in these samples. Studies using saliva have focused on detecting lectins and RNA molecules, while stool analysis has explored several proteins, TERT promoter methylation (DNA), or microbiota profiles.

In patients with peritoneal involvement, an increase in certain exosome-derived miRNAs in peritoneal fluid, such as miR-21-5p, miR-92a-3p, miR-223-3p, and miR-342-3p, has been detected[179]. Furthermore, previous studies have observed that the levels of various miRNAs correlate with OS and RFS.

It is worth noting that despite the scarcity of studies on non-blood samples, molecules with diagnostic value have been detected, which could also be used in disease monitoring. These samples present potential value as a source of molecules for determining prognosis, treatment, or patient management[194]. As mentioned earlier, analyzing these fluids can offer heightened sensitivity as they are in direct proximity to tumor cells, potentially yielding distinct information from blood samples. Additionally, certain samples like urine can be self-collected by patients, leading to an improvement in their quality of life and simplifying the process of conducting serial LBs for follow-up purposes.

The use of artificial intelligence

In some clinical specialties, such as radiology or pathology, digitization and artificial intelligence (AI) tools have substantially improved the workflow and diagnostic accuracy of various techniques where they have been integrated[195,196]. Specifically in pathology, the digitization of slides and the utilization of algorithms have opened an almost infinite world of possibilities, facilitating not only the morphological analysis of samples but also the management of large amounts of data. For instance, it is now feasible to identify and quantify cancer cells, intratumoral and peritumoral stromal cells, or inflammatory cells separately[197]. Additionally, algorithms have been developed for standardizing the interpretation of complementary techniques such as PD-L1 expression[198]. In the field of molecular pathology, AI is useful for combining genomic, transcriptomic, proteomic and metabolomic information, and due to its ability to integrate various types of data, it can also serve as a bridge between clinical and molecular features, advancing personalized cancer management[199]. Many of these advancements are potentially applicable to LB. Although published studies on this topic are not abundant, some authors have highlighted the potential of AI-assisted LB, mainly regarding the interpretation of results from cfDNA/ctDNA analyses[200-204]. The application of AI in the study of CTCs would be more similar to current digital pathology, allowing the identification of tumor cells and accompanying stromal or immune cells both by morphology and by the expression of various markers, decreasing inter- and intra-observer variability[205]. Combining our experience as pathologists and the findings of previous studies, we have summarized the potential applications of AI in LB in

Figure 11.

The multi-omics approach

The genomic, transcriptomic, proteomic, and metabolomic information that LB can provide, coupled with the development of high-throughput techniques such as NGS or mass spectrometry, and the ability to integrate vast amounts of data through AI tools, facilitates the integration of LB into the multi-omics era. Consequently, cfDNA/ctDNA emerges as a significant source of genomic data, cfRNA as a source of transcriptomic data, and CTCs or EVs as reservoirs of multiple sources of molecular information. Multi-omics studies of GC tissues have been pivotal in integrating molecular information and elucidating the molecular landscape of GC, as observed in the ACRG or TCGA classifications. In LB, novel biomarkers have been identified that could be useful in the early diagnosis or treatment of GC. These biomarkers encompass a range of elements such as plasma proteins, somatic mutations, genomic or proteomic signatures. Additionally, ongoing multi-omics clinical trials are investigating diverse blood-based biomarkers for diagnosing GC or detecting peritoneal involvement in GC patients[206,207].

Ongoing clinical trials of LB in GC

Several clinical trials are currently underway to assess the efficacy of LB in diagnosing, determining patient prognosis, or assessing treatment response in GC[146,208]. The majority of these studies have been designed in China, with a smaller proportion occurring in European countries and the United States. While most of these trials focus on analyzing ctDNA using blood specimens, some also evaluate CTCs and samples from peritoneal lavage or gastric fluid. The primary objectives of these trials include predicting prognosis, with a smaller subset aimed at assessing response to chemotherapy, immunotherapy, and/or trastuzumab, primarily in the adjuvant or advanced settings.

FUTURE CHALLENGES

In the following section, we outline the primary challenges related to LB, with a focus on GC. It is important to note that while the authors concentrate on GC, many of the challenges discussed are also applicable to other tumor types.

Improve the sensitivity of available techniques

Due to recent technical advances, the sensitivity of LB studies has increased progressively. However, in GC, it is still not sufficient for clinical application, except for limited approvals, such as the use of blood specimens as a rescue sample when a traditional sample is unavailable for specific indications. The strong dependence of LB sensitivity on the disease stage makes it nearly impossible to use it as a screening tool without improving the detection capacity of available techniques.

Reduce false positives and indeterminate results

Deep analyses and broad-spectrum molecular tools, such as whole-genome sequencing, have been more frequently used in LB than in conventional tissue sample analysis in clinical practice. This may lead to the detection of variants of undetermined significance or even false positives. In this regard, some researchers suggest that the use of algorithms or machine learning tools could be useful to improve the quality of the data obtained in LB studies[209].

Increase the number of patients in research studies

A major limitation of applying LB in clinical practice for GC is the poor quality of the available evidence, largely due to the small sample size of the majority of conducted studies. Another added problem is the heterogeneity of the patients included, especially concerning the stage at diagnosis, a factor that, as previously noted, substantially influences technique sensitivity.

Assess geographical variations

Western and Asian GC patients exhibit differences in multiple aspects, including clinical, histological, prognostic, or molecular features. While most studies have been conducted in Asian patients, LB studies in low-prevalence populations, such as Western populations, are necessary to analyze the accuracy and validate different techniques. This requires inter-institutional collaboration and the establishment of shared, high-quality databases to collect a significant number of cases.

Decrease variability between detection methods

Standardization of sample collection, maintenance, processing procedures, and detection and characterization methods for different molecules is essential in LB. However, there is considerable variability in LB technologies, making it almost impossible to compare results between most of the published studies. The development of protocols by scientific societies and responsible entities has the potential to improve the comparability between studies and the quality of the results obtained.

Improve tumor representativity

Although LB is assumed to represent the total tumor burden of the body better than targeted biopsies, it is unclear whether LB may overrepresent a specific cell clone, limiting its value as a broadly representative test. Therefore, the combination of LB with traditional biopsies, imaging techniques, and analytical values, along with the performance of

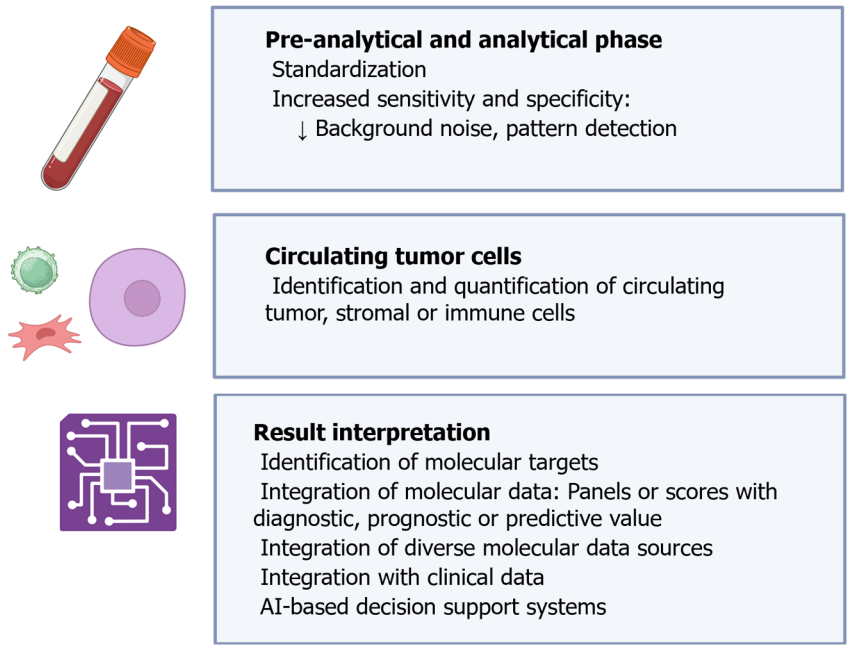

Figure 11 Main applications of artificial intelligence techniques in liquid biopsy. Citation: The authors have obtained permission to use the figure from BioRender.com (Supplementary material)[210].

serial LB studies, may be useful to improve the capture of tumor heterogeneity.

Study of markers beyond CTCs and ctDNA

Most LB studies have focused on the analysis of CTCs or ctDNA. However, exosomes and other cells and molecules present differences and certain advantages compared to CTCs and ctDNA, with great diagnostic, therapeutic and management potential. Therefore, technical improvement, standardization of detection methods, and biomarker studies using these samples are necessary to exploit their full potential.

Improve studies in non-blood samples

In GC and other tumor types, peripheral blood is the most commonly used sample in LB. Despite some advantages associated with non-blood samples, their exploration has been limited. Fewer studies have been published, often with small sample sizes. Additionally, the methods for collecting, maintaining, and processing non-blood samples, as well as the technologies for detecting molecules of interest, exhibit wide variations across studies. It is crucial to acknowledge that the conditions of non-blood samples differ from those of peripheral blood (*e.g.*, pH or density), so procedures commonly used in blood might need to be modified for other sample types. Furthermore, in GC, most studies have been conducted for diagnostic purposes, and the analysis of non-blood samples to detect molecules with prognostic or therapeutic value would also be interesting.

Develop and apply AI techniques

LB can provide a vast amount of information at various molecular levels. In this context, the utilization of AI methodologies offers numerous advantages in both pre-analytical and analytical stages, as well as in result interpretation.

HIGHLIGHTS

LB has emerged as a non- or minimally invasive technique for studying various molecules and cells in bodily fluids, offering advantages in capturing spatial and temporal tumor heterogeneity. It is particularly interesting in patients with advanced tumors and multiple metastatic locations.

Despite its advantages, LB faces challenges, including complex and expensive techniques, lack of standardization and scientific evidence of suboptimal quality due to small sample sizes and procedure variability.

LB presents numerous analytic approaches, serving multiple potential purposes such as early tumor diagnosis, prognostic stratification, treatment monitoring, detection of therapeutic resistance, or analysis of predictive biomarkers.

Various components, including CTCs, cfDNA/ctDNA, RNA, extracellular vesicles, or TEPs can be analyzed in LB, using peripheral blood or other fluids like urine, seminal fluid, saliva, effusions, cerebrospinal fluid, bile, or gastric juice.

CTC analysis offers insights into pre-metastatic cell populations and the genome, transcriptome, proteome, and metabolome of tumor cells. Challenges include enhancing sensitivity, improving capture techniques for better cell viability, identifying more sensitive and specific markers for cell isolation, or developing antigen-independent platforms.

cfDNA/ctDNA is more abundant than CTCs, requires less technological complexity and generally provides greater sensitivity and specificity. Challenges include the use of different platforms depending on the analytic context, the occurrence of false negatives and positives and the inability to conduct transcriptomic, proteomic or metabolomic analyses.

The use of non-blood samples has potential advantages, including greater sensitivity and specificity than peripheral blood samples, depending on the circumstances. Furthermore, some of these samples can be collected by the patients themselves. However, the available evidence on the use of these samples in LB is very limited.

GC is an aggressive tumor that presents clinical, histological, and molecular differences between Western and Asian countries. It is generally diagnosed at advanced stages and has a poor prognosis.

The only curative option for GC is surgery. In advanced tumors, the main therapeutic approach is chemotherapy, and in recent years, a few targeted therapies (anti-HER2 and anti-VEGF/VEGFR) and immunotherapy (anti-PD-L1) have been added to the therapeutic arsenal.

Molecular classifications of GC have been developed and have shown potential prognostic and therapeutic value. Unfortunately, these classifications have not yet been translated into clinical practice.

In GC, there is a need to develop cost-effective screening tools for countries with lower prevalence, improve patient stratification for treatment and clinical trials, increase knowledge about tumor progression mechanisms, and identify new biomarkers with therapeutic value.

CTC studies in GC have shown that both their count and characterization can be useful for detecting GC, determining patient prognosis, monitoring the disease, and identifying therapeutic biomarkers, generally with high specificity.

cfDNA/ctDNA analysis is the predominant LB approach in gastrointestinal tumors. In GC, HER2 status can be assessed using ctDNA in cases where a tissue sample is not available or treatment initiation is urgent.

cfDNA/ctDNA analysis in GC can be useful for determining prognosis and treatment monitoring. Still, for its implementation as a screening tool, the sensitivity of detection techniques needs improvement since its concentration is highly dependent on the tumor stage.

Future advancements in LB should prioritize enhancing technique sensitivity, minimizing false positives, deciphering the pathogenicity of variants of uncertain significance, increasing sample sizes and follow-up times, accounting for geographical disparities, standardizing detection methodologies, developing specific AI techniques, and exploring alternative structures beyond CTCs and cfDNA/ctDNA in both blood and non-blood specimens.

CONCLUSION

In conclusion, LB emerges as a promising tool in GC research, offering significant advantages in terms of reduced invasiveness and enhanced capture of tumor heterogeneity. Despite challenges such as technical complexity and lack of standardization, the diverse analytical approaches in LB open a broad range of possibilities for early diagnosis, prognostic stratification, treatment monitoring, and identification of therapeutic biomarkers. The analysis of CTCs, cfDNA/ctDNA and other structures in blood and non-blood samples provides unique insights into GC and other tumors.

Despite recent advances in the molecular understanding of GC and the application of LB, fundamental challenges persist. These include the standardization of methods and platforms, improvement of sensitivity, reduction of false positives, and the need to increase the number of patients included in research studies to enhance the available evidence. International collaboration and the establishment of high-quality shared databases are essential to comprehensively address these challenges and propel the field forward.

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FOOTNOTES

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MINIREVIEWS

Endoscopic treatment of scarred polyps with a non-thermal device (Endorotor): A review of the literature

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Abstract

Endoscopic resection (ER) of colorectal polyps has become a daily practice in most endoscopic units providing a colorectal cancer screening program and requires the availability of local experts and high-end endoscopic devices. ER procedures have evolved over the past few years from endoscopic mucosal resection (EMR) to more advanced techniques, such as endoscopic submucosal dissection and endoscopic full-thickness resection. Complete resection and disease eradication are the ultimate goals of ER-based techniques, and novel devices have been developed to achieve these goals. The EndoRotor® Endoscopic Powered Resection System (Interscope Medical, Inc., Northbridge, Massachusetts, United States) is one such device. The EndoRotor is a powered resection tool for the removal of alimentary tract mucosa, including post-EMR persistent lesions with scarring, and has both CE Mark and FDA clearance. This review covers available published evidence documenting the usefulness of EndoRotor for the management of recurrent colorectal polyps.

Key Words: EndoRotor; Scarred polyps; Recurrent polyps; Colorectal cancer; Colorectal polyps

Core Tip: Colorectal cancer (CRC) screening was initiated and implemented over the last two decades with a widespread variability in strategies and coverage in different parts of Europe even after the first appearance of the European guidelines for CRC screening. Recurrent or previously manipulated lesions are usually fibrotic with tethering to the muscularis and display the non-lifting sign, making subsequent resection challenging. The EndoRotor® Endoscopic Powered Resection System is a powered debridement device for the removal of alimentary tract mucosa, including post-endoscopic mucosal resection persistent lesions with scarring, and has both CE Mark and FDA clearance. This review covers available published evidence documenting the usefulness of EndoRotor for the management of recurrent scarred colorectal polyps.

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INTRODUCTION

Colorectal cancer (CRC) places an enormous burden on health care services worldwide. Globally, CRC is ranked third in incidence and second in mortality[1]. Despite its increasing trends, more favourable outcomes are observed in many high-incidence countries due to increased colonoscopy screening programs and early removal of any precursor lesions[2,3].

CRC screening has been initiated and implemented over the last two decades, during which there has been widespread variability in strategies and coverage in different parts of Europe even after the first appearance of the European Guidelines for CRC screening[2,4]. These guidelines emphasize detailed quality requirements for the endoscopic unit infrastructure as well as certain competency levels of both the endoscopist and assisting staff[5]. Early detection and complete excision of adenomas are pivotal for minimizing the risk of CRC. Choosing the optimal resection technique is critical in achieving complete excision and is dependent on many factors, including the lesion morphology, size, location, estimated risk of submucosal invasion, and degree of expertise of the endoscopist. Endoscopic resection (ER) choices are explicitly indicated in different guidelines of the Gastroenterological and Endoscopic Societies[6,7]. Endoscopic Mucosal Resection (EMR) is a well-established technique for removing colorectal adenomas[8]. This approach is the standard treatment for sessile polyps 10-20 mm in diameter. For larger polyps, *en bloc* resection is rarely achievable, and piecemeal resection is used. In a large European study, the recurrence rate after EMR of lesions > 20 mm reached 31.7% after 3–6 months[9]. Another report from Australia showed early recurrence rates of 16.0%[8].

With the implementation of CRC screening, an interval cancer problem has emerged, which has been attributed to incomplete resection of the primary lesion in approximately 27% of patients when large colon polyps were first resected [8,10,11]. Recurrent or previously manipulated lesions are usually fibrotic when tethered to the muscularis and display the non lifting sign, making subsequent resection challenging. There are no standards or approved guidelines for ER for these fibrotic and/or recurrent polyps, but there are many techniques reported in the literature, including endoscopic submucosal dissection (ESD), endoscopic full-thickness resection (EFTR), argon plasma coagulation (APC), and endoscopic powered resection (EPR) systems. Although effective, ESD results in an increased risk of perforation due to thermal injury and difficulty in planar identification caused by fibrosis. EFTR is a hybrid surgical approach indicated for lesions < 30 mm where the full thickness of the lumen wall is resected. Recent studies have shown the procedure to be technically successful in approximately 90% of patients; however, the complete resection rate for lesions > 20 mm was significantly lower than that for lesions ≤ 20 mm (58% *vs* 81%, $P = 0.0038$), and serious adverse events such as perforation, appendicitis, and small bowel fistula have been reported[12]. APC can result in thermal injury leading to perforation and is associated with a high recurrence rate[12].

The EndoRotor® EPR System (Interscope Medical, Inc., Northbridge, Massachusetts, United States) is a powered debridement device for the removal of alimentary tract mucosa, including post-EMR persistent lesions with scarring, and has both CE Mark and FDA clearance. This review covers available published evidence documenting the usefulness of EndoRotor for the management of recurrent colorectal polyps.

ENDOROTOR EPR SYSTEM

The EndoRotor EPR System consists of a power console, a beveled tip single-use catheter, foot control pedals, and a specimen trap. The power console contains a motor drive for catheter blade rotation, a peristaltic pump for irrigation, and a dedicated vacuum system. A single-use catheter with an outer diameter of 3.1 mm connects to the power console and is compatible with endoscopes that have a minimum working channel of 3.2 mm. The catheter had demarcation lines to assist with cutting window positioning during the procedure. The console is set to high (1750 rotations/min) or low (1000 rotations/min) speed, and the vacuum is set between 50 and 200 mmHg of negative pressure. The catheter is controlled by the endoscopist using two foot pedals, and the tissue is resected by aspiration into a cutting window with a rotating blade. Resected tissue is simultaneously suctioned through the inner lumen of the catheter and into a specimen trap with a preloaded micron filter that can be sent for histopathological analysis (Figure 1).

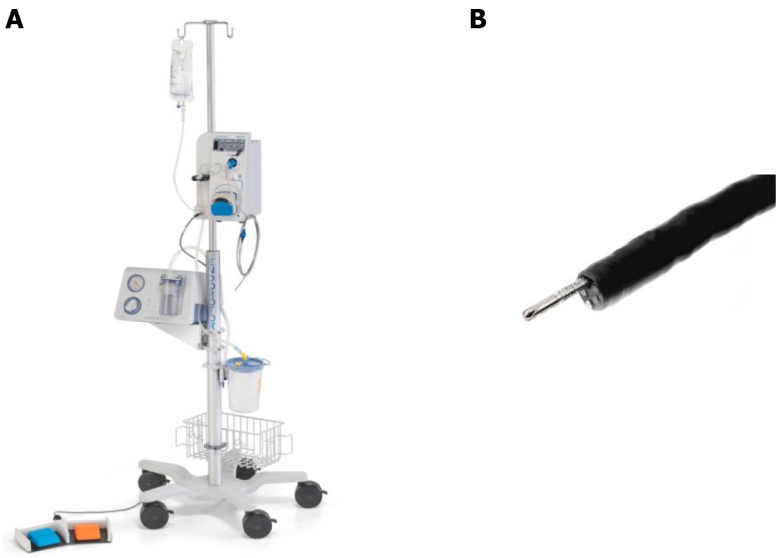

Figure 1 EndoRotor endoscopic powered resection System. A: EndoRotor endoscopic powered resection system; B: Beveled tip single-use catheter.

AVAILABLE PUBLISHED EVIDENCE FOR THE ENDOROTOR EPR SYSTEM

EndoRotor was first evaluated *in vivo* by Hollerbach *et al*[13], who performed several successful gastric (15 Lesions) and colonic (10 Lesions) mucosal resections in German Landrace pigs under general endotracheal anaesthesia with resected areas ranging from 15-70 mm^2. This initial experiment showed the high potential of the EndoRotor EPR System for facilitating safe and effective ER in relatively large mucosal areas in various parts of the gastrointestinal (GI) tract.

Following this animal study, multiple human case reports were published (Table 1). Tillinger[14] successfully removed a scarred recurrent rectal adenoma in two sessions with only minimal bleeding that was endoscopically controlled. Surkunalingam *et al*[15] reported successful resection of recurrent scars on Paris 0-IIa+c lesions over a fold. Despite initial trials of removal by ESD followed by standard EMR, the lesion's location and shape made resection unsuccessful, mandating the use of an innovative approach in which the investigators selected EndoRotor to facilitate resection. Additionally, Stadler *et al*[16] successfully removed a large recurrent laterally spreading granular tumour (LST-G) rectal adenoma *via* both ESD for the non scarred region and EndoRotor for the tough, fibrosed, adherent region. Furthermore, Pellegatta *et al*[17] reported the complete removal of scarred polyps in the rectum, a LST-G of 40 mm, occupying half the circumference with the Kudo IIIL pit pattern in a single setting despite multiple previous EMR and APC trials.

Kandiah *et al*[18], in a prospective pilot study of 19 patients with recurrent scarred colorectal lesions, reported that EndoRotor was a safe and effective technique for the management of these challenging scarred polyps. Investigation of 84% of patients who underwent 1–2 EndoRotor procedures revealed disease eradication, and no serious adverse events occurred.

Emmanuel *et al*[19] used EndoRotor to acquire histological samples from the margins of LST-G lesions post EMR with visually apparently normal lateral margins to evaluate evidence of microscopic adenoma as a possible mechanism of recurrence after ER, especially after piecemeal EMR (pEMR). These authors showed that microscopic residual adenoma in the mucosal bridges of lesion bases could be missed by traditional pEMR, as proven *via* histological examinations of tissue samples retrieved from the EndoRotor. This case series emphasized the potential complementary use of EndoRotor after pEMR to remove any remnants at the base as well as resect the margins.

The largest published clinical human case series with EndoRotor was reported in a retrospective multicentre study from the United States where EndoRotor was evaluated in 40 upper and lower GI lesions, with a previous trial of ER in 85.4% of them. There were 28 colorectal polyps, 25 of which had previously undergone therapeutic intervention. Resection was complete with the Endorotor in all 28 patients. Seventeen (60.7%) patients underwent follow-up endoscopy at least 2 months later, and there was no residual adenomatous tissue in 15 (88.2%) patients[20]. However, this study is subject to selection bias because it was retrospective.

Notably, EndoRotor was evaluated in only a single-arm manner in all the studies, with no comparison to other ER methods, such as EMR, ESD, or ablation, used for the management of difficult adherent GI lesions.

PROS AND CONS OF THE ENDOROTOR EPR SYSTEM

There are several technical advantages to using this cold automated mechanical suction and resection device. First, the absence of heat or ablation of mucosa leads to less scarring, delayed bleeding, or perforation, as expected with other thermal methods[17,18]. Second, there are no concerns about patients with pacemakers because no electric current is used. However, although a single tool was used in most of the reported cases, the Endorotor could also be applied after removing nodular parts of the polyp *via* traditional resection techniques such as EMR or ESD; in that case, the advantages

Table 1 Available published evidence for the EndoRotor endoscopic powered resection system

Ref.	Type	Resected lesions	Comments/reported complications
Hollerbach *et al* [14], 2016	*In vivo* animal study	Gastric (15 lesions; 4 oesophagus, 9 stomach and 2 duodenum) and colonic (10 lesions)	Minimal bleeding (blood loss did not exceed more than 10 – 15 cc of blood); No perforations occurred during "normal" resections. EndoRotor® was significantly faster than current standard techniques, 2 perforations occurred (these serious adverse events only occurred during experiments that were performed deliberately to test the limits of the gastrointestinal wall when excessive force was applied)
Tillinger *et al* [15], 2016	Case report	Circumferential rectal recurrent scarred LST-G mixed lesion 3 cm in length	The whole lesion could be removed in two sessions. Minor bleeding was controlled by means of adrenalin-injection and coagulation with argon plasma. No further complications occurred
Surkunalingam *et al*[16], 2018	Case report	20 m, Paris IIa+c	Prolonged time; The inability to obtain an en-bloc specimen
Emmanuel *et al* [20], 2021	Retrospective study	41 lesions post EMR or pEMR. Anorectum/rectum 8 (19.5), left-sided colon segment 10 (24.4), right-sided colon segment 13 (31.7) Caecum 10 (24.4)	Only one case of postpolypectomy syndrome 1 (2.4)
Kandiah *et al* [19], 2019	Prospective pilot study	19 flat scared polyps (Paris 0-IIa/0-IIb/0-Is) from 7 to 70 mm in the rectum and sigmoid	Cured maximally in 2 sessions. Minor intraprocedural bleeding in 2 cases. There were no perforations, delayed bleeding, postpolypectomy syndrome or complications requiring surgery
Stadler *et al*[17], 2019	Case report	Recurrent rectal adenoma LST-G	No reported complication successful removal of the scarred part in combination with ESD
Pellegatta *et al* [18], 2020	Case report	Scarred polyp in the rectum, a LST-G of 40 mm, hemicirconferential with adenomatous pit pattern (Kudo IIIL)	No recurrence was endoscopically revealed at 6 months' follow-up. Complete removal in one session
Kaul *et al*[21], 2021	Retrospective study	41 lesions; Oesophagus 8 (23.5); Duodenum 5 (14.7); Colon18 (52.9); Rectum 3 (8.8)	Technical success (the ability to complete PED using the EndoRotor device without the use of additional resection modalities) was achieved in 97.6% of lesions (n = 40). Clinical success (no endoscopic and/or histologic evidence of residual/recurrent lesion on follow-up examination) was achieved in 79.2% of patients. Adverse events were reported in 3 patients (8.8%). postprocedural chest pain in one patient with oesophageal lesion. Two patients had delayed bleeding with colonic lesions. Intraprocedural bleeding was observed in 10 patients (29.4%; 4 colon, 5 oesophageal, and 1 duodenum)

LST-G: Laterally spreading granular tumour; EMR: Endoscopic mucosal resection; pEMR: Piecemeal endoscopic mucosal resection; PED: Powered endoscopic debridement.

of cold resection would be lost, at least in part. Third, the alternative to Endorotor for recurrent polyps could be a significantly more invasive technique, mainly full-thickness resection and ESD. Fourth, due to the simultaneous suction and catheter cutting rotation, it is expected that the entire mucosal surface could be removed because of its specific viscoelastic properties against the more fibrous muscularis layer, making it potentially safe for preserving the muscle layer, as no perforations were reported during its use. Fifth, the beveled inner sheath hole is approximately 3 mm^2 in area, allowing the user to resect 2 - 4 mm of tissue per second with each rotation of the inner cannula (1000-1700 rpm), ensuing a very rapid resection. Sixth, the EndoRotor catheters are provided in variable lengths suitable for the scope used by gastroscopy (1240 mm and 1270 mm) or colonoscopy (1890 mm and 1540 mm) to shorten the length of the extraendoscopic catheter for more effective transmission of rotational power. Seventh, the console is easily controlled by 2-foot pedals, one blue to activate rotation of the inner cutting blade of the catheter and then continuous pressing of the orange pedal to activate the suction force throughout the entire cutting process. Once the orange pedal is released, the cutting canula automatically stops for safety reasons. The device has a short learning curve for experienced endoscopists performing EMR and/or ESD. Pathologic tissue samples are simultaneously collected during the cutting operation and automatically transported back to the collection trap, providing a single step of cutting and collecting tissue, thus reducing the resection time.

The EndoRotor EPR System allows adjustment of the suction force (mmHg) and cutting blade speed (revolutions per minute) depending on the nature of the lesion and the preference of the endoscopist. This approach could be helpful for the management of lesions with different degrees of fibrosis, and it would also reduce the need for milder resection parameters at the beginning of the learning curve or in locations with an increased risk of perforation. The manufacturer recommends vacuum levels of 50 to 200 mmHg for resecting endoluminal lesions. In Kandiah *et al*[18] scarred lesions were treated from 100 to 300 mmHg and in Emmanuel *et al*[19] 50 to 100 mmHg when completing EMR margin resections. Studies comparing different settings are lacking. Only one study mentioned the detailed settings used and recommended starting ablation at a high speed (1750 rpm) with 150–200 mmHg suction[21].

The main limitations of the EndoRotor system for the treatment of scarred polyps include the following: (1) The system requires a working channel of 3.2 mm or more; thus, it is impossible to use this system with endoscopes with a reduced channel size. However, this should not be a problem in the management of colorectal polyps, as even paediatric colonoscopes have 3.2 mm channels. However, for its application in upper endoscopy, regular gastroscopy with 2.8 mm

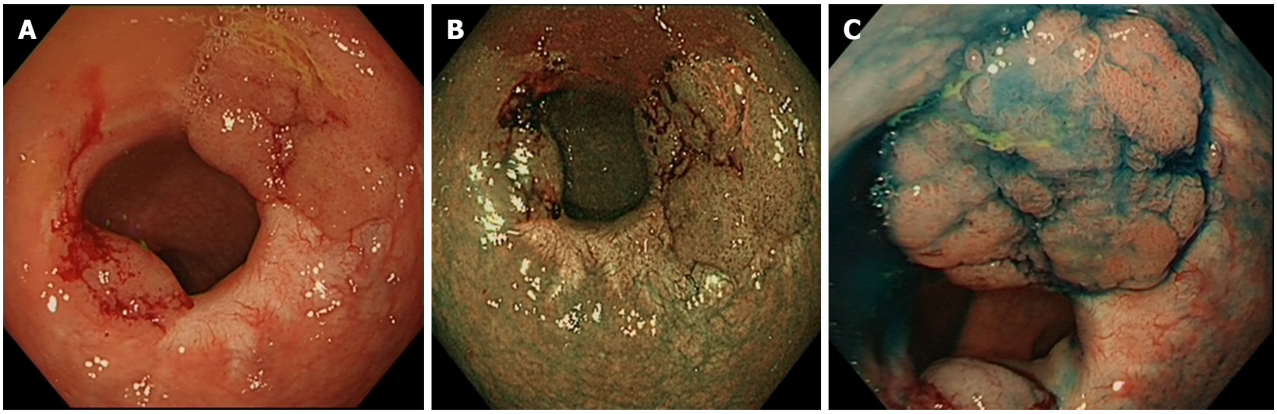

Figure 2 Session one: Examination of two recurrent polyps on the previous resection site with white light narrow-band imaging and Indigo. A: White light; B: Narrow-band imaging; C: Indigo.

channels is not suitable[15]; (2) EndoRotor catheters are relatively stiff, and certain angles of inclination, such as 90°, can be challenging for the operator and the system, while small tilts (30° to 40°) are more convenient[13]. Furthermore, retroflexing the endoscope with the resection catheter inside the working channel is impossible due to the reduced flexion ability of the endoscope with the catheter occupying the whole channel. Therefore, therapy cannot be administered in retroflexion, although this could be solved by lifting just beyond the target lesion to push it into view[19]; (3) Despite the ease of collecting tissue resected, the quality of the collected tissue being similar to that obtained from traditional biopsy forceps does not enable the depth of invasion to be judged. These samples could only confirm the histopathological type and presence, absence, and degree of dysplasia. This leads to consideration of proper patient selection, *e.g.*, previous EMR confirmed a benign lesion that recurred due to persistence. Therefore, EPR is not appropriate in principle for the treatment of suspicious lesions; and (4) EPR is not a one-time procedure, as opposed to ESD/EFTR, but rather requires follow-up every 3-6 months until complete eradication of the polyp can be confirmed, which is typically achieved within 2-3 sessions. Even if the lesion seems to have been entirely treated with an EPR +/- EMR session, it is usually impossible to completely rule out minute residual polyp tissue.

FINANCIAL CONSIDERATIONS AND/OR LESIONS WITH CONFIRMED HGD/CANCER, WHICH WOULD BE BETTER MANAGED BY EN BLOC RESECTION TECHNIQUES (EFTR OR ESD)

The EndoRotor ER system is a relatively expensive tool compared to conventional EMR devices or APC, and retreatment with Endorotor can be required in follow-up procedures. However, the cost of this approach is less than that of EFTR or colectomy for the treatment of polyps. Moreover, being an outpatient, well tolerated, and safe procedure would argue against the primary cost of the EndoRotor EPR System compared to other modalities and taking possible hospital stays into consideration.

Cost is certainly a factor when treating patients, but it must be weighed against patient quality of life and procedural effectiveness. Reimbursement for the endoscopic and surgical management of colorectal polyps varies widely in different settings. Cost-economic studies are required to compare different endoscopic and surgical techniques for the management of residual/recurrent polyps.

