ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2023 November 7; 29(41): 5618-5698

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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Editorial Board Member of *World Journal of Gastroenterology*, Ângelo Zambam de Mattos, MD, MSc, PhD, Adjunct Professor, Attending Doctor, Doctor, Graduate Program in Medicine: Hepatology, Federal University of Health Sciences of Porto Alegre, Porto Alegre 90020-090, Rio Grande do Sul, Brazil. angmattos@hotmail.com

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The *WJG* is now abstracted and indexed in Science Citation Index Expanded (SCIE), MEDLINE, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2023 edition of Journal Citation Reports® cites the 2022 impact factor (IF) for *WJG* as 4.3; Quartile category: Q2. The *WJG*'s CiteScore for 2021 is 8.3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

PUBLICATION DATE
November 7, 2023

PUBLISHING PARTNER
Shanghai Pancreatic Cancer Institute and Pancreatic Cancer Institute, Fudan University
Biliary Tract Disease Institute, Fudan University

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5618-5629

DOI: 10.3748/wjg.v29.i41.5618

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Diet as an epigenetic factor in inflammatory bowel disease

Karina Marangoni, Gilson Dorneles, Daniella Miranda da Silva, Letícia Pereira Pinto, Carina Rossoni, Sabrina Alves Fernandes

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ekine-Afolabi B, United Kingdom; Zhang C, China

Received: July 29, 2023
Peer-review started: July 29, 2023
First decision: September 11, 2023
Revised: September 24, 2023
Accepted: October 25, 2023
Article in press: October 25, 2023
Published online: November 7, 2023

Karina Marangoni, Egas Moniz School of Health and Science, Caparica - Almada, Portugal, Caparica 2820-062, Portugal

Karina Marangoni, National Institute of Sciences and Technology - Theranostics and Nanobiotechnology, Federal University of Uberlandia - MG, Brazil, Uberlândia 38400-902, Brazil

Gilson Dorneles, Corporate Social Responsibility, Hospital Moinhos de Vento, Porto Alegre 90035-004, Brazil

Daniella Miranda da Silva, Postgraduate Program in Gastroenterology, Universidade Federal do Rio Grande do Sul, Porto Alegre 91540-000, Brazil

Daniella Miranda da Silva, Department of Nutrition, Uniasselvi - Group Vitru, Santa Catarina 89082-262, Brazil

Letícia Pereira Pinto, Sabrina Alves Fernandes, Postgraduate Program in Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre 90050-170, Brazil

Carina Rossoni, Faculty of Medicine, Institute of Environmental Health, University of Lisbon, Lisboa 1649-026, Portugal

Carina Rossoni, Master in Physical Activity and Health, Polytechnic Institute of Beja, Beja 7800-000, Portugal

Carina Rossoni, Degree in Nutrition Sciences, Lusófona University, Lisboa 1749-024, Portugal

Corresponding author: Sabrina Alves Fernandes, PhD, Associate Research Scientist, Postdoc, Research Scientist, Researcher, Senior Researcher, Postgraduate Program in Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre, Sarmento Leite 245, Porto Alegre 90050-170, Brazil. sabrinaafernandes@gmail.com

Abstract

Inflammatory bowel disease (IBD) has as a main characteristic the exacerbation of the immune system against enterocytes, compromising the individual's intestinal microbiota. This inflammatory cascade causes several nutritional deficiencies, which further compromise immunological functioning and, as a result, worsen the prognosis. This vicious cycle can be interrupted as the patient's dietary pattern meets their needs according to their clinical condition, acting directly on the inflammatory process of IBD through the interaction of food, intestinal microbiota, and epigenome. Specific nutritional intervention for IBD has a crucial role in pre-

venting and managing disease activity. This review addresses epigenetic modifications through dietary compounds as a mechanism for modulating the intestinal microbiota of patients with IBD.

Key Words: Inflammatory bowel disease; Epigenetics; Nutrition; Nutrigenetics

Core Tip: Inflammatory bowel disease is an autoimmune disease that oscillates between phases of active disease and remission. In any of these situations there are epigenetic mechanisms involved, which are modified by lifestyle, with diet being one of the factors of epigenetic modulation.

Citation: Marangoni K, Dorneles G, da Silva DM, Pinto LP, Rossoni C, Fernandes SA. Diet as an epigenetic factor in inflammatory bowel disease. *World J Gastroenterol* 2023; 29(41): 5618-5629
URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5618.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5618

INTRODUCTION

Epigenetic modifications are heritable alterations in gene expression that do not involve changes in DNA sequence but affect how genes are turned on and off, modulating the risk and severity of many diseases, including inflammatory bowel disease (IBD)[1,2]; such modifications include DNA methylation, histone modifications, and non-coding RNA molecules [such as microRNAs (miRNAs)][3].

These epigenetic mechanisms can be activated and modified through the individual's genetic inheritance and/or environmental factors, including the individual's diet, which requires additional attention[4]. Alongside an exponential growth in IBD cases, we observe obesity and overweight individuals due to a Westernized diet, rich in sugars and fats but deficient in vitamins and minerals[5]. The nutritional status and disease lead to malnutrition, commonly observed in patients with IBD[6,7]. Given the role of diet as a modulator of epigenetic parameters in patients with IBD, this review aims to present some micronutrients and their importance in preventing and/or treating malnutrition in these individuals.

Epigenetics in the pathogenesis of IBD

Epigenetics is classically defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. However, this broad definition allows several mechanisms to be classified as epigenetic or 'above the genome'[4]. Some events are defined as fixed epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNA molecules (such as miRNAs), which translate to phenotypes and can be transmitted down the cell lineage or across generations. These types of epigenetic preservations represent common patterns of epigenetic inheritance.

Environmental factors, including diet, gut microbiota composition, and exercise, can elicit phenotype changes through gene expression without changing the genetic sequence[8]. DNA methylation and histone modifications are two central epigenetic mechanisms that impact gene transcription and cell fate. On the other hand, the impact on the gene transcription and translation by non-coding RNAs, including miRNAs, is also linked to epigenetic control associated with a widely complex system of non-coding RNA regulation[9]. MiRNAs are a class of small, non-coding RNA molecules that play a crucial role in epigenetics. These tiny molecules, typically consisting of 18 to 25 nucleotides, do not encode proteins themselves but act as potent post-transcriptional regulators[10]. These small molecules can influence gene expression by binding to specific messenger RNA (mRNA) molecules, leading to either degradation or translational repression of the target mRNA. Through this mechanism, miRNAs can fine-tune the expression of a wide array of genes, thereby having significant control over various cellular processes[11].

The role of miRNAs in regulating transcriptional processes is paramount to maintaining cellular homeostasis and responding to external stimuli. When miRNAs bind to their target mRNA sequences, they prevent the translation of those mRNA molecules into functional proteins, effectively downregulating the expression of the corresponding genes. This regulation is essential in diverse biological phenomena, including development, cell proliferation, differentiation, and immune responses[12]. By modulating gene expression, miRNAs influence various signaling pathways and cellular networks, acting as crucial epigenetic regulators. Dysregulation of miRNAs has been associated with numerous diseases, such as cancer, gastrointestinal tract diseases, neurodegenerative disorders, and cardiovascular conditions, highlighting their significance in understanding the molecular basis of complex human biology[13].

DNA methylation is a genetic modification that influences gene activity through cytosine methylation and transformation at the 5-carbon position. Single-base resolution maps of DNA methylation in human cell lines indicate that approximately 5% of all cytosines are methylated under normal physiological conditions[14]. Cytosine methylation predominantly occurs in the CpG dinucleotides sequence, where the guanidine base follows methylated cytosines. The primary function of DNA methylation is to actively silence genes and DNA regions, repressing the gene transcription in

the gene promoter region by inhibiting the binding of transcription factors or transcriptional events[15]. The regulation of DNA methylation occurs by the *de novo* DNA methyltransferases DNMT3a and DNMT3b. Across cell divisions, DNA methylation events are maintained by DNMT1. Active removal of methyl groups from cytosines is also hypothesized to occur by specific but yet to be identified factors[15].

The mechanism of DNA methylation in IBD includes specific patterns such as global DNA hypomethylation. Studies have shown that patients with Crohn's disease (CD) and ulcerative colitis (UC) exhibit global DNA hypomethylation in various cell types, including intestinal epithelial cells and immune cells[16]. This hypomethylation can lead to genomic instability and altered gene expression, contributing to disease progression. On the other hand, promoter hypermethylation occurs when there is increased DNA methylation in specific promoter regions, which can result in gene silencing. Several genes involved in inflammation regulation, immune response, and epithelial barrier function in bowel diseases were found to be hypermethylated[17,18]. For example, genes encoding anti-inflammatory cytokines, such as interleukin-10, have been observed to have increased promoter methylation in IBD patients, potentially impairing their ability to control inflammation. However, some genes, including the nuclear transcription factor-kappa B signaling pathway, present hypomethylation status in IBD, leading to their overexpression, contributing to increased bowel inflammation[19].

In IBD patients, alterations in DNA methylation patterns have been identified in genes related to intestinal permeability, mucin production, and tight junction proteins[19,20]. These changes can disrupt the integrity of the gut barrier, leading to increased immune activation and inflammation.

Regarding histone modifications, another frequent epigenetic mechanism, the chromatin structure, has the function to protect and maintain the DNA organization. Histones are essential chromatin proteins and facilitate DNA packaging into nucleosomes[21]. Histone proteins can be subdivided into five major classes in humans (H1, H2A, H2B, H3, and H4), all of which possess tails that can be post-translationally modified to alter the accessibility of the DNA. Histone modifications include acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination in the histone tail which can occur mainly by lysine modifications[22]. The effect of histone modifications on transcription is highly diverse and depends on the type of modification, either silencing or activating gene transcription. Histone proteins and the adjacent tail can be modified to orchestrate DNA transcription, but the extension of this modification results in transcriptional activation or inactivation, depending on the type of modification[23]. Methylation of H3 is associated with transcriptional activation, whereas lysine 36 methylation results in transcriptional repression. Histone lysine acetylation activates gene transcription by histone lysine acetyltransferases (HATs), whereas histone deacetylases (HDACs) remove acetyl groups from lysine residues[22].

Phosphorylation of histone proteins at specific serine or threonine residues can affect gene expression by influencing chromatin structure and protein-protein interactions. In bowel diseases, abnormal histone phosphorylation events have been observed, particularly in inflammation. For instance, phosphorylation of histone H3 at serine 10 (H3S10ph) has been associated with increased expression of pro-inflammatory genes in colonic epithelial cells during colitis[24]. Regarding the histone acetylation pathways, inflammatory disturbances are mediated by the dysregulation of HDACs and HATs, directly impacting the balance of histone deacetylation and acetylation status. Increased expression and activity of HDACs have been observed in IBD, leading to histone deacetylation and transcriptional repression of anti-inflammatory genes, contributing to the perpetuation of chronic inflammation. On the other hand, reduced expression or function of HATs in IBD decreases histone acetylation, suppressing genes involved in maintaining intestinal barrier integrity, immune homeostasis, and immunoregulation[25]. Thus, altered activities of HDACs and HATs induce aberrant cytokine production, imbalanced T cell responses, and impaired epithelial barrier function in IBD, highlighting their crucial roles in disease pathogenesis[26].

In IBD, increased histone acetylation in certain gene promoters has also been observed, leading to the upregulation of pro-inflammatory genes, contributing to chronic inflammation[27]. Furthermore, alterations in histone methylation marks, such as H3K4me3 and H3K27me3, have been associated with altered gene expression profiles in the intestinal epithelium of patients with IBD[28].

It is important to note that specific histone modifications in IBD can vary depending on the individual, the disease subtype, and the stage. The effects of these modifications may depend on the genetic context and environmental factors, influencing several aspects of the pathophysiology of the disease, including inflammation, epithelial barrier integrity, and immune response.

Nutritional aspects of IBD

The incidence of IBD appears to be increasing worldwide in both developed and developing countries[6]. Today, 0.1% of Brazilians live with this chronic condition, as verified through the temporal analysis performed by Quaresma *et al*[29], which also showed a decrease in the incidence of CD but an increase in UC. Although we recognize genetic predisposition as a determining factor in the pathogenesis of IBD, other factors, such as environmental ones, are increasingly recognized as contributing to the risk of developing IBD. Notably, the incidence of IBD follows global trends in terms of lifestyle, industrialization, and the Western diet[7].

Alongside the rise of new cases of IBD in adults and children, malnutrition is also a prevalent nutritional characteristic, which varies from 65%-75% in CD and 18%-62% in UC. Furthermore, we often see overweight and obesity associated with malnutrition in patients with IBD.

This panorama regarding the nutritional condition of patients with IBD reflects the Westernized dietary pattern and the numerous deficiencies in the absorptive process and intestinal selectivity. Among the risk factors related to diet, the intake of ultra-processed foods, additives, and emulsifiers[30-32] is mentioned, since these reduce bacterial diversity and increase intestinal permeability and inflammatory mechanisms[33]. Considering these dietary risk factors associated with a higher incidence of IBD, it is important to prioritize preventive interventions through existing dietary guides, which

warn to avoid ultra-processed foods and increase the intake of fresh and minimally processed foods. The latter promotes diet-microbiome interaction through food groups and short-chain fatty acid (SCFA)-producing bacteria, which benefit from ingesting these foods, thus contributing to the necessary dietary changes in preventing IBD[33].

The key element that links diet to the emergence or worsening of IBD is dysbiosis. The chronic consumption of increased amounts of animal protein, saturated fats, and refined carbohydrates, a Westernized dietary pattern, results in dysbiosis and changes in the microbiota, with an increase in pathogenic bacteria, such as *Bacterioides spp.* and *Ruminococcus torques*[34].

Dysbiosis disrupts the intestinal barrier, making the mucous layer thinner and more permeable to pathogens and antigens, resulting in persistent inflammation. On the other hand, a diet rich in vegetables and fiber reduces intestinal pH and prevents the growth of potentially pathogenic bacteria, ensuring membrane selectivity[32].

People living with IBD should be encouraged to make dietary changes, as these play a significant role in the etiology and management of the disease, promoting the induction and maintenance of clinical remission associated with IBD therapies, in addition to preventing malnutrition and/or obesity, thus avoiding severe nutritional deficiencies, which compromise the course of the disease[35-38].

The most common nutritional deficiencies in IBD patients are folate and fat-soluble vitamins. Patients who undergo extensive bowel resection have an increased risk of vitamin B12 malabsorption. Other key micronutrients that can be deficient and should be monitored are calcium, selenium, magnesium, zinc, and iron[39,40].

Nutri-epigenetic modulator in IBD

Nutri-epigenetics is a field that explores the interaction between nutrition, gene expression, and disease development [41]. In the context of IBD, which includes conditions like CD and UC, there is emerging evidence suggesting that certain dietary factors can influence the epigenetic modifications that contribute to the development and progression of the disease. These modifications are believed to result from a complex interplay between environmental, immunological, and genetic factors[42] (Figure 1).

While diet alone may not be the underlying cause of IBD, it plays a crucial role in developing and managing this complex disease, significantly influencing the symptoms, flare-ups, and overall disease management[43]. Several dietary factors have been investigated in the context of IBD and their potential impact on epigenetic modifications, biased gene expression, and impact on health outcomes. Here are some critical points regarding diet as an epigenetic modulator of IBD (Table 1).

Methyl donor micronutrients

Certain nutrients found in the diet can act as methyl donors or cofactors for enzymes involved in epigenetic processes. Methyl donors provide methyl groups (-CH3) for various biochemical reactions in the body, including DNA methylation, histone modifications, and RNA processing. These nutrients are involved in one-carbon metabolism and are crucial for maintaining proper epigenetic regulation, gene expression, and overall health[44,45]. Some essential methyl donor nutrients are discussed below.

Vitamin B9 (folate): Vitamin B9 (folate) is an essential water-soluble vitamin found in foods such as leafy green vegetables (spinach, cabbage, lettuce, and broccoli), legumes (bean, lentil, and pea), citrus fruits (orange and grapefruit), liver (chicken and beef), and fortified grains (wheat flour). Folate is an important component in metabolism and crucial in various biological processes, including DNA synthesis, repair, and methylation[46,47]. Regarding IBD, folate deficiency has been associated with alterations in DNA methylation patterns. Studies have shown that patients with IBD often exhibit lower levels of folate, and this deficiency can contribute to the dysregulation of DNA methylation in the intestinal epithelial cells[48]. Impaired methylation can lead to the aberrant expression of genes involved in inflammation, immune response, and intestinal barrier function, which are all critical aspects in the pathogenesis of IBD. By influencing DNA methylation, folate supplementation may restore usual gene expression patterns and improve the symptoms of IBD[49]. However, it is important to note that the relation between folate and IBD is complex, and the effects of folate supplementation may vary depending on individual factors. While folate deficiency can be detrimental, excessive folate intake may also have adverse effects, particularly in individuals with specific genetic variations.