Case illustration

As we are currently actively applying this technique for the treatment of recurrent colorectal polyps, we would like to report a case in which two recurrent rectal polyps were found on a previous polypectomy site in an 85-year-old male who underwent transanal endoscopic operation and two EMRs for a large LSTGM polyp in the rectum over a period of 3 years. Both recurrent polyps were laterally spreading tumour-nongranular and were separated by scar tissue. Histology confirmed low-grade dysplasia and tubulovillous adenoma of both polyps.

In the initial session, the patient underwent exclusive EndoRotor treatment (Figure 2).

A follow-up examination three months later revealed the presence of a residual 15 mm flat

An elevated (IIa) lesion was subsequently resected *via* the EMR and EndoRotor (Figure 3).

At the second follow-up, which was conducted six months after the initial presentation, 75% in the size of the original polyp was observed, prompting its resection *via* EndoRotor and Pulsed Argon Plasma Coagulation was used (Figure 4). During the follow-up 6 months after the last intervention, the patient exhibited a healthy scar under both white light and narrow-band imaging assessments (Figure 5) which was confirmed to be histologically normal tissue.

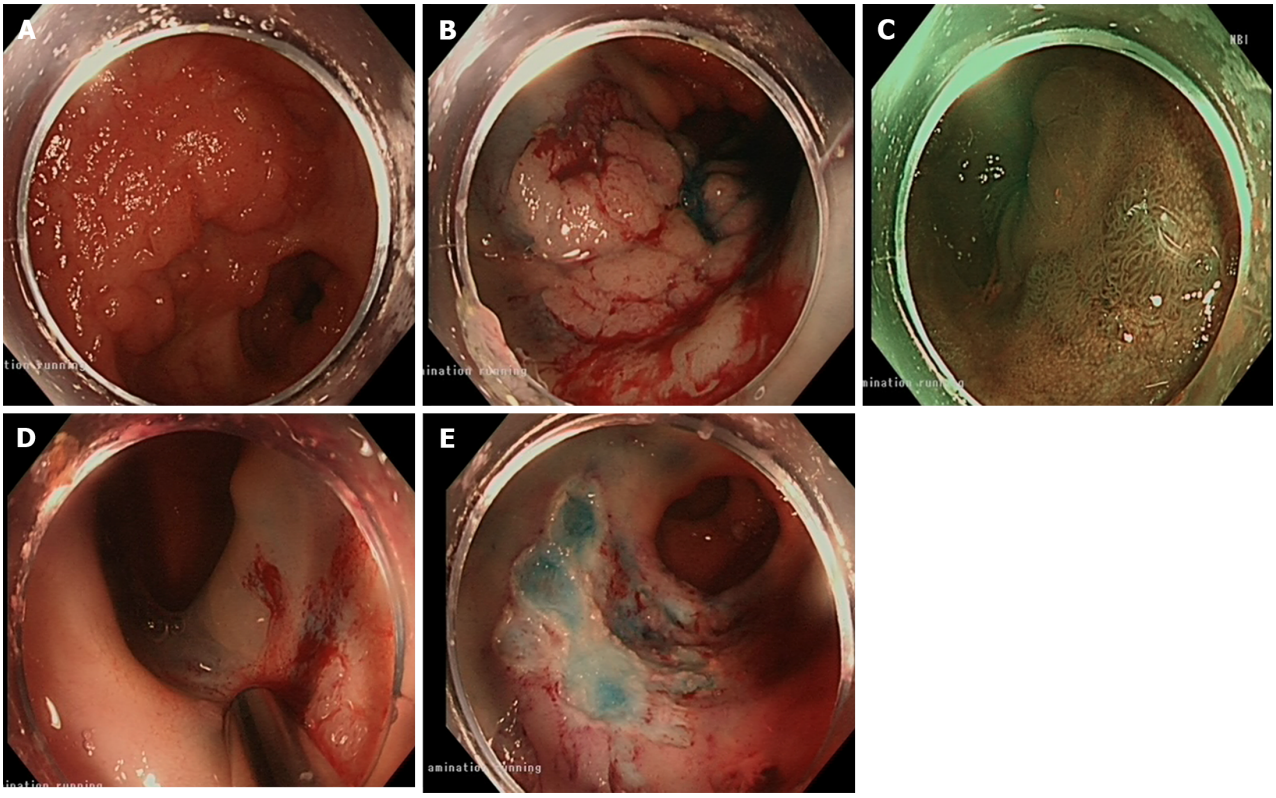

Figure 3 Session two: Examination of a residual 15 mm flat elevated lesion with white light and narrow band imaging; application of EndoRotor; resection site after EndoRotor and endoscopic mucosal resection. A and B: White light; C: Narrow band imaging; D: EndoRotor; E: Resection site after EndoRotor and endoscopic mucosal resection.

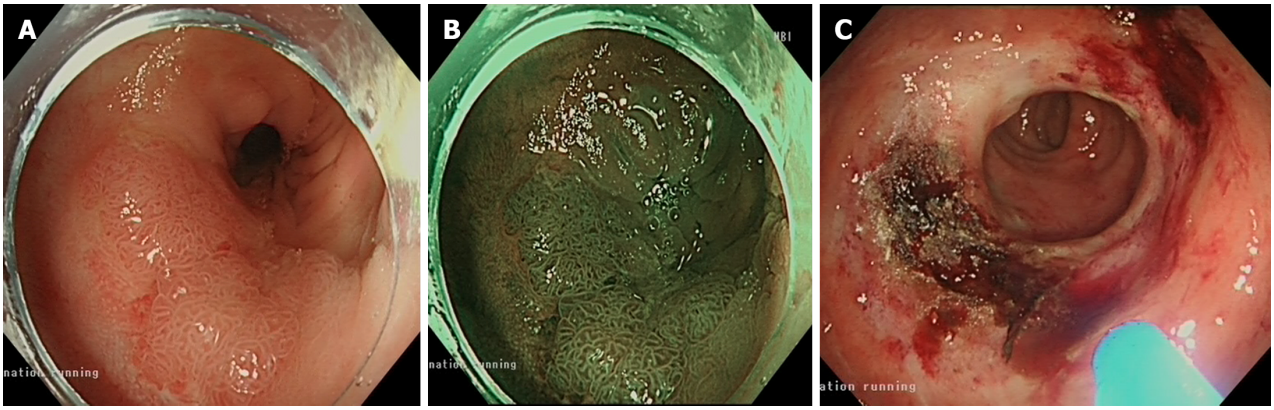

Figure 4 Session three: Examination of the residual polyp with white light and narrow band imaging and resection site after EndoRotor and argon plasma coagulation. A: White light; B: Narrow band imaging; C: Resection site after EndoRotor and argon plasma coagulation.

CONCLUSION

EndoRotor is a novel tool for endoscopists performing EMR and/or ESD and should be considered a treatment option for patients with recurrent colorectal lesions. Where the FTRD offers a solution for small adherent recurrent lesions, larger flat lesions could be problematic and only amenable to ablation, ESD or EndoRotor resection. Ablative therapy does not allow the collection of tissue samples. ER (ESD) is a highly technically demanding procedure with a long learning curve. EndoRotor use should be discussed in multidisciplinary MDT meetings where all options (endoscopic and surgical) are considered, as well as through discussion with the patient. Despite the paucity of evidence, EndoRotor showed high safety and efficacy in the resection of recurrent scarred lesions with previously known pathology. Moreover, this technique could be a valuable addition to tertiary referral endoscopy centres for difficult cases.

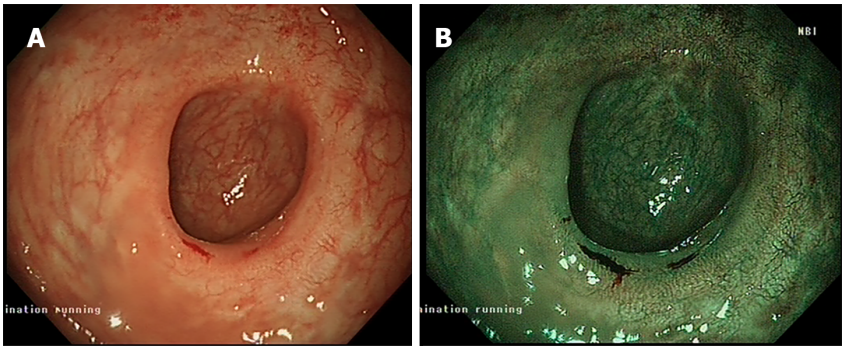

Figure 5 Session four: Examination of the scar tissue *via* the white light and narrow band imaging methods. A: White light; B: Narrow band imaging.

FOOTNOTES

Author contributions: Zaghloul M and Parra-Blanco A designed the review; Zaghloul M reviewed the literature and wrote the manuscript; Parra-Blanco A, Sansone S, Rehman H and Argyriou K reviewed the manuscript; all authors read and approved the final manuscript.

Conflict-of-interest statement: Dr. Parra-Blanco reports other from Inter scope, during the conduct of the study; other from Lumendi and CREO medical, other from Puramatrix, outside the submitted work.

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ORIGINAL ARTICLE

Retrospective Study

Predictive value of red blood cell distribution width and hematocrit for short-term outcomes and prognosis in colorectal cancer patients undergoing radical surgery

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Abstract

BACKGROUND

Previous studies have reported that low hematocrit levels indicate poor survival in patients with ovarian cancer and cervical cancer, the prognostic value of hematocrit for colorectal cancer (CRC) patients has not been determined. The prognostic value of red blood cell distribution width (RDW) for CRC patients was controversial.

AIM

To investigate the impact of RDW and hematocrit on the short-term outcomes and long-term prognosis of CRC patients who underwent radical surgery.

METHODS

Patients who were diagnosed with CRC and underwent radical CRC resection between January 2011 and January 2020 at a single clinical center were included. The short-term outcomes, overall survival (OS) and disease-free survival (DFS) were compared among the different groups. Cox analysis was also conducted to identify independent risk factors for OS and DFS.

RESULTS

There were 4258 CRC patients who underwent radical surgery included in our study. A total of 1573 patients were in the lower RDW group and 2685 patients were in the higher RDW group. There were 2166 and 2092 patients in the higher hematocrit group and lower hematocrit group, respectively. Patients in the higher RDW group had more intraoperative blood loss ($P < 0.01$) and more overall complications ($P < 0.01$) than did those in the lower RDW group. Similarly, patients in the lower hematocrit group had more intraoperative blood loss ($P = 0.012$), longer hospital stay ($P = 0.016$) and overall complications ($P < 0.01$) than did those in the higher hematocrit group. The higher RDW group had a worse OS

and DFS than did the lower RDW group for tumor node metastasis (TNM) stage I (OS, $P < 0.05$; DFS, $P = 0.001$) and stage II (OS, $P = 0.004$; DFS, $P = 0.01$) than the lower RDW group; the lower hematocrit group had worse OS and DFS for TNM stage II (OS, $P < 0.05$; DFS, $P = 0.001$) and stage III (OS, $P = 0.001$; DFS, $P = 0.001$) than did the higher hematocrit group. Preoperative hematocrit was an independent risk factor for OS [$P = 0.017$, hazard ratio (HR) = 1.256, 95% confidence interval (CI): 1.041-1.515] and DFS ($P = 0.035$, HR = 1.194, 95%CI: 1.013-1.408).

CONCLUSION

A higher preoperative RDW and lower hematocrit were associated with more postoperative complications. However, only hematocrit was an independent risk factor for OS and DFS in CRC patients who underwent radical surgery, while RDW was not.

Key Words: Colorectal cancer; Red blood cell distribution width; Survival; Short-term outcomes

Core Tip: This was the first study to show that low hematocrit could predict worse overall survival and disease-free survival in colorectal cancer (CRC) patients who underwent radical surgery. This study investigated the association between red blood cell distribution width (RDW) or hematocrit and short-term outcomes in CRC patients, which has rarely been reported previously. In conclusion, a preoperative higher RDW and lower hematocrit were associated with more postoperative complications.

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INTRODUCTION

According to global cancer statistics[1], there were approximately 1.93 million new cases and 0.94 million deaths from colorectal cancer (CRC) worldwide in 2020, and the incidence of this disease was estimated to increase in the next decade [2,3]; additionally, CRC will impose a heavy burden on the economy[4]. Radical surgery is the most important treatment for CRC patients[5-7]; however, many patients suffer from postoperative complications and reoccurrence after surgery. To improve the prognosis of CRC patients after surgery, various risk factors for postoperative complications and long-term prognosis have been were identified[8,9]. Many hematological indicators, such as hemoglobin[10,11], platelet counts [12,13], and the neutrophil-to-lymphocyte ratio[14,15], have also been identified because of the convenience of easy access and low cost.

The red blood cell distribution width (RDW) reflects the degree of variation in erythrocyte volume and is usually used to identify different types of anemia[16,17]. Several studies have demonstrated that RDW plays a diagnostic role in CRC patients[18-20]; moreover, many scholars have found that a high RDW is a negative predictor of overall survival (OS) and disease-free survival (DFS) independently[21-25]. The underlying mechanisms are mainly associated with tumor-related chronic inflammation and malnutrition, which accelerate tumor progression while affecting iron metabolism and suppressing the production of red blood cells, further leading to a high RDW. However, a previous study showed the opposite results: RDW was not an independent risk factor[26]. As a result, the prognostic value of RDW for CRC patients is controversial. In addition, few studies have focused on the impact of RDW on the short-term outcomes in CRC patients after radical surgery[27].

Since tumor-related chronic inflammation could lead to high RDWs, we suspected that hematocrit, another indicator of anemia, might also be related to the prognosis of CRC patients. Although previous studies have reported that low hematocrit levels indicate poor survival in patients with ovarian cancer[28] and cervical cancer[29], the prognostic value of hematocrit for CRC patients has not been determined. Therefore, this study was to explore the effect of RDW and hematocrit on the outcomes of CRC patients who underwent radical surgery.

MATERIALS AND METHODS

Patients

Patients who were diagnosed with CRC and underwent radical CRC resection were included from January 2011 to January 2020 in our single clinical center. The study was approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University (2022-K205), and all patients signed informed consent forms. This study was conducted in accordance with the World Medical Association Declaration of Helsinki as well.

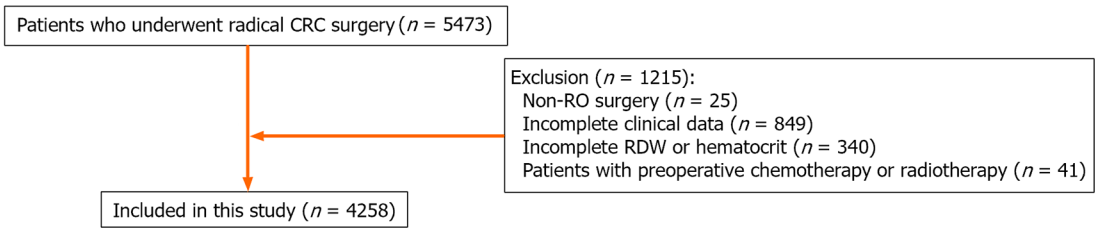

Figure 1 Flow chart of patient selection. CRC: Colorectal cancer; RDW: Red blood cell distribution width.

Inclusion and exclusion criteria

CRC patients who underwent radical CRC surgery were included (n = 5473). The exclusion criteria were as follows: (1) Non-R0 surgery (n = 25); (2) Incomplete clinical data (n = 849); (3) Incomplete RDW or hematocrit (n = 340); and (4) Patients with preoperative chemotherapy or radiotherapy (n = 41). Finally, a total of 4258 CRC patients were included in this study (Figure 1).

Data collection

The baseline characteristics collected were as follows: Age, sex, body mass index (BMI), smoking, drinking, hypertension, type 2 diabetes mellitus (T2DM), coronary heart disease (CHD), surgical method, tumor location, tumor node metastasis (TNM) stage and tumor size. The short-term outcomes included operation time, intra-operative blood loss, postoperative hospital stay, overall complications and major complications. The long-term prognosis was estimated by OS and DFS. All the data were collected from electronic medical record system, outpatient visit and telephone interviews.

Definitions

Based on the AJCC 8th Edition, we identified the TNM stage[30]. The postoperative complications were classified on the basis of the Clavien-Dindo[31] classification and major complications were ≥ grade III. OS was defined as the time from surgery to death or lost follow-up and DFS was calculated from the date of surgery to the date of recurrence or death.

Treatment and follow-up

All patients underwent radical surgery according to standard principles and R0 resection was confirmed by pathology. Patients were regularly followed up every six months in the first three years and every year in the next years.

Optimal cut-off and groups

The RDW and hematocrit were tested within a week before surgery. The value of RDW and hematocrit was expressed as a percentage. We used X-tile software to identify the optimal cut-off[32]. The optimal cut-off values for RDW and hematocrit were 14.4 and 37.7, respectively. Accordingly, patients were divided into the higher RDW group (RDW > 14.4) and the lower RDW group (RDW ≤ 14.4) as well as the higher hematocrit group (hematocrit > 37.7) and the lower hematocrit group (hematocrit ≤ 37.7).

Statistical analysis

An independent-sample t-test was used to compare the difference continuous variables that were expressed as the mean ± SD. χ^2 tests or Fisher's exact tests were used for categorical variables that were expressed as absolute values and percentages. Based on the Kaplan-Meier method, we estimated OS and DFS. In order to compare the OS and DFS between the different groups at different tumor stages, we used log-rank test. To determine independent risk factors for overall complications, logistic regression analysis was conducted. Analysis of Cox regression was conducted to identify independent risk factors for OS and DFS. Statistical significance was determined by a bilateral P value less than 0.05 using SPSS (version 22.0).

RESULTS

Patients

There were 4258 CRC patients who underwent radical surgery included in our study. Patients were divided into different groups according to the optimal cutoff values for RDW and hematocrit. There were 1573 patients in the lower RDW group and 2685 patients in the higher RDW group. The higher RDW group was older (P < 0.01), more likely to be female (P < 0.01), had a lower BMI (P < 0.01), a lower percentage of alcohol consumption (P < 0.01), a higher incidence of T2DM (P = 0.035) and CHD (P < 0.01), and a greater incidence of open surgery (P < 0.01), colon cancer (P < 0.01), TNM stage II (P < 0.01), TNM stage IV (P < 0.01), and tumor size ≥ 5 cm (P < 0.01) (Table 1).

After grouping patients according to hematocrit, there were respectively 2166 and 2092 patients in the higher hematocrit group and lower hematocrit group, respectively. The lower hematocrit group was older (P < 0.01); was more likely to be female (P < 0.01); had a lower BMI (P < 0.01); had a lower rate of smoking (P < 0.01) and drinking (P < 0.01),

Table 1 Comparison between the higher red blood cell distribution width group and the lower red blood cell distribution width group

Characteristics	Higher RDW (*n* = 1573)	Lower RDW (*n* = 2685)	*P* value
RDW	17.3 ± 3.3	13.0 ± 0.6	< 0.01[a]
Age, yr	64.1 ± 12.8	62.2 ± 11.7	< 0.01[a]
Sex			< 0.01[a]
Male	873 (55.5)	1640 (61.1)	
Female	700 (44.5)	1045 (38.9)	
BMI, kg/m^2	22.3 ± 3.3	22.9 ± 3.1	< 0.01[a]
Smoking	570 (36.2)	1047 (39.0)	0.074
Drinking	434 (27.6)	881 (32.8)	< 0.01[a]
Hypertension	434 (27.6)	679 (25.3)	0.099
T2DM	222 (14.1)	319 (11.9)	0.035[a]
CHD	90 (5.7)	91 (3.4)	< 0.01[a]
Open surgery	281 (17.9)	282 (10.5)	< 0.01[a]
Tumor location			< 0.01[a]
Colon	940 (59.8)	1068 (39.8)	
Rectum	633 (40.2)	1617 (60.2)	
TNM stage			< 0.01[a]
I	218 (13.9)	585 (21.8)	
II	727 (46.2)	1020 (38.0)	
III	538 (34.2)	976 (36.4)	
IV	90 (5.7)	104 (3.8)	
Tumor size			< 0.01[a]
< 5 cm	739 (47.0)	1716 (63.9)	
≥ 5 cm	834 (53.0)	969 (36.1)	
Operation time (min)	230.3 ± 81.9	225.8 ± 84.1	0.087
Blood loss (mL)	116.4 ± 171.8	93.3 ± 131.0	< 0.01[a]
Hospital stay (d)	11.5 ± 7.7	11.2 ± 9.2	0.283
Overall complications	400 (25.4)	538 (20.0)	< 0.01[a]
Major complications	42 (2.7)	60 (2.2)	0.370

[a]*P* value < 0.05.

Variables are expressed as the mean ± SD, *n* (%). RDW: Red blood cell distribution width; T2DM: Type 2 diabetes mellitus; BMI: Body mass index; CHD: Coronary heart disease; TNM: Tumor node metastasis.

and had a higher incidence of T2DM ($P < 0.01$), CHD ($P < 0.01$), open surgery ($P < 0.01$), colon cancer ($P < 0.01$), or TNM stage II-IV ($P < 0.01$) (Table 2).

Short-term outcomes

Patients in the higher RDW group had greater intraoperative blood loss ($P < 0.01$) and more overall complications ($P < 0.01$) than did those in the lower RDW group. Similarly, patients in the lower hematocrit group had more intraoperative blood loss ($P = 0.012$), longer hospital stays ($P = 0.016$) and more overall complications ($P < 0.01$) than did those in the higher hematocrit group (Tables 1 and 2).

Multivariate logistic regression analysis of the overall complications showed that age [$P < 0.01$, odds ratio (OR) = 1.018, 95% confidence interval (CI): 1.011-1.025], T2DM ($P = 0.018$, OR = 1.297, 95%CI: 1.045-1.610), smoking ($P = 0.004$, OR = 1.255, 95%CI: 1.075-1.464), and open surgery ($P < 0.01$, OR = 2.056, 95%CI: 1.691-2.500) were independent risk factors. However, RDW ($P > 0.05$) and hematocrit ($P > 0.05$) were not identified as independent indicators of overall complications (Table 3).

Table 2 Comparison between the higher hematocrit group and the lower hematocrit group

Characteristics	Higher hematocrit (*n* = 2166)	Lower hematocrit (*n* = 2092)	*P* value
Hematocrit	42.1 ± 3.0	32.2 ± 4.4	< 0.01[a]
Age, yr	61.2 ± 11.3	64.6 ± 12.7	< 0.01[a]
Sex			< 0.01[a]
Male	1580 (72.9)	933 (44.6)	
Female	586 (27.1)	1159 (55.4)	
BMI, kg/m^2	23.2 ± 3.1	22.2 ± 3.2	< 0.01[a]
Smoking	1023 (47.2)	594 (28.4)	< 0.01[a]
Drinking	839 (38.7)	476 (22.8)	< 0.01[a]
Hypertension	540 (24.9)	573 (27.4)	0.068
T2DM	220 (10.2)	321 (15.3)	< 0.01[a]
CHD	68 (3.1)	113 (5.4)	< 0.01[a]
Open surgery	222 (10.2)	341 (16.3)	< 0.01[a]
Tumor location			< 0.01[a]
Colon	782 (36.1)	1226 (58.6)	
Rectum	1384 (63.9)	866 (41.4)	
TNM stage			< 0.01[a]
I	485 (22.4)	318 (15.2)	
II	847 (39.1)	900 (43.0)	
III	750 (34.6)	764 (36.5)	
IV	84 (3.9)	110 (5.3)	
Tumor size			< 0.01[a]
< 5 cm	1415 (65.3)	1040 (49.7)	
≥ 5 cm	751 (34.7)	1052 (50.3)	
Operation time (min)	228.1 ± 85.4	226.8 ± 81.1	0.593
Blood loss (mL)	96.3 ± 138.0	107.6 ± 157.1	0.012[a]
Hospital stay (d)	11.0 ± 7.5	11.6 ± 9.8	0.016[a]
Overall complications	419 (19.3)	519 (24.8)	< 0.01[a]
Major complications	53 (2.4)	49 (2.3)	0.823

[a]*P* value < 0.05.

Variables are expressed as the mean ± SD, *n* (%). T2DM: Type 2 diabetes mellitus; BMI: Body mass index; CHD: Coronary heart disease; TNM: Tumor node metastasis.

Table 3 Univariate and multivariate logistic regression analysis of the overall complications

Risk factors	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, yr	1.024 (1.018-1.030)	< 0.01[a]	1.018 (1.011-1.025)	< 0.01[a]
Sex (male/female)	0.901 (0.777-1.045)	0.166		
BMI, kg/m^2	0.973 (0.951-0.995)	0.018[a]	0.983 (0.959-1.007)	0.160
Hypertension (yes/no)	1.358 (1.158-1.592)	< 0.01[a]	1.128 (0.944-1.347)	0.186
T2DM (yes/no)	1.553 (1.270-1.900)	< 0.01[a]	1.297 (1.045-1.610)	0.018[a]

Tumor location (colon/rectum)	0.966 (0.835-1.117)	0.638		
Tumor stage (IV/III/II/I)	1.053 (0.963-1.151)	0.257		
Smoking (yes/no)	1.173 (1.012-1.360)	0.034[a]	1.255 (1.075-1.464)	0.004[a]
Drinking (yes/no)	0.989 (0.845-1.157)	0.893		
CHD (yes/no)	1.807 (1.314-2.484)	< 0.01[a]	1.365 (0.977-1.908)	0.069
Tumor size (≥ 5/< 5), cm	1.214 (1.049-1.404)	0.009[a]	1.059 (0.910-1.234)	0.459
Surgical methods (open/laparoscopic)	2.250 (1.860-2.721)	< 0.01[a]	2.056 (1.691-2.500)	< 0.01[a]
RDW (lower/higher)	0.735 (0.634-0.852)	< 0.01[a]	0.887 (0.749-1.050)	0.163
Hematocrit (lower/higher)	1.376 (1.189-1.591)	< 0.01[a]	1.165 (0.981-1.383)	0.082

[a]*P* value < 0.05.

RDW: Red blood cell distribution width; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; CHD: Coronary heart disease.

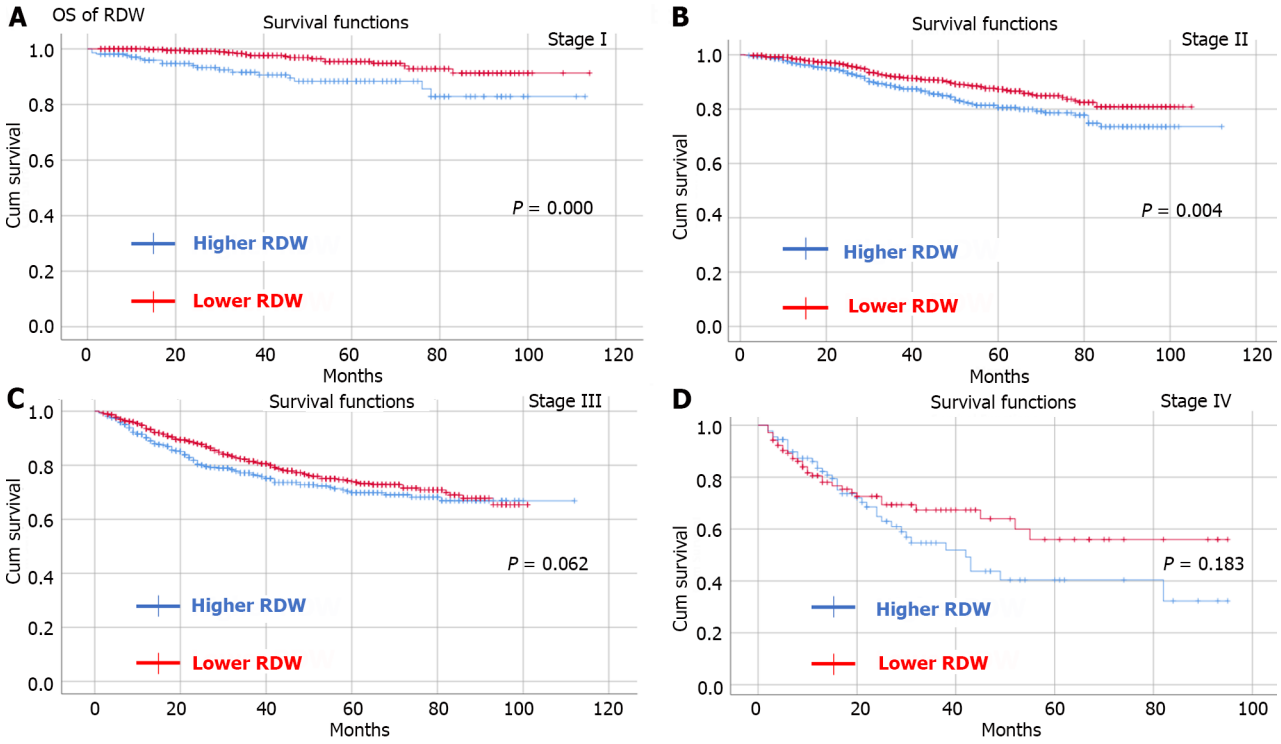

Figure 2 Kaplan-Meier survival curve for the impact of preoperative red blood cell distribution width on the overall survival of patients in tumor node metastasis stage I-IV. A: Kaplan-Meier survival curve for the impact of preoperative red blood cell distribution width (RDW) on the overall survival (OS) of patients in tumor node metastasis (TNM) stage I; B: Kaplan-Meier survival curve for the impact of preoperative RDW on the OS of patients in TNM stage II; C: Kaplan-Meier survival curve for the impact of preoperative RDW on the OS of patients in TNM stage III; D: Kaplan-Meier survival curve for the impact of preoperative RDW on the OS of patients in TNM stage IV. RDW: Red blood cell distribution width.

Kaplan-Meier curves for patients in different tumor stages

By observing OS and DFS, the median follow-up time was 35 (1-114) months. Comparing OS and DFS between the higher RDW group and the lower RDW group as well as between the higher hematocrit group and the lower hematocrit group at different TNM stages. The results showed that the higher RDW group had worse OS (Figure 2) and DFS (Figure 3) than did the lower RDW group for TNM stage I (OS, $P < 0.05$; DFS, $P = 0.001$) and stage II (OS, $P = 0.004$; DFS, $P = 0.01$); moreover, the lower RDW group; the lower hematocrit group had worse OS (Figure 4) and DFS (Figure 5) for TNM stage II (OS, $P < 0.05$; DFS, $P = 0.001$) and stage III (OS, $P = 0.001$; DFS, $P = 0.001$) than did the higher hematocrit group.

Analysis of Cox regression for OS and DFS

To determine independent risk factors for OS and DFS, Cox analysis was conducted. Independent risk factors for OS included age [$P < 0.01$, hazard ratio (HR) = 1.038, 95%CI: 1.030-1.046], tumor stage ($P < 0.01$, HR = 2.167, 95%CI: 1.943-2.416), tumor size ($P = 0.014$, HR = 1.235, 95%CI: 1.044-1.459), preoperative hematocrit ($P = 0.017$, HR = 1.256, 95%CI:

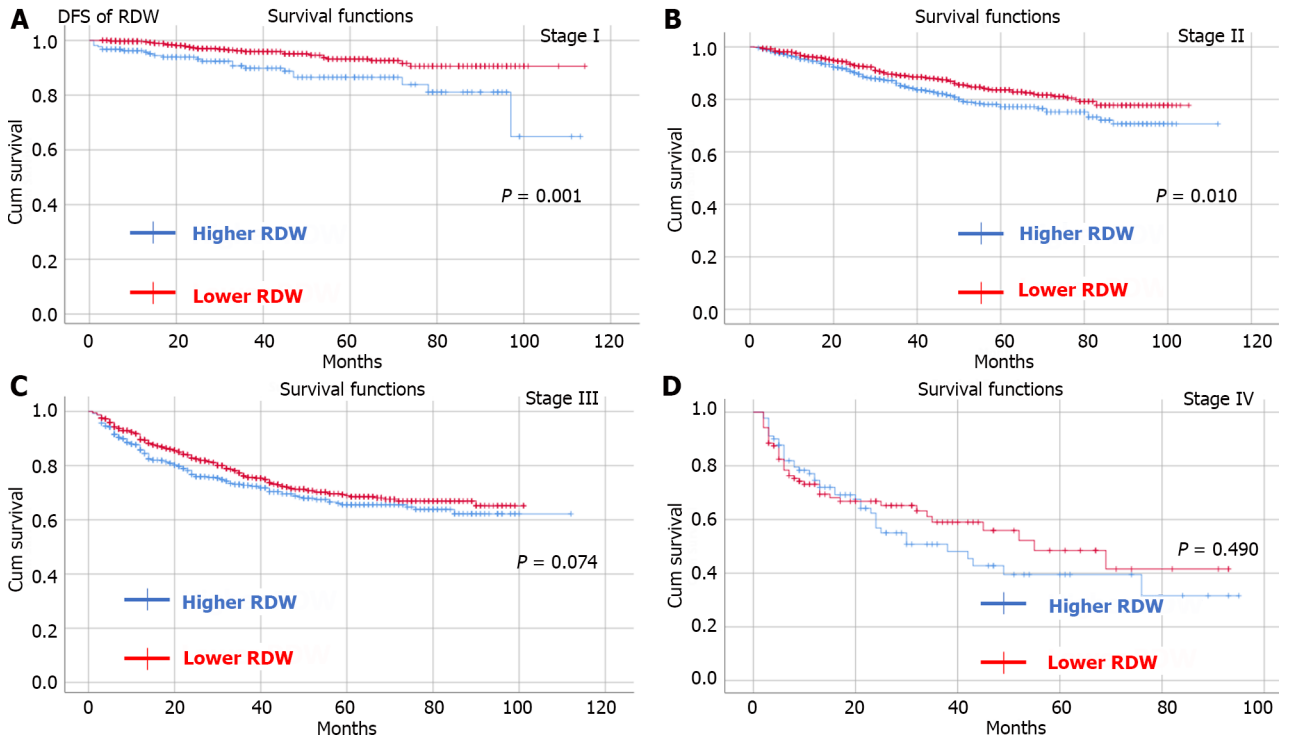

Figure 3 Kaplan-Meier survival curve for the impact of preoperative red blood cell distribution width on the disease-free survival of patients in tumor node metastasis stage I-IV. A: Kaplan-Meier survival curve for the impact of preoperative red blood cell distribution width (RDW) on the disease-free survival (DFS) of patients in tumor node metastasis (TNM) stage I; B: Kaplan-Meier survival curve for the impact of preoperative RDW on the DFS of patients in TNM stage II; C: Kaplan-Meier survival curve for the impact of preoperative RDW on the DFS of patients in TNM stage III; D: Kaplan-Meier survival curve for the impact of preoperative RDW on the DFS of patients in TNM stage IV. RDW: Red blood cell distribution width.