Vitamin B12 (cobalamin): Vitamin B12 (cobalamin) is another water-soluble vitamin found primarily in animal-based foods like meat, fish (especially cold-water fish like salmon, trout, tuna, and sardines), shellfish, eggs, dairy products like milk, cheese, and yogurt, and fortified products like morning cereal and vegetable milk (soy, almond, or oat). Vitamin B12 contributes with folate to donate methyl groups for DNA methylation and other essential cellular processes[50].

Methionine: Methionine is an essential amino acid found in protein-rich foods such as meat, fish, eggs, seafood, nuts, soy-based food like tofu, legumes (beans, lentils, and chickpeas), dairy products, oats, and whole grains. It acts as a precursor for S-adenosylmethionine (SAM), the primary methyl donor for DNA and histone methylation[50]. SAM is a molecule formed from methionine and adenosine triphosphate. It is the primary methyl donor in numerous cellular processes, including DNA, RNA, protein, and histone methylation[51].

Betaine (trimethylglycine): Betaine (trimethylglycine) is a naturally occurring compound found in foods like beets, spinach, broccoli, shellfish, and whole grains (wheat and rye) that acts as a methyl donor in reactions that convert homocysteine to methionine, which can be used for DNA and protein methylation[52].

Choline: Choline is an important nutrient involved in various biological processes, including lipid metabolism and cell membrane structure. Choline can be converted to betaine, and both choline and betaine behave as methyl donors in the

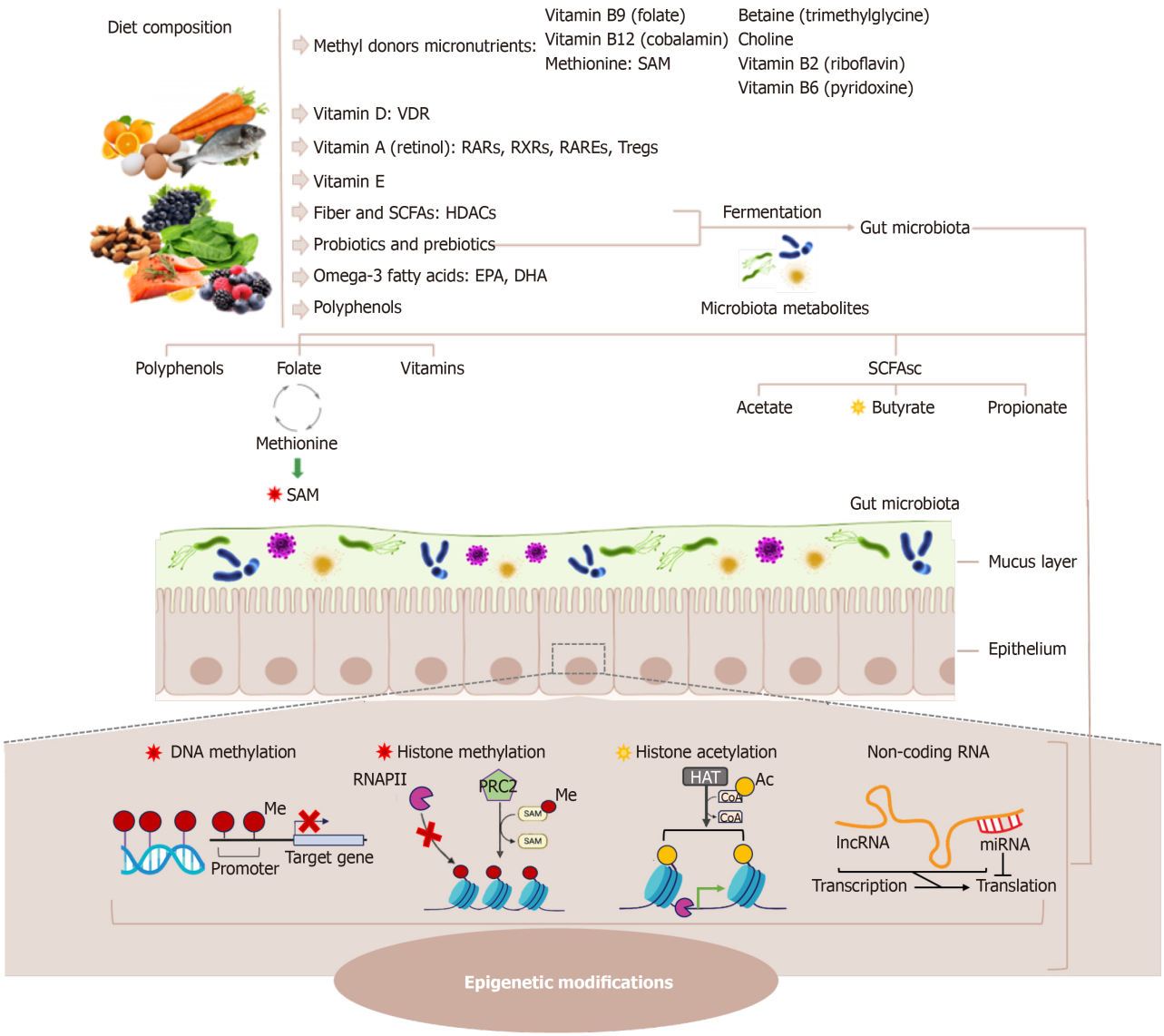
Table 1 Nutrients and their effect on epigenetic modifications in the context of inflammatory bowel disease

Nutrient(s)	Dietary sources	Biological activities	Epigenetic modifications	Ref.
Vitamin B (folate)	Leafy green vegetables, legumes, citrus fruits, and fortified grains	Methyl donor, DNA synthesis and repair	DNA methylation	[47-50]
Vitamin B12 (cobalamin)	Animal-based foods like meat, fish, eggs, and dairy products	Methyl donor	DNA methylation	[51]
Methionine	Protein-rich foods such as meat, fish, and dairy products	Methyl donor	DNA and histone methylation	[51, 52]
Betaine (trimethylglycine)	Beets, spinach, and whole grains	Methyl donor	DNA and protein methylation	[53]
Choline	Eggs, liver, peanuts, and cruciferous vegetables	Methyl donor, lipid metabolism, and cell membrane structure	DNA methylation	[53]
Vitamins B2 (riboflavin) and B6 (pyridoxine)	Whole grains, nuts, seeds, poultry, fish, and leafy green	FAD and FMN precursors	DNA methylation	[54]
Vitamin D	Fatty fish, eggs	VDRs ligand	DNA and histone methylation	[55-60]
Vitamin A	Leafy green vegetables, orange and yellow vegetables, tomato, fruits, and vegetable oils	RARs and RXRs ligand	DNA methylation, histone methylation and acetylation	[61-64]
Vitamin E	Plant-based oils, nuts, seeds, fruits, and vegetables	Antioxidant	DNA and histone methylation	[64-68]
SCFAs	High-fiber diets	Inhibition of HDACs activity	Histone acetylation	[69-73]
Lactobacillus rhamnosus GG	Fermented foods	Anti-inflammatory	DNA methylation	[78]
EPA and DHA	Salmon, mackerel, and sardines, flaxseeds, and walnuts	Anti-inflammatory	DNA methylation, histone methylation and acetylation, non-coding RNA	[81-83]
Polyphenols	Plant-based foods, such as fruits (berries), vegetables, nuts, seeds, green tea, cocoa, and olive oil	Anti-inflammatory and antioxidant	DNA methylation and non-coding RNA	[84, 85]

FAD: Flavin adenine dinucleotide; FMN: Flavin mononucleotide; VDRs: Vitamin D receptors; RARs: Retinoic acid receptors; RXRs: Retinoid X receptors; SCFAs: Short-chain fatty acids; HDACs: Histone deacetylases; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

body. Good sources of choline include eggs, liver, cold-water fish such as salmon and tuna, peanuts, dairy products, walnuts, almonds, sunflower seeds, and cruciferous vegetables[52].

Vitamins B2 (riboflavin) and B6 (pyridoxine): Vitamins B2 (riboflavin) and B6 (pyridoxine) are not primary donors like folate, vitamin B12, betaine, or choline, but they indirectly contribute to one-carbon metabolism and influence methylation reactions. B2 is an essential water-soluble vitamin, as it is a precursor for two important coenzymes, flavin adenine dinucleotide and flavin mononucleotide. These coenzymes are involved in redox reactions, energy metabolism, and various enzymatic reactions, including those related to the methylation process. Moreover, B2 plays an indirect role by synthesizing and recycling the methyl donor methionine. Conversion of homocysteine to methionine requires a methyl group from 5-methyltetrahydrofolate derived from folate metabolism. Additionally, vitamin B2 is necessary for converting folate to its active form, 5-methyltetrahydrofolate, thus indirectly supporting one-carbon metabolism and methylation reactions. Regarding B6, it represents a group of water-soluble compounds that include pyridoxine, pyridoxal, and pyridoxamine. These compounds are converted to their active form, pyridoxal 5′-phosphate (PLP), in the body. PLP is a coenzyme involved in various enzymatic reactions, including those related to amino acid metabolism. Similar to B2, vitamin B6 indirectly supports methylation reactions by converting methionine to SAM, the primary methyl donor for DNA and histone methylation. PLP is specifically required for the enzymatic reaction that converts methionine to SAM. Hence, a balanced diet that includes sources of B2 and B6, such as whole grains, nuts, seeds, avocado, potatoes, banana, dairy products, poultry, fish, and leafy green[45] vegetables, can help ensure sufficient levels of these nutrients [45]. Although methyl donors can alter DNA methylation patterns, little is known about the necessary doses and the exact period of dietary exposure or depletion that contributes to changes in epigenetic marks[45]. Therefore, more systematic studies are needed to provide more consistent findings.



DOI: 10.3748/wjg.v29.i41.5618

Figure 1 Schematic representation of the interplay of specific dietary constituents with the gut microbiota that interacts with the mammalian epigenome through the production of epigenetic substrates or regulators of chromatin-modifying enzymes. This process leads to epigenetic modifications that affect the immune response, compromising the epithelial barrier and defense mechanisms, resulting in chronic inflammation, as observed in inflammatory bowel disease. VDR: Vitamin D receptor; SCFAs: Short-chain fatty acids; HDACs: Histone deacetylases; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; RARs: Retinoic acid receptors; RXRs: Retinoid X receptors; RAREs: Retinoic acid response elements; Tregs: Regulatory T cells.

VITAMIN D

Vitamin D is a fat-soluble vitamin that is crucial in various physiological processes. Adequate levels of vitamin D, which can be obtained through sun exposure and dietary sources like fatty fish, cod liver oil, eggs, liver, and fortified foods (milk, orange juice, and morning cereals), have been linked to a lower risk of IBD[53,54]. Growing evidence suggests that vitamin D can affect DNA methylation patterns and histone modifications, leading to changes in gene expression profiles. Vitamin D acts as a ligand for the vitamin D receptor (VDR), which is present in various cell types, including immune cells. Upon binding to the VDR, vitamin D can influence the activity of enzymes involved in DNA methylation and histone modification, thereby altering gene expression[55,56].

Recent findings demonstrated that vitamin D deficiency is prevalent in individuals with IBD, and low vitamin D levels have been associated with increased disease activity and a higher risk of flare-ups[57]. Supplementing vitamin D in IBD patients has been shown to benefit disease activity and reduce inflammation. Furthermore, studies revealed that vitamin D supplementation can modulate epigenetic processes, altering IBD patients' DNA methylation patterns in immune cells and intestinal tissues. Thus, these changes in DNA methylation can affect the expression of genes involved in inflammation and immune regulation[58]. However, the exact mechanisms through which vitamin D influences epigenetic processes in IBD are still being elucidated.

VITAMIN A

Vitamin A, also known as retinol, is a crucial nutrient that plays a significant role in various biological processes, including immune function and inflammation. This fat-soluble vitamin is found in the form of beta-carotene in food sources like liver, carrots, sweet potato, pumpkin, spinach, kale, mango, melon, egg yolks, fatty fish, dairy products, and other orange vegetables (peppers and yellow zucchini). Their effects on gene expression through both genomic and non-genomic pathways were already demonstrated previously[59]. Retinoic acid, the active form of vitamin A, acts as a ligand for nuclear receptors known as retinoic acid receptors and retinoid X receptors. When retinoic acid binds to these receptors, it can regulate gene expression by interacting with specific DNA sequences called retinoic acid response elements in the promoter regions of targeted genes[60].

Recent findings demonstrated that retinoic acid affects DNA methylation patterns, leading to changes in gene expression profiles associated with immune regulation and inflammation. Additionally, retinoic acid can influence histone modifications, such as acetylation and methylation, which can further impact gene expression and cellular processes involved in IBD[61]. Furthermore, vitamin A has been shown to promote the development and function of regulatory T cells (Tregs) in the gut. Tregs are crucial in maintaining immune tolerance and preventing excessive inflammatory responses. Vitamin A helps to induce the expression of the transcription factor Foxp3, which is essential for Treg development and function. Epigenetic modifications, including DNA methylation and histone modifications, have been implicated in regulating Foxp3 expression and Treg function[62]. The therapeutic benefits of vitamin A as an epigenetic modulator, concerning its continuous use as a nutritional supplement, depend on our further understanding of its epigenetic effects on health and disease through different generations.

VITAMIN E

Vitamin E is a fat-soluble vitamin with antioxidant properties that protects cells from oxidative damage. While vitamin E is primarily known for its antioxidant effects, it also has some influence on epigenetic processes[62]. There were limited direct studies on the influence of vitamin E on epigenetic processes in IBD. However, research in other contexts suggests that vitamin E could impact epigenetic mechanisms.

Some studies in different disease models and cellular systems have shown that vitamin E can affect DNA methylation and histone modifications, both of which are crucial epigenetic mechanisms. These changes in epigenetic marks could lead to altered gene expression patterns and potentially influence disease processes[63-66].

While these studies provide preliminary evidence of the potential influence of vitamin E on epigenetic processes in IBD, additional research is needed to fully understand the mechanisms involved and establish a clear cause-and-effect association. Further studies, including clinical trials, are necessary to determine the optimal dosage and period of vitamin E supplementation and its effects on epigenetic modifications in individuals with IBD. It is important to emphasize that vitamin E supplementation should be approached with caution, especially at high doses, as excessive intake may have adverse effects. On the other hand, the consumption of foods rich in vitamin E can be encouraged, such as sunflower seeds, almonds, nuts, vegetable oils (wheat oil, sunflower oil, and wheat germ oil), avocado, hazelnuts, salmon, kiwi, man-go, and tomatoes.

Fiber and SCFAs

High-fiber diets have been associated with several beneficial effects in IBD, such as reduced inflammation, improved gut barrier function, and modulation of the gut microbiota[67]. Soluble fiber can dissolve in water, forming thick gels, and is subject to fermentation by colonic bacteria. Examples of highly fermentable soluble fibers with significant solubility and viscosity are beta-glucans and pectin, naturally present in whole grains like oats and barley and fruits such as apples. Non-viscous soluble fibers that undergo fermentation by the gut microbiota include inulin, gum acacia, resistant starch, polydextrose, and corn fiber. Fructans of the inulin-type are naturally found in agave (a succulent plant), artichokes, asparagus, bananas, chicory root, garlic, onions, and leeks. Resistant starches (found in legumes, seeds, raw and cooked potatoes, green bananas, and whole grains) and polydextrose are not absorbed in the small intestine due to their specific physical and chemical characteristics, rendering them inaccessible to α-amylase[35].

One of the mechanisms by which high-fiber diets can influence epigenetic processes is the production of SCFAs. SCFAs are generated by the fermentation of dietary fiber by gut bacteria, such as butyrate, propionate, and acetate. They have been shown to have anti-inflammatory effects and can act as signaling molecules that modulate gene expression through epigenetic modifications[28].

Butyrate, a type of SCFA, has been shown to inhibit the activity of HDACs, enzymes involved in epigenetic regulation. HDACs remove acetyl groups from histone proteins, leading to a more condensed chromatin structure and reduced gene expression. By inhibiting HDACs, butyrate and other SCFAs can promote a more relaxed chromatin structure, allowing increased gene expression of anti-inflammatory genes[62,28,68]. Furthermore, high-fiber diets can also influence the gut microbiota composition. Certain beneficial bacteria in the gut, such as *Faecalibacterium prausnitzii*, have been associated with the production of anti-inflammatory metabolites and a healthy gut environment[69,70].