1.041-1.515) and overall complications (P < 0.01, HR = 1.608, 95%CI: 1.357-1.904) ; Independent risk factors for DFS included age (P < 0.01, HR = 1.027, 95%CI: 1.020-1.033), tumor stage (P < 0.01, HR = 2.093, 95%CI: 1.900-2.307), preoperative hematocrit (P = 0.035, HR = 1.194, 95%CI: 1.013-1.408) and overall complications (P < 0.01, HR = 1.510, 95%CI: 1.293-1.763). However, RDW was not an independent risk factor for OS (P = 0.396) or DFS (P = 0.308) (Tables 4 and 5).

DISCUSSION

In this retrospective study, 4258 CRC patients who underwent radical surgery were divided into different groups according to the optimal cutoff values for RDW and hematocrit. The prognostic value of the RDM and hematocrit for the short-term outcomes and prognosis (including OS and DFS) was investigated.

In terms of short-term outcomes, previous studies have reported that RDW and hematocrit could predict postoperative complications in brain surgery[33], cardiac surgery[34,35] and so on, but few studies have focused on CRC patients. In our study, we found that a higher RDW and lower hematocrit were associated with greater intraoperative blood loss and more postoperative complications. Nevertheless, neither RDW nor hematocrit was an independent risk factor for overall complications. However, further studies are needed to validate the roles of RDW and hematocrit in determining surgical complications in CRC patients.

Many studies have shown that RDW is a predictor of the long-term prognosis of CRC patients. Several researchers have conducted a propensity matching score of 5135 CRC patients and found that patients with higher RDWs had worse OS and DFS[21]. However, the study enrolled only patients with TNM stage I-II disease, and multivariate analysis was lacking. One study reported that RDW was a negative predictor of OS and DFS in patients with TNM stage I-III disease [22], and another study reported that found preoperative RDW could predict the OS[24]; however, the sample sizes of these studies were relatively small, and many confounding factors, such as T2DM and CHD, were missed, which might cause bias. Furthermore, a study of 591 patients revealed that a higher RDW was associated with worse OS in patients with TNM stage I disease; however, RDW was not an independent risk factor[25].

Most studies have attributed the prognostic value of RDW to tumor-related chronic inflammation[25,36,37]. Tumor development of tumor, reoccurrence, and metastasis have been shown to interact with the systemic inflammatory response[38], and the latter is associated with overproduction of cytokines such as interleukins and tumor necrosis factor, which might influence iron metabolism and suppress the production of red blood cells[39]. Thus, RDW might represent systemic inflammation and tumor burden. In our study, although the higher RDW group had significantly worse OS and DFS than did the lower RDW group for TNM stage I and stage II disease, RDW could not predict the OS or DFS

Table 4 Univariate and multivariate analysis of overall survival

Risk factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.044 (1.036-1.051)	< 0.01[a]	1.038 (1.030-1.046)	< 0.01[a]
Sex (female/male)	0.861 (0.730-1.015)	0.074		
BMI (kg/m^2)	0.959 (0.935-0.984)	0.001[a]	0.997 (0.972-1.023)	0.840
T2DM (yes/no)	1.303 (1.038-1.636)	0.022[a]	0.941 (0.745-1.189)	0.610
Tumor site (colon/ rectum)	1.422 (1.091-1.853)	0.009[a]	0.984 (0.831-1.165)	0.851
Tumor stage (IV/III/II/I)	2.202 (1.979-2.452)	< 0.01[a]	2.167 (1.943-2.416)	< 0.01[a]
Smoking (yes/no)	1.081 (0.918-1.273)	0.351		
Drinking (yes/no)	1.070 (0.901-1.271)	0.438		
Hypertension (yes/no)	1.055 (0.879-1.266)	0.564		
CHD (yes/no)	1.355 (0.939-1.957)	0.105		
Tumor size (≥ 5 cm/< 5 cm)	1.532 (1.305-1.799)	< 0.01[a]	1.235 (1.044-1.459)	0.014[a]
RDW (lower/higher)	0.664 (0.565-0.780)	< 0.01[a]	0.925 (0.774-1.107)	0.396
Hematocrit (lower/higher)	1.693 (1.437-1.995)	< 0.01[a]	1.256 (1.041-1.515)	0.017[a]
Overall complications (yes/no)	1.903 (1.611-2.248)	< 0.01[a]	1.608 (1.357-1.904)	< 0.01[a]

[a]*P* value < 0.05.

RDW: Red blood cell distribution width; HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; T2DM: Type 2 diabetes mellitus.

Table 5 Univariate and multivariate analysis of disease-free survival

Risk factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.031 (1.025-1.038)	< 0.01[a]	1.027 (1.020-1.033)	< 0.01[a]
Sex (female/male)	0.861 (0.730-1.015)	0.058		
BMI (kg/m^2)	0.975 (0.953-0.997)	0.029[a]	1.003 (0.981-1.026)	0.784
T2DM (yes/no)	1.127 (0.911-1.396)	0.271		
Tumor site (colon/ rectum)	1.126 (0.975-1.300)	0.107		
Tumor stage (IV/III/II/I)	2.120 (1.926-2.334)	< 0.01[a]	2.093 (1.900-2.307)	< 0.01[a]
Smoking (yes/no)	1.093 (0.944-1.267)	0.234		
Drinking (yes/no)	1.104 (0.946-1.288)	0.208		
Hypertension (yes/no)	1.036 (0.879-1.220)	0.676		
CHD (yes/no)	1.281 (0.917-1.791)	0.147		
Tumor size (≥ 5 cm/< 5 cm)	1.363 (1.180-1.574)	< 0.01[a]	1.115 (0.962-1.294)	0.149
RDW (lower/higher)	0.713 (0.616-0.824)	< 0.01[a]	0.920 (0.783-1.080)	0.308
Hematocrit (lower/higher)	1.509 (1.304-1.747)	< 0.01[a]	1.194 (1.013-1.408)	0.035[a]
Overall complications (yes/no)	1.705 (1.463-1.987)	< 0.01[a]	1.510 (1.293-1.763)	< 0.01[a]

[a]*P* value < 0.05.

RDW: Red blood cell distribution width; HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; CHD: Coronary heart disease.

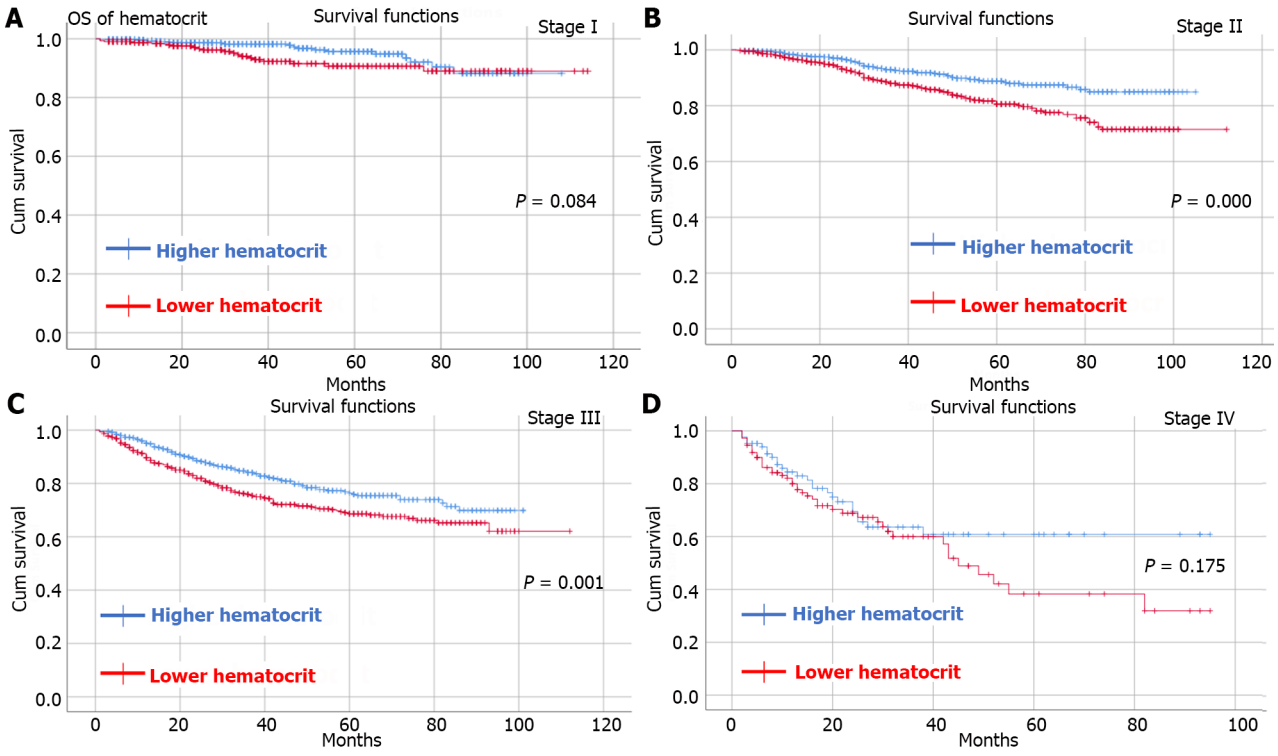

Figure 4 Kaplan-Meier survival curve for the impact of preoperative hematocrit on the overall survival of patients in tumor node metastasis stage I-IV. A: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the overall survival (OS) of patients in tumor node metastasis (TNM) stage I; B: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the OS of patients in TNM stage II; C: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the OS of patients in TNM stage III; D: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the OS of patients in TNM stage IV.

independently, which was similar to the conclusion conclusions of previous studies[26].

In contrast, we found that low hematocrit could predict worse OS and DFS in CRC patients after radical surgery. In our study, patients with stage II-III disease were more likely to have worse OS and DFS. The predictive value of hematocrit has been reported for gynaecological tumours[28,29] and renal carcinoma[40], but the underlying mechanism has remained unclear. The hematocrit was used to measure the volume of red blood cells in whole blood and estimate the oxygen-carrying ability of blood, and a lower hematocrit often indicated anemia. It was also demonstrated that anemia was associated with worse survival in CRC patients after surgery[41,42] because anemia could cause hypoxia, which might promote tumor development[43]. Moreover, the relationship between anemia and tumor-related chronic inflammation, as mentioned above, might further explain the prognostic value of hematocrit.

Interestingly, although both RDW and hematocrit are hematocrit were erythrocyte-related parameters and reflect the status of anemia, we found that hematocrit was an independent risk factor for OS and DFS, while RDW was not. In addition to the bias caused by our research design, there might be several unknown mechanisms that need to be further investigated.

To our knowledge, this was the first study to show that low hematocrit could predict worse OS and DFS in CRC patients after radical surgery. A relatively large sample size of 4258 patients were included in this study. Moreover, we investigated the association between RDW or hematocrit and short-term outcomes in CRC patients, which has rarely been reported previously. This study has several limitations. First, the retrospective nature of this single-center study might cause inaccurate baseline information and bias. Second, chemotherapy information was lacking for TNM III-IV patients, which might affect the analysis of the survival. Therefore, there is a need for multicenter prospective studies to further examine the prognostic role of RDW and hematocrit.

CONCLUSION

In conclusion, a preoperative higher RDW and lower hematocrit were associated with more postoperative complications. However, only hematocrit was an independent risk factor for OS and DFS in CRC patients who underwent radical surgery, while RDW was not.

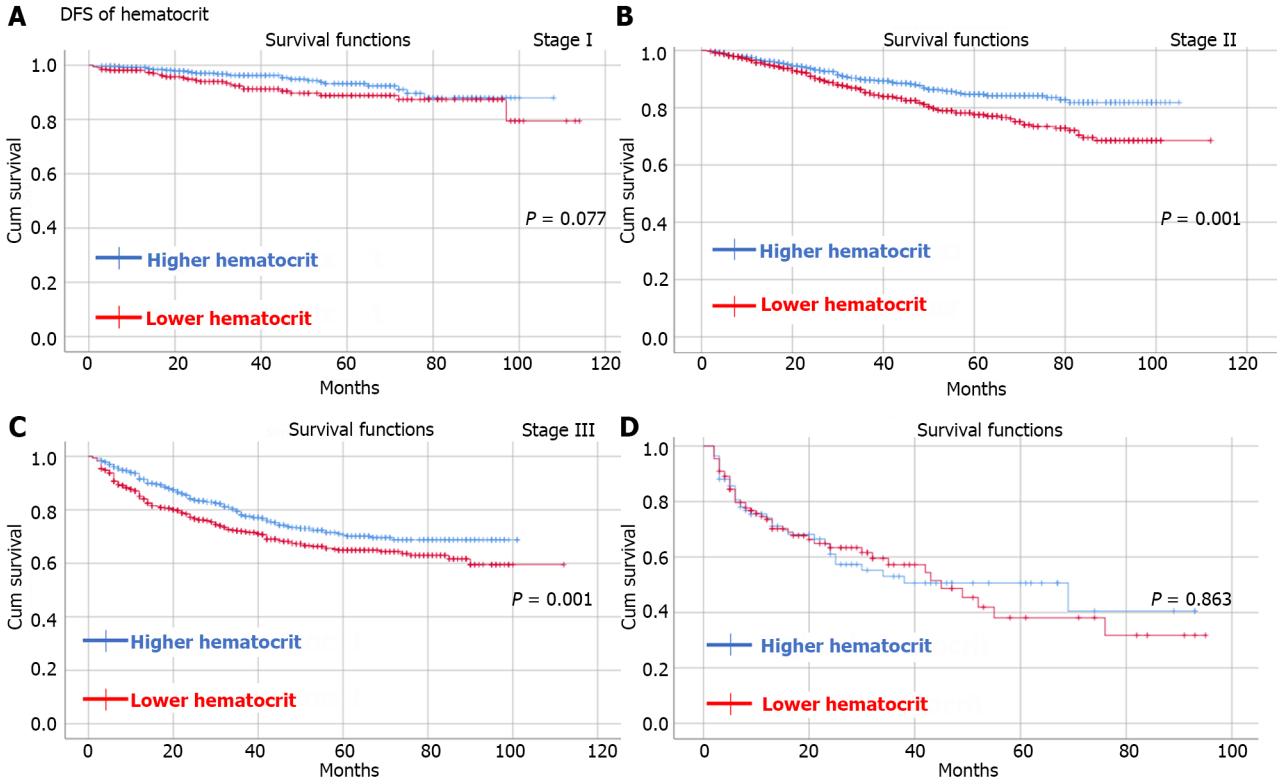

Figure 5 Kaplan-Meier survival curve for the impact of preoperative hematocrit on the disease-free survival of patients in tumor node metastasis stage I-IV. A: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the disease-free survival (DFS) of patients in tumor node metastasis (TNM) stage I; B: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the DFS of patients in TNM stage II; C: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the DFS of patients in TNM stage III; D: Kaplan-Meier survival curve for the impact of preoperative hematocrit on the DFS of patients in TNM stage IV.

ARTICLE HIGHLIGHTS

Research background

The prognostic value of red blood cell distribution width (RDW) for colorectal cancer (CRC) patients is controversial and the prognostic value of hematocrit for CRC patients has not been determined.

Research motivation

This was the first study to show that low hematocrit could predict worse overall survival (OS) and disease-free survival (DFS) in CRC patients after radical surgery.

Research objectives

The objective of this study was to explore the effect of RDW and hematocrit on the outcomes of CRC patients who underwent radical surgery.

Research methods

Patients who were diagnosed with CRC and underwent radical CRC resection between January 2011 and January 2020 at a single clinical center were included. An independent-sample *t*-test was used to compare the difference continuous variables that were expressed as the mean ± SD. χ^2 tests or Fisher's exact tests were used for categorical variables that were expressed as absolute values and percentages. The short-term outcomes, OS and DFS were compared among the different groups. To determine independent risk factors for overall complications, logistic regression analysis was conducted. Analysis of Cox regression was conducted to identify independent risk factors for OS and DFS.

Research results

There were 4258 CRC patients who underwent radical surgery included in our study. The higher RDW group had a worse OS and DFS than did the lower RDW group for tumor node metastasis (TNM) stage I (OS, $P < 0.05$; DFS, $P = 0.001$) and stage II (OS, $P = 0.004$; DFS, $P = 0.01$) than the lower RDW group; the lower hematocrit group had worse OS and DFS for TNM stage II (OS, $P < 0.05$; DFS, $P = 0.001$) and stage III (OS, $P = 0.001$; DFS, $P = 0.001$) than did the higher hematocrit group. RDW ($P > 0.05$) and hematocrit ($P > 0.05$) were not identified as independent indicators of overall complications. Preoperative hematocrit was an independent risk factor for OS [$P = 0.017$, hazard ratio (HR) = 1.256, 95% confidence interval (CI): 1.041-1.515] and DFS ($P = 0.035$, HR = 1.194, 95%CI: 1.013-1.408). However, RDW was not an independent

risk factor for OS (P = 0.396) or DFS (P = 0.308).

Research conclusions

This was the first study to show that low hematocrit could predict worse OS and DFS in CRC patients after radical surgery. A preoperative higher RDW and lower hematocrit were associated with more postoperative complications. However, only hematocrit was an independent risk factor for OS and DFS in CRC patients who underwent radical surgery, while RDW was not.

Research perspectives

Further multicenter prospective studies are needed to investigate the prognostic role of RDW and hematocrit.

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FOOTNOTES

Co-first authors: Dong Peng and Zi-Wei Li.

Author contributions: Peng D and Li ZW have contributed equally to this work. Peng D and Li ZW wrote the original draft; all authors contributed to data collection; Peng D and Liu F contributed to the data analysis; Peng D and Liu XR led the quality assessments; Peng D, Li ZW, and Wang CY revised the manuscript; and all authors have agreed on the journal to which the manuscript will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Institutional review board statement: The study was approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University (2022-K205), this study was conducted in accordance with the World Medical Association Declaration of Helsinki as well.

Informed consent statement: All patients signed informed consent.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Retrospective Study

Assessing recent recurrence after hepatectomy for hepatitis B-related hepatocellular carcinoma by a predictive model based on sarcopenia

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Abstract

BACKGROUND

Sarcopenia may be associated with hepatocellular carcinoma (HCC) following hepatectomy. But traditional single clinical variables are still insufficient to predict recurrence. We still lack effective prediction models for recent recurrence (time to recurrence < 2 years) after hepatectomy for HCC.

AIM

To establish an interventable prediction model to estimate recurrence-free survival (RFS) after hepatectomy for HCC based on sarcopenia.

METHODS

We retrospectively analyzed 283 hepatitis B-related HCC patients who underwent curative hepatectomy for the first time, and the skeletal muscle index at the third lumbar spine was measured by preoperative computed tomography. 94 of these patients were enrolled for external validation. Cox multivariate analysis was performed to identify the risk factors of postoperative recurrence in training cohort. A nomogram model was developed to predict the RFS of HCC patients, and its predictive performance was validated. The predictive efficacy of this model was evaluated using the receiver operating characteristic curve.

RESULTS

Multivariate analysis showed that sarcopenia [Hazard ratio(HR) = 1.767, 95%CI: 1.166-2.678, $P < 0.05$], alpha-fetoprotein ≥ 40 ng/mL (HR = 1.984, 95%CI: 1.307-

3.011, $P < 0.05$), the maximum diameter of tumor > 5 cm (HR = 2.222, 95%CI: 1.285-3.842, $P < 0.05$), and hepatitis B virus DNA level ≥ 2000 IU/mL (HR = 2.1, 95%CI: 1.407-3.135, $P < 0.05$) were independent risk factors associated with postoperative recurrence of HCC. Based on the sarcopenia to assess the RFS model of hepatectomy with hepatitis B-related liver cancer disease (SAMD) was established combined with other the above risk factors. The area under the curve of the SAMD model was 0.782 (95%CI: 0.705-0.858) in the training cohort (sensitivity 81%, specificity 63%) and 0.773 (95%CI: 0.707-0.838) in the validation cohort. Besides, a SAMD score ≥ 110 was better to distinguish the high-risk group of postoperative recurrence of HCC.

CONCLUSION

Sarcopenia is associated with recent recurrence after hepatectomy for hepatitis B-related HCC. A nutritional status-based prediction model is first established for postoperative recurrence of hepatitis B-related HCC, which is superior to other models and contributes to prognosis prediction.

Key Words: Alpha-fetoprotein; Hepatitis B virus; Hepatectomy; Hepatocellular carcinoma; Nomogram; Predictive models; Recurrence; Recurrence-free survival; Risk factors; Sarcopenia

Core Tip: Our focus on the factors that can intervene or improve the adverse outcomes of postoperative recurrence in patients with hepatitis B-related hepatocellular carcinoma (HCC) and establish a more effective model for predicting recurrence. Our study found Sarcopenia is remarkably associated with recent recurrence after hepatectomy for hepatitis B-related HCC. The SAMD model based on sarcopenia established in this study emphasizes the assessment and monitor of sarcopenia in hepatitis B-related HCC patients and effectively assists clinicians in closely to identify and monitor high-risk populations to improve the recurrence outcome of hepatitis B-related HCC patients after surgery through multi angle intervention measures.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. For patients with early-stage HCC, hepatic resection, and liver transplantation offer the most reasonable expectation for curative treatment[1]. Unfortunately, the prognosis of HCC patients following curative resection remains dismal due to the high postoperative recurrence rate. It is reported that 40%-70% of HCC cases present with disease recurrence within 5 years[2,3]. Currently, there is no effective therapy to prevent the recurrence of HCC, which makes early identification and timely treatment of the high-risk populations for HCC recurrence crucial to improving the prognosis of HCC.

Recurrence after hepatectomy for HCC is associated with many factors. Several prognostic scoring systems and models, such as assessment for surveillance interval score (AS score)[4], early recurrence after surgery for liver tumor (ERASL-pre), and model of recurrence after liver transplant (pre-MORAL)[5,6], have been developed to predict the risk of HCC recurrence preoperatively. However, it is difficult to guide clinically targeted interventions to factors related to the tumor. Nutritional status is crucial in determining the prognosis of HCC patients. Objective nutritional assessment and timely nutritional intervention may improve patient prognosis. Sarcopenia is a representative indicator of nutrition, associated with morbidity and mortality in various pathology, including colorectal, gastric, and liver[7,8]. Studies have also found that sarcopenia is a risk factor for the recurrence of HCC after curative treatment[9,10]. It's worth noting that measuring the skeletal muscle index (SMI) of the third lumbar spine (L3) by abdominal computed tomography (CT) is the most objective method for assessing sarcopenia in liver disease[11]. This study aims to establish a comprehensive prediction model for HCC recurrence based on sarcopenia to improve the prediction of early recurrence risk in HCC patients undergoing hepatectomy, which can provide a reference for the formulation of comprehensive treatment plans for patients.

MATERIALS AND METHODS

Study population

All patients who underwent primary hepatectomy for HCC at the Guizhou Provincial People's Hospital between January 2017 and September 2021 were retrospectively collected. The inclusion criteria included: (1) First hepatectomy for HCC;

(2) no extrahepatic metastasis; (3) above 18 years old; and (4) history of chronic hepatitis B with positive hepatitis B surface antigen. The exclusion criteria included: (1) Data incomplete; (2) history of hepatectomy for liver malignancies; (3) history of transcatheter arterial chemoembolization for HCC; (4) history of chemotherapy for HCC; (5) particular subtypes of HCC confirmed by resected specimens (such as intrahepatic cholangiocarcinoma and combined hepatocellular-cholangiocarcinoma); and (6) patients complicated with other malignant tumors. Detailed patient information is shown in Figure 1. Specifically, 189 patients in the training cohort were hospitalized at the second ward of hepatobiliary surgery; Moreover, 94 patients hospitalized in the third ward of hepatobiliary surgery at the Guizhou Provincial People's Hospital were enrolled for external validation.

Clinical data

The demographic data and laboratory parameters of all patients were extracted by reviewing the medical records. All biochemical and pathological indicators were determined in our laboratory. The laboratory parameters included alpha-fetoprotein (AFP), alanine-aminotransferase (ALT), total bilirubin, albumin (ALB), international normalized ratio (INR), leukocyte, platelet count (PLT), serum Hepatitis B virus deoxyribonucleic acid (HBV DNA). The pathological characteristics of HCC including the degree of differentiation, liver capsular invasion, and microvascular invasion (MVI) were recorded. The maximum tumor diameter and the third lumbar-skeletal muscle area (SMA) were measured by CT before hepatectomy. Sarcopenia was assessed by the L3-SMI. Existing prediction models: AS score = 0.176 × age (year) + 17.279 × INR–21.887[4]; ERASL-pre score = 0.818 × Gender (0: Female, 1: Male) + 0.447 × Albumin-Bilirubin (ALBI) grade (0: Grade 1; 1: Grade 2 or 3) + 0.100 × ln (Serum AFP in μg/L) + 0.580 × ln (Tumor size in cm) + 0.492 × Tumor number (0: Single; 1: Two or three; 2: Four or more)[5]; pre-MORAL score = tumor size > 3 cm (Score: 3) + AFP ≥ 200 ng/mL (Score: 4) + and the neutrophil-lymphocyte ratio > 5 (Score: 6)[6]; Cut-offs to generate the risk groups: AS score ≥ 9.26, ERASL-pre score > 3.521 (high), ERASL-pre score > 3.521 (high), and pre-MORAL score > 7 (high). Use these models to rate in our dataset.

Surgery and definition

In our institute, the selection of patients with HCC for hepatectomy was based on considering tumor factor, liver functional status, and patient factor. A curative resection was defined as a complete resection of all macroscopically evident tumors. The absence of tumor cells along the parenchymal transection line was confirmed histologically. No tumors remained on CT or serological features in the remnant liver at 2 months after the operation. HCC recurrence was defined as the recurrence of HCC after radical treatment of HCC. Recent recurrence [time to recurrence (TTR) < 2 years] and forward recurrence (TTR ≥ 2 years) by TTR[12]. All recurrent tumors were new lesions diagnosed by radiological features typical of HCC in the CT scans[13].

Using CT images, SMA and SMI at the L3 vertebra could be calculated and analyzed to evaluate skeletal muscle mass. Data at CT were obtained at baseline. L3-SMI was calculated using the following formula: L3-SMI = L3 SMA (cm^2)/the square of the patient's height (m^2). The diagnostic criteria of sarcopenia were determined based on the Japan Society of Hepatology *Guidelines for Sarcopenia in Liver Disease*[14]. L3-SMI of female and male patients were < 38 cm^2/m^2 and < 42 cm^2/m^2, respectively.

Assessment of recurrence

The follow-up imaging results of patients after HCC resection were reviewed every 3-6 months. Tumor recurrence was defined as new lesions in the liver detected by liver ultrasound, CT, or magnetic resonance imaging, with elevated serum AFP.

Ethics statement

The present study was approved by the Ethics Committee of Guizhou Provincial People's Hospital and performed according to the ethical guidelines of the 1975 Declaration of Helsinki. The requirement for informed consent was waived due to the retrospective nature of the study and the anonymity of the data.

Statistical analysis

Categorical variables were presented in number and percentage (%), and variables were compared using the chi-square test. Continuous variables are presented as mean ± standard deviation or median (interquartile range). Quantitative variables in normal distribution were compared by student's *t*-test or Mann-Whitney nonparametric U test. recurrence-free survival (RFS) was calculated by the Kaplan-Meier method, and RFS curves were compared using the log-rank test. The cox regression model performed univariate and multivariate analyses with a stepwise selection of variables. The time-dependent area under the receiver operating characteristic curve ROC). Based on the selected independent predictors, nomograms were constructed, and assessment calibration curves were plotted to assess the calibration of the model. Statistical analysis was performed using IBM SPSS 23.0 software (IBM, Armonk, NY, United States), GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, United States), and the R statistical programming version 3.3.1 (Vienna, Austria, http://www.r-project.org). A value of $P < 0.05$ was considered statistically significant.

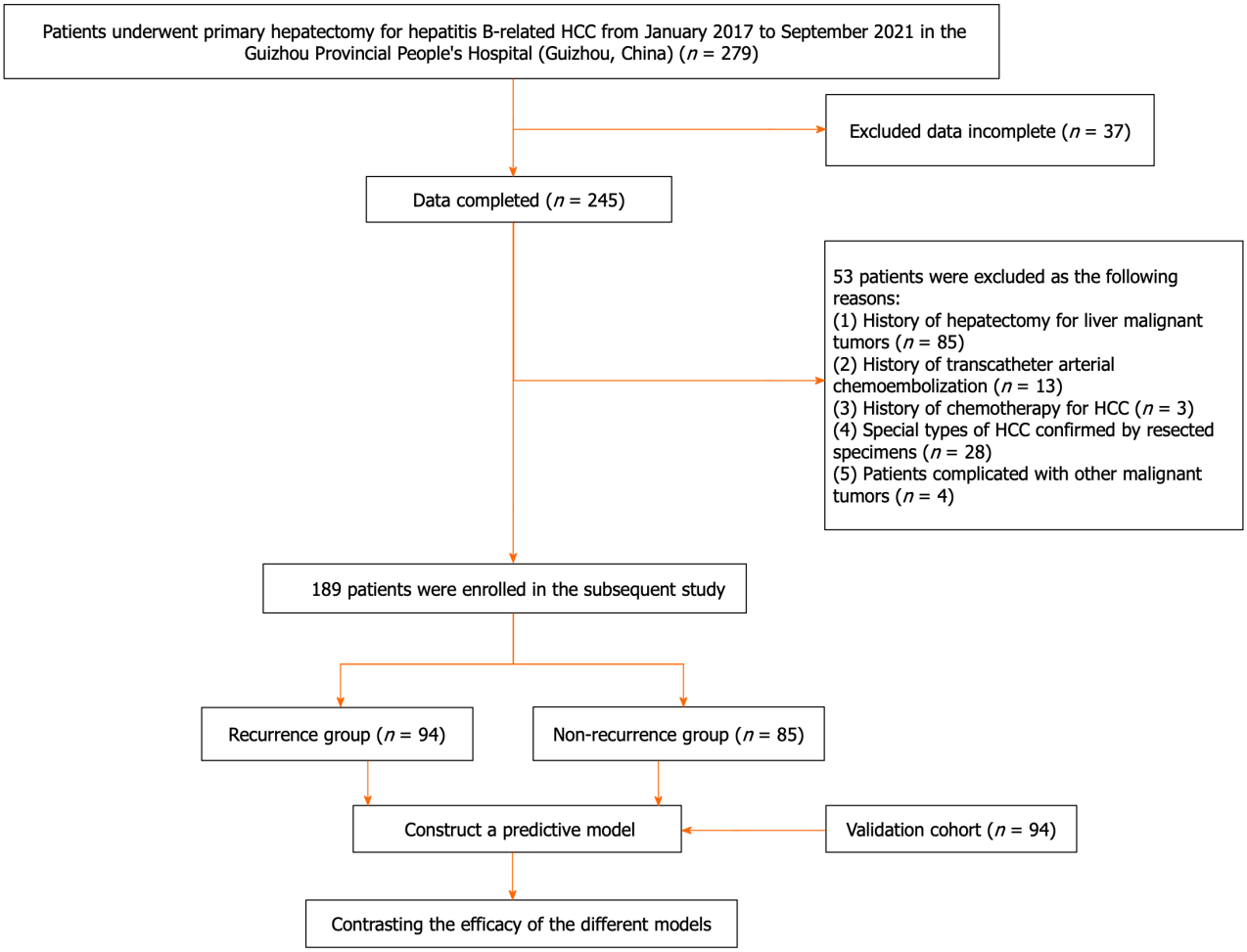

Figure 1 Flowchart of patients. HCC: Hepatocellular carcinoma.

RESULTS

Characteristics of patients

A total of 283 patients were recruited into the training and validation cohorts, including 241 men (85.2%) and 42 women (14.8%), with a mean age of 52.71 ± 11.15 years. The follow-up results showed that 144 (50.9%) patients developed recurrence within 2 years after hepatectomy, and the median RFS was 7.67 months (95%CI: 6.59-8.75), The baseline characteristics of the training and validation cohorts are presented in Table 1. All baseline characteristics were comparable between the two cohorts ($P > 0.05$). Among the 189 patients enrolled in the training cohort, 104 (55%) patients experienced HCC recurrence with the median RFS of 6 months (95%CI: 7.18-11.05), and the remaining 85 (45%) patients were assigned to the non-recurrence group. Further, as shown in Table 2, the recurrence rate was higher in patients with preoperative sarcopenia (66.7% *vs* 50.3%, $P < 0.05$) and HBV-DNA ≥ 2000 IU/mL (71% *vs* 47.2%, $P < 0.05$). There were significant differences in tumor differentiation, MVI and the maximum diameter of tumor between the recurrence group and the non-recurrence group (all $P < 0.05$). There was no significant difference in age, gender, liver capsular invasion, liver cirrhosis, ALT, albumin, total bilirubin, leukocyte, INR, PLT between the recurrence group and the non-recurrence group ($P > 0.05$).