Probiotics and prebiotics

Probiotics are live microorganisms that can be consumed through fermented foods or supplements and, when administered in adequate amounts, provide health benefits to the host. Some foods are naturally rich in probiotics or are fermented to contain these microorganisms like yogurt, kefir, kimchi, sauerkraut, pickles, aged cheese, and some

fermented soy products such as miso and tempeh. Probiotics have been extensively studied for their potential therapeutic effects in various health conditions, including IBD. While probiotics have shown promise in improving symptoms and reducing inflammation in IBD, their specific influence on epigenetic processes in this context is an area of ongoing research[71].

Some studies have explored the effects of probiotics on epigenetic modifications in the context of IBD. They found that the probiotic treatment led to changes in DNA methylation patterns at specific gene loci, suggesting a potential epigenetic mechanism underlying the beneficial effects of probiotics in IBD[72-74].

Other studies evaluated the effects of a specific probiotic strain, *Lactobacillus rhamnosus* GG, on DNA methylation patterns in a mouse model of colitis. They observed that the probiotic treatment altered DNA methylation in the colon tissue, potentially contributing to its anti-inflammatory effects[75].

Although these studies provide insights into the potential epigenetic effects of probiotics in IBD, it is important to note that the exact mechanisms by which probiotics influence epigenetic processes are still not fully understood. Further research is needed to elucidate the specific molecular pathways involved to determine the clinical implications of these findings.

In addition to probiotics, there also are prebiotics, which are non-digestible fibers that promote the growth of beneficial gut bacteria. Both have shown promise in modulating epigenetic processes and reducing inflammation associated with IBD[76]. A recent review showed that dietary prebiotics, as well as their microbial metabolites, directly target enzymatic activities and/or modulate the expression of enzymes involved in epigenetic gene regulation, with potential implications for health status and susceptibility to diseases[77].

Although the exact mechanisms by which prebiotics influence epigenetic processes in IBD are not yet fully understood, these studies provide evidence of their potential to modulate gene expression through epigenetic modifications. Further research is needed to elucidate the specific mechanisms and identify the most effective prebiotic interventions for individuals with IBD.

Omega-3 fatty acids

Omega-3 fatty acids, particularly eicosapentaenoic acid and docosahexaenoic acid, are polyunsaturated fatty acids commonly found in fatty fish (such as salmon, mackerel, and sardines), flaxseeds, and walnuts. They have anti-inflammatory properties and have been studied for their potential therapeutic effects on IBD. Several studies have indicated that omega-3 fatty acids can modulate epigenetic processes associated with inflammation in IBD by reducing DNA methylation, potentially upregulating the expression of anti-inflammatory genes and downregulating the expression of pro-inflammatory genes, leading to beneficial effects on IBD[78,79].

In addition to DNA methylation, omega-3 fatty acids can also affect other epigenetic mechanisms, such as histone modi-fications and expression of miRNAs, which are small RNA molecules that can post-transcriptionally regulate gene expression, although the specific mechanisms are still being investigated[80].

Overall, omega-3 fatty acids have the potential to modulate epigenetic processes in IBD, leading to reduced inflammation and improved outcomes. However, further research is needed to fully understand the precise mechanisms and determine the optimal dosages and treatment strategies for using omega-3 fatty acids in managing IBD.

Polyphenols

Polyphenols are a group of bioactive compounds found in various plant-based foods, such as fruits (berries, apple, and grape), green leafy vegetables (spinach, kale, and broccoli), onion, garlic, nuts, hazelnuts, almonds, seeds, green tea, black tea, coffee, cocoa, whole grains (oats, barley, and buckwheat), and olive oil. These compounds have been studied for their potential health benefits, including their ability to modulate epigenetic processes and potentially exert beneficial effects in IBD.

These compounds have been shown to have anti-inflammatory and antioxidant properties, and they can modulate various signaling pathways involved in immune response and inflammation. One of the well-studied epigenetic effects of polyphenols is their ability to influence DNA methylation. Polyphenols can affect DNA methyltransferase enzymes, which are responsible for adding or removing methyl groups from DNA molecules. By modulating these enzymes, polyphenols can potentially alter DNA methylation patterns, leading to changes in gene expression[81,82].

Moreover, polyphenols can also affect histone modifications, which regulate chromatin structure and gene expression. They can influence enzymes responsible for adding or removing acetyl, methyl, or other chemical groups to histone proteins, thereby modulating the accessibility of DNA and influencing gene expression patterns. Additionally, polyphenols have been shown to regulate the expression of miRNAs[83-85].

While promising evidence suggests the epigenetic influence of polyphenols in IBD, it is important to note that research in this area is still evolving, and more studies are needed to fully understand the specific mechanisms and effects of different polyphenols in IBD. It is important to note that the field of nutri-epigenetics in IBD is still in its early stages. Understanding the interplay between dietary factors and epigenetic mechanisms in IBD is an active area of research. While evidence suggests their involvement, more studies are needed to elucidate the specific mechanisms and determine the potential therapeutic implications of targeting these factors in managing IBD. It is essential for individuals with IBD to work closely with healthcare professionals, such as gastroenterologists and registered dietitians, to ensure optimal nutrient intake and personalized management strategies.

CONCLUSION

In conclusion, evidence has shown that dietary composition should be the primary basis for managing patients with IBD due to its role in intestinal health. Moreover, the impact of diet on the epigenetic process of microbiota modulation is clear. However, further clinical trials involving diets, supplements, and epigenetic and inflammatory markers are still needed.

FOOTNOTES

Author contributions: All authors contributed to the literature review and writing of the article; Fernandes SA conceived the research project and critically reviewed the manuscript; and all authors read and approved the final manuscript.

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

Country/Territory of origin: Brazil

ORCID number: Karina Marangoni 0000-0002-9761-3668; Gilson Dorneles 0000-0001-6524-3204; Daniella Miranda da Silva 0000-0001-9489-7047; Letícia Pereira Pinto 0000-0001-8543-1587; Carina Rossoni 0000-0002-6494-4639; Sabrina Alves Fernandes 0000-0001-8504-603X.

S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Wang JJ

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5630-5640

DOI: 10.3748/wjg.v29.i41.5630

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Application of extended criteria donor grafts in liver transplantation for acute-on-chronic liver failure: A retrospective cohort study

Jin-Long Gong, Jia Yu, Tie-Long Wang, Xiao-Shun He, Yun-Hua Tang, Xiao-Feng Zhu

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gupta T, India; Vij M, India

Received: August 23, 2023
Peer-review started: August 23, 2023
First decision: September 11, 2023
Revised: September 24, 2023
Accepted: October 23, 2023
Article in press: October 23, 2023
Published online: November 7, 2023

Jin-Long Gong, Department of Hepatobiliary Surgery, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, Changsha 410005, Hunan Province, China

Jin-Long Gong, Jia Yu, Tie-Long Wang, Xiao-Shun He, Yun-Hua Tang, Xiao-Feng Zhu, Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

Jia Yu, Department of Gastroenterology Surgery, The First Affiliated Hospital of University of South China, Hengyang 421005, Hunan Province, China

Corresponding author: Xiao-Feng Zhu, MD, PhD, Professor, Surgeon, Organ Transplant Center, The First Affiliated Hospital, Sun Yat-Sen University, No. 58 Zhongshan Road, Guangzhou 510080, Guangdong Province, China. zhuxiaof@mail.sysu.edu.cn

Abstract

BACKGROUND

There is no consensus on the usage of extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients.

AIM

To summarize the experience of using ECD livers in ACLF-LT.

METHODS

A retrospective cohort study was conducted, enrolling patients who underwent LT at the First Affiliated Hospital of Sun Yat-Sen University from January 2015 to November 2021. The patients were divided into ECD and non-ECD groups for analysis.

RESULTS

A total of 145 recipients were enrolled in this study, of which ECD and non-ECD recipients accounted for 53.8% and 46.2%, respectively. Donation after cardiac death (DCD) recipients accounted for the minority compared with donation after brain death (DBD) recipients (16.6% *vs* 83.4%). Neither overall survival nor graft survival significantly differed between ECD and non-ECD and DCD and DBD recipients. ECD grafts were associated with a significantly higher incidence of early allograft dysfunction (EAD) than non-ECD grafts (67.9% *vs* 41.8%, P = 0.002). Postoperative outcomes between DCD and DBD recipients were compa-

rable (P > 0.05). ECD graft (P = 0.009), anhepatic phase (P = 0.034) and recipient gamma glutamyltransferase (P = 0.016) were independent risk factors for EAD. Recipient preoperative number of extrahepatic organ failures > 2 (P = 0.015) and intraoperative blood loss (P = 0.000) were independent predictors of poor post-LT survival.

CONCLUSION
Although related to a higher risk of EAD, ECD grafts can be safely used in ACLF-LT. The main factors affecting post-LT survival in ACLF patients are their own severe preoperative disease and intraoperative blood loss.

Key Words: Extended criteria donor; Acute-on-chronic liver failure; Liver transplantation

Core Tip: This manuscript is intended to summarize a Chinese single center experience of using extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients. In this paper, we found that under ECD grafts are associated with a higher risk of early allograft dysfunction than non-ECD grafts but can be safely used in ACLF recipients as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.

Citation: Gong JL, Yu J, Wang TL, He XS, Tang YH, Zhu XF. Application of extended criteria donor grafts in liver transplantation for acute-on-chronic liver failure: A retrospective cohort study. *World J Gastroenterol* 2023; 29(41): 5630-5640
URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5630.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5630

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a complex clinical syndrome characterized by the failure of extrahepatic organ(s) that has an extremely high short-term mortality and a 90-d transplant-free mortality above 50%[1]. Liver transplantation (LT) is the only curative treatment option for various end-stage liver diseases and has been reported to bring strong survival benefits to ACLF patients[2]. However, the current supply of acceptable donor livers is far from sufficient to meet the demands of the growing number of recipients. In an effort to reduce waiting list mortality, extended criteria donor (ECD) livers, also known as marginal livers, are increasingly being used in LT[3,4].

Usually, ECD livers are mainly defined as livers from donors with advanced age, macrovesicular steatosis, donation after cardiac death (DCD), and other unfavorable characteristics that indicate suboptimal quality[5,6]. The use of livers with ECD in ACLF-LT remains controversial. On the one hand, ECD livers were historically considered to be related to poor graft function and even poor survival; on the other hand, although transplanted with ECD grafts, recipients with high model for end-stage liver disease (MELD) scores or severe ACLF also obtained strong survival benefits, with 1-year post-LT survival rates reaching 78.1%[7,8]. The impact of the increased use of ECD grafts in ACLF patients needs to be further researched[9].

To our knowledge, there are still no studies published based on experiences at a Chinese hospital. In this study, we aimed to investigate the perioperative and long-term outcomes of ACLF patients in terms of whether they were ECD or non-ECD recipients.

MATERIALS AND METHODS

Patients

A retrospective cohort study was conducted. We recruited patients who underwent LT at the First Affiliated Hospital of Sun Yat-Sen University from January 2015 to November 2021 for our study. The inclusion criteria were as follows: Underwent LT; met the ACLF diagnostic criteria; and age ≥ 18 years. The exclusion criteria were complicated with hepatocellular carcinoma or other hepatobiliary cancer, combined transplantation with other organ (s), cases of ischemia-free LT (IFLT)[10] in a prospective randomized controlled study (registration number: ChiCTR1900021158) conducted during the same period, living donor LT, and incomplete medical records. Of note, there is still no unified definition of ACLF. Considering the unique epidemiological background in our country in which ACLF is mainly caused by hepatitis B virus infection, we adopted the diagnostic criteria recently proposed by the Chinese Group on the Study of Severe Hepatitis B[11] in this study; that is, regardless of the presence of cirrhosis, patients with chronic hepatitis B, total bilirubin (TB) ≥ 12 mg/dL and international normalized ratio ≥ 1.5 should be diagnosed with ACLF.

Donor and recipient clinical characteristics

The clinical parameters of both donors and recipients were extracted from electronic medical records. The baseline

characteristics of recipients were based on the last examination before LT. The severity of ACLF was measured by the MELD score and the number of extrahepatic organ failures (OFs) at the time before LT. Extrahepatic OF was defined by previous reports and included kidney[12], coagulation[13], circulatory system[14], respiratory system[15], and hepatic encephalopathy[16]. The baseline characteristics of donors were based on the last examination before organ procurement. There is still no precise definition of an ECD liver; with reference to previous reports[5,6] and the experience of our center, ECD was defined in this study as meeting any of the following criteria: Age > 65 years, body mass index (BMI) > 30 kg/m^2, macrovesicular steatosis ≥ 30%, serum sodium > 165 mmol/L, serum alanine aminotransferase (ALT)/aspartate aminotransferase (AST) > 120 U/L, serum TB > 51 μmol/L, cold-ischemia time (CIT) > 12 ho, split, DCD.

Outcomes

The primary endpoint events of interest were graft survival (from LT to re-LT or death) and overall survival (OS), from LT to death. The patients were followed up until December 2021. The secondary outcomes mainly included rates of early allograft dysfunction (EAD)[17], acute kidney injury (AKI)[17] and other perioperative characteristics [intraoperative blood loss/transfusion, operative time, and intensive care unit (ICU) stay].

Statistical analysis

The patients were divided into ECD and non-ECD recipient groups for analysis. As the main factor of concern in ECD, DCD recipients were also analyzed as a subgroup for comparison with donation after brain death (DBD) recipients. Statistical analyses were conducted using SPSS (version 23.0, IBM). Continuous measurement data are expressed as the mean ± SD, and differences between groups were detected by Student's t test. Enumeration data are expressed as numbers (percentages), and differences were detected by the χ^2 test. OS and graft survival were calculated by the Kaplan-Meier method and compared through the log-rank test. Univariate and multivariate logistic/Cox regression analyses were performed to identify the risk factors and independent risk factors for EAD/OS, and variables showing univariate significance or considered clinically relevant were entered into multivariate analysis[18]. Statistical significance was defined as $P < 0.05$.

RESULTS

Baseline characteristics of recipients

Ultimately, 145 patients were enrolled in our study, of whom 78 (53.8%) were in the ECD group and 67 (46.2%) were in the non-ECD group (Table 1). The severity of ACLF was quantified by the MELD score and OFs, and both quantitative tools showed no significant difference in the severity of the preoperative disease between these two groups ($P = 0.579$ and 0.547 and 0.591 and 0.547, respectively). Other demographic indicators, such as age, sex, blood type, and preoperative biochemical tests, also proved to have nonsignificant differences ($P > 0.05$). In addition, the numbers of IFLT cases were approximately similar in both groups ($P = 0.170$).

This finding indicates that there is no significant bias between these two groups in the allocation and usage of ECD grafts, and the probability of being assigned an ECD liver is approximately equal for patients with severe or mild ACLF.

Baseline characteristics of donor livers

Compared with non-ECD, ECD accounted for more than half of the total grafts [Table 2, 67 (46.2%) *vs* 78 (53.8%)]. The specific types of ECD grafts are shown in Table 2. Liver grafts were defined as ECD mainly because ALT/AST was greater than 120 U/L (29.5%/46.2%), followed by DCD (30.8%) and high serum sodium (17.9%). A total of 11.5% of grafts were classified as ECD due to BMI > 30 kg/m^2, and 9% were classified due to TB > 51 μmol/L. Notably, macrovesicular steatosis (2.6%), advanced age (1.3%), prolonged CIT (6.4%), and split (1.3%) only accounted for a very small proportion of ECD livers.

Primary outcomes

During the follow-up period, only one patient underwent retransplantation. Neither OS nor graft survival significantly differed between patients in the ECD and non-ECD groups and in the DCD and DBD groups (Figure 1). In ECD *vs* non-ECD recipients, the 1-, 3-, and 5-year OS rates were 87.0%, 83.7%, and 81.4% *vs* 86.0%, 83.8% and 83.8% ($P = 0.901$), respectively, and the 1-, 3-, and 5-year graft survival rates were 87.0%, 83.8% and 81.5% *vs* 86.0%, 83.8% and 83.8% ($P = 0.902$). In DCD and DBD recipients, the 1-, 3-, and 5-year OS rates were equal to the graft survival rates (83.3%, 83.3%, and 75.8% *vs* 87.1%, 83.5% and 83.5%, respectively) ($P = 0.631$ and 0.633, respectively).

Secondary outcomes

ECD recipients demonstrated a significantly higher postoperative EAD incidence than non-ECD recipients (67.9% *vs* 41.8%, $P = 0.002$). Except for EAD, there were no significant differences ($P > 0.05$) in the other secondary endpoints between these two groups (Table 3).

Then, we divided the patients into DCD and DBD groups, and all secondary endpoints, including EAD, AKI, blood loss and other perioperative indicators, showed no significant differences (Table 4, $P > 0.05$).