Risk factors for HCC recurrence after hepatectomy

Univariate analysis showed that preoperative sarcopenia, tumor differentiation, the maximum diameter of tumor, HBV-DNA level, and AFP ≥ 40 ng/mL, tumor differentiation, MVI were related to HCC recurrence after hepatectomy (AFP cut-off value was analyzed by the ROC curve). Further multivariate analysis revealed that preoperative sarcopenia, AFP ≥ 40 ng/mL, the maximum diameter of tumor > 5 cm, and HBV-DNA level ≥ 2000 IU/mL were independent risk factors of HCC recurrence after hepatectomy, regardless of whether pathological factors were included in the multivariate analysis (Table 3).

Construction and validation of the prediction model

The four available risk factors found to be independent predictors of RFS after hepatectomy were used to develop a SAMD model, and a visual nomogram was established (Figure 2A). The total SAMD score was calculated using the corresponding score obtained from the vertical line of each risk factor coordinate axis (corresponding to the last

Table 1 Baseline characteristics of patients in the training and validation cohorts of hepatocellular carcinoma patients

Characteristic	Total (*n* = 283)	Training cohort (*n* = 189)	Validation cohort (*n* = 94)	*P* value
Age (yr)	52.71 ± 11.15	52.86 ± 11.65	52.43 ± 10.12	0.760
Gender, *n* (%)				0.467
Male	241 (85.2)	163 (86.2)	78 (83.0)	
Female	42 (14.8)	26 (13.8)	16 (17.0)	
Sarcopenia, *n* (%)	91 (32.2)	54 (28.6)	37 (39.4)	0.067
Liver cirrhosis, *n* (%)	169 (59.7)	105 (55.6)	64 (68.1)	0.053
MVI, *n* (%)	126 (44.5)	89 (47.1)	37 (39.6)	0.218
Tumor differentiation, *n* (%)				0.530
Poor	56 (19.8)	33 (17.5)	23 (24.5)	
Moderate	128 (45.2)	81 (42.9)	47 (50.0)	
Well	99 (35.0)	75 (39.6)	24 (25.5)	
Liver capsular invasion	160 (56.5)	112 (59.3)	45 (51.1)	0.190
Maximum diameter of tumor, *n* (%)				0.062
≤ 3 cm	72 (25.4)	40 (21.2)	32 (34.0)	
3-5 cm	62 (21.9)	43 (22.8)	19 (20.2)	
> 5 cm	149 (52.7)	106 (56.1)	43 (45.7)	
HBV-DNA, *n* (%)				0.130
< 2000 IU/mL	173 (61.1)	127(67.2)	46 (48.9)	
≥ 2000 IU/mL	110 (38.9)	62 (32.8)	48 (51.1)	
AFP ≥ 40 ng/mL	158 (55.8)	106 (56.1)	52 (55.3)	0.903
ALT, U/L	36 (26-58)	36 (26-51.75)	38.5 (26.75-63.5)	0.191
Total bilirubin (mmol/L)	14.80 (11.15-22.02)	14.80 (11.15-22.02)	19.8 (11.95-30.4)	0.051
Albumin, g/L	40.2 (35.80-43.70)	40.52 (35.43-43.98)	40.2 (36.32-43.02)	0.796
INR	1.02 (0.95-1.09)	1.01 (0.94-1.08)	1.04 (0.96-1.12)	0.146
Leukocyte, 10^9/L	5.49 (4.24-7.14)	5.38 (4.37-6.49)	6.13 (4.04-8.05)	0.054
PLT, 10^9/L	154 (105-207)	162 (109-220)	137 (94-185)	0.012
Recurrence	144 (50.9)	104 (55)	40 (42.6)	0.221

MVI: Microvascular invasion; HBV-DNA: Hepatitis B virus deoxyribonucleic acid; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; INR: International normalized ratio; PLT: Platelet count.

coordinate axis) to get the 1- and 2-year RFS in each patient. To evaluate the SAMD model, we plotted a calibration curve based on the actual probability of postoperative recurrence and the predicted probability of postoperative recurrence. The calibration curves indicated a good consistency between the predicted and observed results in both the training cohort and validation cohort (Figure 2B). Meanwhile, an online calculator was developed to permit easy clinical application: https://yuchch.shinyapps.io/DynNomappRFS/. We can use Dynamic Nomogram to Predicted Survey at the Follow Up.

Predictive value of the model

Furthermore, we compared the SAMD model with other preoperative models and assessed their prognostic values by analyzing the AUC (Figure 3A). The AUC of the SAMD model for predicting 2-year RFS was 0.782 (95%CI: 0.705-0.858) (P < 0.001). The sensitivity and specificity were 81% and 63%. The AUC of the verification cohort for predicting 2-year RFS was 0.773 (95%CI: 0.707-0.838), reflecting a good accuracy of the nomogram. For other models, the AUC of the AS score was 0.555 (95%CI: 0.472-0.637, P = 0.195), and the sensitivity and specificity were 37.9% and 77.7%, respectively. The AUC of the ERASL-pre model. and the pre-MORAL model was 0.623 (95%CI: 0.543-0.702, P = 0.004) and 0. 587 (95%CI: 0.506-0.669, P = 0.038), respectively. The sensitivity and specifcity were 55.8% and 68.1% for the ERASL-pre model and 81.1% and 61.7% for the pre-MORAL model, respectively. From all the above, our nomogram exhibits favorable clinical practicality and is a promising clinical decision-making tool. The SAMD model significantly outperforms other existing prediction systems.

Table 2 Baseline characteristics of and biochemical data of hepatocellular carcinoma patients in the training cohort

Characteristic	Total (*n* = 189)	Recurrence group (*n* = 104)	Non-recurrence group (*n* = 85)	*P* value
Age (yr)	52.86 ± 11.65	51.92 ± 11.42	54 ± 11.88	0.751
Gender, *n* (%)				0.579
Male	163 (86.2)	91 (87.5)	72 (84.7)	
Female	26 (13.8)	13 (12.5)	13 (15.3)	
Sarcopenia, *n* (%)	54 (28.6)	36 (66.7)	18 (33.3)	0.042
Liver cirrhosis, *n* (%)	105 (55.6)	55 (52.4)	50 (47.6)	0.414
MVI, *n* (%)	89 (47.1)	56 (62.9)	33 (37.1)	0.04
Tumor differentiation, *n* (%)				0.008
Poor	33 (17.5)	22 (66.7)	11 (33.3)	
Moderate	81 (42.9)	51 (63.0)	30 (37.0)	
Well	75 (39.6)	31 (41.3)	44 (58.7)	
Liver capsular invasion	112 (59.3)	67 (59.8)	45 (40.2)	0.11
Maximum diameter of tumor, *n* (%)				0.003
≤ 3 cm	40 (21.2)	16 (40.0)	25 (60.0)	
3-5 cm	43 (22.8)	18 (41.9)	25 (58.1)	
> 5 cm	106 (56.1)	70 (66.0)	38 (34.0)	
HBV-DNA, *n* (%)				0.002
< 2000 IU/mL	127 (67.2)	60 (47.2)	67 (52.8)	
≥ 2000 IU/mL	62 (32.8)	44 (71.0)	20 (29.0)	
AFP ≥ 40 ng/mL	106 (56.1)	70 (66.0)	36 (34.0)	0.001
ALT, U/L	36 (26-51.75)	36 (28-52.75)	36 (22.25-51)	0.481
Total bilirubin (mmol/L)	14.80 (11.15-22.02)	15.90 (12.15-23.15)	14.3 (10.55-19.70)	0.051
Albumin, g/L	40.52 (35.43-43.98)	39.05 (34.65-43.5)	41 (36.62-44.48)	0.096
INR	1.01 (0.94-1.08)	1.0 (0.95-1.07)	1.01 (0.93-1.09)	0.846
Leukocyte, 10^9/L	5.38 (4.37-6.49)	5.38 (4.24-6.79)	5.39 (4.5-6.36)	0.921
PLT, 10^9/L	162 (109-220)	147 (100-200)	176 (116-234)	0.056

MVI: Microvascular invasion; HBV-DNA: Hepatitis B virus deoxyribonucleic acid; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; INR: International normalized ratio; PLT: Platelet count.

Finally, the patients were divided into the high-risk and low-risk recurrence groups by the preselected cut-off point of SAMD score ≥ 110. Further, the RFS rates predicted by different models were compared by the kaplan-meier curve (Figure 3B). Meanwhile, according to hazard ratio (HR) by Cox regression, patients in the high-risk group with a SAMD score ≥ 110, ERASL-pre score > 3.521, pre-MORAL score > 7, and AS score ≥ 9.26 had higher likelihood of recurrence events than those patients in the low-risk group with the scores below cut-off points, and the HR were 4.228-fold, 2.053-fold, 1.802-fold, and 1.506-fold, respectively. It is proved that a SAMD score ≥ 110 can better distinguish the high-risk group for postoperative HCC recurrence.

DISCUSSION

Surgical resection is the best option for HCC patients to get a cure[15]. However, it cannot be ignored that a high recurrence rate after surgery. In our study, the recent recurrence rate is as high as 55%. Therefore, it is necessary to analyze the risk factors affecting the recurrence of early-stage HCC patients and establish a predictive model to make the prediction of recurrence risk. This study indicated that preoperative sarcopenic, AFP ≥ 40 ng/mL, the maximum diameter of tumor > 5 cm, and HBV-DNA level ≥ 2000 IU/mL were independent prognostic predictors for recurrence after hepatectomy. More importantly, in predicting the prognosis of HCC patients undergoing hepatectomy, the sarcopenia-based nomogram named SAMD model showed superior discrimination over other indicators, indicating that this

Table 3 Univariate and multivariate Cox proportional hazard models of hepatocellular carcinoma recurrence after hepatectomy involving whether pathological factors in the training cohort

Variable	Univariate analysis			Multivariate analysis without pathological factors			Multivariate analysis with pathological factors		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Male	1.038	0.58-1.858	0.9						
Age (yr)	0.991	0.975-1.007	0.255						
Sarcopenia	1.61	1.073-2.416	0.022	1.814	1.196-2.751	0.005	1.819	1.199-2.759	0.005
Liver cirrhosis (yes, no)	0.888	0.593-1.328	0.562						
Liver capsular invasion	1.289	0.863-1.927	0.215						
ALT (U/L)	1	0.997-1.002	0.746						
Total bilirubin (mmol/L)	0.994	0.975-1.014	0.577						
Albumin (g/L)	0.971	0.942-1.001	0.061						
INR	1.055	0.951-1.171	0.312						
MVI	1.663	1.13-2.449	0.01						
Tumor differentiation									
Well	Ref.								
Moderate	1.764	1.127-2.76	0.013						
Poor	1.93	1.115-3.34	0.019						
Maximum diameter of tumor									
≤ 3 cm	Ref.			Ref.			Ref.		
3-5 cm	1.063	0.542-2.087	0.858	1.134	0.576-2.233	0.715	1.151	0.585-2.268	0.684
> 5 cm	2.202	1.277-3.796	0.005	2.286	1.322-3.952	0.003	2.228	1.288-3.852	0.004
HBV-DNA									
< 2000 IU/mL	Ref.			Ref.			Ref.		
≥ 2000 IU/mL	2.007	1.358-2.966	< 0.001	2.191	1.468-3.272	< 0.001	2.162	1.45-3.223	< 0.001
AFP ≥ 40 ng/mL	1.92	1.269-2.904	0.002	2.032	1.322-3.952	< 0.001	1.823	1.198-2.773	0.005

MVI: Microvascular invasion; HBV-DNA: Hepatitis B virus deoxyribonucleic acid; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; INR: International normalized ratio; PLT: Platelet count.

nomogram may be helpful for clinical monitoring and timely intervention.

Previous studies have shown that recurrence after hepatectomy is associated with characteristics of tumors themselves, surgery-related factors, and patient's state including large tumor size, satellite lesions, poorly differentiated tumors, vascular invasion, age, male gender, and so on[16-19]. In this study, MVI, tumor differentiation degree, and tumor diameter were statistically different between the recurrence and non-recurrence groups. Since many risk factors of HCC recurrence can not be modified (such as age and pathological type), our focus should be on factors that can be intervened or improved preoperatively, which is even more attractive for clinical treatment guidance.

Our study found that 28.6% patients presented with sarcopenia, 66.7% of which experienced postoperative recurrence in the training cohort. Sarcopenia is accepted as one of the independent risk factors for postoperative recurrence[10]. Patients without sarcopenia can typically achieve a more prolonged RFS during follow-up. dietary supplementation may substantially improve muscle mass in cancer patients[20]. The mechanism underlying the association between sarcopenia and HCC recurrence is unclear but may be related to the tumor microenvironment (inflammation and immunity) and cytokine (myokines and adipokines)[21]. It seems promising to improve the prognosis through nutritional intervention. However, correcting the nutritional status takes a long time, and delaying surgical intervention in patients with cancer may worsen the prognosis and result in tumor progression. Therefore, we should strengthen the preoperative and postoperative monitoring of sarcopenia, supplement nutritional treatment, and implement long-term monitoring of nutritional status. Since every patient after hepatectomy of HCC requires an abdominal CT examination in follow-up. Determining sarcopenia by calculating abdominal CT SMI is feasible and objective, which shortens the patient's time and does not increase additional evaluation costs.

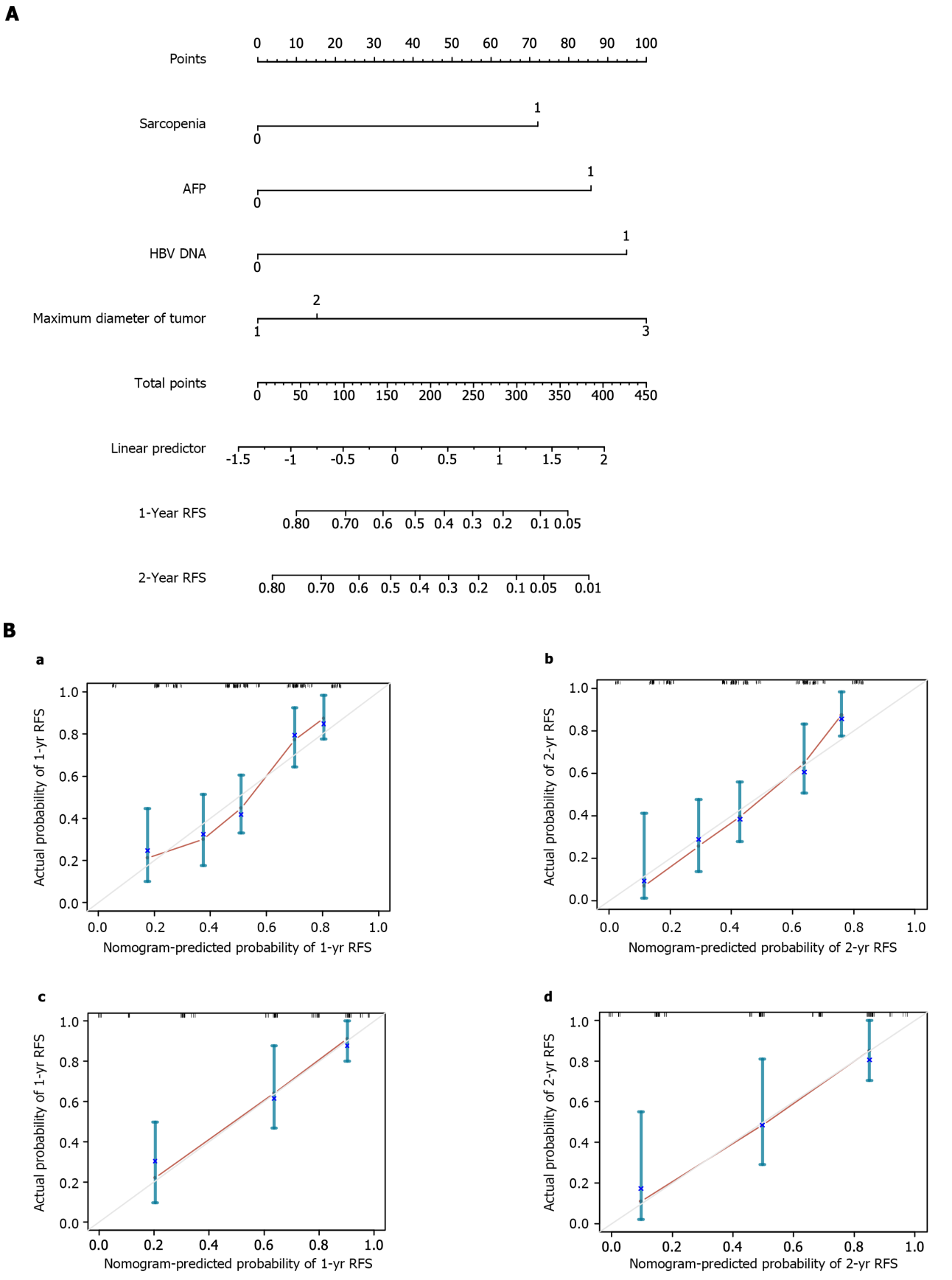

Figure 2 Predicting the probability of 1- and 2-yr recurrence-free survival in hepatocellular carcinoma patients at 1- and 2-yr after hepatectomy using a visualized SAMD model's nomogram (SAMD model: Sarcopenia, alpha-fetoprotein ≥ 40 ng/mL, the maximum diameter of tumor > 5 cm, and hepatitis B virus level ≥ 2000 IU/mL). A: Calibration plots of predicted 1- and 2-yr recurrence-free survival based on the cox regression model in the training and validation cohorts; B: Training cohort (a and b); validation cohort (c and d). AFP: Alpha-fetoprotein; RFS: Recurrence-free

survival; HBV: Hepatitis B virus; SAMD: Based on the sarcopenia to assess the recurrence-free survival model of hepatectomy with hepatitis B-related liver cancer disease.

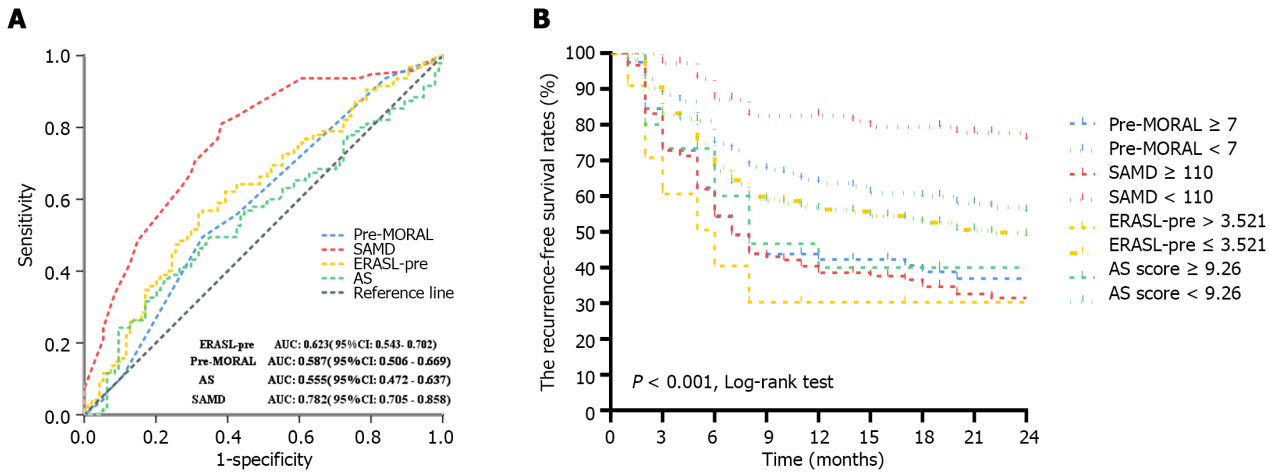

Figure 3 Comparison of receiver operating characteristic curves and recurrence-free survival rates between SAMD model and other models in predicting 2-year recurrence in hepatocellular carcinoma patients after hepatectomy. SAMD model [sarcopenia, alpha-fetoprotein (AFP) ≥ 40 ng/mL, the maximum diameter of tumor > 5 cm, and hepatitis B virus deoxyribonucleic acid level ≥ 2000 IU/mL]; assessment for surveillance interval score (age and international normalized ratio); pre-model of recurrence after liver transplantation (tumor size > 3 cm, AFP ≥ 200 ng/mL, and Neutrophil-to-lymphocyte ratio > 5); the pre-operative model, early recurrence after surgery for liver tumor: (Gender, albumin-bilirubin grade, AFP, tumor size, and tumor number). A: Receiver operating curve; B: Recurrence-free survival rates. pre-MORAL: Pre-model of recurrence after liver transplantation; ERASL-pre: The pre-operative model, early recurrence after surgery for liver tumor; AS: Assessment for Surveillance interval score; SAMD: Based on the sarcopenia to assess the recurrence-free survival model of hepatectomy with hepatitis B-related liver cancer disease.

On the other hand, HBV infection is the leading cause of HCC worldwide, accounting for 33% of cases[22], which is also still one of the main factors causing HCC in China[5]. Many studies have shown that viral load plays a crucial role in the prognosis of HCC[23,24]. In this study, we observed a more significant recurrence rate in patients with high viral load. HBV DNA levels ≥ 2000 IU/mL were an independent prognostic factor for HCC recurrence after curative hepatectomy. At the same time, we also observed that although the recurrence rate is low in low-level viremia, 47.2% of patients still have a recurrence. Studies have shown that low-level viremia patients with a relatively low viral load can still benefit from effective antiviral therapy, significantly reducing HCC recurrence after hepatic resection[25]. Herein, it is recommended to use a highly sensitive real-time quantitative PCR method to detect HBV DNA, with a lower quantitative limit of 10-20 IU/mL or even lower, thereby initiating antiviral therapy or adjusting the treatment schedule promptly.

Several studies have proposed the prediction models of HCC recurrence, such as the preoperative (ERASL-pre) and postoperative (ERASL-post) risk models[5] based on gender, ALBI score, serum AFP, and tumor volume and quantity. The pre-MORAL and postoperative post-MORAL models[6] can predict recurrence after liver transplantation. The RETREAT scale can also assess the risk of recurrence after liver transplantation[5]. The AS score evaluates the risk of recurrence after liver resection and radiofrequency ablation[4]. However, the current prediction models didn't consider nutritional factors. Therefore, we compared the SAMD model with other preoperative prediction models and revealed that the SAMD model was more capable of predicting postoperative recurrence. Furthermore, compared for the different models prediction of RFS between the low- and high-risk groups, which revealed that the RFS rate in the high-risk patients with a SAMD model was lower than that in patients with an AS score and pre-MORAL. Patients in the high-risk group of the SAMD scores, the ERASL-pre scores, the Pre-MORAL scores and the AS scores had higher likelihood of recurrence events, and the hazard regression ratio are 4.228-fold, 2.053-fold, 1.802-fold and 1.506-fold, respectively. It is proved that a SAMD score was better to distinguish the high-risk group of postoperative recurrence of HCC than other model. Besides, a SAMD score < 110 can better distinguish the low-risk group of postoperative HCC recurrence, which is also significantly outperforms other prediction models. The RFS curve of the SAMD model indicates that the application of this model may extend the monitoring interval for low-risk patients, reduce patient economic costs, and improve social benefits.

However, the present study also has some limitations. Firstly, as a retrospective single-center study, this study has a limited sample size. This could limit the generalizability of the findings to a broader population. Extensive prospective studies with different geographical locations, ethnic backgrounds, or healthcare systems are needed to verify the model's efficacy. Secondly, the study does not seem to account for potential confounding factors that could influence the results, such as the patient's overall health status, lifestyle factors, or other comorbidities. Patients with liver cirrhosis and those without liver cirrhosis were included in this study. Although these groups were well-balanced, different grades of liver cirrhosis and varying degrees of portal hypertension might introduce selection bias and impact our results. On the other hand, limitations in statistical methods may also affect the effectiveness of the model. In addition, only the hepatitis B

population was selected in this study, lacking the discussion of other causes.

CONCLUSION

In conclusion, we revealed that sarcopenia before hepatectomy is associated with recent recurrence after hepatectomy for hepatitis B-related HCC patients, and the prediction model based on liver nutrition was first established for postoperative recurrence of hepatitis B-related HCC. the SAMD model based on sarcopenia has favorable performance in predicting RFS in patients undergoing hepatectomy for hepatitis B-related HCC. It is helpful for the comprehensive clinical intervention in such patients. In the future, we need to further validate and apply this model, and conduct prospective studies to explore the impact of nutritional interventions on patient survival outcomes.

ARTICLE HIGHLIGHTS

Research background

The recurrence of hepatocellular carcinoma (HCC) has a significant impact on the survival outcomes of patients, and early prediction and intervention can help improve patient survival outcomes. Nutritional factors have always been a hot topic of concern and are prone to intervention.

Research motivation

Sarcopenia is one of the effective indicators for evaluating nutritional status in chronic liver disease, which was reported that sarcopenia as a negative prognostic factor in patients with HCC. Hence, it is necessary to incorporate them into models for predicting early recurrence of HCC to screen out high-risk groups, as they may require more aggressive intervention.

Research objectives

This study aimed to construct a nutrition-based model to estimate recurrence-free survival (RFS) after hepatectomy for hepatitis B-related HCC based on sarcopenia.

Research methods

According to the inclusion and exclusion criteria, 283 patients with hepatitis B-related HCC were eventually enrolled in this retrospective study: 189 patients in the training cohort and 94 patients in the validation cohort. Skeletal muscle index at the third lumbar spine was evaluated according to abdominal computed tomography scans before hepatectomy. Independent predictors of disease recrudescence were evaluated with univariate and multivariate Cox proportional hazard models in training cohort, and A nomogram model was developed to predict the RFS of HCC patients. Its predictive performance was validated in the validation cohort. Furthermore, we compared the predictive model with other preoperative models and assessed their prognostic values by analyzing the time-dependent area under the receiver operating characteristic curve (tdAUROC).

Research results

Our data demonstrated that among 144 (50.9%) patients developed recurrence within 2 years after hepatectomy, and the median RFS was 7.67 months (95%CI: 6.59-8.75). Multivariate analysis showed that sarcopenia, alpha-fetoprotein ≥ 40 ng/mL, the maximum diameter of tumor > 5 cm, and hepatitis B virus DNA level ≥ 2000 IU/ mL were independent risk factors associated with postoperative recurrence of HCC. The SAMD model predicting the RFS of HCC patients was established based on the above factors. The area under the curve of the SAMD model was 0.782 (95%CI: 0.705-0.858) in the training cohort (sensitivity 81%, specifcity 63%) and 0.773 (95%CI: 0.707-0.838) in the validation cohort. Besides, a SAMD score ≥ 110 was better to distinguish the high-risk group of postoperative recurrence of HCC compared to other models. Further multicenter studies are warranted to validate our findings.

Research conclusions

Our study highlights the strong correlation between sarcopenia and recent recurrence after hepatectomy for hepatitis B-related HCC. A predictive model based on sarcopenia for assessing recent recurrence after liver resection for hepatitis B-associated hepatocellular carcinoma was developed for the first time.

Research perspectives

The SAMD model based on sarcopenia has favorable performance in predicting RFS in patients undergoing hepatectomy for hepatitis B-related HCC. It is helpful for the comprehensive clinical intervention in such patients. In the future, we need to further validate and apply this model, and conduct prospective studies to explore the impact of nutritional interventions on patient survival outcomes.

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FOOTNOTES

Co-first authors: Hong Peng and Si-Yi Lei.

Author contributions: Peng H and Lei SY contributed equally to this work; Peng H and Lei SY conceptualized and designed the study; Fan W, Dai Y, Zhang Y and Chen G were the hepatobiliary surgery specialists; Fan W, Dai Y, Zhang Y and Chen G, Liu TZ and Xu JH acquired the data; Lei SY and Huang Y analyzed and interpreted the data; Xiong TT, Wang XF and Luo XH provided fund support; Lei SY drafted the manuscript; Peng H and Luo XH critically revised the manuscript for important intellectual content; Peng H , Fan W, Zhang Y and Luo XH provided administrative, technical, or material support; Peng H and Luo XH supervised the study. All authors made a significant contribution to this study and approved the final manuscript.

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Institutional review board statement: The 1975 Declaration of Helsinki's ethical principles were followed by the research design. The ethics committee of the Guizhou Provincial People's Hospital authorized the investigation, which attests to this (Approval No: 2023-009).

Informed consent statement: Due to the study's retrospective character, an exemption from the informed consent criteria was authorized.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets generated during and analyzed during the current study are not publicly available due to participant privacy issues but are available from the corresponding author at Luoxinhua1972@126.com upon reasonable request.

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ORIGINAL ARTICLE

Retrospective Study

Treatment patterns and survival outcomes in patients with non-metastatic early-onset pancreatic cancer

Le-Tian Zhang, Ying Zhang, Bi-Yang Cao, Chen-Chen Wu, Jing Wang

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Abstract

BACKGROUND

The incidence of patients with early-onset pancreatic cancer (EOPC; age ≤ 50 years at diagnosis) is on the rise, placing a heavy burden on individuals, families, and society. The role of combination therapy including surgery, radiotherapy, and chemotherapy in non-metastatic EOPC is not well-defined.

AIM

To investigate the treatment patterns and survival outcomes in patients with non-metastatic EOPC.

METHODS

A total of 277 patients with non-metastatic EOPC who were treated at our institution between 2017 and 2021 were investigated retrospectively. Overall survival (OS), disease-free survival, and progression-free survival were estimated using the Kaplan–Meier method. Univariate and multivariate analyses with the Cox proportional hazards model were used to identify prognostic factors.

RESULTS

With a median follow-up time of 34.6 months, the 1-year, 2-year, and 3-year OS rates for the entire cohort were 84.3%, 51.5%, and 27.6%, respectively. The median

OS of patients with localized disease who received surgery alone and adjuvant therapy (AT) were 21.2 months and 28.8 months, respectively (P = 0.007). The median OS of patients with locally advanced disease who received radiotherapy-based combination therapy (RCT), surgery after neoadjuvant therapy (NAT), and chemotherapy were 28.5 months, 25.6 months, and 14.0 months, respectively (P = 0.002). The median OS after regional recurrence were 16.0 months, 13.4 months, and 8.9 months in the RCT, chemotherapy, and supportive therapy groups, respectively (P = 0.035). Multivariate analysis demonstrated that carbohydrate antigen 19-9 level, pathological grade, T-stage, N-stage, and resection were independent prognostic factors for non-metastatic EOPC.

CONCLUSION

AT improves postoperative survival in localized patients. Surgery after NAT and RCT are the preferred therapeutic options for patients with locally advanced EOPC.

Key Words: Pancreatic cancer; Early-onset; Non-metastatic; Multimodal treatment; Radiotherapy; Overall survival

Core Tip: Young adults are an important subgroup of the pancreatic cancer (PC) patient population. This article describes the comprehensive treatment patterns and survival outcomes for patients with non-metastatic early-onset PC (EOPC) from a high-volume center. We demonstrated that adjuvant therapy significantly improves postoperative survival in patients with limited EOPC. We also found that radiotherapy-based combination therapy achieved favorable outcomes in patients with locally advanced and postoperative recurrence. Our findings support an aggressive multimodal treatment strategy for these unique patients.

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INTRODUCTION

Pancreatic cancer (PC) is a clinically challenging disease with a 5-year survival rate of only 12.5%[1] because of its insensitivity to therapy and rapid progress. It is estimated that PC will become the second-leading cause of cancer-related deaths by 2030[2]. The incidence and mortality rate of PC tend to increase in young people in many countries[3-5]. Early-onset PC (EOPC) is generally defined as PC diagnosed before the age of 50 years and accounts for approximately 4%-18%. Although EOPC is less common than late-onset PC, it greatly increases the burden on individuals, families, and society of PC patients.

A study reported that EOPC is responsible for 20%-30% of the total number of years of life lost due to the disease[6]. Several studies have demonstrated that smoking, obesity, diabetes, and alcohol consumption are key modifiable risk factors for EOPC[7]. According to older studies, the clinicopathological features of young patients with PC are generally similar to those of older patients[8]. Genomic studies have shown that EOPC has a unique molecular genetic profile with a lower incidence of *KRAS* mutations and a higher incidence of pathogenic germline variants[9-11].

Population-based studies have shown that patients with EOPC often experience multimodal and more intense regimens[12,13]. Patients with non-metastatic EOPC are likely to benefit from local plus systemic therapy. However, very little data exist regarding the treatment outcomes of non-metastatic EOPC. Clinical guidelines do not provide treatment recommendations for young PC patients, and the optimal therapy remains unclear. This study investigated the clinical features, treatment patterns, and survival outcomes of patients with non-metastatic EOPC treated with multimodal therapy at a high-volume center in Beijing, China.

MATERIALS AND METHODS

Patients

Between January 2017 and December 2021, 277 patients with non-metastatic EOPC who had been treated at the Chinese PLA General Hospital were retrospectively enrolled in our study. PC was diagnosed based on clinical, radiological, and pathological findings and was confirmed by multidisciplinary consultation. The inclusion criteria were as follows: (1) Initial consultation between January 2017 and December 2021; (2) ≤ 50 years and ≥ 18 years of age; (3) Clinical or pathological diagnosis of pancreatic adenocarcinoma; and (4) An Eastern Cooperative Oncology Group performance status score ≤ 2. The exclusion criteria were as follows: (1) Metastatic disease; (2) Pathological subtype of non-adenocarcinoma; (3) History of malignancies at other sites; and (4) Loss to follow-up. The detailed patient selection process is

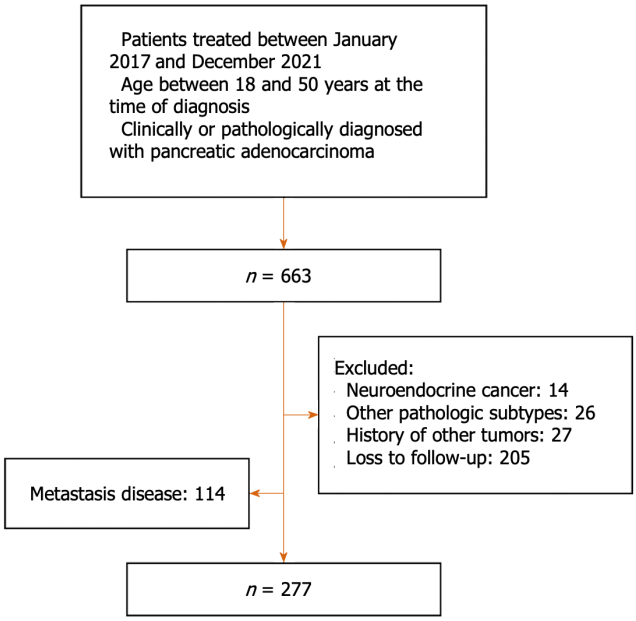

Figure 1 Patient selection.

shown in Figure 1. The study protocol was approved by the Medical Ethics Committee of Chinese PLA General Hospital. Patient consent was waived, given the retrospective nature of the study.

Treatment

Radical resection was the primary treatment for localized (resectable/borderline resectable) EOPC. Preoperative neoadjuvant therapy (NAT) generally involved 4-6 cycles of gemcitabine plus nab-paclitaxel (commonly referred to as GnP) or S-1 (an oral drug of fluorouracil) plus nab-paclitaxel (commonly referred to as SnP). Adjuvant therapy (AT) generally involved six cycles of a single or multiagent regimen based on S-1. For patients with locally advanced disease, treatment included surgery after NAT, radiotherapy-based combination therapy (RCT), and chemotherapy. Individualized radiotherapy target volumes were designed according to the tumor size, lymph node involvement, and adjacent organs at risk. Treatment doses of 50 Gy to the planning target volume and 60-70 Gy to the gross tumor target volume were prescribed with 30 fractions in intensity-modulated radiation therapy and 5 fractions in stereotactic body radiation therapy (SBRT). The first-line chemotherapy regimens mainly included GnP, SnP, and 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX). Immunotherapy mainly included immune checkpoint inhibitors. Targeted therapies included poly ADP-ribose polymerase inhibitors, epidermal growth factor receptor inhibitors, and vascular endothelial growth factor receptor inhibitors.

Data collection and follow-up

Patient demographic, clinical, pathological, and serological data were collected from the database and confirmed by chart review. The patients were restaged according to the National Comprehensive Cancer Network (commonly known as NCCN) Guidelines[14] and the American Joint Committee on Cancer 8th edition staging system. The primary endpoint was overall survival (OS). The secondary endpoints included tumor disease-free survival (DFS) and progression-free survival (PFS). OS was defined as the time from diagnosis to death or last follow-up. DFS or PFS was measured from the start of treatment to tumor recurrence or progression, last follow-up, or death. Recurrence and progression were assessed by experienced oncologists according to the Response Evaluation Criteria in Solid Tumors guidelines (version 1.1)[15]. The last follow-up was confirmed up to July 1, 2023.

Statistical analysis

Statistical analyses were conducted using R software (version 4.2.0). Clinical characteristics and treatment patterns were summarized using medians and ranges for continuous variables and frequencies for categorical descriptors. OS, DFS, and PFS were estimated using the Kaplan-Meier method and compared between subgroups using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazard model. Statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics and treatment

A total of 277 patients with non-metastatic EOPC were enrolled in this study. The patient characteristics are presented in

Table 1. The median age of all patients was 46 years (range: 20-50 years), and 68.6% were males. Tumors in the head of the pancreas accounted for 69.4%. The initial symptoms often presented with abdominal pain (49.1%), jaundice (30%), new-onset diabetes (4.3%), back pain (3.2%), and no symptoms (10.1%). History of tobacco, alcohol, obesity, diabetes, and chronic pancreatitis accounted for 36.8%, 27.9%, 8.9%, 5.9%, and 2.9%, respectively. Patients with baseline carbohydrate antigen 19-9 (CA19-9) ≥ 150 U/mL accounted for 26.1%. Among the 222 patients with pathological grading, poor differentiation adenocarcinoma accounted for 50.3%. Localized and locally advanced disease accounted for 77.6% and 22.4%, respectively. Overall, 78.7% of patients were treated with tumor resection, 74.7% with chemotherapy, 27.1% with radiotherapy, 31.0% with immunotherapy, and 19.9% with targeted therapy.

Survival

With a median follow-up time of 34.6 months, 167 patients died due to tumor progression. The estimated median OS (mOS) for patients with non-metastatic EOPC was 24.8 months 95%CI: 21.6-27.4 months (Figure 2A). The corresponding 1-year, 2-year, and 3-year OS rates were 84.3% (95%CI: 79.9%-88.9%), 51.5% (95%CI: 45.3%-58.5%), and 27.6% (95%CI: 21.8%-34.8%), respectively. The mOS was 25.8 months (95%CI, 22.1-28.7 months) for patients with localized disease and 19.9 months (95%CI: 17.1-29.9 months) for patients with locally advanced disease (Figure 2B).

Treatment outcomes in localized disease

Among the 215 patients with localized disease, all except 11 underwent pancreatic tumor resection. Among them, 80 (39.2%), 10 (4.9%), and 120 (58.8%) patients received surgery alone, NAT, and AT, respectively (Table 2). The mOS for the NAT/AT group was 28.8 months (95%CI: 24.8-33.7 months), which was significantly longer than that for the surgery alone group (21.2 months, 95%CI: 16.6-26.5 months, $P = 0.007$; Figure 3A). The median DFS for the NAT/AT group was 11.7 months (95%CI: 9.8-13.2 months), which was similar to the surgery alone group (9.2 months, 95%CI: 6.8-11.7 months, $P = 0.28$; Figure 3B).

Treatment outcomes in locally advanced disease

Of the 62 patients with localized disease, 14 (22.6%), 29 (46.8%), and 19 (30.6%) underwent surgery after NAT, RCT, and chemotherapy, respectively (Table 2). The mOS of the surgery group, RCT group, and chemotherapy group was 25.6 months, 28.5 months, and 14.0 months ($P = 0.002$), respectively (Figure 3C). The median PFS for each of the three groups was 10.6 months, 14.0 months, and 7.4 months ($P = 0.21$), respectively (Figure 3D).

Treatment outcomes in patients with recurrence

Definite recurrence occurred in 161 of the 218 patients who underwent resection, including isolated regional recurrence (operative area and lymph nodes; 39.7%, 64/161) and distant metastasis with or without regional recurrence (60.3%, 97/161). The mOS after recurrence was 13.2 months (95%CI: 10.4-17.1 months) for regional recurrence patients and 10.6 months (95%CI: 8.2-11.5 months) for distant metastases (Figure 4A). There were 19 patients each with regional recurrence treated with RCT and chemotherapy, 1 patient with repeat surgical resection, and the remaining patients with supportive treatment. The mOS after regional recurrence was 16.0 months, 13.4 months, and 8.9 months in the RCT, chemotherapy, and supportive therapy groups, respectively ($P = 0.035$; Figure 4B). The numbers of patients with distant metastases who received chemotherapy, RCT, surgical resection, and supportive therapy were 45, 10, 2, and 40, respectively. The mOS after distant metastasis was 11.5 months, 10.9 months, and 5.0 months in the RCT, chemotherapy, and supportive therapy groups, respectively ($P < 0.001$; Figure 4C).

Prognostic factors

According to the univariate analysis, baseline CA19-9 level, pathological grade, T-stage, N-stage, and resection were found to be associated with OS. On multivariate analysis, lower CA19-9 level, well and moderate pathological grade, lower T-stage, N0-stage, and resection were independent prognostic factors for OS (Table 3).

DISCUSSION

The present study analyzed the treatment patterns, survival outcomes, and prognostic factors of 227 patients with non-metastatic EOPC using real-world data from a high-volume center in China. The mOS of all patients was 24.8 months, and the 1-year, 2-year, and 3-year OS rates were 84.3%, 51.5%, and 27.6%, respectively. The mOS for patients with localized and locally advanced disease was 25.8 months and 19.9 months, respectively. Compared with a retrospective population-based Dutch database study, younger patients had significantly longer survival than patients of all ages (mOS: 8 months)[16]. The 1-year OS in our cohort was better than that of the EOPC cohort from the National Cancer Database (stage I/II: 72.4%, stage III: 47.6%)[12]. These findings suggest that modern multimodal therapy can provide survival benefits.

Surgical resection is the only potential curative treatment for PC. AT can eradicate occult metastatic disease in patients with localized disease. NAT may lead to downstaging before surgery and facilitating a margin-negative resection. We found that 60.8% of patients with localized disease received NAT and/or AT based on fluorouracil or gemcitabine. The mOS was significantly better than that of patients who underwent surgery alone (28.8 months *vs* 21.2 months, $P = 0.007$), and the median DFS tended to improve (11.7 months *vs* 9.2 months, $P = 0.28$). The benefit of AT in patients with PC was demonstrated in the CONKO-001 trial[17]. Patients who received postoperative gemcitabine single-agent chemotherapy

Table 1 Clinical characteristics in patients with non-metastatic early-onset pancreatic cancer

Characteristics	*n* (%)
Age (yr)	
Median (range)	46 (20-50)
< 45	115 (41.5)
≥ 45	162 (58.5)
Sex	
Male	190 (68.6)
Female	87 (31.4)
Tumor site	
Body and tail	85 (30.6)
Head	193 (69.4)
Clinical manifestation	
Abdominal pain	136 (49.1)
Jaundice	83 (30.0)
New-onset diabetes	12 (4.3)
Back pain	9 (3.2)
No symptoms	28 (10.1)
Others	9 (3.2)
History of tobacco	102 (36.8)
History of alcohol	72 (26.0)
Obesity	19 (6.9)
Pre-existing diabetes	10 (3.6)
History of chronic pancreatitis	6 (2.2)
Baseline CA19-9 (U/mL)	
≥ 150	126 (51.2)
< 150	120 (48.8)
Unknown	31
Pathological grade	
Well	12 (5.4)
Moderate	110 (49.5)
Poor	100 (45.0)
Unknown	55
T-stage	
1	33 (11.9)
2	124 (44.8)
3	56 (20.2)
4	62 (22.4)
X	2 (0.7)
N-stage	
0	172 (62.1)
1	95 (34.3)
2	10 (3.6)

Clinical stage	
Localized	215 (77.6)
Locally advanced	62 (22.4)
Resection	218 (78.7)
Chemotherapy	207 (74.7)
Radiotherapy	75 (27.1)
Immunotherapy	86 (31.0)
Targeted therapy	55 (19.9)

CA19-9: Carbohydrate antigen 19-9; X: No assessment.

Table 2 Treatment details based on clinical stage

Treatment	*n* (%)
Localized disease	215 (77.6)
Resection	204 (94.9)
Neoadjuvant and/or adjuvant therapy	124 (60.8)[1]
Surgery alone	80 (39.2)
Nonsurgical therapy	11 (5.1)
Locally advanced disease	62 (22.4)
Surgery after neoadjuvant therapy	14 (22.6)
Radiotherapy-based combination therapy	29 (46.8)[2]
Chemotherapy	19 (30.6)

[1]10 neoadjuvant therapy, 120 adjuvant therapy.
[2]6 intensity-modulated radiotherapy, 23 stereotactic body radiotherapy.

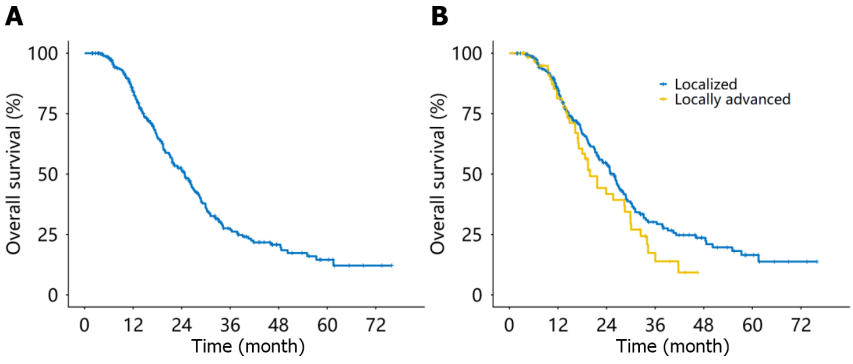

Figure 2 Overall survival of patients. A: Overall survival (OS) of 277 patients with non-metastatic early-onset pancreatic cancer; B: OS in patients with localized and locally advanced disease.

had significantly better OS and DFS than patients who received surgery-alone. The PRODIGE 24 trial further compared adjuvant chemotherapy with modified FOLFIRINOX to gemcitabine[18]. After a median follow-up of 30.5 months, the mOS was 54.4 months in the modified FOLFIRINOX arm and 35.0 months in the gemcitabine arm. The modified FOLFIRINOX had much greater toxicity than other regimens and might be ideal for younger patients with good performance status. In addition, the PREOPANC trial demonstrated that gemcitabine-based neoadjuvant chemoradiotherapy improved OS in resectable and borderline resectable PC compared with upfront surgery[19]. It suggests that early interventional radiotherapy is an effective treatment option in localized patients.

For locally advanced disease, the NCCN guidelines recommend radiotherapy as an optional localized treatment[14]. Our previous studies showed that definitive radiotherapy for inoperable non-metastatic PC patients had favorable and encouraging survival outcomes (mOS: 18 months)[20]. This strategy is also applicable to patients with EOPC. We found

Table 3 Univariate and multivariate analyses for overall survival

Characteristics	Univariable analysis		Multivariable analysis	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Sex				
Male	Reference	N/A	N/A	N/A
Female	0.74 (0.53-1.05)	0.089	N/A	N/A
Age (yr)				
≥ 45	Reference	N/A	N/A	N/A
< 45	0.82 (0.60-1.12)	0.217	N/A	N/A
Site				
Body and tail	Reference	N/A	N/A	N/A
Head	0.83 (0.60-1.15)	0.271	N/A	N/A
Baseline CA19-9 in U/mL				
> 150	Reference	N/A	N/A	N/A
≤ 150	0.62 (0.44-0.87)	0.005[a]	0.67 (0.48-0.95)	0.025[a]
Unknown	1.05 (0.66-1.66)	0.841	1.17 (0.72-1.91)	0.532
Pathology grade				
Well and moderate	Reference	N/A	N/A	N/A
Poor	1.62 (1.15-2.28)	0.006[a]	1.56 (1.08-2.26)	0.017[a]
Unknown	1.45 (0.96-2.19)	0.076	0.94 (0.52-1.70)	0.834
T-stage				
1	Reference	N/A	N/A	N/A
2	1.40 (0.82-2.39)	0.220	1.38 (0.80-2.39)	0.252
3	1.88 (1.06-3.36)	0.031[a]	2.17 (1.18-3.98)	0.012[a]
4	1.78 (0.99-3.20)	0.053[a]	1.35 (0.67-2.72)	0.400
X	2.45 (0.56-10.72)	0.234	2.35 (0.50-11.10)	0.282
N-stage				
0	Reference	N/A	N/A	N/A
1-2	1.85 (1.36-2.51)	< 0.001[a]	1.88 (1.36-2.60)	< 0.001[a]
Clinical stage				
Localized	Reference	N/A	N/A	N/A
Locally advanced	1.34 (0.93-1.92)	0.117	N/A	N/A
Resection				
No	Reference	N/A	N/A	N/A
Yes	0.62 (0.44-0.89)	0.009[a]	0.52 (0.29-0.93)	0.027[a]
Chemotherapy				
No	Reference	N/A	N/A	N/A
Yes	1.02 (0.74-1.41)	0.916	N/A	N/A
Radiotherapy				
No	Reference	N/A	N/A	N/A
Yes	0.81 (0.57-1.14)	0.223	N/A	N/A
Immunotherapy				
No	Reference	N/A	N/A	N/A

Yes	1.01 (0.73-1.40)	0.959	N/A	N/A
Targeted therapy				
No	Reference	N/A	N/A	N/A
Yes	0.85 (0.59-1.23)	0.385	N/A	N/A

[a]$P < 0.05$.

CA19-9: Carbohydrate antigen 19-9; N/A: Not applicable.

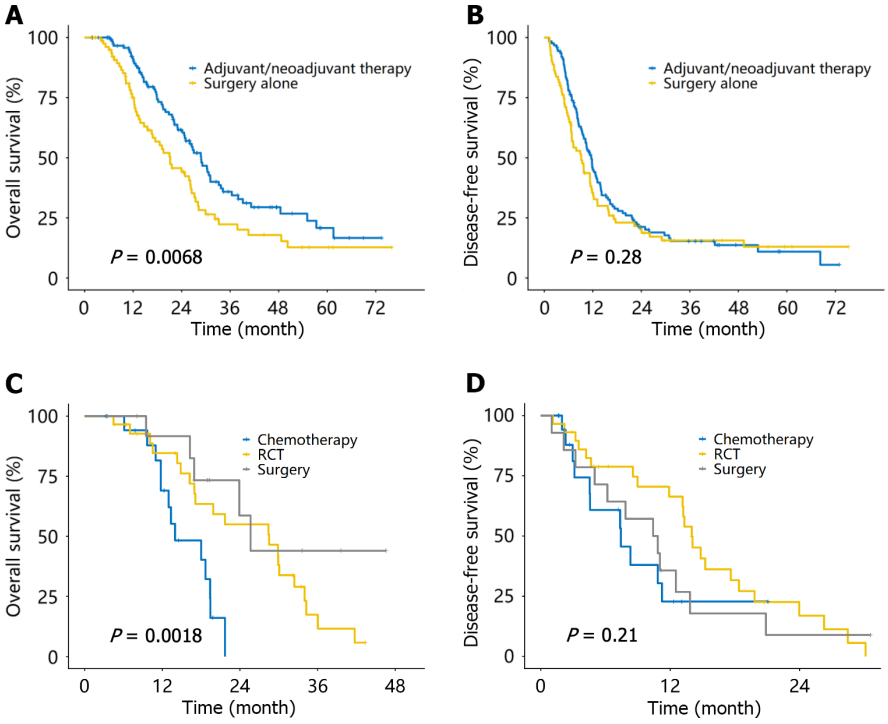

Figure 3 Treatment outcomes in localized and locally advanced disease. A: Overall survival (OS) with surgery alone and adjuvant therapy (AT)/neoadjuvant therapy (NAT) in patients with localized disease; B: Disease-free survival with surgery alone and AT/NAT in patients with localized disease; C: OS with chemotherapy, radiotherapy-based combination therapy (RCT), and surgery in patients with locally advanced disease; D: Progression-free survival with chemotherapy, RCT, and surgery in patients with locally advanced disease. RCT: Radiotherapy-based combination therapy.

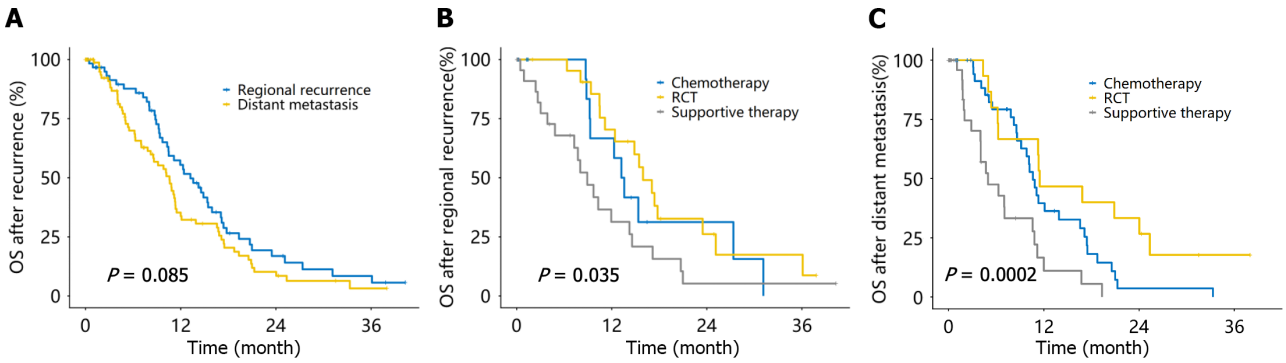

Figure 4 Survival in patients with postoperative recurrence. A: Overall survival (OS) in patients with regional recurrence and distant metastasis; B: Treatment outcome with chemotherapy, radiotherapy-based combination treatment (RCT), and supportive therapy in patients with regional recurrence; C: Treatment outcome with chemotherapy, RCT, and supportive therapy in patients with distant metastasis. RCT: Radiotherapy-based combination therapy.

that nearly half of the patients with locally advanced disease received RCT. Compared to surgery and chemotherapy, RCT achieved the longest median PFS among the three groups, and the mOS was similar to that of pancreatectomy. A meta-analysis of SBRT for the treatment of locally advanced PC showed a 1-year survival rate of 51.6%, an mOS of 17 months (range: 5.7-47.0 months), and the incidence of serious adverse events of no more than 10%[21]. This finding suggests that SBRT can achieve satisfactory efficacy and safety for the treatment of inoperable PC. However, efficacy of SBRT in EOPC still needs to be further validated in clinical trials.

The increasing use of NAT and advances in surgical techniques have rendered some locally advanced patients eligible for surgical resection. In our study, approximately 20% of patients with locally advanced disease underwent pancreatectomy after NAT, with an mOS of 25.6 months. An international dual-center study showed that EOPC patients who underwent pancreatectomy with American Joint Committee on Cancer III-T4 tumors had an mOS of 29.5 months [22]. Even with locally advanced disease, patients can achieve satisfactory results at high-volume centers by NAT combined with surgery.

Several studies have shown that the use of a multidrug regimen of modified FOLFIRINOX, GnP, and SnP prolongs survival in patients with advanced PC[23-25]. In our study, locally advanced patients in the chemotherapy group were treated primarily with a multiagent regimen based on gemcitabine or fluorouracil, with an mOS of 14 months. Our result is similar to survival outcomes reported in previous studies.

Although AT and NAT significantly improve survival in patients with non-metastatic EOPC, regional or systemic recurrence occurred in two-thirds of patients, with mOS after recurrence of 13.2 months and 10.6 months, respectively. There is no consensus based on high-quality evidence on which intervention is most appropriate for patients with postoperative recurrence. A phase II trial evaluated the efficacy of radiotherapy plus chemotherapy or targeted immunotherapy in patients with locally recurrent PC with *KRAS* mutations and PD-L1 immunohistochemistry positivity, with a mOS of 14.9 months in the SBRT plus pembrolizumab and trametinib group and 12.8 months in the SBRT plus gemcitabine group[26]. Another ongoing randomized controlled trial is evaluating the efficacy of additional SBRT in patients with locally recurrent disease compared with the current standard of care alone (NCT04881487)[27]. In general, distant recurrent disease is treated the same as primary metastatic disease. The NCCN guidelines recommend that if distant recurrence occurs during the 1st 6 months of AT, an alternative chemotherapy regimen that is different from the original regimen is administered. Otherwise, repeating systemic therapy as previously administered or switching to any other systemic regimen is recommended[14]. These are consistent with our findings that multimodal combination therapy significantly prolonged survival in patients with postoperative recurrence compared to supportive care. For patients with isolated regional recurrence, localized treatments such as radiotherapy demonstrated a trend toward prolonged survival. In general, supportive treatment and active home care for patients can effectively improve quality of life and reduce the burden on patients and families[28].

Our series demonstrated that CA19-9 Level, pathological grade, T-stage, N-stage, and resection were independent prognostic factors in patients with non-metastatic EOPC. The serum CA19-9 level is the primary serologic marker for PC diagnosis and follow-up[29]. We found that EOPC patients with baseline serum CA19-9 < 150 U/mL had significantly longer survival (hazard ratio: 0.67, 95%CI: 0.48-0.95). Pathology grades of moderately and poorly differentiated tumors were found in 49.5% and 45.0% of patients, respectively, which is consistent with other findings that concluded that EOPC is more aggressive[30].

Several studies showed that EOPC also affects prognosis through molecular genetic features. A study from the Memorial Sloan Kettering Cancer Center found that EOPC patients had a higher proportion of *KRAS* wildtype (15.9% *vs* 5.4%)[11]. Both *KRAS* wildtype and pathogenic germline variants were associated with better clinical outcomes in PC patients. Our study did not find that targeted therapy and immunotherapy improved survival in non-metastatic EOPC. However, a retrospective analysis of the Know Your Tumor programme showed that 26% of PC had actionable mutations and that patients with matched targeted therapy had a significantly better prognosis than patients who receive nonspecific treatment[31]. Therefore, extensive genetic testing in patients with EOPC is beneficial in identifying patients with actionable mutations and for guiding targeted therapy.

However, the limitations of this study need to be recognized. First, the data were extracted from a single tertiary referral center. This limited the diversity of the patient groups included, which may have led to bias. Second, this was a retrospective study with no available family history or molecular genetic information. Additionally, due to the diversity of chemotherapy regimens and radiotherapy parameters, the prognostic impact of different treatment details remains to be clarified in further prospective studies.

CONCLUSION

In this series, the survival outcomes of patients with non-metastatic EOPC receiving multimodal therapy were satisfactory. AT significantly improved postoperative survival in patients with localized EOPC. RCT and surgery after NAT are the preferred therapeutic options for patients with locally advanced disease. Patients with postoperative recurrence undergoing multimodal therapy can achieve good outcomes; however, the role of radiotherapy needs to be further confirmed in randomized controlled trials. As an important subgroup of PC, our findings supported an aggressive multimodal therapeutic strategy for these unique patients and emphasized the need to make treatment recommendations for PC based on age.

ARTICLE HIGHLIGHTS

Research background

The incidence of early-onset pancreatic cancer (EOPC) is showing an increasing trend worldwide. Pancreatic cancer (PC) is insensitive to monotherapy and has a poor prognosis.

Research motivation

There are few studies on EOPC. The role of combination therapies, including surgery, radiotherapy, and chemotherapy, in non-metastatic EOPC is unclear.

Research objectives

To explore the survival outcomes of combination therapy in patients with non-metastatic PC.

Research methods

A total of 277 patients with non-metastatic EOPC who received antitumor therapy in a tertiary care hospital were retrospectively collected. Survival curves were plotted using the Kaplan-Meier method. Univariate and multivariate analyses using Cox proportional hazards modeling were performed to determine prognostic factors.

Research results

With a median follow-up time of 34.6 months, the 1-year, 2-year, and 3-year overall survival (OS) rates for the cohort were 84.3%, 51.5%, and 27.6%, respectively. The median OS of patients with localized disease who received surgery alone and adjuvant therapy (AT) was 21.2 months and 28.8 months, respectively (P = 0.007). The median OS of patients with locally advanced disease who received radiotherapy-based combination therapy (RCT), surgery after neoadjuvant therapy (NAT), and chemotherapy was 28.5 months, 25.6 months, and 14.0 months, respectively (P = 0.002). The median OS after regional recurrence was 16.0 months, 13.4 months, and 8.9 months in the RCT, chemotherapy, and supportive therapy groups, respectively (P = 0.035). Multivariate analysis demonstrated that carbohydrate antigen 19-9 Level, pathological grade, T-stage, N-stage, and resection were independent prognostic factors for non-metastatic EOPC.

Research conclusions

AT improves postoperative survival in localized patients. NAT after surgery and RCT are the preferred treatment options for patients with locally advanced EOPC.

Research perspectives

This study proposed that patients with EOPC should be treated with aggressive multimodal therapy. However, multicenter randomized controlled studies are needed to further understand this subject.

FOOTNOTES

Co-first authors: Le-Tian Zhang and Ying Zhang.

Author contributions: Wang J was involved in the study conception, design and supervision; Zhang LT and Zhang Y contributed equally to this work in design of the research, collection and analysis of the data, and writing of the first draft of the manuscript; Cao BY and Wu CC contributed to conceiving the research and analyzing the data. Zhang LT and Zhang Y contributed equally to this study, so they are the co-first authors of this paper.

Institutional review board statement: This study was reviewed and approved by the Medical Ethics Committee of Chinese PLA General Hospital.

Informed consent statement: As the study used anonymous and pre-existing data, the informed consent from patients was waived.

Conflict-of-interest statement: All authors declare having no potential conflicts of interest related to this study.

Data sharing statement: The dataset is available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Clinical Trials Study

Early proactive monitoring of DNA-thioguanine in patients with Crohn's disease predicts thiopurine-induced late leucopenia in NUDT15/TPMT normal metabolizers

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Abstract

BACKGROUND

Thiopurine-induced leucopenia significantly hinders the wide application of thiopurines. Dose optimization guided by nudix hydrolase 15 (*NUDT15*) has significantly reduced the early leucopenia rate, but there are no definitive biomarkers for late risk leucopenia prediction.

AIM

To determine the predictive value of early monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides (6TGN) for late leucopenia under a *NUDT15*-guided thiopurine dosing strategy in patients with Crohn's disease (CD).

METHODS

Blood samples were collected within two months after thiopurine initiation for

detection of metabolite concentrations. Late leucopenia was defined as a leukocyte count < 3.5×10^9/L over two months.

RESULTS

Of 148 patients studied, late leucopenia was observed in 15.6% (17/109) of *NUDT15*/thiopurine methyltransferase (*TPMT*) normal and 64.1% (25/39) of intermediate metabolizers. In patients suffering late leucopenia, early DNATG levels were significantly higher than in those who did not develop late leucopenia ($P = 4.9 \times 10^{-13}$). The DNATG threshold of 319.43 fmol/μg DNA could predict late leucopenia in the entire sample with an area under the curve (AUC) of 0.855 (sensitivity 83%, specificity 81%), and in *NUDT15/TPMT* normal metabolizers, the predictive performance of a threshold of 315.72 fmol/μg DNA was much more remarkable with an AUC of 0.902 (sensitivity 88%, specificity 85%). 6TGN had a relatively poor correlation with late leucopenia whether in the entire sample ($P = 0.021$) or *NUDT15*/*TPMT* normal or intermediate metabolizers ($P = 0.018$, $P = 0.55$, respectively).

CONCLUSION

Proactive therapeutic drug monitoring of DNATG could be an effective strategy to prevent late leucopenia in both *NUDT15*/*TPMT* normal and intermediate metabolizers with CD, especially the former.

Key Words: Thiopurine-induced late leucopenia; DNA-thioguanine; 6-thioguanine nucleotide; Proactive therapeutic drug monitoring; Crohn's disease

Core Tip: This study pioneeringly explores if early proactive monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides stable concentration within two months could predict late leucopenia susceptible population under a nudix hydrolase 15 (NUDT15) genotype-guided thiopurines dosing strategy in patients with Crohn's disease. We first provide evidence indicating that early proactive monitoring of DNATG during the initial stages of thiopurine therapy could be an effective strategy to discern patients susceptible to developing late leukopenia, especially in NUDT15/thiopurine methyltransferase normal metabolizers, with a concentration threshold of 315.72 fmol/μg DNA.

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INTRODUCTION

Thiopurines, mercaptopurine (MP), and azathioprine (AZA) have been widely used in inflammatory bowel disease (IBD) for 70 years[1]. However, up to 10%-30% of patients discontinue therapy primarily due to adverse effects. Thiopurine-induced leucopenia is one of the most common and potentially fatal toxicities, particularly in Asian populations[2].

Thiopurine methyltransferase (TPMT), a key enzyme in thiopurine metabolism, is involved in the inactive conversion of thiopurines. *TPMT* polymorphisms are significantly associated with thiopurine-induced leucopenia and have been a landmark discovery in genomics to guide the clinical application of thiopurines. However, the guiding value of *TPMT* for thiopurine clinical usage is very limited in Asia, because the mutation frequency of *TPMT***3C*, as the most prevalent variant in Asia, is only 1%-3%[3,4]. Until 2014, a genome-wide association study in Korea revealed a common missense variant, nudix hydrolase 15 (*NUDT1*)**3* (rs116855232, R139C, p.Arg139Cys), which strongly increased individual susceptibility to thiopurine-induced leucopenia, bringing the gospel to Asian patients for safe use of thiopurines[5].

Thiopurine-induced leucopenia occurs at any time during treatment, ranging from early (within two months) to late onset (over two months)[6,7]. Thiopurine-induced severe, early-onset leucopenia, we now know, can be attributed largely to *NUDT15* deficiency. Since its introduction, the highly predictive value of *NUDT15***3* for thiopurine-induced leucopenia, mainly for early leucopenia (within two months), has been consistently confirmed, but so far, no definitive conclusion has been reached concerning late leucopenia (over two months)[5,8-10]. In a prospective study, we showed that halved dosage for *NUDT15***3* heterozygous patients reduced the incidence of early leucopenia by approximately 50%, but it did not reduce the risk of late leucopenia[9]. Similar findings were also reported in a Japanese study[10]. As thiopurine-induced late leucopenia is common, occurring in as high as 20%-40% of IBD patients[5,8,10], regular follow-up and therapeutic drug monitoring (TDM) are crucial during long-term thiopurine maintenance therapy.