Identification of independent risk factors for EAD and OS

We enrolled all potential clinical parameters in univariable and multivariable logistic regression analyses to identify risk

Table 1 Baseline characteristics of recipients, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
MELD	30.73 ± 5.96	30.19 ± 5.60	0.579
MELD			0.547
> 30	40 (51.3)	31 (46.3)	
≤ 30	38 (48.7)	36 (53.7)	
OFs			0.591
0	25 (32.1)	28 (41.8)	
1	33 (42.3)	20 (29.9)	
2	11 (14.1)	9 (13.4)	
3	7 (9)	8 (11.9)	
4	2 (2.6)	2 (3)	
OFs			0.547
> 2	9 (11.5)	10 (14.9)	
≤ 2	69 (88.5)	57 (85.1)	
Age (yr)			0.365
> 60	4 (5.1)	6 (9)	
≤ 60	74 (94.9)	61 (91)	
Sex			0.730
Male	69 (88.5)	58 (86.6)	
Female	9 (11.5)	9 (13.4)	
BMI (kg/m^2)			0.611
> 30	5 (6.4)	3 (4.5)	
≤ 30	73 (93.6)	64 (95.5)	
Blood group			0.411
O	29 (37.2)	28 (41.8)	
A	24 (30.8)	20 (29.9)	
B	22 (28.2)	13 (19.4)	
AB	3 (3.8)	6 (9)	
WBC (× 10^9/L)	6.84 ± 3.56	6.35 ± 3.52	0.411
N/L	6.27 ± 6.34	7.00 ± 6.12	0.482
Hb (g/L)	94.27 ± 22.29	89.45 ± 19.06	0.167
GGT (U/L)	49.51 ± 26.39	50.12 ± 32.49	0.901
ALB (g/L)	36.87 ± 4.94	38.37 ± 4.99	0.071
ALT (U/L)	85.33 ± 96.36	60.88 ± 75.02	0.088
AST (U/L)	114.47 ± 103.80	92.42 ± 82.42	0.163
PLT (× 10^9/L)	63.50 ± 33.83	62.49 ± 38.57	0.867
Fib (g/L)	1.02 ± 0.47	1.12 ± 0.43	0.176
IFLT	2 (2.6)	5 (7.5)	0.170

MELD: Model for end-stage liver disease; ECD: Extended criteria donor; OFs: Organ failures; BMI: Body mass index; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen; IFLT: Ischemia-free liver transplantation.

Table 2 Baseline characteristics of donor livers, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
DCD/DBD	24 (30.8)/54 (69.2)	0/ 67 (100)	0.000
Split	1 (1.3)	0	0.352
Macrovesicular steatosis			0.187
≥ 30	2 (2.6)	0	
< 30	76 (97.4)	67 (100)	
Age (yr)			0.352
> 65	1 (1.3)	0	
≤ 65	77 (98.7)	67 (100)	
BMI (kg/m^2)			0.004
> 30	9 (11.5)	0	
≤ 30	69 (88.5)	67 (100)	
Na (mmol/L)			0.000
> 165	14 (17.9)	0	
≤ 165	64 (82.1)	67 (100)	
ALT (U/L)			0.000
> 120	23 (29.5)	0	
≤ 120	55 (70.5)	67 (100)	
AST (U/L)			0.000
> 120	36 (46.2)	0	
≤ 120	42 (53.8)	67 (100)	
TB (μmol/L)			0.012
> 51	7 (9.0)	0	
≤ 51	71 (91.0)	67 (100)	
CIT (h)			0.035
> 12	5 (6.4)	0	
≤ 12	73 (93.6)	67 (100)	
Reason of death			0.969
Trauma	41 (52.6)	35 (52.2)	
Other	37 (47.4)	32 (47.8)	

DCD: Donation after cardiac death; DBD: Donation after brain death; ECD: Extended criteria donor; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; CIT: Cold-ischemia time.

factors and independent risk factors for EAD. The results showed that ECD graft (P = 0.002), anhepatic phase (P = 0.003), operation time (P = 0.005) and recipient gamma glutamyltransferase (GGT), (P = 0.027) were risk factors for EAD. Then, ECD graft (P = 0.009), anhepatic phase (P = 0.034) and recipient GGT (P = 0.016) were shown to be independently associated with EAD (Table 5).

All potential factors that may be related to OS were included in the Cox regression analysis. Univariable analysis showed that blood loss (P = 0.000), EAD (P = 0.048), and OFs > 2 (P = 0.011) were risk factors for OS; then, multivariable analysis further demonstrated that blood loss (P = 0.000) and OFs > 2 (P = 0.015) were independent risk factors for OS (Table 6). Patients complicated with preoperative OFs > 2 (P = 0.007) or intraoperative blood loss > 2 L (median = 2 L, P = 0.038) were associated with significantly poorer post-LT survival (Figure 2).

Table 3 Secondary outcomes of recipients between extended criteria donor and non-extended criteria donor grafts, *n* (%)

	ECD (*n* = 78)	Non-ECD (*n* = 67)	*P* value
EAD	53 (67.9)	28 (41.8)	0.002
AKI	34 (43.6)	30 (44.8)	0.886
Blood loss (mL)	2.55 ± 1.70	2.82 ± 2.93	0.489
RBC transfused (U)	7.89 ± 3.93	9.13 ± 6.18	1.148
Plasma transfused (U)	9.77 ± 4.28	10.23 ± 4.51	0.534
Operative time (h)	7.48 ± 1.23	7.35 ± 1.37	0.554
ICU stay (d)	4.68 ± 4.95	4.63 ± 6.27	0.958

EAD: Early allograft dysfunction; ECD: Extended criteria donor; AKI: Acute kidney injury; ICU: Intensive care unit; RBC: Red blood cell.

Table 4 Secondary outcomes of recipients between donation after cardiac death and donation after brain death grafts, *n* (%)

	DCD (*n* = 24)	DBD (*n* = 121)	*P* value
EAD	17 (70.8)	64 (52.9)	0.106
AKI	11 (45.8)	53 (43.8)	0.855
Blood loss (mL)	2.53 ± 1.81	2.70 ± 2.44	0.747
RBC transfused (U)	8.61 ± 3.77	8.43 ± 5.35	0.876
Plasma transfused (U)	9.17 ± 5.03	10.14 ± 4.24	0.319
Operative time (h)	7.67 ± 1.46	7.37 ± 1.26	0.298
ICU stay (d)	5.25 ± 5.00	4.54 ± 5.70	0.571

DCD: Donation after cardiac death; DBD: Donation after brain death; ECD: Extended criteria donor; AKI: Acute kidney injury; ICU: Intensive care unit; RBC: Red blood cell.

DISCUSSION

The impact of the increased use of ECD grafts in ACLF patients has not yet been evaluated well[9]. To our knowledge, this is the first report from China that summarizes the experiences of using ECD grafts in ACLF-LT patients.

In our study, both OS and graft survival between ECD and non-ECD recipients and DCD and DBD recipients were not significantly different. This was approximately consistent with the conclusions of recent studies based on adult recipients (regardless of primary disease)[19] and high-acuity patients (MELD ≥ 35)[20]. For severe ACLF recipients[8], a marginal donor liver (donor risk index[21] above 1.7) was considered an independent risk factor for 1-year post-LT survival. However, as the authors pointed out in this article, although transplanted with marginal livers, it was clear that patients still obtain strong survival benefits, with 1-year post-LT survival rates reaching 78.1%. In our study, ECD recipients had a 5-year post-LT survival rate above 80%. Compared with the poor prognosis of 90-d transplant-free mortality above 50% [1], ECD grafts undoubtedly provide an important life-saving option for ACLF patients. Moreover, refusing ECD and continuing to wait for an ideal graft means a prolonged waiting time, which also means an increased risk of worse preoperative disease and higher post-LT mortality[22]. Consequently, it may be better for ACLF patients to accept an existing ECD graft rather than waiting for a prospective ideal liver.

In our opinion, the reason why there was no significant difference in survival between ECD and non-ECD patients was mainly due to the inevitable selection bias in clinical practice. As shown in Table 2, advanced age, prolonged CIT and macrovesicular steatosis, which have been widely recognized as the strongest prognostic risk factors[23], only accounted for 1.3%, 6.4% and 2.6% of our ECD grafts, respectively. This indicates that the ECD grafts actually adopted in our clinical practice may be relatively safe, and those grafts empirically judged as "high risk" were abandoned. Nevertheless, ECD recipients still showed a significantly higher incidence of EAD than non-ECD recipients.

ECD grafts, anhepatic phase and recipient GGT were proven to be significantly associated with EAD in our further research. The importance of shortening the anhepatic phase in transplantation is self-evident and has been proven by previous studies[24,25]. Our research emphasized the importance of surgical techniques once again. Traditionally, high serum GGT has been considered a biomarker of hepatobiliary diseases. Recent studies have shown its predictive role in carcinogenesis, tumor progression and many other life-threatening diseases[26]. The potential of donor GGT in predicting EAD[27] and graft survival[28] has also been reported, but few studies have focused on its role in recipients. Our study found that a high preoperative recipient serum GGT level was significantly correlated with post-LT EAD but did not

Table 5 Univariable and multivariable logistic analysis of risk factors for early allograft dysfunction

Variables	OR	Univariate analysis		OR	Multivariate analysis	
		HR (95%CI)	*P* value		HR (95%CI)	*P* value
Donor characteristics						
HBV (positive *vs* negative)	1.076	0.522-2.220	0.843			
Death of trauma (yes *vs* no)	1.331	0.689-2.568	0.395			
ECD (yes *vs* no)	2.953	1.497-5.826	0.002	2.712	1.286-5.720	0.009
Operation characteristics						
Blood loss (L)	1.080	0.925-1.262	0.330			
Anhepatic phase (min)	1.036	1.012-1.060	0.003	1.031	1.002-1.060	0.034
Operation time (h)	1.487	1.124-1.967	0.005	1.271	0.915-1.767	0.153
IFLT (yes *vs* no)	0.121	0.014-1.031	0.053	0.114	0.011-1.218	0.072
Recipient characteristics						
BMI (> 30 *vs* ≤ 30)	0.779	0.187-3.244	0.732			
MELD (> 30 *vs* ≤30)	1.455	0.753-2.812	0.265			
OFs (> 2 *vs* ≤ 2)	0.861	0.327-2.264	0.761			
WBC ($\times 10^9$/L)	1.039	0.945-1.142	0.432			
N/L	0.997	0.946-1.051	0.906			
Hb (g/L)	1.006	0.990-1.022	0.444			
GGT (U/L)	1.015	1.002-1.029	0.027	1.017	1.003-1.032	0.016
ALB (g/L)	1.052	0.984-1.126	0.139			
ALT (U/L)	1.002	0.998-1.006	0.288			
AST (U/L)	1.001	0.997-1.004	0.650			
PLT ($\times 10^9$/L)	1.001	0.992-1.010	0.800			
Fib (g/L)	0.692	0.332-1.441	0.325			

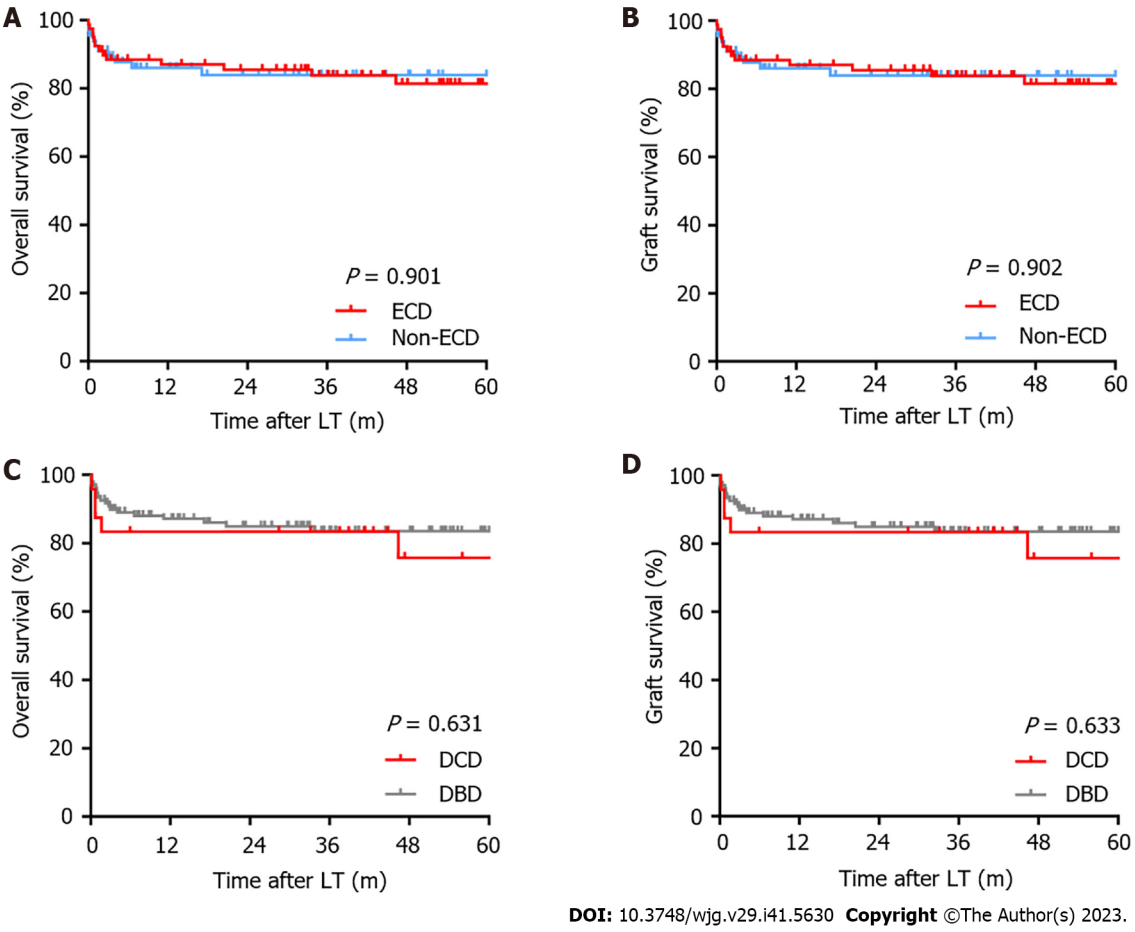
OR: Odds ratio; CI: Confidence interval; HBV: Hepatitis B virus; ECD: Extended criteria donor; BMI: Body mass index; IFLT: Ischemia-free liver transplantation; MELD: Model for end-stage liver disease; OFs: Organ failures; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen.

affect survival. This may be due to its critical role in the modulation of redox equilibria[29], and high serum GGT may reflect worse preoperative disease in recipients. At the same time, it should be noted that the determinants of prognosis in LT are numerous and complex.

Our analysis showed that only preoperative recipient OFs and intraoperative blood loss were independently associated with OS. This finding indicates that under our current ECD experience, the post-LT survival of ACLF patients mainly depends on the severity of their own preoperative disease and intraoperative conditions. The MELD score is widely accepted as a tool to quantify the severity of end-stage liver disease and to allocate donor livers. However, in recent years, a growing number of studies have found that MELD or MELD-Na underestimates the severity of ACLF, mainly because it fails to capture the two key pathophysiological features of ACLF: Extrahepatic OF and systemic inflammation[30,31]. Our study also showed that OFs may reflect the severity of ACLF more accurately than MELD.

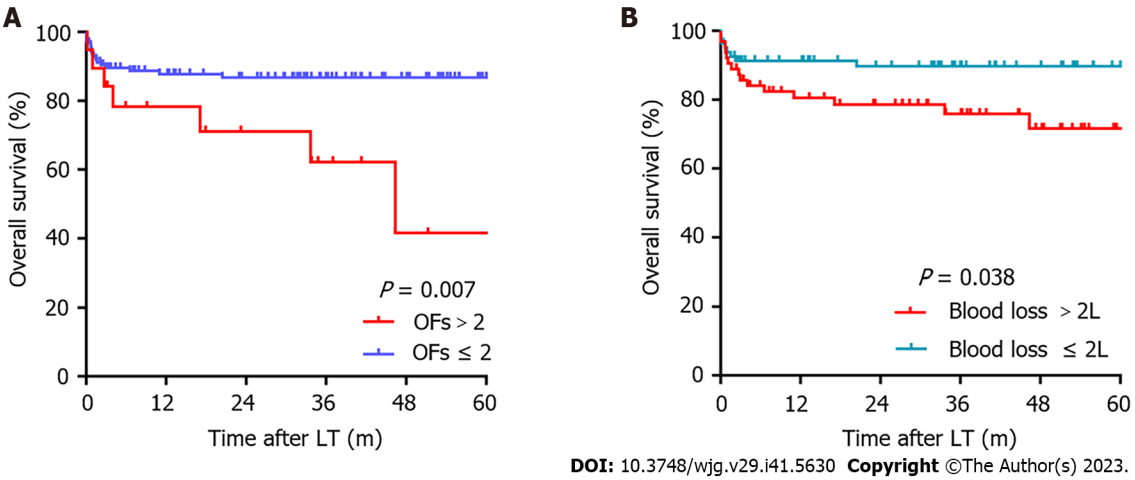
There are limitations in our study. The first is that the boundary of ECD remains undetermined. It should be recognized that the definition of ECD is not a simple concept of yes or no but should be linearly quantified. The application value of the Donor Risk Index[21] in China is limited due to unique ethnic characteristics. What is the safe boundary of an acceptable ECD graft? Unfortunately, we have not been able to establish a quantitative formula thus far in our country, and this will be the focus of our future research. Second, our results need to be further confirmed by a larger sample study.

In conclusion, our experience suggests that ECD grafts are associated with a higher risk of EAD than non-ECD grafts but can be safely used in ACLF recipients, as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.



DOI: 10.3748/wjg.v29.i41.5630

Figure 1 Kaplan-Meier analysis. A: Overall survival between extended criteria donor (ECD) and non-ECD groups; B: Graft survival between ECD and non-ECD groups; C: Overall survival between donation after cardiac death (DCD) and donation after brain death (DBD) groups; D: Graft survival between DCD and DBD groups. LT: Liver transplantation; ECD: Extended criteria donor; DCD: Donation after cardiac death; DBD: Donation after brain death.



DOI: 10.3748/wjg.v29.i41.5630

Figure 2 Kaplan-Meier analysis of overall survival. A: Organ failures; B: Blood loss. OFs: Organ failures.

CONCLUSION

In conclusion, our experience suggests that ECD grafts are associated with a higher risk of EAD than non-ECD grafts but can be safely used in ACLF recipients as they do not affect post-LT survival. The main factors affecting the prognosis of ACLF recipients are the severity of their own preoperative disease and intraoperative blood loss.

Table 6 Univariable and multivariable Cox analysis of risk factors for overall survival

Variables	OR	Univariate analysis		OR	Multivariate analysis	
		HR (95%CI)	*P* value		HR (95%CI)	*P* value
Donor characteristics						
HBV (positive *vs* negative)	1.478	0.646-3.383	0.355			
Death of trauma (yes *vs* no)	0.848	0.379-1.898	0.688			
ECD (yes *vs* no)	1.053	0.465-2.386	0.901			
No. of ECD features (≥ 2 *vs* < 2)	0.840	0.279-2.531	0.757			
Operation characteristics						
Blood loss (L)	1.271	1.137-1.421	0.000	1.276	1.123-1.449	0.000
Anhepatic phase (min)	1.009	0.989-1.028	0.381			
Operation time (h)	1.002	0.746-1.345	0.990			
IFLT (yes *vs* no)	0.822	0.111-6.107	0.848			
EAD (yes *vs* no)	2.726	1.009-7.365	0.048	2.481	0.914-6.737	0.075
AKI (yes *vs* no)	1.882	0.830-4.269	0.130			
Recipient characteristics						
BMI (> 30 *vs* ≤ 30)	0.831	0.112-6.166	0.856			
MELD (> 30 *vs* ≤ 30)	1.528	0.678-3.444	0.307			
OFs (> 2 *vs* ≤ 2)	3.191	1.309-7.780	0.011	3.042	1.245-7.432	0.015
WBC ($\times 10^9$/L)	1.007	0.897-1.129	0.912			
N/L	1.040	0.992-1.090	0.102			
Hb (g/L)	0.984	0.963-1.005	0.128			
GGT (U/L)	0.986	0.968-1.005	0.142			
ALB (g/L)	1.044	0.963-1.131	0.300			
ALT (U/L)	0.992	0.984-1.001	0.070			
AST (U/L)	0.998	0.992-1.004	0.463			
PLT ($\times 10^9$/L)	0.999	0.987-1.010	0.813			
Fib (g/L)	0.874	0.345-2.211	0.775			

OR: Odds ratio; CI: Confidence interval; HBV: Hepatitis B virus; ECD: Extended criteria donor; EAD: Early allograft dysfunction; BMI: Body mass index; IFLT: Ischemia-free liver transplantation; AKI: Acute kidney injury; MELD: Model for end-stage liver disease; OFs: Organ failures; WBC: White blood cell; Hb: Hemoglobin; GGT: Gamma glutamyltransferase; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelets; Fib: Fibrinogen.

ARTICLE HIGHLIGHTS

Research background

There is no consensus on the usage of extended criteria donor (ECD) grafts in liver transplantation (LT) for acute-on-chronic liver failure (ACLF) patients.

Research motivation

It was intended to summarize the experience of using ECD livers in ACLF-LT.

Research objectives

Our study aimed to summarize the experience of using ECD livers in ACLF-LT, and to provide reference for clinical practice.

Research methods
We conducted a retrospective cohort study to analyze outcomes between ECD and non-ECD recipients.

Research results
There was no significant difference ($P > 0.05$) in survival between ECD and non-ECD recipients after LT, although ECD grafts were associated with a significantly higher incidence of early allograft dysfunction. The most important factors affecting post-LT survival of ACLF patients were extrahepatic organ failures (OFs) > 2 ($P = 0.015$) and intraoperative blood loss ($P = 0.000$).

Research conclusions
ECD grafts can be safely used in ACLF-LT, although related to a higher risk of early allograft dysfunction.

Research perspectives
Due to the unavoidable selection bias in clinical practice, there were only 2 cases of donor liver have diagnosed as macrovesicular steatosis more than 30%. This indicates that the ECD grafts actually adopted in our clinical practice may be relatively safe, and those grafts empirically judged as "high risk" were abandoned. Admittedly, this is a major limitation of our current study, and the next step will be to try to compensate it by including more cases in our study over a longer period or in conjunction with other transplant centers.

FOOTNOTES

Author contributions: Gong JL, Tang YH, and Zhu XF conceived study; Gong JL, Yu J and Wang TL collected data and analyzed data; Yu J and Wang TL prepared figures and tables; Gong JL and Yu J wrote manuscript; He XS, Tang YH, and Zhu XF reviewed manuscript; All authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved for publication by our Institutional Reviewer.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

Data sharing statement: The datasets of the current study are available from the corresponding author upon reasonable request.

Country/Territory of origin: China

ORCID number: Jin-Long Gong 0009-0003-5715-4211; Jia Yu 0000-0001-8274-0421; Tie-Long Wang 0000-0003-3914-6412; Xiao-Shun He 0000-0003-3103-4097; Yun-Hua Tang 0000-0002-9196-7414; Xiao-Feng Zhu 0000-0001-6182-3447.

S-Editor: Qu XL
L-Editor: A
P-Editor: Yu HG

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5641-5656

DOI: 10.3748/wjg.v29.i41.5641

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Long-term efficacy and predictors of pembrolizumab-based regimens in patients with advanced esophageal cancer in the real world

Hong-Chi Wang, Xiang Huang, Jing Chen, Ye Li, Yang Cong, Bao-Lin Qu, Sheng-Qiang Feng, Fang Liu

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Koma YI, Japan; Shimokawa T, Japan

Received: July 27, 2023
Peer-review started: July 27, 2023
First decision: September 26, 2023
Revised: October 7, 2023
Accepted: October 23, 2023
Article in press: October 23, 2023
Published online: November 7, 2023

Hong-Chi Wang, Xiang Huang, Jing Chen, Ye Li, Yang Cong, Bao-Lin Qu, Fang Liu, Department of Radiotherapy, The First Medical Center of Chinese PLA General Hospital, Beijing 100853, China

Sheng-Qiang Feng, Health Service, The Guard Bureau of Joint Staff Department of Chinese PLA, Beijing 100017, China

Corresponding author: Fang Liu, MD, Chief Physician, Department of Radiotherapy, The First Medical Center of Chinese PLA General Hospital, No. 28 Fuxing Road, Haidian District, Beijing 100853, China. liufangfsq@163.com

Abstract

BACKGROUND

Pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with advanced esophageal cancer. Few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease.

AIM

To analyze long-term outcomes of pembrolizumab in locally advanced or metastatic esophageal squamous cell carcinoma (ESCC) in the real world.

METHODS

Patients with advanced ESCC admitted to our center from October 2019 to October 2021 were enrolled in this study. Clinical staging of the patients was based on the 8th edition of the American Joint Committee on Cancer TNM staging system. The patients received different treatments based on clinical stage. In brief, patients with locally advanced and resectable ESCC received neoadjuvant therapy combined with surgery. For those who were not candidates for resection, radical concurrent chemoradiotherapy plus pembrolizumab was more preferable. Patients with metastatic ESCC or who were unsuitable for radiotherapy underwent chemotherapy in combination with pembrolizumab. Long-term survival outcomes such as overall survival (OS), progression-free survival, disease-free survival, long-term adverse effects (AEs), immune maintenance therapy and predictors of immune checkpoint inhibitors (ICIs) efficacy were evaluated.

RESULTS

A total of 55 patients with advanced ESCC were enrolled in this retrospective, observational study. The median age was 61 years (range 44-74), with 47.3% (26/55) of the patients in stage IV and 45.5% of the patients had the tumor (25/55) located in the middle third of the esophagus. The median OS in all patients was not reached. The 12-mo OS rate among all patients was 78.8% and the 18-mo OS rate was 72.7%. 9 patients died due to tumor progression and 7 patients died due to treatment-related complications. The therapeutic effect evaluated at the interim evaluation was significantly reflected in the long-term outcome. Patients with complete response or partial response in all patients (P = 0.005) and in the chemoradiotherapy plus pembrolizumab group (P = 0.007) obtained a better prognosis than non-responders. A total of 20 patients (20/55, 36%) received immune maintenance therapy. Baseline peripheral blood biomarkers of the neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and neutrophil-to-(leukocyte-neutrophil) ratio did not predict the efficacy of ICIs.

CONCLUSION

Pembrolizumab combined with chemotherapy or radiotherapy resulted in favorable long-term survival in patients with locally advanced or metastatic ESCC, with safe and manageable long-term AEs.

Key Words: Esophageal cancer; Pembrolizumab; Radiotherapy; Long-term survival; Chemotherapy; Real-world

Core Tip: Pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with metastatic esophageal cancer. Few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease. Our study showed that this treatment in patients with locally advanced or metastatic esophageal squamous cell carcinoma resulted in favorable long-term survival and manageable long-term adverse effects. Randomized phase III trials should be carried out for further verification.

Citation: Wang HC, Huang X, Chen J, Li Y, Cong Y, Qu BL, Feng SQ, Liu F. Long-term efficacy and predictors of pembrolizumab-based regimens in patients with advanced esophageal cancer in the real world. *World J Gastroenterol* 2023; 29(41): 5641-5656
URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5641.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5641

INTRODUCTION

Esophageal cancer is the seventh most common and the sixth leading cause of malignant tumor death worldwide[1]. In China, the incidence and mortality risk of esophageal cancer rank sixth and fourth, respectively[2]. The majority of esophageal cancer patients in China have esophageal squamous cell carcinoma (ESCC) which accounts for 90% of tissue types, and less than 10% have adenocarcinoma[3,4]. Most patients initially diagnosed with esophageal cancer have advanced disease, some patients have locally advanced disease which is inoperable, and some patients have metastases to other sites. Neoadjuvant chemoradiotherapy (nCRT) followed by surgery is the main treatment for resectable ESCC[5,6]. Radical concurrent chemoradiotherapy is an important treatment strategy for locally advanced unresectable patients[7]. For metastatic ESCC, systemic chemotherapy is the only treatment option[8,9]. In fact, for locally advanced or metastatic ESCC, treatment modalities are limited, progress in long-term survival is slow, and the efficacy is unsatisfactory. Data shows that the 5-year survival rate for locally advanced ESCC is no more than 30%. The 5-year survival rate for metastatic ESCC is less than 10%[10].

In recent years, immune checkpoint inhibitors (ICIs) combined with chemotherapy has made significant progress in the first-line treatment of advanced esophageal cancer[11-15]. In the randomized phase III KEYNOTE-590 study, ICIs therapy targeting programmed cell death protein 1 (PD-1), pembrolizumab combined with chemotherapy showed a significant survival advantage over chemotherapy alone in the first-line therapy. The median overall survival (OS) was more than 12 mo and the median progression-free survival (PFS) was 6.3 mo, significantly better than the median OS of 9.8 mo and the median PFS of 5.8 mo in the chemotherapy alone group. In addition, the safety was reliable[12]. For locally advanced patients treated with neoadjuvant therapy, a multicenter real-world study in China showed that the R0 resection rate reached 97.7% in combination with ICIs, and 25.5% of patients in the ICIs plus chemotherapy group and 42.3% of patients in the ICIs plus chemoradiotherapy group achieved pathologic complete response (pCR)[16]. Furthermore, some single-arm clinical trials have also investigated the application of ICIs combined with chemotherapy or concurrent chemoradiotherapy in the field of neoadjuvant therapy[17-20]. To date, the evidence for neoadjuvant treatment combined with ICIs remains inadequate, and results from large phase III clinical trials and long-term follow-up data are lacking. In unresectable locally advanced ESCC, a recent phase IB clinical study examined the efficacy and safety of the PD-1 inhibitor camrelizumab combined with radical radiotherapy for locally advanced ESCC which was intolerant to concurrent chemoradiotherapy[21]. The results showed that median OS and PFS were 16.7 mo and 11.7 mo, respectively. The 24-mo OS rate and PFS rate were 31.6% and 35.5%, respectively. The objective response rate (ORR) was 74%. Three

randomized phase III studies (KEYNOTE-975, ESCORT-CRT and RATIONALE 311) are currently being conducted to further confirm the value of ICIs combined with concurrent chemoradiotherapy. Although these studies have demonstrated the benefit of ICIs plus chemotherapy or chemoradiotherapy in locally advanced or metastatic ESCC, there is currently a lack of reliable predictors of the efficacy of ICIs in esophageal cancer. Several retrospective studies have explored the predictors of efficacy in subsequent-lines for ESCC, and the results showed that blood cell composition can predict the efficacy of ICIs[22-24]. However, predictive results for first-line treatment of locally advanced or metastatic ESCC are still lacking.

Based on this, our center conducted a real-world clinical study to examine the efficacy and safety of pembrolizumab in neoadjuvant therapy, concurrent chemoradiotherapy and first-line therapy for ESCC[25]. Early results showed that the combination with pembrolizumab demonstrated considerable ORR and acceptable adverse effects (AEs). We here report the long-term survival such as OS, disease-free survival (DFS) ,PFS, long-term toxicities and ICIs completion rates. We also assess the relationship between baseline blood cell composition indices such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), neutrophil-to-(leukocyte-neutrophil) ratio (dNLR) and long-term survival in order to determine the predictive factors of ICIs.

MATERIALS AND METHODS

Study design

This single-arm, single-center, retrospective clinical study was conducted in the First Medical Center of Chinese PLA General Hospital. Patients who were initially diagnosed with locally advanced or metastatic esophageal cancer from October 1, 2019 to October 1, 2021 were included. Clinical staging of the patients was based on the 8th edition of the American Joint Committee on Cancer TNM staging system. According to the different clinical stages and treatment modalities, the patients were divided into different subgroups. ORR was defined as the proportion of total patients with CR or partial response (PR). OS was considered the time from definitive diagnosis to death by any cause. DFS was regarded as the period from definitive diagnosis to disease recurrence or death in operable patients. PFS was defined as the period from definitive diagnosis to disease recurrence or death in patients with inoperable locally advanced or metastatic esophageal cancer. Blood samples were obtained at baseline. The NLR was the total number of neutrophils divided by the lymphocyte count. The PLR was the platelet count divided by the lymphocyte count. The dNLR represented the total number of neutrophils divided by the difference between the total number of white blood cells and neutrophils. The study was approved by the Ethics Committee of the Chinese PLA General Hospital in line with the Declaration of Helsinki (as revised in 2013).

Therapeutic regimen

Neoadjuvant therapy plus surgery: Eligible patients were aged 18-75 years, initially diagnosed with operable locally advanced ESCC (T2-4N0M0 or T2-4N+M0), with Eastern Cooperative Oncology Group performance status score of 0 or 1, and life expectancy of at least 6 mo. Patients with active autoimmune disease, and a history of ICIs or chemotherapy treatment were excluded. The patients underwent neoadjuvant chemotherapy (lobaplatin combined with albumin-paclitaxel) plus ICIs (pembrolizumab), prior to surgery once every 3 wk for 2 or 3 cycles. Surgery was then performed after physical examination, laboratory tests, contrast-enhanced chest computed tomography (CT) and pulmonary function tests.