Thiopurine drugs need to undergo extensive enzymatic conversion into 6-thioguanine nucleotides (6TGN) to exert their pharmacological and cytotoxic action. The role of the 6TGN level as an integral component of thiopurine monitoring to minimize adverse reactions has been investigated for decades[1,11-13]. However, whether 6TGN monitoring can

reduce thiopurine-induced leucopenia is controversial and debatable[8,13-17]. Two studies have recently shown that late DNA-thioguanine (DNATG), a *NUDT15*-associated subcellular DNA-incorporated thiopurine metabolite, was strongly related to thiopurine-induced late leucopenia, compared to 6TGN[3,16].

Therefore, it is necessary to verify whether proactive TDM of DNATG or 6TGN in the initial medication phase can predict the occurrence of late leucopenia. After all, early identification of patients at risk of thiopurine-induced late leucopenia is of greater significance than metabolite concentration monitoring after routine blood tests indicating signs of leucopenia.

Herein, we conducted a prospective observational study to explore whether early proactive TDM of DNATG stable concentration within two months could help predict the leucopenia-susceptible population under a *NUDT15* genotype-guided thiopurine dosing strategy in patients with Crohn's disease (CD)[9], compared with 6TGN concentrations over the same period.

MATERIALS AND METHODS

Patient recruitment and study design

Patients diagnosed with CD from June 2019 to December 2022 were recruited in the Sixth Affiliated Hospital, Sun Yat-sen University. After prescribed thiopurines, patients were clinically followed up within two months when blood specimens were collected for TDM (6TGN, DNATG), and every two to three months thereafter. At each follow-up visit, clinical data, and routine blood tests were recorded. Medication adherence was followed up prospectively for a minimum duration of six months to monitor whether leucopenia occurred.

NUDT15 and *TPMT* genotypes were detected before treatment initiation. Patients with *NUDT15* wild-type were prescribed the target dose of 2.0 mg/kg/d AZA or 1.0 mg/kg/d 6-MP, and heterozygous patients were administered the target dose of 1.0 mg/kg/d AZA or 0.5 mg/kg/d 6-MP. Thiopurines were contraindicated in patients with homozygous mutations. The initial dose of AZA was 1.0 mg/kg/d in the wild-type group or 0.5 mg/kg/d in the heterozygous group and then increased to the target dosage in approximately one to two weeks[9]. The dose of 6-MP in mg/kg body weight was obtained by multiplying the dose of AZA by 0.5 as a conversion factor. DNATG and 6TGN levels were determined within the two-month follow up during which patients were medication stable and did not complain of any thiopurine-induced discomfort. The design of this study is depicted in Figure 1.

The clinical information was recorded at every visit until thiopurine discontinuation due to inefficacy, adverse drug reactions, and poor adherence/loss to follow-up. A white blood cell count < 3.5×10^9/L after two or more months of treatment was defined as thiopurine-induced late leucopenia. The exclusion criteria were as follows: Patients with blood transfusion or administration of cyclosporine or methotrexate; patients with insufficient function of the heart, liver, or kidneys; patients with active infection at the sampling time point; and patients suffering from acute adverse reactions within two months. The present study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. This trial is registered with the Chinese Clinical Trials Register (No. ChiCTR2100050295). All patients recruited provided a written informed consent form to participate in the trial.

Genotyping and TDM

DNA was extracted from peripheral leukocytes according to the manufacturer's instructions (TIANGEN DP349-02, Beijing, China). *TPMT**2 (rs1800462, G238C), **3A* (rs100460 and rs1142345), **3B* (rs100460, G460A), **3C* (rs1142345, A719G), and *NUDT15***3* (rs116855232, C415T), **5* (rs186364861, G52A), and **6* (rs746071566) genotypes were determined by Sanger sequencing. Detailed methods of quantitative detection of DNATG concentration in leukocytes and 6TGN concentration in erythrocytes have been published previously[3].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5 (GraphPad, La Jolla, CA, United States) and R software (version 4.2.1; R Foundation). The continuous values are shown as medians (ranges) or median ± interquartile range (IQR) according to the distribution of the values. The nonparametric Spearman test was used for all correlation analyses. χ^2 and Fisher's exact probability tests were used to analyze categorical data, while Mann-Whitney tests were used to analyze measurement data.

The performance and a target threshold of 6TGN or DNATG were evaluated by computing receiver operating characteristic (ROC) curves and the area under the ROC curves (AUC) using the pROC package in R. Cumulative incidence rates of late leucopenia during follow-up were constructed using the Kaplan-Meier method, and the survival curves were compared using the log-rank test with survival and survminer packages in R. Cox proportional hazards regression was used for both univariate and multivariate analysis of thiopurine-induced late leucopenia. Data visualization was performed in Prism 9.5 and the R packages survminer, rms and, ggplot2. Statistical significance was indicated at the 0.05 level.

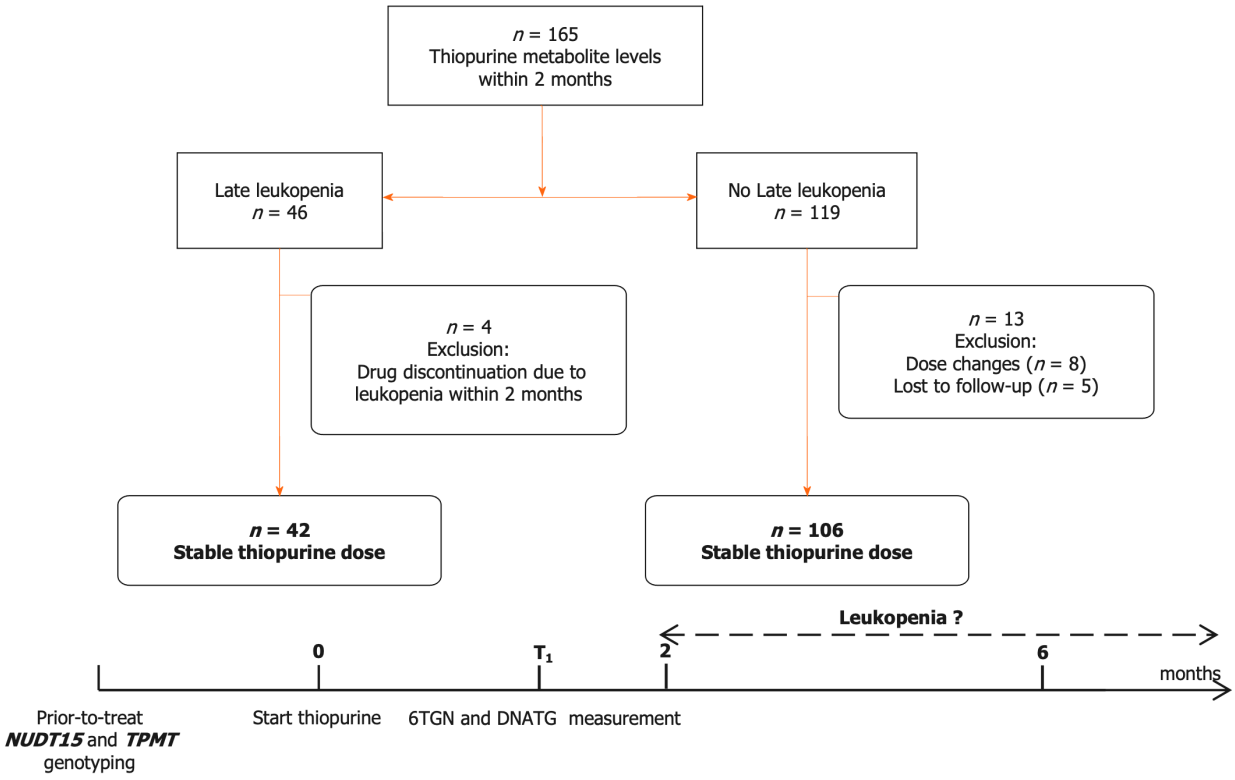

Figure 1 Flow chart of patient enrolment, exclusion, and follow-up. Late leucopenia was defined as a leukocyte count < 3.5 × 10^9/L over two months. The stable dose (2.0 mg/kg/d azathioprine and 1.0 mg/kg/d 6-mercaptopurine, approximately) is reached approximately one to two weeks after the initial administration. NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase; 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine.

RESULTS

Patient characteristics

The flow chart of patient enrollment, exclusion, and follow-up is depicted in Figure 1. A total of 165 patients were recruited in this study, and 17 patients were excluded due to drug discontinuation/dose change/loss of follow-up. A total of 148 patients with DNATG and 6TGN measurements within two months were analyzed in this study. The median patient follow-up time was 13.1 months, with 82.4% of patients followed longitudinally for six months or more. In our study, late leucopenia was observed in 15.6% (17/109) of the *NUDT15/TPMT* normal and 64.1% (25/39) of intermediate metabolizers, consistent with previous observations in similar populations[4,9,15,18]. Of the 148 patients, 3 were heterozygous for *TPMT**3C*, and 37 were heterozygous for low activity *NUDT15* alleles (21 *NUDT15**1/*3*, 14 *NUDT15**1/*2*, 2 *NUDT15**1/*6*). There were no significant differences in sex, age, dose, weight, comedication, or hematologic indices except for platelets (Table 1).

Correlation between early thiopurine metabolite concentrations and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts

There was a significant negative correlation between early DNATG concentration and the minimum peripheral leukocyte, neutrophil, lymphocyte, and, monocyte counts during follow-up (r = -0.3231, P < 0.0001; r = -0.1834, P = 0.0260; r = -0.1793, P = 0.0320; r = -0.2854, P = 0.0004; respectively, Figure 2A-D). Similarly, a marginally significant negative correlation was found between early DNATG concentration and the average peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts during follow-up (r = -0.1806, P = 0.0280; r = -0.1473, P = 0.0740; r = -0.1419, P = 0.0850; r = -0.2392, P = 0.0034; respectively, Figure 2E-H). By contrast, no significant correlation was found between the early 6TGN concentration and the minimum or average peripheral leukocyte, neutrophil, and lymphocyte counts during follow-up, except for monocyte counts (r = -0.2459, P = 0.0026; r = -0.1692, P = 0.0398; respectively, Figure 3).

Association of early thiopurine metabolite concentrations with late leucopenia

Median early DNATG concentrations were much higher in patients who developed late leucopenia than in those who did not [394.9 ± 137.4 fmol/μg DNA (n = 42) *vs* 225.3 ± 139.6 fmol/μg DNA (n = 106), P = 4.9 × 10^{-13}, Figure 4A]. In patients with *TPMT/NUDT15* wild-type (n = 109)], early DNATG concentration [401.1 ± 222.9 fmol/μg DNA (n = 17) *vs* 221.1 ± 140.8 fmol/μg DNA (n = 92), P = 4.9 × 10^{-9}, Figure 5A] was associated with late leucopenia with much higher significance than in heterozygous patients [382.5 ± 143.1 fmol/μg DNA (n = 25) *vs* 240.5 ± 248.3 fmol/μg DNA (n = 14), P = 0.024; 3 for *TPMT**3C*, 21 for *NUDT15**3*, 2 for *NUDT15**6*, 14 for *NUDT15**2*, Figure 5A].

Table 1 Baseline characteristics of the 148 patients included in the study

Characteristics	Patients		*P* value	Odds ratio (95%CI)
	Leucopenia	Nonleucopenia		
Number of subjects (%)	42 (28.4)	106 (71.6)		
Female, *n* (%)	14 (33.3)	22 (20.8)	0.11	1.91 (0.86-4.23)
Age (yr)	29 (12-59)	26 (13-61)	0.03	0.96 (0.92-0.99)
Azathioprine, *n* (%)	37 (26.6)	102 (73.4)	Reference	0.29 (0.07-1.14)
6-mercaptopurine, *n* (%)	5 (55.6)	4 (44.4)	0.08	1.05 (1.01-1.10)
Thiopurine dose (mg)	87.5 (25-100)	75 (50-150)	0.76	0.99 (0.98-1.01)
Azathioprine	75 (25-100)	75 (50-150)		
6-mercaptopurine	50 (37.5-50)	37.5 (25-50)		
Weight (kg)	52 (40-69)	55 (37-79)	0.02	1.05 (1.01-1.10)
Hematologic indices				
Leukocyte count (10^9/L)	6.25 (4.09-16.66)	6.92 (4.09-17.60)	0.83	1.31 (0.11-15.71)
Absolute neutrophil count (10^9/L)	3.98 (1.30-15.55)	4.59 (1.91-14.76)	0.78	0.70 (0.06-8.31)
Absolute lymphocyte count (10^9/L)	1.64 (0.41-4.45)	1.53 (0.27-4.26)	0.79	0.94 (0.57-1.53)
Monocyte count (10^9/L)	0.49 (0.22-1.98)	0.57 (0.10-1.72)	0.66	1.29 (0.42-3.91)
Hemoglobin (g/L)	126 (76-165)	130 (66-166)	0.43	1.01 (0.99-1.02)
Platelets (10^9/L)	287 (122-589)	302 (150-680)	0.10	1.00 (0.99-1.01)
Comedication (%)				
Anti-TNF agents	2 (4.76)	16 (15.09)	0.49	0.46 (0.05-4.09)
Corticosteroids	2 (4.76)	5 (4.72)	0.76	1.41 (0.16-12.47)
Genotypes (phenotypes)				
*NUDT15*1/*1* (NM)	18 (42.86)	93 (87.74)	Reference	
*NUDT15*1/*3* (IM)	13 (30.95)	8 (7.55)	0.95	0.97 (0.34-2.72)
*NUDT15*1/*2* (IM)	9 (21.43)	5 (4.72)	0.61	1.42 (0.37-5.44)
*NUDT15*1/*6* (IM)	2 (4.76)	0	0.99	
*TPMT*1/*1* (NM)	40 (95.24)	105 (99.06)	Reference	
*TPMT*1/*3C* (IM)	2 (4.76)	1 (0.94)	0.19	0.94 (0.47-2.35)

CI: Confidence interval; NM: Normal metabolizer; IM: Intermediate metabolizer; NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase.

By contrast, median early 6TGN concentrations were slightly higher in patients who developed late leucopenia than in those who did not [322.4 ± 210.6 pmol/8 × 10^8 red blood cells (RBCs) (*n* = 42) *vs* 247.5 ± 182.5 pmol/8 × 10^8 RBCs (*n* = 106), *P* = 0.021, Figure 4B]. Notably, the early 6TGN concentration did not exhibit a significant difference in *TPMT*/*NUDT15* heterozygous patients with or without late leucopenia [310.0 ± 180.6 pmol/8 × 10^8 RBCs (*n* = 25) *vs* 249.9 ± 303.9 pmol/8 × 10^8 RBCs (*n* = 14), *P* = 0.55], although it did in *TPMT*/*NUDT15* wild-type patients with or without late leucopenia [333.7 ± 270.7 pmol/8 × 10^8 RBCs (*n* = 17) *vs* 247.5 ± 162.9 pmol/8 × 10^8 RBCs (*n* = 92), *P* = 0.018] (Figure 5B).

Predictive performance of early thiopurine metabolite concentration for late leucopenia

The AUC of the ROC analysis for early DNATG concentrations was 0.855 (sensitivity 83%, specificity 81%) in the total population (Figure 6A), 0.902 (sensitivity 88%, specificity 85%) in *TPMT*/*NUDT15* wild-type (Figure 6B), and 0.72 (sensitivity 79%, specificity 72%) in heterozygous subgroups (Figure 6C) and the cutoff values of DNATG were 319.43, 315.72, and 354.68 fmol/μg DNA, respectively.

The AUC of the ROC analysis for early 6TGN concentration was 0.622 (sensitivity 66%, specificity 60%) in the total population (Figure 6A), 0.680 (sensitivity 71%, specificity 67%) in *TPMT*/*NUDT15* wild-type (Figure 6B), and 0.560 (sensitivity 50%, specificity 64%) in heterozygous subgroups (Figure 6C). Additionally, discontinuations occurred in 35.7% (15/42) of patients who developed late leucopenia (median follow-up: 5.8 months), with 80% (12/15) of them taking the drug for less than a year. Similarly, early DNATG levels were much more impressive in predicting discon-

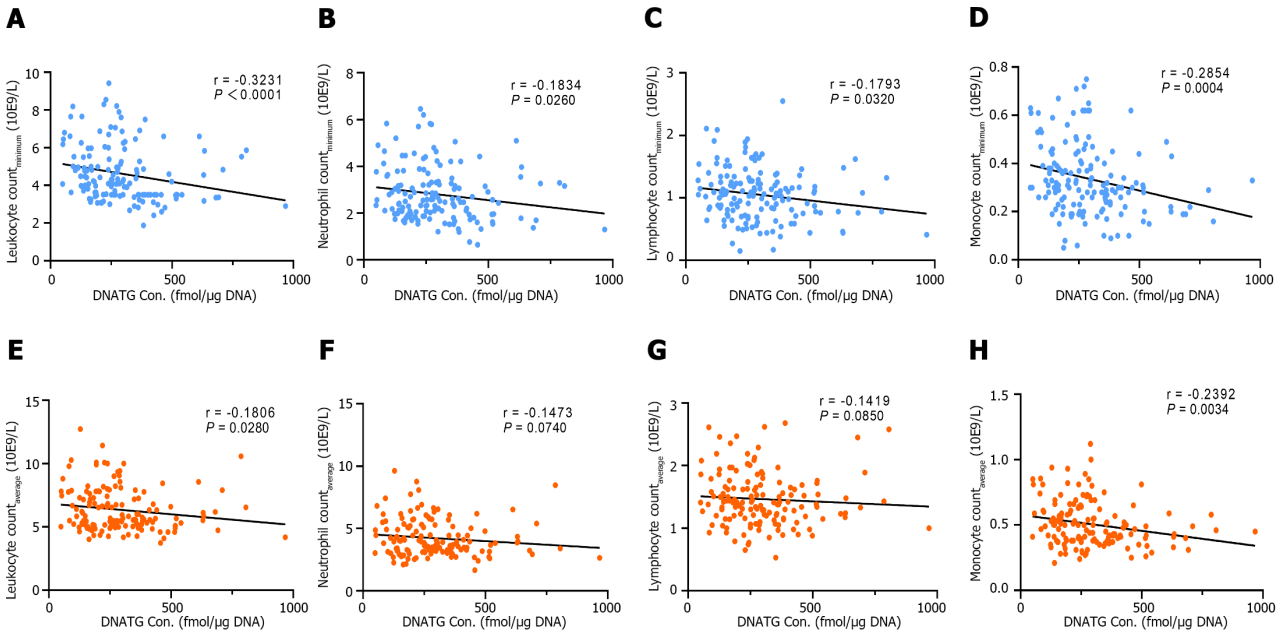

Figure 2 Correlation between early DNA-thioguanine concentration and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. A-D: There was a significant negative correlation between early DNA-thioguanine (DNATG) concentration, and the minimum peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts during follow-up; E-H: A marginally significant negative correlation was found between early DNATG concentration and the average peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. *P* values were determined by the nonparametric Spearman test. DNATG: DNA-thioguanine.

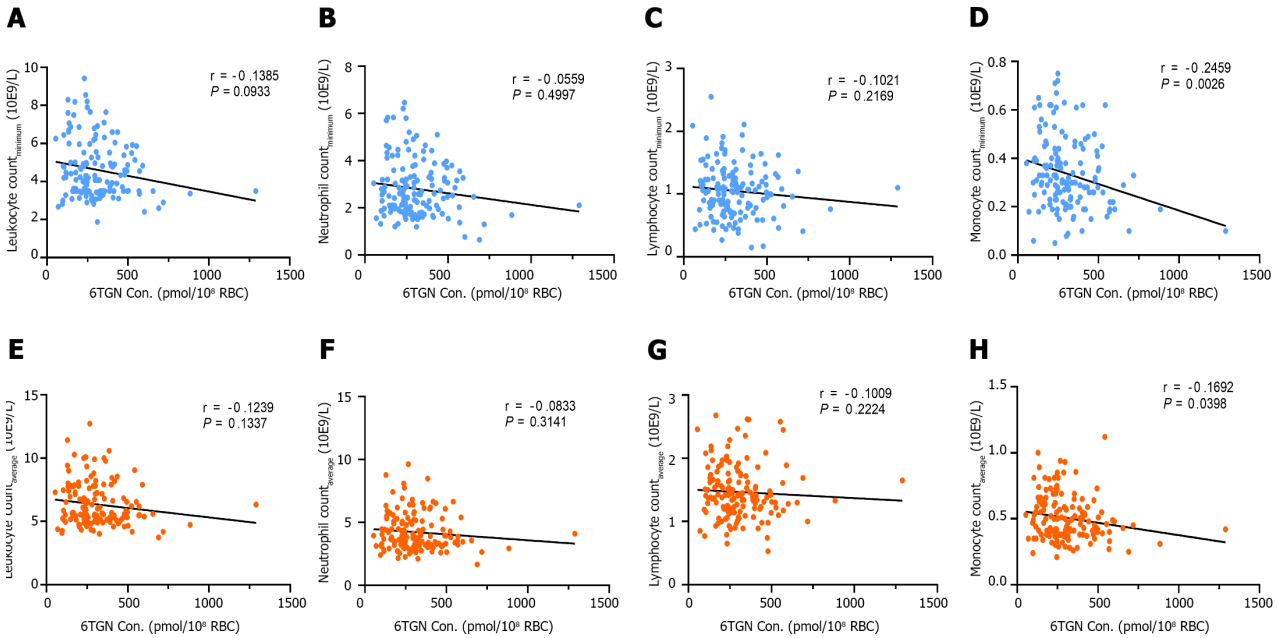

Figure 3 Correlation between early 6-thioguanine nucleotide concentration and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. A-H: There was no significant correlation between early 6-thioguanine nucleotides (6TGN) concentration, and the minimum or average peripheral leukocyte, neutrophil, and lymphocyte counts during follow-up ($P > 0.05$) (A-C, E-G). There was a correlation between early 6TGN concentration and the minimum or average peripheral monocyte counts ($P < 0.05$) (D and H). *P* values were determined by the nonparametric Spearman test. 6TGN: 6-thioguanine nucleotides; RBC: Red blood cell.

tinuation events due to recurrent leucopenia, with an AUC of 0.812 (sensitivity 87%, specificity 77%) in the total population, and 0.864 (sensitivity 100%, specificity 67%) in the *TPMT/NUDT15* wild-type population (Supplementary Figure 1).

Kaplan-Meier survival analysis showed that the cumulative incidence rates of late leucopenia during follow-up were significantly increased in patients with DNATG concentrations greater than 319.43 fmol/μg DNA in the total population and 315.72 fmol/μg DNA in the *TPMT/NUDT15* wild-type population (Figure 7).

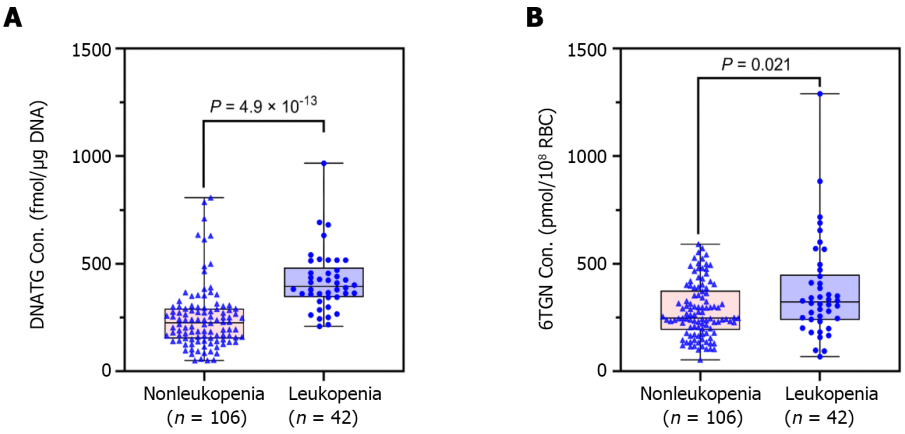

Figure 4 Early DNA-thioguanine concentration was associated with late leucopenia with immense significance compared to 6-thioguanine nucleotides. A: Patients who developed leucopenia had much higher early DNA-thioguanine levels compared with those who did not ($P = 4.9 \times 10^{-13}$); B: Patients who developed leucopenia had slightly higher early 6-thioguanine nucleotides levels compared with those who did not ($P = 0.021$). *P* values were determined by the Mann-Whitney test. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine.

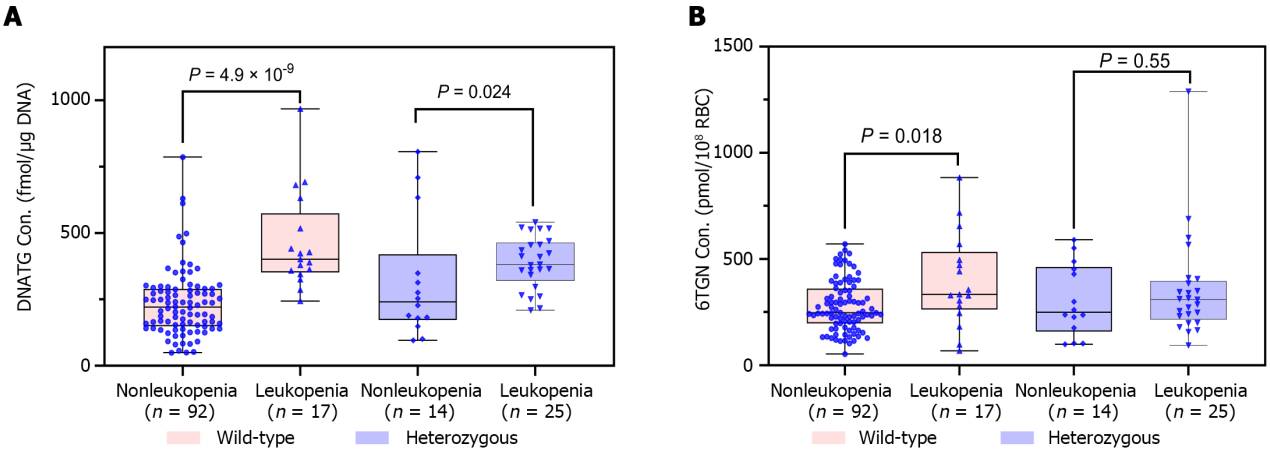

Figure 5 Early DNA-thioguanine concentration was significantly associated with late leucopenia, especially in thiopurine methyltransferase/nudix hydrolase 15 wild-type patients compared to 6-thioguanine nucleotides. A: Early DNA-thioguanine concentration was associated with late leucopenia, especially in nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) wild-type patients ($P = 4.9 \times 10^{-9}$ *vs* $P = 0.024$); B: 6-thioguanine nucleotides was correlated with late leucopenia in *NUDT15/TPMT* wild-type patients ($P = 0.018$) but not in heterozygous subgroups ($P = 0.55$). *P* values were determined by the Mann-Whitney test. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; RBC: Red blood cell.

Multivariable prediction model for thiopurine-induced late leucopenia

Univariate Cox regression analysis of 148 patients on stable thiopurine therapy showed that age, weight, early DNATG and 6TGN concentrations, and *NUDT15* genotypes were relevant determinants for the development of late leucopenia ($P < 0.1$). After discarding the nonsignificant factors, 6TGN concentrations, in the multivariate analysis, the final Cox model included age, early DNATG concentration, and *NUDT15* genotypes ($P < 0.05$). Predictive variables and hazard ratios for the development of leucopenia are presented in Table 2. To improve the feasibility of clinical application, a nomogram based on multivariate analysis results is shown in Figure 8.

DISCUSSION

This is the first time that the DNATG level has been reported to be applicable in the prediction of thiopurine-induced late leucopenia. To date, few studies have been conducted to determine the worth of DNATG level and its correlation with thiopurine-induced late leucopenia in IBD patients. In our previous study, we found that late DNATG levels were significantly correlated with late leucopenia and were a better independent factor for late leucopenia than 6TGN. Therefore, we proposed that proactive quantification of DNATG may be a good way to predict late leucopenia and this prospective observational study was conducted thereafter. In this study, we also found that early DNATG concentrations were consistent with late concentrations in representative patients with multiple follow-up DNATG measurements (Supplementary Figure 2), which further indicated the reliability and feasibility of early proactive quantification of

Table 2 Univariate and multivariate analysis of independent predictors for leucopenia

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.04 (1.01-1.08)	0.005	1.06 (1.02-1.09)	0.002
Weight (kg)	0.95 (0.91-0.99)	0.013	0.96 (0.92-1.00)	0.076
DNATG (fmol/μg DNA)	1.004 (1.002-1.005)	< 0.001	1.004 (1.002-1.005)	< 0.001
NUDT15 genotypes		< 0.001		
*NUDT15**1/*1	Reference		Reference	
*NUDT15**1/*6	17.99 (3.95-81.90)	< 0.001	11.62 (1.51-89.26)	0.018
*NUDT15**1/*3	4.93 (2.41-10.10)	< 0.001	3.21 (1.54-6.71)	0.002
*NUDT15**1/*2	4.50 (2.01- 10.03)	< 0.001	3.25 (1.41-7.50)	0.006
6TGN (pmol/8 × 10^8 RBC)	1.003 (1.001-1.004)	< 0.001	1.000 (0.998-1.002)	0.98
Sex		0.19		
Female	Reference			
Male	0.64 (0.34-1.22)	0.17		
TPMT genotypes		0.31		
*TPMT**1/*1	Reference			
*TPMT**1/*3C	2.38 (0.57-9.87)	0.25		

HR: Hazard ratio; CI: Confidence interval; NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase; 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; RBC: Red blood cell.

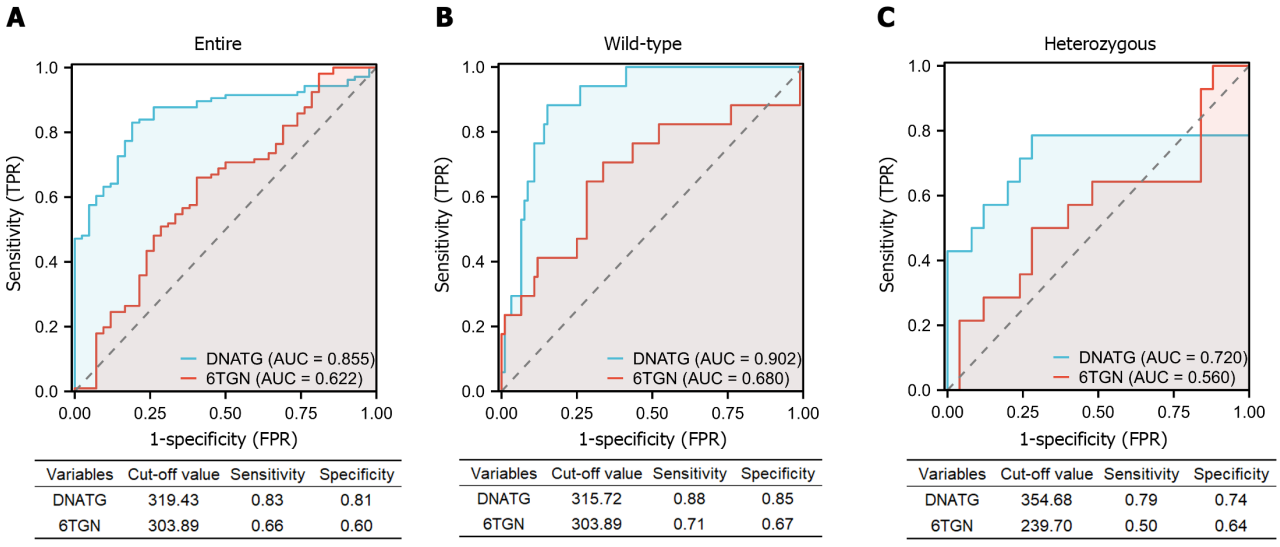

Figure 6 Performance of early DNA-thioguanine and 6-thioguanine nucleotides concentrations in the prediction of late leucopenia in the entire population, nudix hydrolase 15/thiopurine methyltransferase wild-type and heterozygous subgroups. A: Performance in the entire population; B: Performance in nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) wild-type subgroups; C: Performance in NUDT15/TPMT heterozygous subgroups. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; AUC: Area under the curve; FPR: False positive rate; TPR: True positive rate.

DNATG.