Chemoradiotherapy plus pembrolizumab: Patients were aged 18-75 years, locally advanced and inoperable esophageal cancer or limited to supraclavicular lymph node metastasis, with Eastern Cooperative Oncology Group performance status score of 0 or 1, life expectancy of at least 6 mo were included. Patients with active autoimmune disease, and a history of ICIs or chemotherapy treatment were excluded. They received radical chemoradiotherapy plus pembrolizumab. The patients underwent 2-4 cycles of induction therapy with a chemotherapy regimen (lobaplatin combined with albumin-paclitaxel) plus pembrolizumab. Radical radiotherapy or chemoradiotherapy was then given and an external irradiation technique was used. The total dose of radiotherapy was 54 Gy/30 F, 1.8 Gy each time, 5 times a week. On this basis, primary esophageal lesions and metastatic lymph nodes received 63 Gy/30 F. Pembrolizumab could be discontinued during radiotherapy due to safety concerns. After radiotherapy, pembrolizumab was used as maintenance therapy for a total of 2 years. Treatment was suspended if disease progression or intolerable toxicity occurred.

Chemotherapy plus pembrolizumab: Patients were aged 18-75 years, diagnosed with metastatic esophageal cancer or unsuitable for radiotherapy, with adequate organ function, Eastern Cooperative Oncology Group performance status score of 0 or 1, life expectancy of at least 6 mo were included. Patients with active autoimmune disease, and a history of ICI or chemotherapy treatment were excluded. A chemotherapy regimen (lobaplatin combined with albumin-paclitaxel) plus pembrolizumab was administered every 3 wk for a total of 4 cycles, and then pembrolizumab was given as maintenance therapy for 2 years.

Follow-up

Follow-up began at the time of the patient's diagnosis and treatment in our hospital. The last follow-up was on December 1, 2022. Contrast-enhanced chest and abdominal CT, upper gastrointestinal contrast, ultrasound, and laboratory tests were routinely performed during the follow-up. Gastroscopy, positron emission tomography-CT and chest magnetic resonance imaging were also performed when necessary. Follow-up was conducted every 3 mo during the first 2 years

and then every 6 mo thereafter. The patient's physical condition and long-term AEs were assessed by consultation, telephone and other methods.

Statistical analysis

SAS 9.4 was used for all statistical analyses. The Kaplan-Meier method was used to estimate OS, DFS, PFS and their corresponding 95% confidence intervals (CIs). We divided patients into 3 subgroups according to the different treatment modalities (neoadjuvant treatment plus ICIs, radical chemoradiotherapy plus ICIs, chemotherapy plus ICIs). For the analysis of predictors of immunotherapy efficacy, we used a median cutoff value of 2.43 for NLR, 139.7 for PLR, and 1.72 for dNLR. The group with a larger cutoff value than the median cutoff value was defined as the high group, while the group with a smaller value than the median cutoff value was defined as the low group.

RESULTS

Patient characteristics

A total of 55 patients with ESCC were enrolled in this study from October 1, 2019 to October 1, 2021 (Table 1). The majority of patients were male (43/55, 78.2%) and 12 patients were female. The median age was 61 years (range 44-74), with 47.3% (26/55) of the patients in stage IV and 45.5% of the patients had the tumor (25/55) located in the middle third of the esophagus.

Therapeutic regimen received

Patients received different therapeutic regimens according to clinical stage. Among them, 21 patients received neoadjuvant treatment plus pembrolizumab followed by surgery. 20 patients with locally advanced inoperable and partial stage IV with supraclavicular lymph node metastasis were treated with radical chemoradiotherapy combined with pembrolizumab. The remaining patients who had metastatic esophageal cancer or were unsuitable for chemoradiotherapy received chemotherapy plus pembrolizumab.

Long-term efficacy

The median OS in all patients was not reached. The 12-mo OS rate in all patients was 78.8% and the 18-mo OS rate was 72.7% (Figure 1A). 9 patients died due to tumor progression and 7 died due to treatment-related complications. In the subgroup analysis, the 12-mo OS rate was 65% and the 18-mo OS rate was 60% in the chemoradiotherapy plus pembrolizumab group (Figure 1B). The 12-mo OS rate in the neoadjuvant treatment plus pembrolizumab group was 95% and the 18-mo OS rate was 89.7% (Figure 1C). In the chemotherapy plus pembrolizumab group, the 12-mo OS rate was 75% and the 18-mo OS rate was 66.7% (Figure 1D). The median OS for the 3 subgroups was not reached (Table 2). The 12-mo PFS rate was 55%, the 18-mo PFS rate was 50% and the median PFS was 17 mo in the chemoradiotherapy plus pembrolizumab group (Figure 2A). The 12-mo DFS rate was 85%, the 18-mo DFS rate was 75% and the median DFS was not reached in the neoadjuvant treatment plus pembrolizumab group (Figure 2B). The 12-mo PFS rate was 67.7%, the 18-mo PFS rate was 67.7% and the median PFS was not reached in the chemotherapy plus pembrolizumab group (Figure 2C, Tables 3 and 4).

In addition, the therapeutic effect assessed at the interim evaluation was significant in the long-term outcome. Patients with ORR (CR or PR) in all patients (P = 0.005) (Figure 3A) and in the chemoradiotherapy plus pembrolizumab group (P = 0.007) (Figure 3B) obtained a better prognosis than non-responders. However, we did not find a tendency for benefit in the neoadjuvant therapy followed by surgery group (Figure 3C) and chemotherapy plus pembrolizumab group (Figure 3D).

Safety and patterns of recurrence

In the chemoradiotherapy plus pembrolizumab group, 8 patients died (4 due to esophageal fistula, 1 due to liver failure, 2 due to tumor progression, and 1 due to lung infection). 5 patients developed disease progression (4 patients had recurrence in the radiotherapy targeted area of supraclavicular lymph node metastasis, esophageal lesion, mediastinal lymph node and 1 patient had liver metastasis). In the neoadjuvant treatment plus pembrolizumab group, 4 patients died, including 3 patients who died from tumor progression and 1 patient from a treatment-related complication. 7 patients had disease recurrence and metastasis, among whom 2 patients had local recurrence and 5 patients developed distant metastases. In the chemotherapy plus pembrolizumab group, 4 patients died (two from lung metastases and two from liver metastases) (Table 5).

10 patients in the chemoradiotherapy plus pembrolizumab group (10/20, 50%), 3 patients in the neoadjuvant treatment plus pembrolizumab group (3/21, 14.3%) and 7 patients in the chemotherapy plus pembrolizumab group (7/14, 50%) received immune maintenance therapy. Rash occurred in 3 patients (3/20, 15%), 2 patients developed hypothyroidism (2/20, 10%), and 3 patients experienced pneumonia (3/20, 15%). To date, 6 patients have stopped immune maintenance therapy due to AEs (6/20, 30%) (Table 5).

Impact of NLR, PLR, and dNLR on clinical outcomes

Figure 4 showed the relationship between the baseline NLR (Figure 4A), PLR (Figure 4B), dNLR (Figure 4C) and long-term survival outcomes following ICIs. These results suggested that baseline NLR < 2.43, dNLR < 1.72 and PLR < 139.7 indicated a trend in OS benefit compared with NLR > 2.43, dNLR > 1.72, and PLR > 139.7, although there were no statist-

Table 1 Patient baseline characteristics (*n* = 55)

Characteristics	*n* (%)
Age (yr)	
Median	61
Range	44-74
Sex	
Male	43 (78.2)
Female	12 (21.8)
Tumor location	
Upper esophagus	19 (34.5)
Middle esophagus	25 (45.5)
Lower esophagus	11 (20)
Clinical stage	
II	10 (18.2)
III	19 (34.5)
IV	26 (47.3)
Subgroups	
Chemoradiotherapy plus pembrolizumab (group A)	21 (38.2)
Neoadjuvant therapy plus surgery (group B)	20 (36.4)
Chemotherapy plus pembrolizumab (group C)	14 (25.5)

Table 2 Summary of overall survival

	All patients (n = 55)	Group A (*n* = 20)	Group B (*n* = 21)	Group C (*n* = 14)
Patients with event	16 (29.1%)	8 (40.0%)	4 (19.0%)	4 (28.6%)
Patients without event	39 (70.9%)	12 (60.0%)	17 (81.0%)	10 (71.4%)
Time to event (mo)				
Median	-	-	-	-
95%CI	27.0, -	9.0, -	27.0, -	12.0, -
25% and 75%-ile	15.50, -	10.50, -	27.00, -	12.50, -
Min-max	0.5-39	5-26	2.6-39	0.5-35
12 mo probability (95%CI)	78.8 (65.1-87.7)	65.0 (40.3-81.5)	95.0 (69.5-99.3)	75.0 (40.8-91.2)
18 mo probability (95%CI)	72.7 (58.3-82.9)	60.0 (35.7-77.6)	89.7 (64.8-97.3)	66.7 (33.7-86.0)

Group A: Chemoradiotherapy plus pembrolizumab. Group B: Neoadjuvant therapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab. CI: Confidence interval.

ically significant differences. The *P* values were 0.457, 0.474 and 0.238, respectively.

DISCUSSION

Our previous results showed that PD-1 inhibitor plus chemotherapy or chemoradiotherapy had a good ORR and manageable safety[25]. We used lobaplatin and albumin-paclitaxel as the chemotherapy regimen instead of cisplatin, as cisplatin has AEs on renal function. The trial proved that lobaplatin had favorable results in ESCC[26]. The present study reported the results of long-term follow-up.

Table 3 Summary of progression-free survival

	Group A (*n* = 20)	Group C (*n* = 14)
Patients with event	11 (55.0%)	4 (28.6%)
Patients without event	9 (45.0%)	10 (71.4%)
Time to event (mo)		
Median	17	-
95%CI	8.0, -	9.0, -
25% and 75%-ile	8.50, -	12.0, -
Min-max	5-26	0.5-35
12 mo probability (95%CI)	55.0 (31.3-73.5)	67.7 (34.9-86.5)
18 mo probability (95%CI)	50.0 (27.1-69.2)	67.7 (34.9-86.5)

Group A: Chemoradiotherapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab. CI: Confidence interval.

Table 4 Summary of disease-free survival

	Group B (*n* = 21)
Patients with event	7 (33.3%)
Patients without event	14 (66.7%)
Time to event (mo)	
Median	-
95%CI	5.0, -
25% and 75%-ile	17.50, -
Min-max	2.6-27
12 mo probability (95%CI)	85.0 (60.4-94.9)
18 mo probability (95%CI)	75.0 (50.0-88.7)

Group B: Neoadjuvant therapy plus pembrolizumab. CI: Confidence interval.

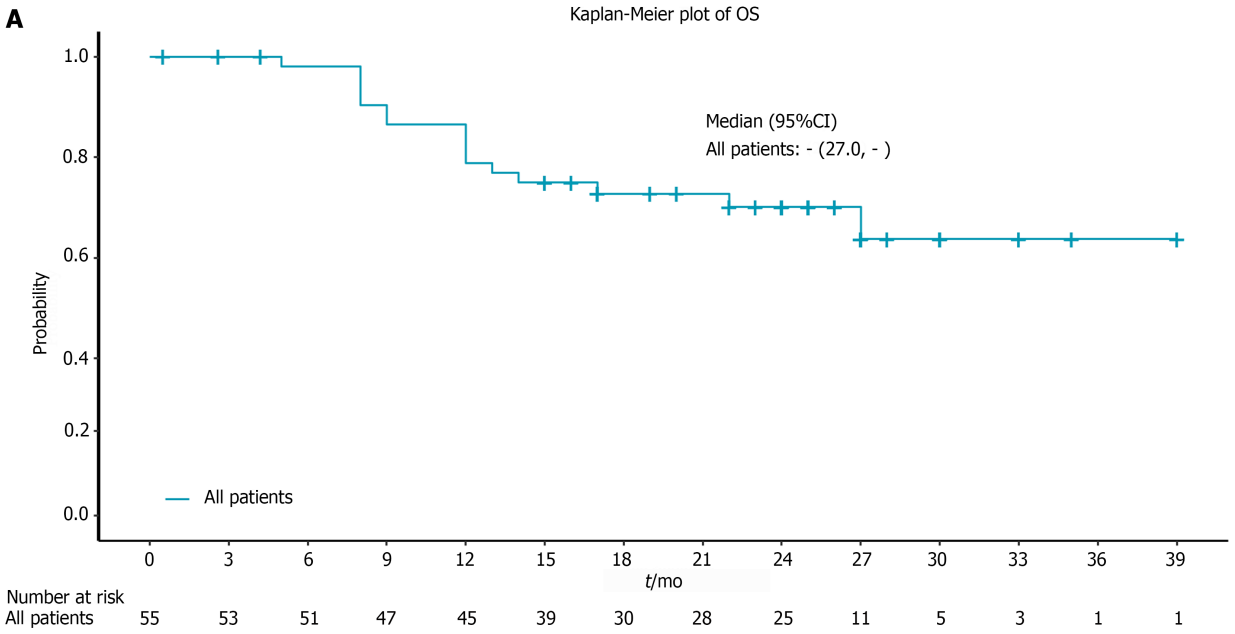
Table 5 Patterns of recurrence and immune maintenance therapy

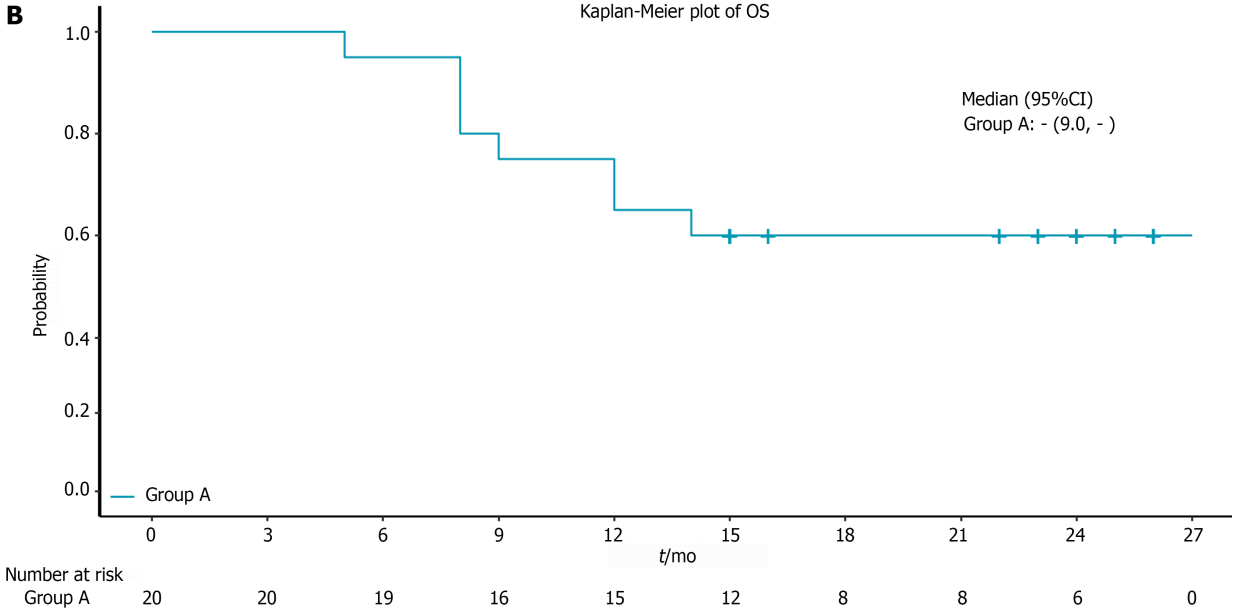
	Patterns of recurrence		Immune maintenance therapy
	Local	Distant organ	
Group A (20)	4 (20%)	1 (5%)	10 (50%)
Group B (21)	2 (9%)	5 (24%)	3 (14.3%)
Group C (14)	2 (14.3%)	4 (28.6%)	7 (50%)

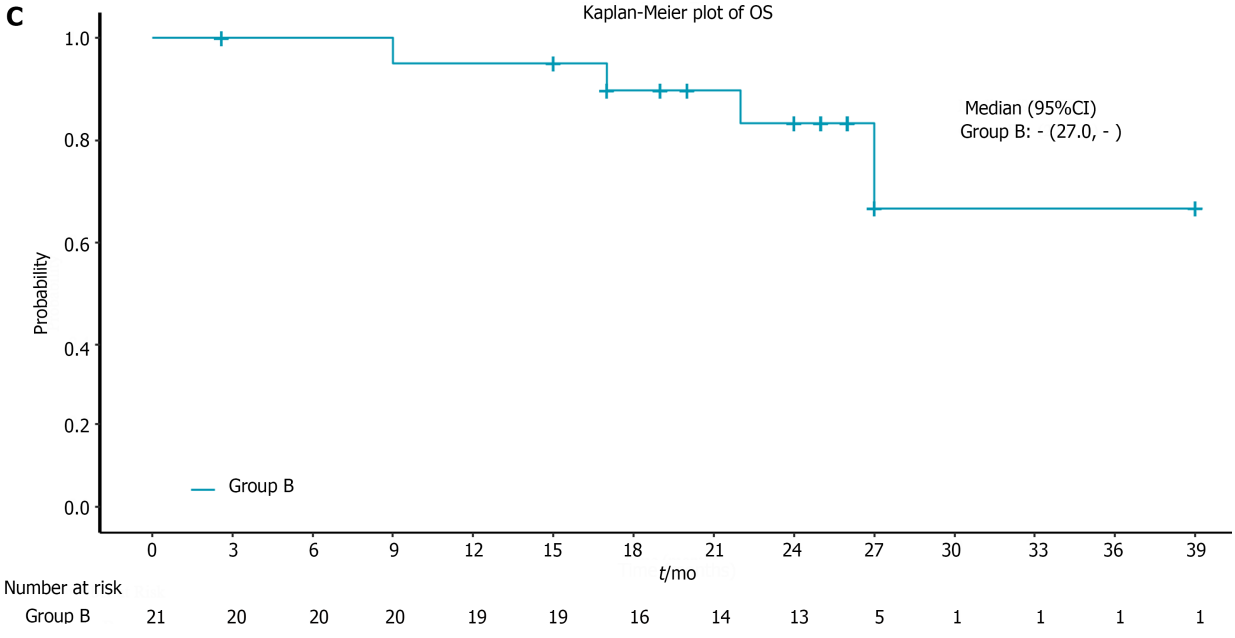
Group A: Chemoradiotherapy plus pembrolizumab. Group B: Neoadjuvant therapy plus pembrolizumab. Group C: Chemotherapy plus pembrolizumab.