In the previous decade, investigations on individualized thiopurines were mainly focused on genotypes of *TPMT* and *NUDT15*. The Clinical Pharmacogenetics Implementation Consortium guidelines recommend initial thiopurine dose adjustment based on *TPMT* and *NUDT15* genotypes[19]. Numerous studies have consistently reported that *NUDT15* variants serve as the sole or major factors linked to thiopurine-induced early leucopenia, exhibiting a sensitivity and specificity higher than 90%, while *TPMT* variants demonstrate a sensitivity of 12.1% and a specificity of 97.6%[5]. In addition, some studies have also shown an association between *NUDT15* variants and late leucopenia[8]. In the present

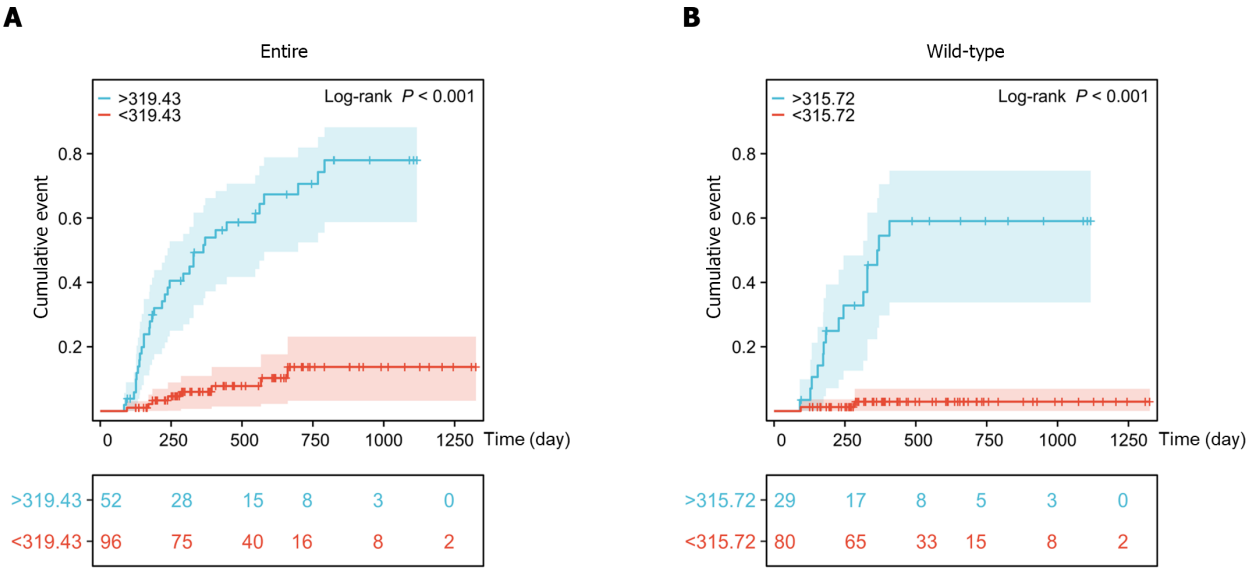

Figure 7 The cumulative events of leucopenia with corresponding DNA-thioguanine thresholds in the entire and thiopurine methyltransferase/nudix hydrolase 15 wild-type populations. A: Cumulative incidence rates were significantly greater in patients with DNA-thioguanine (DNATG) concentrations of more than 319.43 fmol/μg DNA in the entire population; B: Cumulative incidence rates were significantly greater in patients with DNATG concentrations of more than 315.72 fmol/μg DNA in the thiopurine methyltransferase/nudix hydrolase 15 wild-type population. Both log-rank $P < 0.001$. Kaplan-Meier survival analysis followed by the log-rank test was employed to estimate cumulative leucopenia events.

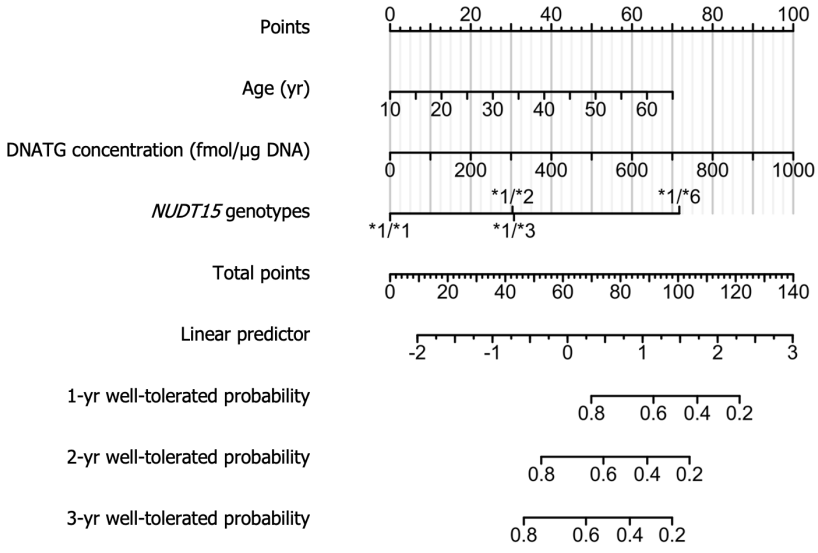

Figure 8 Nomogram based on the multivariate analysis results shown in Table 2. For example, a 65-year-old patient with the wild-type nudix hydrolase 15 (NUDT15)*1/*1, having a DNA-thioguanine (DNATG) concentration of 200 fmol/μg DNA, would score 70 for age, 20 for DNATG concentration, and 0 for the NUDT15 genotype, resulting in a total score of 90. Correspondingly, the estimated 1-year, 2-year, and 3-year well-tolerated (no occurrence of late leukopenia) probabilities are approximately 0.6, 0.4, and 0.3, respectively. DNATG: DNA-thioguanine; NUDT15: Nudix hydrolase 15.

study, we verified that *NUDT15* was a significant independent factor for late risk, and therefore, the importance of pretreatment for the determination of the *NUDT15* genotype cannot be overemphasized.

Nonetheless, a large portion of thiopurine-induced leucopenia cannot be explained by *NUDT15*/*TPMT* deficiency, which was manifested by the variation in leucopenia in patients receiving optimized thiopurines based on the genotype of *NUDT15*/*TPMT*[9]. Meanwhile, a high incidence of late leucopenia (10%-20%) can still be seen in the *NUDT15*/*TPMT* wild-type population[3,4,9,18]. It is very important to identify biomarkers for risk in the populations mentioned above.

TDM of 6TGN has been employed in some clinical settings as a strategy to predict thiopurine-induced toxicity for over two decades although the value of 6TGN TDM is still controversial[20-22]. Kang *et al*[23] reported that the 6-TGN level (goal < 167.1 pmol/8 × 10^8 RBCs) could be used to adjust AZA doses to reduce late thiopurine-induced leucopenia in *NUDT15* intermediate metabolizers.

However, a large number of studies have shown that 6TGN levels are not significantly different between groups with and without leucopenia[8,13-16]. In our previous study[4], late 6TGN levels were significantly associated with late

leucopenia in patients with wild-type *NUDT15 R139C* (P = 0.006, median 413.0 *vs* 279.7 pmol/8 × 10^8 RBCs), while no significant association was found between 6TGN levels and leucopenia in patients with *NUDT15 R139C* variants (P = 0.26). Similarly, in the present study, the early concentrations of 6TGN exhibited no significant difference in *TPMT*/*NUDT15* heterozygous patients with or without late leucopenia (P = 0.55), although it did in the entire sample and *TPMT*/*NUDT15* wild-type patients with or without late leucopenia (P = 0.021, 0.018, respectively). This discrepancy may be because in the study performed by Kang *et al*[23], 6TGN concentrations were monitored regularly, and doses were adjusted accordingly so that 6TGN levels would be within the therapeutic range of 235-450 pmol/8 × 10^8 RBCs[24]. In our study, the doses were only adjusted based on genotypes before thiopurine administration.

Furthermore, our current study found that compared to 6TGN, early DNATG levels were more significantly correlated with thiopurine-induced late leucopenia (P = 4.9 × 10^{-13} *vs* P = 0.021). The DNATG threshold of 319.43 fmol/μg DNA predicted late leucopenia in the entire sample with an AUC of 0.855 (sensitivity 83%, specificity 81%), and in the *NUDT15/TPMT* normal metabolizers, the predictability of a threshold of 315.72 fmol/μg DNA was much more impressive with an AUC of 0.902 (sensitivity 88%, specificity 85%). Notably, early DNATG levels were much more impressive in predicting discontinuation events due to recurrent leucopenia with an AUC of 0.864 (sensitivity 100%, specificity 67%) in the *TPMT*/*NUDT15* wild-type population.

In recent years, proactive and reactive TDM strategies in IBD management have been under intense discussion. Reactive TDM in non- or partial responders with active IBD aims to guide treatment changes and routine proactive TDM in patients with quiescent disease aims to reach a target trough concentration during routine clinical care[20]. Many studies have suggested that proactive TDM can provide more clinical benefits than reactive TDM for anti-tumor necrosis factor biologics[25-28]. One Danish study reported that DNATG levels in neutrophils (P < 0.0001) and 6TGN in erythrocytes (P = 0.01) on methotrexate/6-MP maintenance therapy for childhood acute lymphoblastic leukemia, are predictors of neutrophil nadirs within two weeks following high-dose methotrexate infusion[29]. In the present study, it is heartening that early proactive monitoring of DNATG is of very high sensitivity (88%) and specificity (85%) to predict late leucopenia in *NUDT15*/*TPMT* wild-type subgroups that would not benefit from the *NUDT15* genotype-guided dosing strategy.

In general, pretreatment determination of the *NUDT15* genotype is necessary to identify Asian patients with IBD who are susceptible to thiopurine-induced leucopenia. Proactive DNATG therapeutic monitoring is very important, especially for many patients with wild-type *NUDT15*/*TPMT*, to avoid late risk.

There are several shortcomings in our study. First, this was a prospective observational study with relatively small sample sizes. In our study, once leucopenia occurred, patients discontinued the thiopurines, switched therapies, or followed a strategy of empiric dose adaptation due to the patient's wishes and physician discretion. We did not fully determine the benefits of reactive DNATG-TDM-guided dose adaptation decisions with *NUDT15*-guided initial medication. Further randomized clinical trials are warranted to probe whether routine reactive DNATG monitoring, compared with empiric treatment changes, helps to reduce the incidence of drug withdrawal due to late leucopenia in IBD patients.

CONCLUSION

In this prospective study of CD patients receiving thiopurines under *NUDT15* genotype-guided dosing strategy, we demonstrated that in addition to the *NUDT15* genotype, early proactive monitoring of DNATG was highly sensitive in predicting thiopurine-induced late leucopenia. With the cutoff value of 319.43 fmol/μg DNA, late leucopenia cases could be predicted with 83% sensitivity and 81% specificity, and with the cutoff value of 357.05 fmol/μg DNA, the discontinuation of thiopurines could be predicted with 87% sensitivity and 77% specificity. DNATG levels were more significantly associated with late leucopenia than 6TGN levels, which was manifested especially in patients with *NUDT15*/*TPMT* wild-type, with a cutoff value of 315.72 fmol/μg DNA (sensitivity 88%, specificity 85%). This study provides empirical evidence indicating that early monitoring of DNATG levels, offers a predictive capability to discern patients susceptible to developing late leucopenia during the initial stages of thiopurine therapy, especially for many patients with wild-type *NUDT15*/*TPMT*.

ARTICLE HIGHLIGHTS

Research background

Thiopurine-induced leucopenia significantly hinders the use of thiopurines. Genotype-guided dose optimization has significantly reduced the early leucopenia rate, but there are no definitive biomarkers for late risk prediction.

Research motivation

Can we identify definitive predictors for late risk as early as possible under current genotype-guided thiopurine dosing strategy?

Research objectives

To determine the predictive value of early monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides (6TGN) for late leucopenia in patients with Crohn's disease (CD).

Research methods

Blood samples were collected within two months after thiopurine initiation for detection of metabolite concentrations. Late leucopenia was defined as a leukocyte count < 3.5×10^9/L over two months.

Research results

In patients suffering late leucopenia, early DNATG levels were significantly higher than those who did not ($P = 4.9 \times 10^{-13}$). DNATG threshold of 319.43 fmol/μg DNA could predict late leucopenia in the entire sample collection with an area under the curve (AUC) of 0.855 (sensitivity 83%, specificity 81%), and in the nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) normal metabolizers, the prediction performance of a threshold of 315.72 fmol/μg DNA was much more remarkable with an AUC of 0.902 (sensitivity 88%, specificity 85%). 6TGN had a relatively poor correlation with late leucopenia whether in the entire sample collection ($P = 0.021$) or NUDT15/TPMT normal or intermediate metabolizers ($P = 0.018$, $P = 0.55$, respectively).

Research conclusions

Early DNATG concentration was significantly correlated with thiopurine-induced late leucopenia in CD patients, especially in NUDT15/TPMT normal metabolizers.

Research perspectives

Proactive therapeutic drug monitoring to keep DNATG concentration below 320 fmol/μg DNA could be an effective strategy to prevent thiopurine-induced late leucopenia in CD patients.

FOOTNOTES

Author contributions: Yang T, Chao K, and Zhu X contributed equally to this work. Huang M and Gao X contributed to the study conceptualization and supervision and funding acquisition; Chao K and Gao X contributed to the acquisition and curation of data; Mao J and Li P helped to collect and process the blood samples; Guan SX and Xie W monitored the project progress and contributed to the writing and revision of the manuscript; Zhu X, Wang XD, and Guan YP helped to detect the genotypes and revise the manuscript; Yang T and Chan S detected drug concentrations; Yang T administered the project, analyzed the data, and wrote the manuscript; and all authors have read and approved the final manuscript.

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Clinical trial registration statement: This trial is registered with the Chinese Clinical Trials Register, No. ChiCTR2100050295.

Informed consent statement: All patients recruited provided a written informed consent form to participate in the trial.

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ORIGINAL ARTICLE

Basic Study

ALKBH5 suppresses autophagic flux *via* N6-methyladenosine demethylation of *ZKSCAN3* mRNA in acute pancreatitis

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Abstract

BACKGROUND

Increasing evidence has demonstrated that N6-methyladenosine (m^6A) RNA modification plays an essential role in a wide range of pathological conditions. Impaired autophagy is a critical hallmark of acute pancreatitis (AP).

AIM

To explore the role of the m^6A modification of *ZKSCAN3* in the regulation of autophagy in AP.

METHODS

The AP mouse cell model was established by cerulein-treated mouse pancreatic acinar cells (MPC-83), and the results were confirmed by the levels of amylase and inflammatory factors. Autophagy activity was evaluated by specific identification of the autophagy-related microstructure and the expression of autophagy-related genes. *ZKSCAN3* and *ALKBH5* were knocked down to study the function in AP. A m^6A RNA binding protein immunoprecipitation assay was used to study how the m6A modification of *ZKSCAN3* mRNA is regulated by *ALKBH*.

RESULTS

The increased expression of amylase and inflammatory factors in the supernatant and the accumulation of autophagic vacuoles verified that the AP mouse cell model was established. The downregulation of *LAMP2* and upregulation of *LC3-II/I* and *SQSTM1* demonstrated that autophagy was impaired in AP. The expression of *ZKSCAN3* was upregulated in AP. Inhibition of *ZKSCAN3* increased the expression of *LAMP2* and decreased the expression of the inflammatory factors, *LC3-II/I* and *SQSTM1*. Furthermore, *ALKBH5* was upregulated in AP. Knockdown of *ALKBH5* downregulated *ZKSCAN3* expression and restored

decreased autophagic flux in AP. Notably, the bioinformatic analysis revealed 23 potential m^{6}A modification sites on *ZKSCAN3* mRNA. The m^{6}A modification of *ZKSCAN3* mRNA was significantly decreased in AP. Knockdown of *ALKBH5* increased the modification of *ZKSCAN3* mRNA, which confirmed that *ALKBH5* upregulated *ZKSCAN3* expression in a m^{6}A-dependent manner.

CONCLUSION

ALKBH5 inhibits autophagic flux through m^{6}A demethylation of *ZKSCAN3* mRNA in AP, thereby aggravating the severity of the disease.

Key Words: Acute pancreatitis; Autophagy; *ZKSCAN3*; N6-methyladenosine; *ALKBH5*

Core Tip: Acute pancreatitis (AP) is a common emergency in digestive system. Impaired autophagy is one of important pathogenic mechanisms of AP, however, its regulatory mechanism remains unclear. N6-methyladenosine modification and *ZKSCAN3* are crucial regulatory factors of autophagy, but their roles in AP are not well-defined. This study confirmed that the demethylase *ALKBH5* can inhibit autophagy flux by upregulating *ZKSCAN3*, thereby exacerbating the inflammatory severity of AP. The findings of this study provided new insights into the autophagy regulation mechanism and offered a novel direction for early intervention in AP.

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INTRODUCTION

Acute pancreatitis (AP) is one of the most common digestive emergencies. The global prevalence and incidence of AP are approximately 76/100000 and 34/100000, respectively, and the number of new cases is increasing at an annual rate of 3% [1-3]. With the progression of therapeutic concepts and interventions, the prognosis of AP has significantly improved. However, due to the unclear pathogenesis of AP, clinicians are still unable to effectively intervene specifically in local or systemic inflammation. The pathogenesis of AP is complex and multifactorial and induces significant and sustained pathological disruption[4,5]. Therefore, further study of the mechanism underlying the progression of AP will provide insight into the development of future therapeutic strategies.

Autophagy is a highly conserved catabolic process in which abnormal biomolecules and organelles are degraded and degradation products are recycled. Autophagy plays an important role in maintaining cellular homeostasis. The entire autophagy process is defined as autophagic flux, and disrupted integrity of the process is called impaired autophagy[6]. Studies have shown that impaired autophagy plays an important role in the development of various diseases, such as neurodegeneration, inflammation, infection, tumors, and metabolic disorders[7-9]. In recent years, the important role of autophagy in AP has been gradually recognized. The basal level of autophagy in the mouse exocrine pancreas is significantly greater than that in the endocrine pancreas and other organs[10]. In experimental pancreatitis, interfering with the expression of upstream regulatory molecules or autophagy-related genes can induce inflammatory changes in exocrine pancreatic cells[11]. Impaired autophagy in AP manifests as activation of the initial stage but blockade of the degradation stage, resulting in harmful factors such as abnormal zymogen granules and disrupted organelles that cannot be effectively degraded[10]. Although impaired autophagy can mediate abnormal zymogen activation, inflammation, and cell death in pancreatic acinar cells[12], the specific regulatory mechanism involved is still unclear.

ZKSCAN3 is a zinc finger DNA-binding protein that simultaneously contains KRAB and SCAN domains; it is also a recognized inhibitory factor of autophagy[13,14]. Studies have shown that *ZKSCAN3* can inhibit the transcription of numerous autophagy-related genes, such as *LC3* and *WIPI2*, thereby suppressing a series of autophagy steps in various diseases[15,16]. However, the role of *ZKSCAN3* in autophagy in AP has not yet been determined.

The N6-methyladenosine (m^{6}A) modification of RNA plays an important role in the autophagy regulatory network. This process is reversible and involves mainly methyltransferases, demethylases, and methylated RNA-binding proteins [17,18]. ALKBH5 is a crucial demethylase that plays a key role in various diseases[19,20]. In ovarian cancer, the overexpression of ALKBH5 promotes the formation of the BCL-2-Beclin1 complex, and inhibits autophagy[21]. In silica-related pneumonia, *ALKBH5* can mediate autophagic flux blockade through the Slam7 pathway[22]. However, in myocardial ischemia-reperfusion injury, *ALKBH5* plays a role in promoting autophagic flux[23]. Although the role of m^{6}A modification in impairing autophagy has been demonstrated in various tumors and inflammatory diseases, there is no experimental research on m^{6}A modification in AP. Recent bioinformatics studies have shown that decreased m^{6}A levels are related to the occurrence of severe AP[24], but whether this change is related to *ALKBH5*-mediated impaired autophagy in AP is unclear.

Clarifying the regulatory mechanism of autophagy in AP is crucial for early intervention. However, research on the autophagy and its regulatory mechanism in AP has not been illustrated. Therefore, in this article we aimed to explore the role and mechanism of action of *ALKBH5* in *ZKSCAN3* regulated autophagy. We verified the results at the cellular level through a series of molecular biology experiments, which provided a novel perspective on the research of pathogenesis and molecular mechanism of AP and highlighted new targets for therapeutic intervention.

MATERIALS AND METHODS

Cell culture

Mouse pancreatic acinar cells (MPC-83) were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 37 °C incubator with 5% CO_2. The control groups were not treated, and the AP groups were pretreated with cerulein (10 nM) for 24 h.

Cell transfection

MPC-83 cells were seeded in 6-well plates and maintained at 37 °C and 5% CO_2. *ALKBH5* and *ZKSCAN3*-siRNA (50 nM) (RiboBio, Gunagzhou, China) were transfected into MPC-83 cells. After 48 h of transfection, the cells were treated with cerulein (10 nM) for 24 h.

Quantitative real-time RT-PCR

Total RNA was extracted from cells using the TRIzol method. All mRNAs were reverse transcribed using the PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa, Kyoto, Japan). Reverse transcription and quantitative real-time RT-PCR were performed with SYBR® Premix Ex Taq™ (TaKaRa, Kyoto, Japan). The results were normalized to that of β-actin and calculated *via* the relative quantification ($2^{-\Delta\Delta Ct}$) method. The primers used were purchased from Sangon Company (Table 1).

ELISA

The supernatant of MPC-83 cells was collected. The levels of interleukin 6 (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were assessed using ELISA kits (Neobioscience, Shenzhen, China).

Western blot

Cell lysates were prepared using lysis buffer composed of 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40. The lysates were centrifuged to collect the supernatants. An equal amount of protein was denatured in SDS sample buffer and separated on 8% or 10% polyacrylamide gels based on the molecular weight of the target proteins. The separated proteins were then transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk in TBST (TBS containing 0.05% Tween 20), incubated with primary antibodies, and subsequently incubated with secondary antibodies conjugated to alkaline phosphatase. Protein expression was detected by chemiluminescence. The antibodies used were against *ALKBH5* (ab195377, Abcam, Britain), *ZKSCAN3* (ab223477, Abcam, Britain), *LC3* (Proteintech, Wuhan, China), *LAMP-2* (Proteintech, Wuhan, China) and *SQSTM1* (Proteintech, Wuhan, China).

Immunofluorescence

After cell fixation, the cells were treated with 0.2% Triton X-100 at room temperature. The cells were then blocked with blocking solution. Subsequently, the cells were treated with primary and secondary antibodies. DAPI dye was added to the cells, which were subsequently incubated in the dark. The cells were mounted on slides using anti-fade mounting medium, and fluorescence was observed using a fluorescence microscope. The antibodies used were against *LC3* (Proteintech, Wuhan, China) and *LAMP-2* (Proteintech, Wuhan, China).

Transmission electron microscopy

The specimens were cut and fixed in a 2.5% glutaraldehyde solution with Millonig's phosphate buffer (pH = 7.3). The samples were washed three times with Millonig's phosphate buffer at 10-minute intervals. The dehydration process was performed at room temperature using a graded series of acetone (50%, 70%, and 90%) at 10-min intervals, followed by two washes with 100% acetone at 15-min intervals. The samples were then soaked and embedded in a mixture of acetone and resin (1:1) for 12 h, followed by polymerization overnight at 37 °C using 100% resin. To solidify the sample resin, the specimens were further polymerized at 37 °C overnight, followed by an additional 12 h at 60 °C. Ultrathin sections of 50-100 nm were obtained from the specimens using an ultramicrotome and a diamond knife. The sections were then stained with 3% uranyl acetate and lead nitrate, after which they were examined and photographed using a Hitachi HT-7700 electron microscope.

M^{6}A RNA binding protein immunoprecipitation assay

The M^{6}A RNA binding protein immunoprecipitation kit was purchased from RiboBio. RNA was fragmented using RNA fragmentation buffer. Magnetic beads for m^{6}A were prepared using magnetic beads A/G and an anti-m^{6}A antibody. RNA immunoprecipitation was conducted by mixing the fragmented RNA with anti-m6A magnetic beads. The RNA was washed with elution buffer to remove it from the magnetic beads.

Table 1 Primer sequences for qPCR

Genes	Sequence
ALKBH5	Forward 5′- CTTTGCTTCGGCTGCAAGTT -3′
	Reverse 5′- CCGGCGTTCCTTAATGTCCT -3′
ZKSCAN3	Forward 5′- CAGAGTAGGGTGGAAAGCC -3′
	Reverse 5′- AAGGTATGAAGGTCGGGTG -3′
Primer 1	Forward 5′- CCAGGCGGTTCTATTGC -3′
	Reverse 5′- TGGCTTTCCACCCTACTCT -3′
Primer 2	Forward: 5′- CAGAGTAGGGTGGAAAGCC-3′
	Reverse 5′- AGGTATGAAGGTCGGGTG-3′
Primer 3	Forward 5′- TGGTTCGGGATGGCTAG-3′
	Reverse 5′- AACAGCACTGCCTTGGAG-3′
β-actin	Forward 5′- GTGGCCGAGGACTTTGATTG-3′
	Reverse 5′- CCTGTAACAACGCATCTCATATT-3′

Website for m⁶A site prediction

Potential m⁶A binding sites on *ZKSCAN3* mRNA were analyzed *via* a website (http://www.cuilab.cn/sramp/).

Statistical analysis

All the statistical analyses were performed in GraphPad Prism 8. Independent sample *t* tests were used to compare the means of two samples, while one-way ANOVA was used for analyzing and comparing the means of more than two groups of samples. *P* values < 0.05 were considered to indicate statistical significance. The experimental results are presented as the mean ± SD.

RESULTS

Impaired autophagy in the AP mouse cell model

To construct a cell model of AP, MPC-83 cells were treated with 10 nM cerulein for 24 h. The levels of amylase and the inflammatory factors IL-1β, IL-6, and TNF-α in the supernatant were measured *via* ELISA. The results showed that the levels of amylase and inflammatory factors were significantly greater in the cerulein-treated group (Figure 1A and B), indicating that the AP cell model was successfully established.

The expression levels of autophagy-related marker proteins were detected by western blotting, which showed that the ratio of *LC3B-II/I* was increased in the AP group, indicating an increase in autophagosomes. The expression of *LAMP-2* was decreased in the AP groups, indicating impaired lysosomal synthesis. The expression of the selective autophagy receptor *SQSTM1* was increased, indicating inhibited substrate degradation (Figure 1C). Transmission electron microscopy (TEM) revealed the accumulation of circular autophagic vacuoles in the AP group, indicating impaired degradation and accumulation of autophagosomes and autolysosomes (Figure 1D). Furthermore, immunofluorescence staining revealed that *LC3* was significantly increased in the AP groups (Figure 1E), while *LAMP-2* expression was decreased (Figure 1F). These results demonstrated that autophagosome formation is activated, while lysosomal synthesis and function are impaired, leading to decreased substrate degradation efficiency and accumulation of autophagic vacuoles, suggesting impaired autophagy.

ZKSCAN3 is upregulated and promotes the release of inflammatory factors in AP

To investigate the role of *ZKSCAN3* in AP, qPCR, and western blot were used to detect the expression levels of *ZKSCAN3*. The results showed that the mRNA and protein expression levels of *ZKSCAN3* were significantly increased in the AP group (Figure 2A and B). Three different siRNAs were used to knock down the expression of *ZKSCAN3*, and siRNA-2 had the most significant interference effect (Figure 2C and D). Subsequent experiments were performed using siRNA-2 to knock down *ZKSCAN3*. After the inhibition of *ZKSCAN3*, the cells were treated with cerulein to construct the AP cell model. The levels of inflammatory factors IL-1β, IL-6, and TNF-α in the knocking down (KD) group were significantly lower than those in the negative control (NC) group (Figure 2E). These results suggest that *ZKSCAN3* is upregulated and promotes the release of inflammatory factors in the AP mouse cell model.

ZKSCAN3 impaired autophagic flux in AP

To investigate the role of *ZKSCAN3* in autophagic flux in AP, western blot was used to detect the expression of autophagy marker proteins (Figure 3A). The *LC3B-II/I* ratio was decreased in the KD group, demonstrating the increased

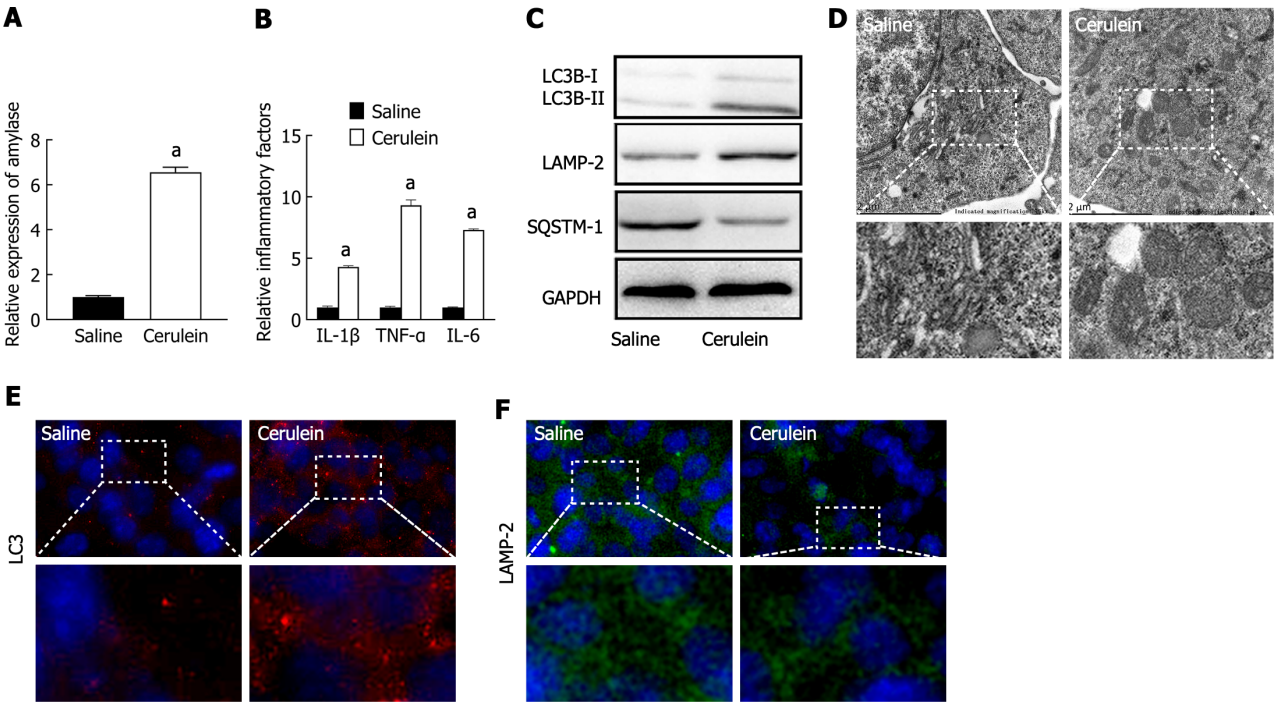

Figure 1 Impaired autophagy in a mouse acute pancreatitis cell model. A: The levels of amylase in the supernatant were detected by ELISA; B: The levels of inflammatory factors in the supernatant were detected by ELISA; C: The expression of autophagy-related proteins was detected by western blot; D: The microstructure of intracellular autophagy was observed by transmission electron microscopy; E: The expression of *LC3* was detected by immunofluorescence (magnification × 800); F: The expression of *LAMP-2* was detected by immunofluorescence (magnification × 800). $^{a}P < 0.05$ *vs* saline group. TNF: Tumor necrosis factor; IL: Interleukin.

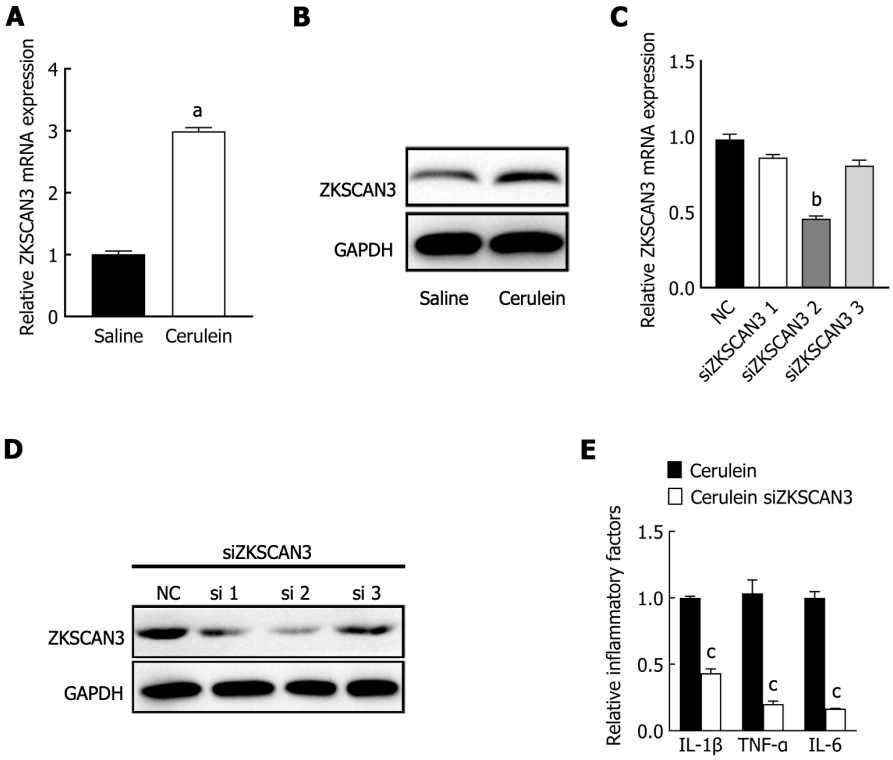

Figure 2 The expression and function of *ZKSCAN3* in acute pancreatitis. A: The expression of *ZKSCAN3* mRNA was detected by qPCR; B: The expression level of *ZKSCAN3* protein was detected by western blot; C: The expression level of *ZKSCAN3* mRNA treated with three different siRNAs was detected by qPCR; D: The expression level of *ZKSCAN3* protein in the MPC-83 cell line treated with three different siRNAs; E: After interfering with the expression of *ZKSCAN3*, the expression level of inflammatory factors in the mouse acute pancreatitis cell model was detected by ELISA. $^{a}P < 0.05$ *vs* saline group; $^{b}P < 0.05$ *vs* negative control group; $^{c}P < 0.05$ *vs* cerulein group. NC: Negative control. TNF: Tumor necrosis factor; IL: Interleukin.

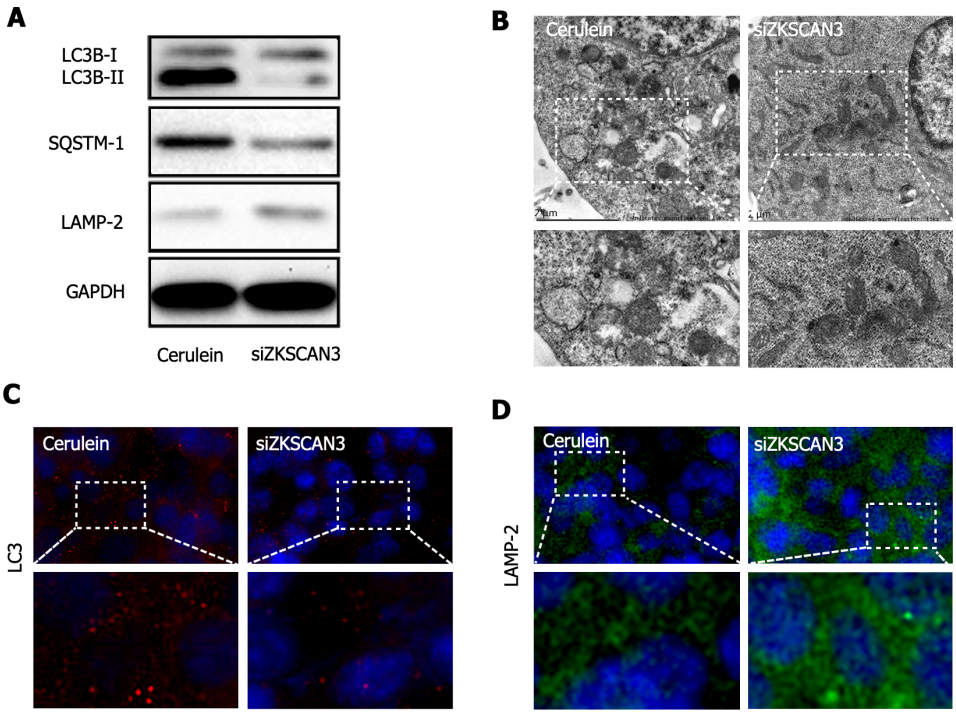

Figure 3 *ZKSCAN3* inhibits autophagic flux in acute pancreatitis. A: The expression of autophagy related proteins in the negative control group and knockdown group was detected by western blot; B: The extent of autophagy was observed by transmission electron microscopy; C: The expression of *LC3* was detected by immunofluorescence (magnification × 800); D: The expression of *LAMP-2* was detected by immunofluorescence (magnification × 800).

clearance of autophagolysosomes. The expression of *LAMP-2* increased, suggesting a reduction in lysosomal biogenesis impairment, and the expression of *SQSTM1* decreased, indicating an improvement in substrate degradation efficiency. TEM revealed a significant reduction in autophagosome accumulation in the KD group (Figure 3B). Immunofluorescence staining revealed decreased expression of *LC3* in the KD group (Figure 3C) and increased expression of LAMP-2 (Figure 3D). These results suggest that *ZKSCAN3* inhibits autophagic flux in AP and that KD *ZKSCAN3* expression can impair the blockade of autophagic flux.

ALKBH5 is upregulated in AP and promotes the release of inflammatory factors

M^{6}A methylation is widely involved in autophagy and contributes to the pathogenesis of human disease. The expression and function of *ALKBH5*, a primary m^{6}A demethylase in AP, have not yet been determined. We detected *ALKBH5* expression in the AP mouse cell model by qPCR and western blot analysis. The level of *ALKBH5* was upregulated in the AP mouse cell model (Figure 4A and B). Furthermore, three different siRNAs were used to knock down *ALKBH5* expression in MPC-83 cells, and siRNA-3 had the most effective interference effect (Figure 4C and D); therefore, siRNA-3 was used for subsequent experiments. The expression of the inflammatory factors IL-1β, IL-6, and TNF-α was significantly reduced (Figure 4E). These results suggest that *ALKBH5* was upregulated in the mouse AP cell model and promoted the release of inflammatory factors.

ALKBH5 inhibited autophagic flux by promoting *ZKSCAN3* expression

In the AP mouse cell model, knockdown of *ALKBH5* downregulated the mRNA and protein expression of *ZKSCAN3* (Figure 5A), indicating that *ALKBH5* promotes *ZKSCAN3* expression. Furthermore, the expression of *LC3B-II/I* and *SQSTM1* decreased, while *LAMP-2* expression was increased (Figure 5B), indicating that the knockdown of *ALKBH5* rescued the blockade of autophagic flux in AP. TEM confirmed that autophagic vacuole accumulation was reduced after the expression of *ALKBH5* was inhibited (Figure 5C). Immunofluorescence revealed that the immunoreactivity of the *LC3* protein decreased (Figure 5D), while the immunoreactivity of the *LAMP-2* protein increased in the AP mouse cell model transfected with the *ALKBH5* target siRNA (Figure 5E). These results suggested that *ALKBH5* promoted *ZKSCAN3* expression, resulting in the blockade of autophagic flux in AP.

ALKBH5 regulated *ZKSCAN3* expression in a m^{6}A-dependent manner

Considering that *ALKBH5* is a well-known m^{6}A demethylase, we further investigated the role of m^{6}A modification in the regulation of *ZKSCAN3* by *ALKBH5*. Biological software analysis revealed 23 potential m^{6}A binding sites on *ZKSCAN3* mRNA, including 6 highly credible sites, 7 highly credible sites, 6 moderately credible sites, and 4 sites with low credibility (Table 2, Figure 6A). Additionally, we constructed a secondary structure diagram of highly credible m^{6}A binding sites (Figure 6B).

To confirm the role of m^{6}A modification in the relationship between *ALKBH5* and *ZKSCAN3*, MeRIP-qPCR was performed with specific primers aimed at identifying potential m^{6}A sites, and the enrichment of m^{6}A-modified *ZKSCAN3*

Table 2 m^{6}A sites of *ZKSCAN3* mRNA

Number	Position	Sequence context	Confidence
1	34	GUGCCCCGCCCCCCGGGGUCGGACUUUCGACACUUUUGUGACUGC	High
2	131	ACAGCUACAGUGAAACGGGAGAACUGCUUGGUUCGGGAUGGCUAG	High
3	180	UCAAGGGAAAGCACAACCUUGGACUCACACUCUGCAGAGGACCAG	Very high
4	198	UUGGACUCACACUCUGCAGAGGACCAGAUGGAGCUACUGGUCAUA	High
5	229	AGCUACUGGUCAUAAAGGUGGAACAAGAAGAGGCCUCCCCCUUGG	Moderate
6	534	GUGGCGCUGCUGGAGUACUUGGACAGGCAGCUGGAUGACACACCU	High
7	589	CAGAUGAUGACGAUGGGCAGGAACUCCUUUGCUCCAAGGCAGUGC	Moderate
8	824	CCCAGUCCUUUCCCCCAGAUGGACAGAGCAGGAUUCAUCUCAGAU	Very high
9	849	GAGCAGGAUUCAUCUCAGAUGAACCUCUACAAAGAUGGAAUGCAG	Moderate
10	906	AGCCUGGUUUCCCUGGAUCAGGACAUGCAGACUAAGGUUAGGGAC	Very high
11	914	UUCCCUGGAUCAGGACAUGCAGACUAAGGUUAGGGACUUGCCUCG	Moderate
12	927	GACAUGCAGACUAAGGUUAGGGACUUGCCUCGAGCUGAAGAAUAC	Very high
13	954	CCUCGAGCUGAAGAAUACAGGGACCAAAAGCCUGAGCAGACAGUG	High
14	971	CAGGGACCAAAAGCCUGAGCAGACAGUGUGCUUCCUGGGUGAAGA	Low
15	993	ACAGUGUGCUUCCUGGGUGAAGACACUGUCCCGAUUCCUACAGGU	Low
16	1347	GAAAAGCCCUACGAGUGUGAUGACUGUGGGAAAACCUUCACUCAG	High
17	1387	CUCAGAGCUGCAGCCUCCUUGAACAUCACAGAAUUCACACUGGGG	Low
18	1471	GGCGUAGCUCACAUCUUCUGAGACAUCAGAGGACCCAUACUGGGG	Moderate
19	1633	GUAGGAUUACAAGCCUUAUUGAACACCAAAAAGUACACACUGGUG	Low
20	1745	GAGAAGACACACGGGGAAGAAAACUUCUGUCACAGUGACCCCUGC	Moderate
21	1803	GUUGGUGUUCAACUGUCAUUGAACUGAAGCCACUCUGUAGUUCUU	High
22	1830	AGCCACUCUGUAGUUCUUAAUGACUGCAGAAGUCAUAGGCUGGGG	Very high
23	1985	ACAAGAGUCCUCACCCAUUGGAACUAAAUGGGCUUCCUGACUGUC	Very high

mRNA in the AP group was significantly lower (Figure 7). This finding suggested that *ALKBH5* can decrease the m^{6}A modification of *ZKSCAN3*.

DISCUSSION

This study is the first to reveal the regulatory roles of *ZKSCAN3* and m^{6}A modification in impairing autophagy in AP. We found that *ALKBH5* upregulated *ZKSCAN3* expression by demethylating *ZKSCAN3* inhibited autophagy, and promoted the release of inflammatory factors in a mouse cell model of AP.

Impaired autophagy is one of the key pathogenic mechanisms in AP; this process affects the functions of various organelles, such as mitochondria and the endoplasmic reticulum, and disrupts the homeostasis of acinar cells[12,25]. Usually, autophagy degrades dysfunctional mitochondria during AP. Inhibition of autophagic flux by knocking out the *ATG5* and *ATG7* genes impaired the clearance of damaged mitochondria, further affecting generation the efficiency of ATP generation in acinar cells[26,27]. Moreover, autophagy maintains the stability of endoplasmic reticulum function. Knocking out the IκB kinase α gene leads to impaired autophagy, and the accumulated *SQSTM1* further causes the accumulation of misfolded proteins in the endoplasmic reticulum, triggering endoplasmic reticulum stress and ultimately inducing AP[28]. Therefore, impaired autophagy may trigger or exacerbate other cellular pathological factors in AP. Furthermore, other pathological factors can also induce impaired autophagy. In arginine-treated mice, abnormal mitochondrial membrane leads to disrupted energy metabolism, which inhibits autophagic flux[29]. In ethanol-induced AP, endoplasmic reticulum stress causes folding and transport disorders of autophagy-related proteins[30]. Therefore, autophagy is interconnected with other pathological events during AP. Early autophagy-related intervention may help alleviate the malignant cycle caused by pathological factors.

ZKSCAN3 is currently recognized as a key autophagy inhibitor[15,31]. It affects the progression of various diseases by inhibiting autophagic flux. In hepatocellular carcinoma (HCC), *ZKSCAN3* inhibits autophagy, leading to decreased degradation of local adhesion proteins and reducing the metastasis of HCC[32]. In addition, impaired autophagy

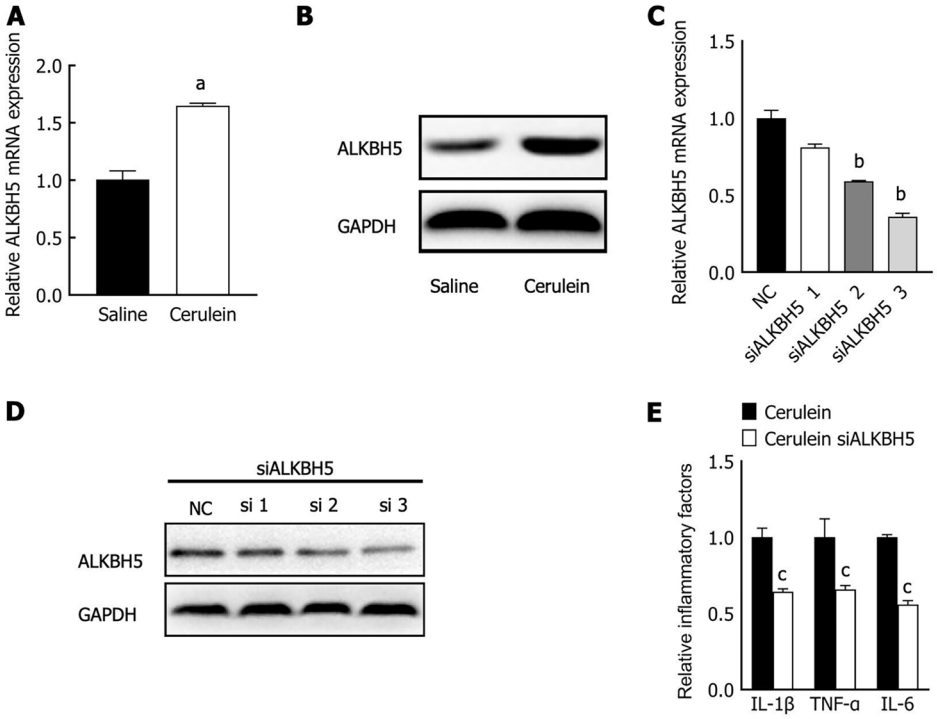

Figure 4 The expression and function of *ALKBH5* in acute pancreatitis. A: qPCR was used to detect *ALKBH5* mRNA expression in acute pancreatitis (AP) group and control group; B: The expression level of the *ALKBH5* protein in AP group and control group was detected by western blot; C: qPCR was used to detect *ALKBH5* mRNA expression in the MPC-83 cell line treated with different siRNAs; D: western blot was used to detect *ALKBH5* protein expression in MPC-83 cell line treated with three different siRNA; E: After interfering with the expression of *ALKBH5*, the expression level of inflammatory factors in the mouse AP cell line was detected by ELISA. $^{a}P < 0.05$ *vs* saline group; $^{b}P < 0.05$ *vs* negative control group; $^{c}P < 0.05$ *vs* cerulein group. NC: Negative control; TNF: Tumor necrosis factor; IL: Interleukin.

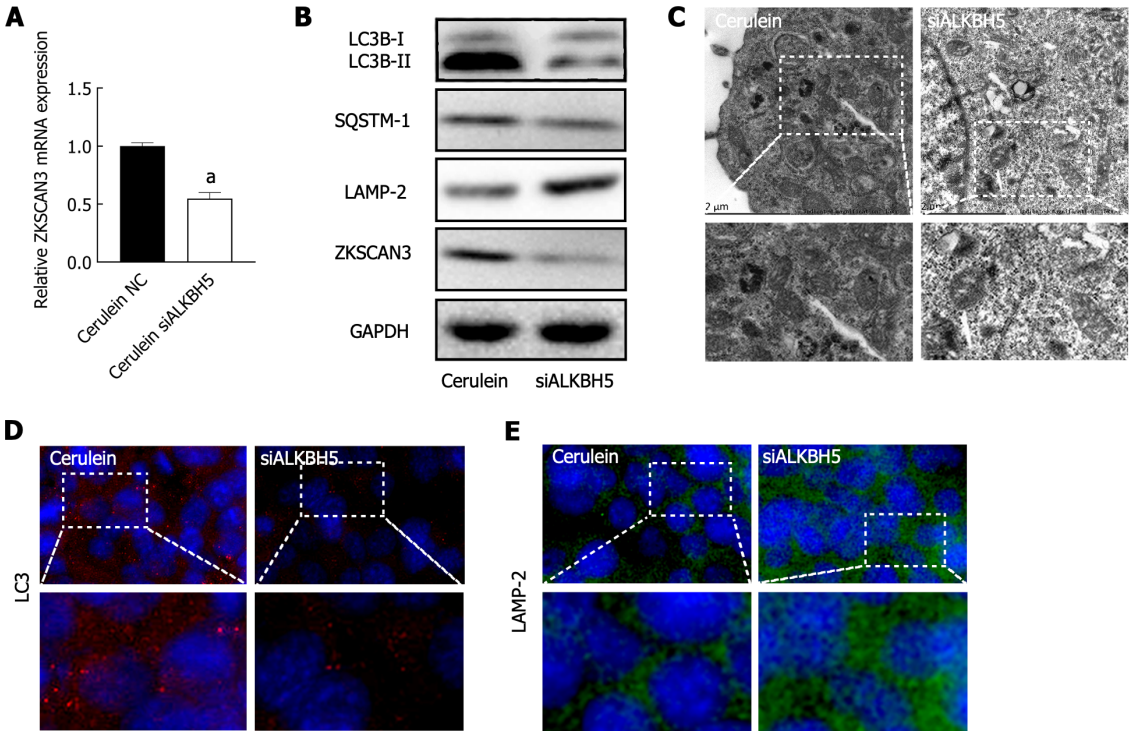

Figure 5 *ALKBH5* promoted the expression of *ZKSCAN3* and inhibited autophagic flux. A: After interfering with *ALKBH5* expression, the expression level of *ZKSCAN3* mRNA was detected by qPCR; B: The expression levels of *ZKSCAN3* protein and autophagy related proteins were detected by western blot; C: Autophagic microstructure was observed by transmission electron microscopy; D: *LC3* was detected by immunofluorescence (magnification × 800); E: *LAMP-2* was detected by immunofluorescence (magnification × 800). $^{a}P < 0.05$ *vs* negative cerulein group. NC: Negative control.

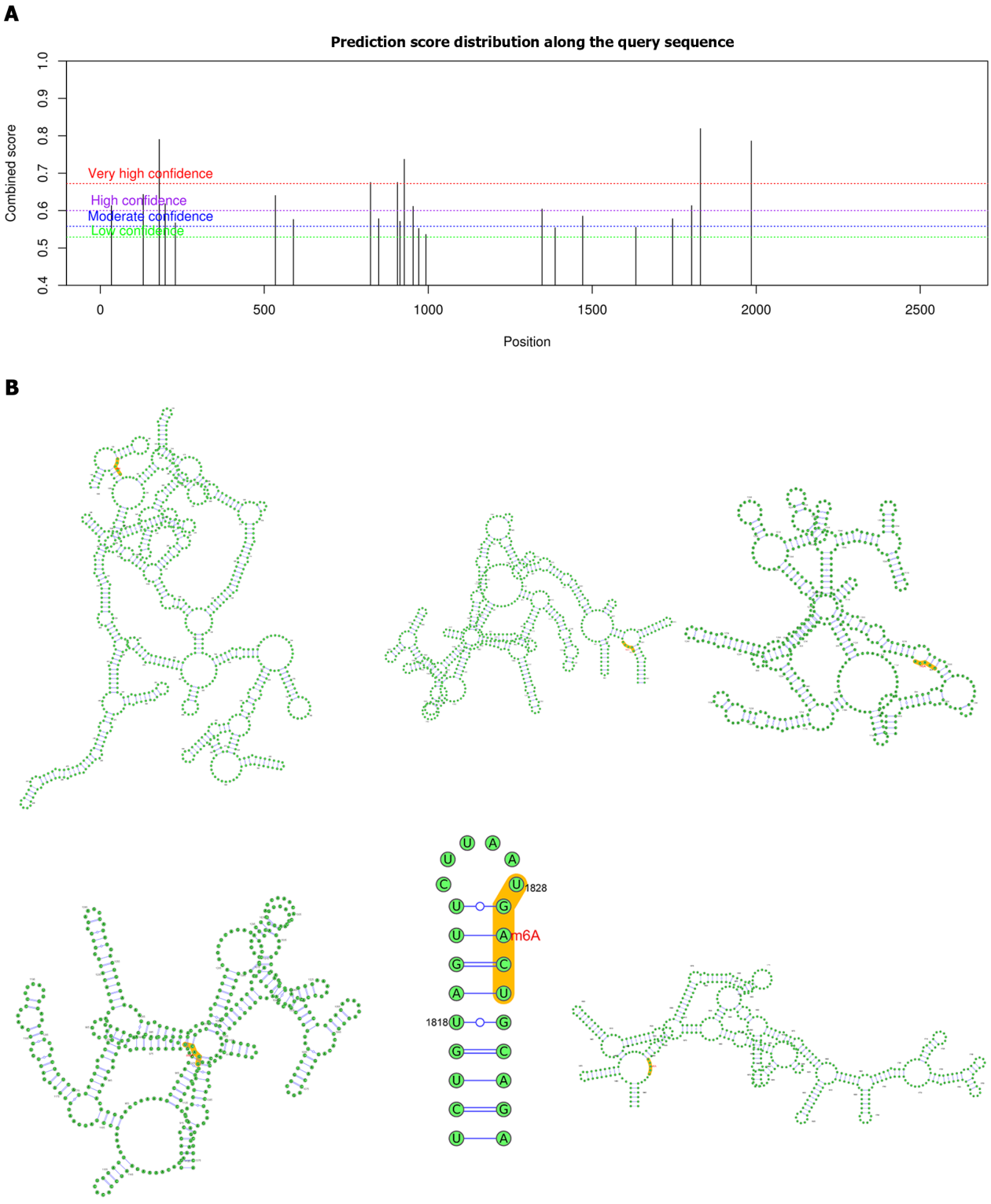

Figure 6 Prediction of m^{6}A binding sites in *ZKSCAN3*. A: Potential m^{6}A binding sites on *ZKSCAN3* mRNA were analyzed *via* a website (http://www.cuilab.cn/sramp/); B: Diagram of the secondary structure of highly credible m^{6}A binding sites. m^{6}A: N6-methyladenosine.

mediated by *ZKSCAN3* is closely related to sepsis-induced immunosuppression[33]. However, the role of *ZKSCAN3* in the pathogenesis of AP is still uncertain. Our study first confirmed the high expression of *ZKSCAN3* in cerulein-treated MPC-83 cells and the inhibitory effect on autophagy.

ZKSCAN3 functions mainly through nucleoplasmic translocation; when activated, it moves into the nucleus to suppress the transcription of autophagy related genes[15]. However, the upstream regulatory mechanism of *ZKSCAN3* is unclear. A study revealed that SIRT1 deacetylates the lysine residues of *ZKSCAN3* and promotes its shuttling between the nucleus and cytoplasm[34]. PKC and BRAF inhibitors can activate the inhibition of *ZKSCAN3 via* phosphorylation[35,36]. Although a few studies have revealed the upstream molecular mechanisms of *ZKSCAN3*, there is no related research focusing on this gene in AP.

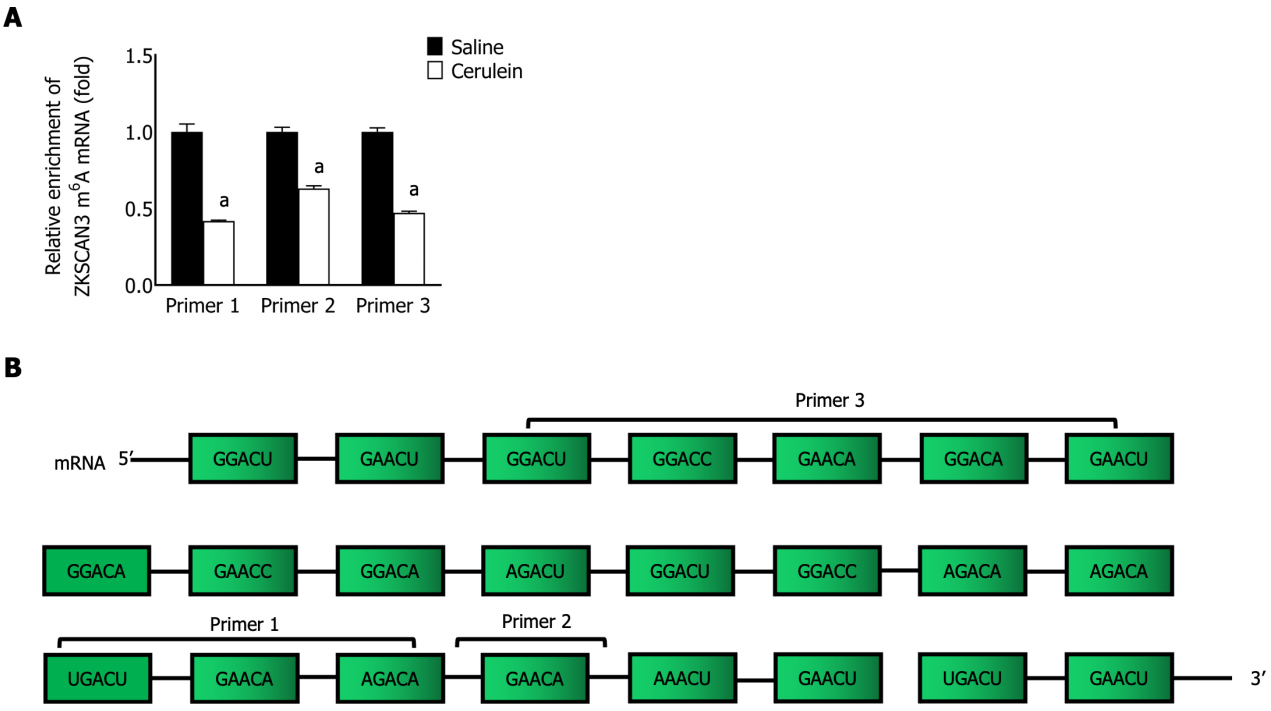

Figure 7 Enrichment levels of m^{6}A modifications on *ZKSCAN3* mRNA. A: qPCR was used to detect the m^{6}A modification level of *ZKSCAN3* mRNA; B: Schematic diagram of the sequences of primers used for *ZKSCAN3* mRNA. $^aP < 0.05$ *vs* saline group.

As an important component of epigenetics, m^{6}A modification plays an important regulatory role in autophagy. METTL3 promotes the binding of the RNA-binding protein HNRNPD to the precursor mRNA of TFEB, thus inhibiting autophagy[23]. In HCC, loss of METTL3 increases the stability of the FOXO3 mRNA 3'-UTR modification through a YTHDF1-dependent mechanism and activates autophagy[37]. Moreover, in testicular stromal cells, human chorionic gonadotropin activates autophagy flow by upregulating the expression of *ALKBH5* and inhibiting the translation of the m^{6}A-mediated protein PPM1A, thereby increasing testosterone secretion[38]. Therefore, m^{6}A modification is widely involved in the regulation of autophagy in physiological and pathological processes. Among different types of diseases, the same type of m^{6}A modification has different effects on autophagy, which is related to downstream molecular targets and the pathological and physiological stages of disease[39]. Currently, the regulatory role of m^{6}A modification in autophagy has been well documented in various disorders[40], but its role in AP has rarely been studied. Bioinformatics study has shown that m^{6}A-modified noncoding RNAs may participate in the pathological changes observed in AP, but there is still a lack of relevant experimental evidence[24]. This research is the first to demonstrate that *ALKBH5* upregulates the expression of *ZKSCAN3* by demethylation, thereby inhibiting autophagy in AP.

This study has several limitations. First, the experimental subjects were cell models, and further *in vivo* animal experiments need to be conducted. In addition, there are significant differences in homology between animal and human tissues. However, due to the lack of human pancreatic exocrine cell lines and a stable extraction method, experimental research on AP cannot be performed in depth in human tissue[41,42]. In clinical practice, identifying pancreatic, peripancreatic or infected necrotic tissues is difficult due to pancreatic juice corrosion or infection. Therefore, human pancreatic exocrine cell lines and tissues are essential for mechanistic research on AP in the future.

CONCLUSION

In summary, we first revealed the important roles of *ZKSCAN3* and m^{6}A modification in AP. In cerulein-treated MPC-83 cells, *ALKBH5* upregulates *ZKSCAN3* expression by demethylation, thereby inhibiting autophagic flux and aggravating the severity of AP. The results obtained in this study provide important insights into the mechanism of autophagy regulation in AP and offer reference value for future in-depth exploration and early intervention.

ARTICLE HIGHLIGHTS

Research background

The incidence of acute pancreatitis (AP) is increasing annually, and its mortality rate is high. Impaired autophagy is a key factor in the occurrence and development of AP. Therefore, it is crucial to clarify the regulatory mechanism of autophagy in AP.

Research motivation
Evidence has shown that *ALKBH5* and *ZKSCAN3* can regulate autophagy in a variety of diseases, but there are no relevant studies on AP.

Research objectives
We aimed to explore the regulatory functions and mechanisms of autophagy mediated by *ALKBH5* and *ZKSCAN3* in AP.

Research methods
The AP mouse cell line was constructed with cerulein, and the levels of inflammatory factors were detected *via* ELISA. Similarly, the expression of *ALKBH5*, *ZKSCAN3* and autophagy-related proteins was detected *via* qPCR, western blot, and immunofluorescence. Microscopic manifestations of autophagy in the cell model were observed *via* transmission electron microscopy. Additionally, RNA binding protein immunoprecipitation was used to analyze the interaction between *ALKBH5* and *ZKSCAN3*.

Research results
The expression of *ALKBH5* and *ZKSCAN3* was upregulated in the AP model, and the trend toward increased expression of autophagy-related genes suggested that autophagic flux was blocked in AP. Autophagy was improved by inhibiting the expression of *ALKBH5* and *ZKSCAN3*. *ZKSCAN3* mRNA has m^{6}A binding sites, and *ALKBH5* can upregulate its expression by demethylating *ZKSCAN3*, which inhibits autophagic flux, thereby aggravating inflammation in AP.

Research conclusions
ALKBH5 suppresses autophagic flux by demethylating the m^{6}A site on *ZKSCAN3* mRNA, consequently promoting the onset and progression of AP.

Research perspectives
We proved that *ALKBH5* inhibits autophagy by upregulating *ZKSCAN3*, thereby promoting the occurrence and development of AP and providing new ideas for future research on autophagy regulation and early drug intervention in AP.

FOOTNOTES

Co-corresponding authors: Shuai Zhu and Geng-Wen Huang.

Author contributions: Zhu S and Huang GW conceived, designed and refined the study protocol; Zhang T finished the experiments, collected and analyzed the data and drafted the manuscript; Zhu S and Huang GW reviewed and revised the manuscript. All authors had access to the study data and reviewed and approved the final manuscript. Zhu S and Huang GW contributed equally to this work as co-corresponding authors. The reasons for designating Zhu S and Huang GW as co-corresponding authors are twofold. First, co-corresponding authors jointly conceived the overall design of the study and revised the manuscript. Second, they jointly provided financial support for the study. In summary, we believe that designating Zhu S and Huang GW as co-corresponding authors accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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LETTER TO THE EDITOR

Hepatic recompensation according to Baveno VII criteria *via* transjugular intrahepatic portosystemic shunt

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Abstract

Transjugular intrahepatic portosystemic shunt is a therapeutic modality done through interventional radiology. It is aimed to decrease portal pressure in special situations for patients with decompensated liver disease with portal hypertension. It represents a potential addition to the therapeutic modalities that could achieve hepatic recompensation in those patients based on Baveno VII criteria.

Key Words: Decompensated liver cirrhosis; Hepatic recompensation; Baveno VII; Portal hypertension

Core Tip: Liver cirrhosis is a complication of chronic liver disease. Hepatic decompensation follows a period of compensation if the etiology of the chronic liver disease is not eliminated and the liver inflammation is persistent. Hepatic recompensation is a novel term in which decompensation reverses after the clearance of the etiological factors in some patients. The use of a transjugular intrahepatic portosystemic shunt is a potential addition to achieve recompensation in a subset of patients with portal hypertension as demonstrated in published research.

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TO THE EDITOR

The topic of hepatic recompensation, according to Baveno VII criteria, is a novel and promising topic for patients with decompensated liver cirrhosis. It was previously thought that reaching the stage of decompensation in liver cirrhosis is a point of no return. This topic opens a new hope for patients with decompensated liver cirrhosis and may improve their clinical outcome if the appropriate therapeutic measures are taken.

The Baveno VII concept of hepatic recompensation necessitates meeting the following criteria: (1) Addressing the primary cause of cirrhosis through removal, suppression, or cure; (2) Achieving resolution of ascites, encephalopathy, and ensuring the absence of recurrent variceal hemorrhage for a minimum of 12 months; and (3) Demonstrating stable improvement in liver function tests [1].

We read the interesting manuscript of Gao *et al*[2], who presented a retrospective evaluation of 64 patients who underwent transjugular intrahepatic portosystemic shunt (TIPS) for bleeding varices or refractory ascites.

It was interesting that one-third of the patient population achieved hepatic recompensation as per Baveno VII criteria.

The concept of Baveno VII is novel, and a comparison of previous studies discussing hepatic recompensation is limited by the heterogenicity of the definition of hepatic recompensation. More studies are needed to accurately define the rate of recompensation under its criteria especially with different etiologies[3]. The criteria for recompensation for chronic hepatitis B patients were validated in a multicenter prospective study[4]. Most of the previous studies focused on etiological removal, suppression, or cure before measuring recompensation. Interestingly, this study adds a therapeutic intervention for portal hypertension in addition to targeting the etiology of cirrhosis.

Baveno VII criteria put a condition of removal, suppression, or cure of the etiology of liver cirrhosis. The study mentioned that all patients received essential medication or lifestyle interventions in line with EASL guidelines to achieve the removal or suppression of the primary cause of cirrhosis. The primary disease was referred to in the study as a collective viral hepatitis, alcohol, and others. There was no mention in the study of the specific viral etiologies, whether hepatitis B virus, hepatitis C virus, or coinfections, and the nature of the other diseases. It would have increased the value of the study if those specific details had been highlighted, including the follow-up of the etiologic cause during the post-intervention year to ensure the continuous state of suppression or cure. The follow-up only included liver function tests, Child-Pugh score, and Model for End-Stage Liver Disease score for 1 year, which ensured stable improvement as per BAVENO VII criteria.

TIPS is a therapeutic intervention not aimed directly at the clearance of the etiological factor of liver cirrhosis. It has specific indications during chronic liver disease, including bridging to liver transplantation, acute variceal bleeding, and refractory ascites. It has documented complications as well, such as hemorrhage, encephalopathy, TIPS dysfunction, and liver failure[5]. To prove it carries additional benefits to achieve recompensation, it needs a controlled study where the isolated effect of TIPS is measured, but this poses an ethical challenge as mentioned in the study limitations.

Regarding the clearance of the etiologic diagnosis, the impact of therapy other than for viral and alcohol-related liver disease remains to be further studied [3]. Double-blinded studies will pose an ethical challenge, as giving no intervention to clear the etiology for decompensated cases will deprive those patients of the potential of their disease improvement.

FOOTNOTES

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