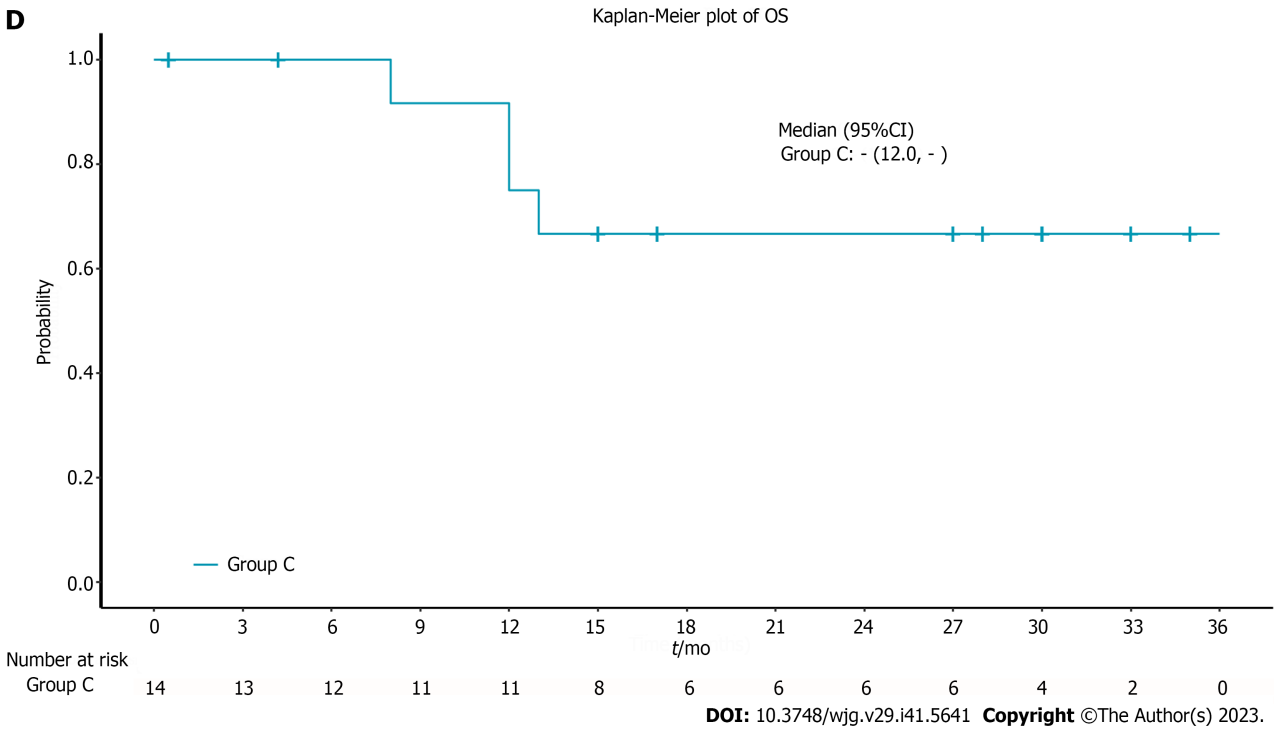
For advanced ESCC, especially locally advanced disease, neoadjuvant chemotherapy plus immunotherapy followed by surgery or chemoradiotherapy combined with immunotherapy warrants further studies, as current clinical studies are confined to phase I-II trials, and long-term follow-up data are lacking. In the present study, relatively good long-term outcomes were achieved with tolerable side effects, and evidence for PD-1 inhibitor combined with chemotherapy or radiotherapy used in ESCC has been provided.

In this study, 21 patients received neoadjuvant therapy plus pembrolizumab followed by surgery. The results demonstrated that the 12-mo DFS rate was 85%, the 18-mo DFS rate was 75%, the 12-mo OS rate was 95% and the 18-mo OS rate was 89.7%. The median OS or DFS was not reached. The results of the NEOCRTEC 5010 study indicated that the 1-year OS rate in the nCRT group was 90% and the 2-year OS rate was 75.1%[5]. Our results were similar to those of the NEOCRTEC 5010 trial. However, during a median follow-up of 24 mo in our study, patients were found to have local


A
Kaplan-Meier plot of OS
Median (95%CI)
All patients: - (27.0, -)
Probability
All patients
t/mo
Number at risk
All patients 55 53 51 47 45 39 30 28 25 11 5 3 1 1

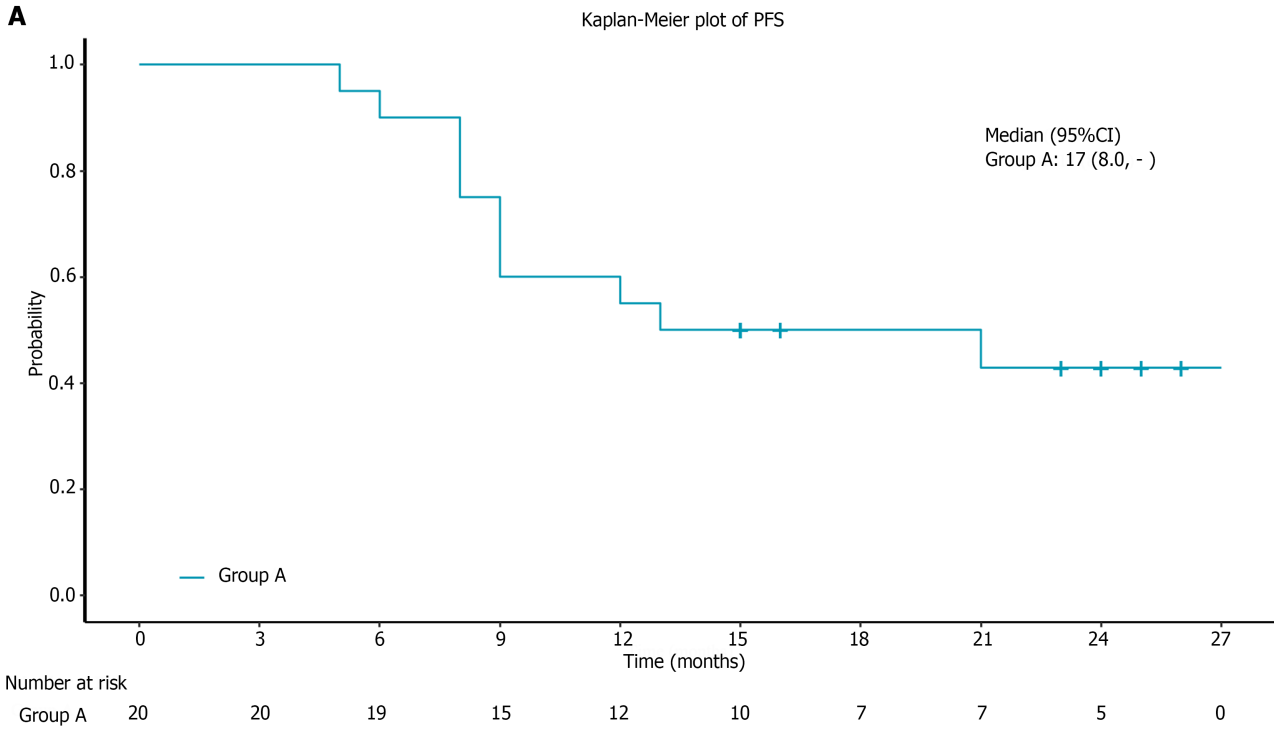

B
Kaplan-Meier plot of OS
Median (95%CI)
Group A: - (9.0, -)
Probability
Group A
t/mo
Number at risk
Group A 20 20 19 16 15 12 8 8 6 0


C
Kaplan-Meier plot of OS
Median (95%CI)
Group B: - (27.0, -)
Probability
Group B
t/mo
Number at risk
Group B 21 20 20 20 19 19 16 14 13 5 1 1 1 1



DOI: 10.3748/wjg.v29.i41.5641

Figure 1 Kaplan-Meier plot of overall survival. A: Kaplan-Meier plot of overall survival (OS) in all patients; B: Kaplan-Meier plot of OS in group A; C: Kaplan-Meier plot of OS in group B; D: Kaplan-Meier plot of OS in group C. Group A: Chemoradiotherapy plus pembrolizumab; Group B: Neoadjuvant therapy plus pembrolizumab; Group C: Chemotherapy plus pembrolizumab; OS: Overall survival; CI: Confidence interval.



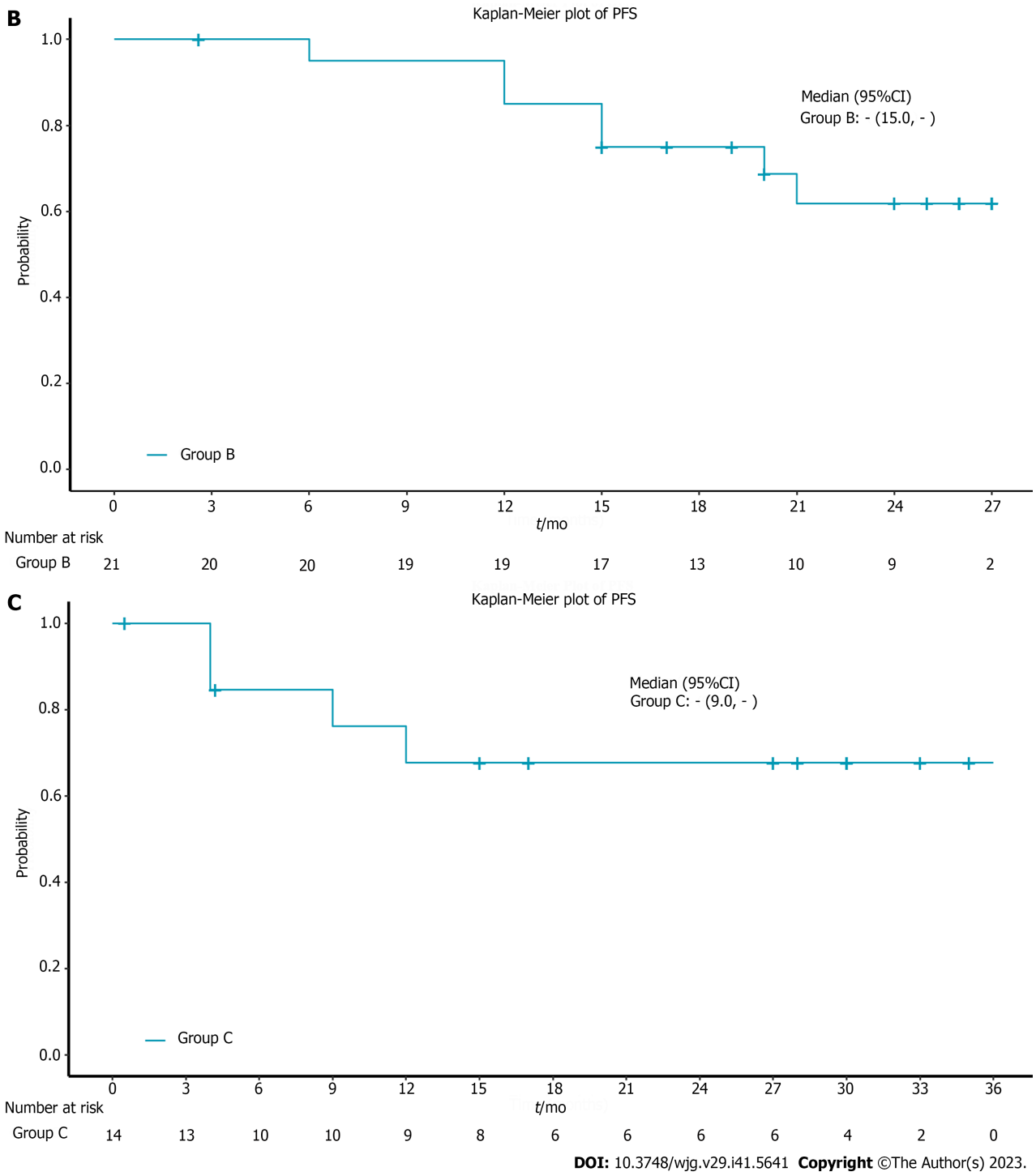


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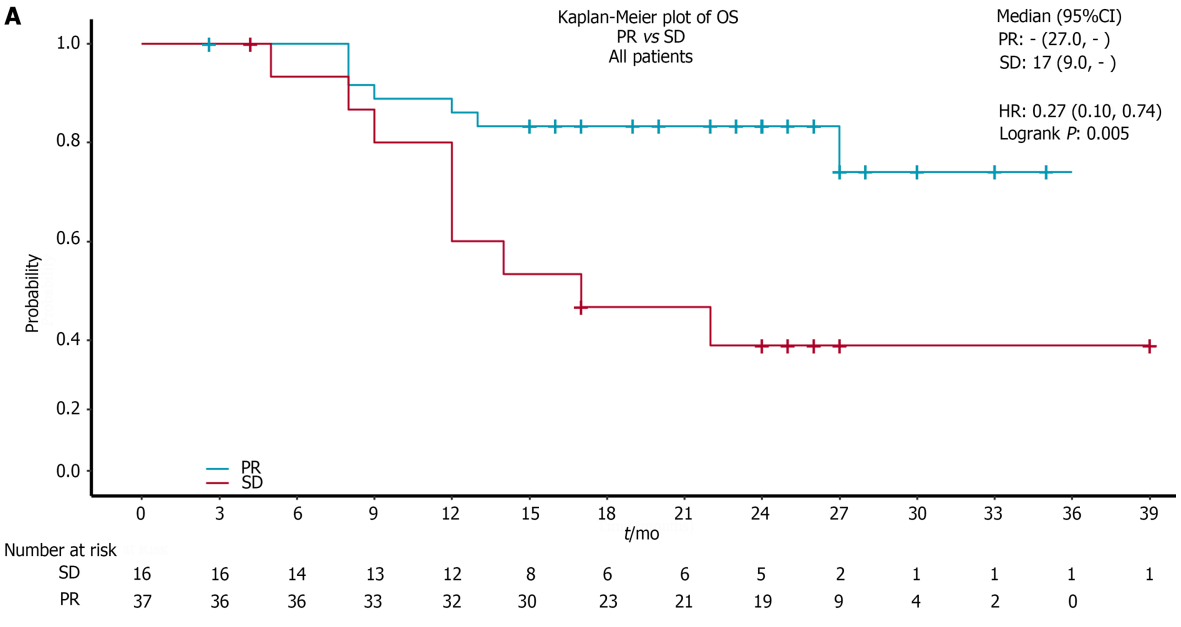
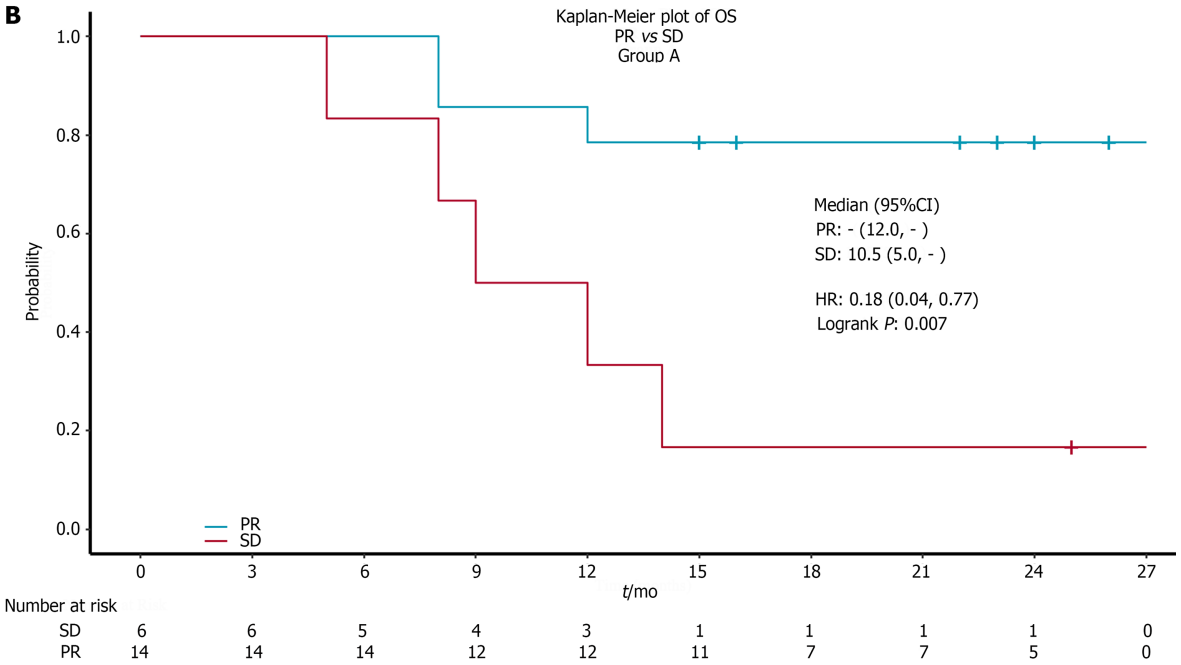
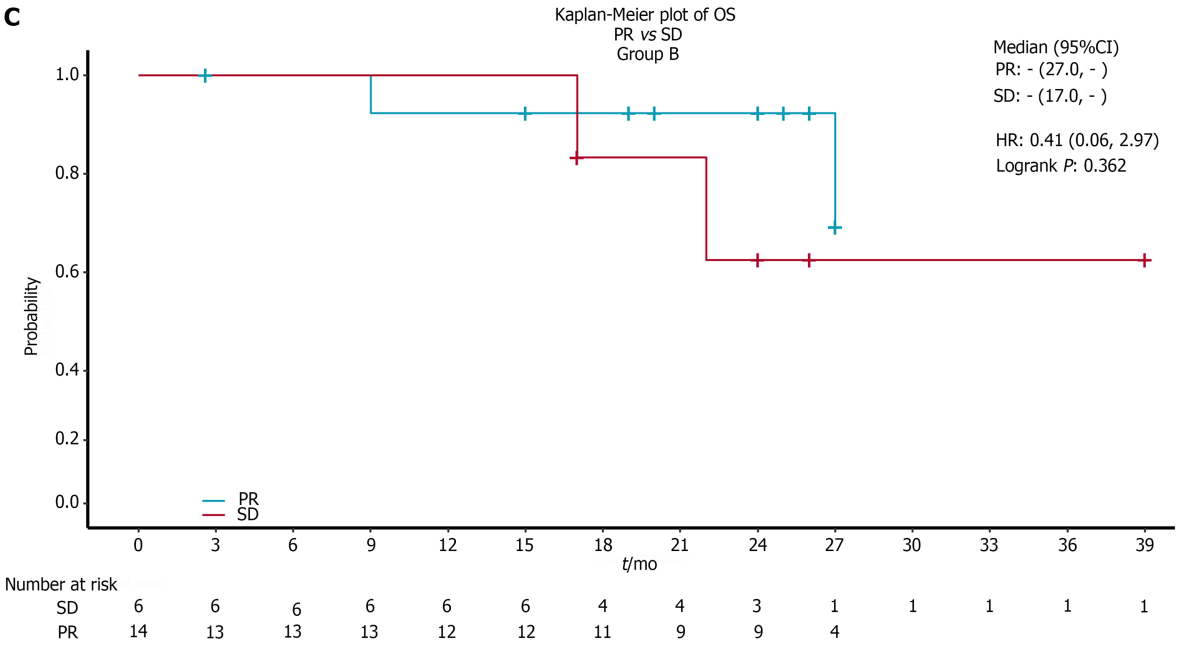
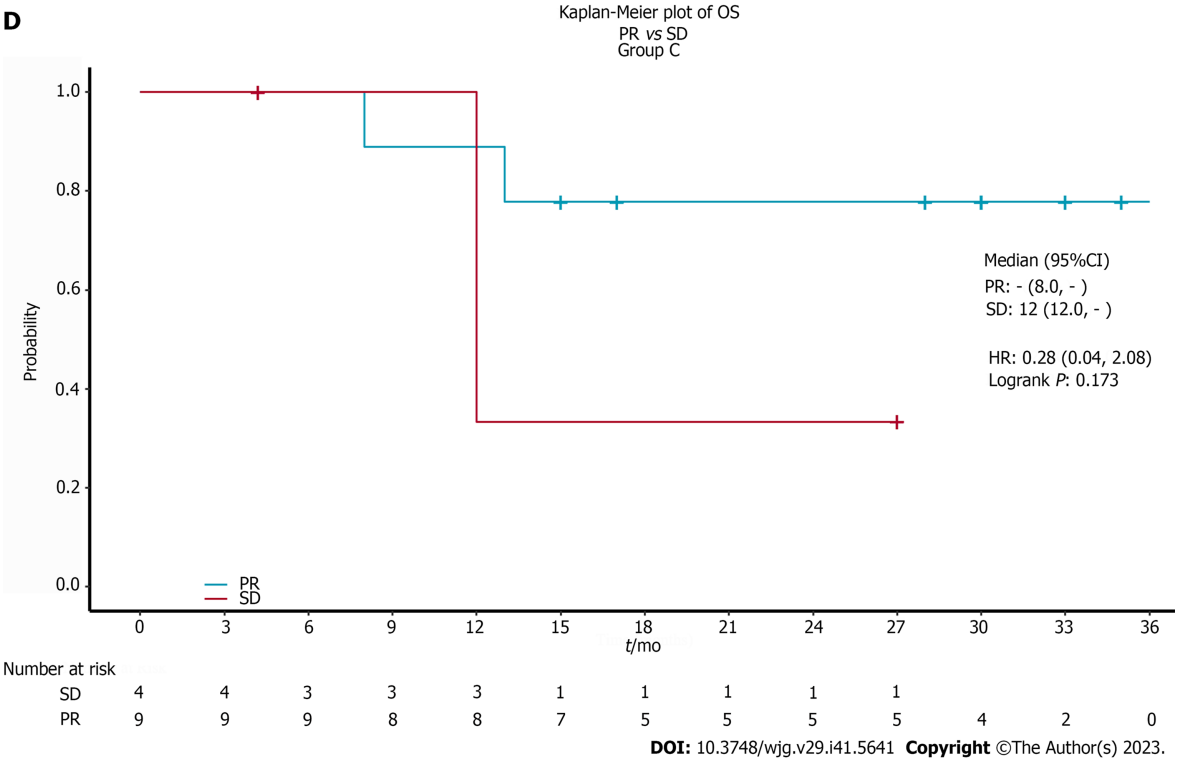
Figure 2 Kaplan-Meier plot of progression-free survival. A: Kaplan-Meier plot of progression-free survival (PFS) in group A; B: Kaplan-Meier plot of disease-free survival in group B; C: Kaplan-Meier plot of PFS in group C. Group A: Chemoradiotherapy plus pembrolizumab; Group B: Neoadjuvant therapy plus pembrolizumab; Group C: Chemotherapy plus pembrolizumab; PFS: Progression-free survival; CI: Confidence interval; DFS: Disease-free survival.

recurrence in mediastinal lymph nodes, anastomotic stoma and retroperitoneal lymph nodes. Lung, pleural effusion, and supraclavicular lymph node metastases were found in 23.8% of patients (5/21). The 10-year pattern of recurrence and metastasis in the CROSS study showed that the proportion of isolated local recurrence in the neoadjuvant group was 8% (15/178). The percentage of patients with both local recurrence and distant metastasis were 13% (23/178). In addition, the ratio of patients with simple distant metastasis was 27% (48/178)[27]. In our study, the recurrence pattern was dominated by distant metastasis, but there was still a high local recurrence rate. Therefore, it remains unclear whether the local recurrence risk with neoadjuvant chemotherapy plus ICIs is non-inferior to neoadjuvant concurrent chemoradiotherapy. Furthermore, the relatively high recurrence rate in the short follow-up period in our study requires further verification in large clinical trials. In the CHECKMATE-577 trial, the median DFS for patients who did not reach pCR after nCRT was significantly better in the maintenance treatment group with nivolumab than in the placebo group[28]. In our study, the postoperative immune maintenance rate was only 14.3% (3/21), which may also be one of the reasons for the increased


A
Kaplan-Meier plot of OS
PR vs SD
All patients
Median (95%CI)
PR: - (27.0, -)
SD: 17 (9.0, -)
HR: 0.27 (0.10, 0.74)
Logrank P: 0.005
Probability
PR
SD
t/mo
Number at risk
SD 16 16 14 13 12 8 6 6 5 2 1 1 1 1
PR 37 36 36 33 32 30 23 21 19 9 4 2 0


B
Kaplan-Meier plot of OS
PR vs SD
Group A
Median (95%CI)
PR: - (12.0, -)
SD: 10.5 (5.0, -)
HR: 0.18 (0.04, 0.77)
Logrank P: 0.007
Probability
PR
SD
t/mo
Number at risk
SD 6 6 5 4 3 1 1 1 1 0
PR 14 14 14 12 12 11 7 7 5 0


C
Kaplan-Meier plot of OS
PR vs SD
Group B
Median (95%CI)
PR: - (27.0, -)
SD: - (17.0, -)
HR: 0.41 (0.06, 2.97)
Logrank P: 0.362
Probability
PR
SD
t/mo
Number at risk
SD 6 6 6 6 6 6 4 4 3 1 1 1 1 1
PR 14 13 13 13 12 12 11 9 9 4



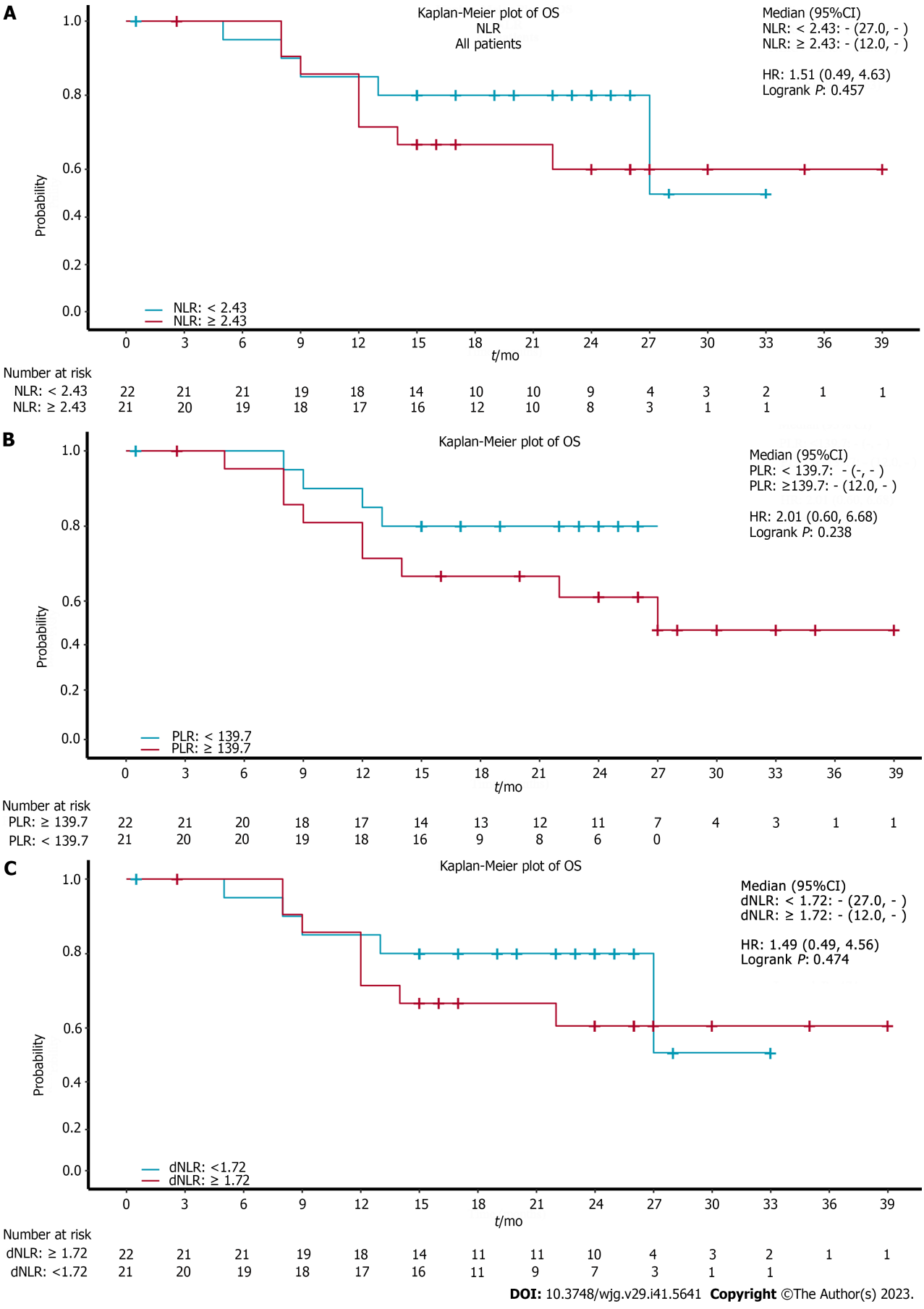
DOI: 10.3748/wjg.v29.i41.5641

Figure 3 Kaplan-Meier plot of overall survival in partial response and stable disease. A: Kaplan-Meier plot of overall survival (OS) in partial response (PR) and stable disease (SD) at the midterm evaluation in all patients; B: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group A; C: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group B; D: Kaplan-Meier plot of OS in PR and SD at the midterm evaluation in group C. PR: Partial response; SD: Stable disease; HR: Hazard ratio; OS: Overall survival; group A: Chemoradiotherapy plus pembrolizumab; group B: Neoadjuvant therapy plus pembrolizumab; group C: Chemotherapy plus pembrolizumab.

rate of distant metastasis. In the future, neoadjuvant therapy for locally advanced esophageal cancer requires continuous optimization of protocols to reduce the risk of distant metastasis and improve survival. In addition, on the premise of ensuring local control, eliminating radiotherapy to reduce AEs is also worth further exploration.

20 patients with unresectable locally advanced or limited supraclavicular lymph node metastases received chemoradiotherapy combined with pembrolizumab. The results showed that the 12-mo OS rate was 65%, the 18-mo OS rate was 60% and the median OS was not reached. The 12-mo PFS rate was 55%, the 18-mo PFS rate was 50% and the median PFS was 17 mo. The median survival time following radical concurrent chemoradiotherapy recommended by current guidelines was 18 mo, and the 2-year survival rate was about 40%[29]. The long-term survival in the radical chemoradiotherapy plus pembrolizumab group in our study was slightly better than that in the standard radical concurrent chemoradiotherapy group. The addition of ICIs to chemoradiotherapy likely increased the efficacy and prolonged survival. However, randomized phase III studies are needed to verify this. A phase IB clinical study is currently examining the efficacy and safety of the PD-1 inhibitor camrelizumab combined with radical radiotherapy in patients with locally advanced ESCC who are intolerant to concurrent chemoradiotherapy[21]. The median OS and PFS were 16.7 mo and 11.7 mo, respectively. The 24-mo OS rate and PFS rate were 31.6% and 35.5%, respectively. The ORR rate was 74%. Our results showed a more beneficial outcome, probably because we used the combination of radiotherapy and chemotherapy, which strengthened the intensity of treatment and improved survival outcomes. Studies have shown that the incidence rate of esophageal fistula caused by radiotherapy and chemotherapy is approximately 15%, of which T4 and esophageal stenosis increase the risk of fistula with a poor prognosis[30]. In our study, 20% (4/20) patients died from esophageal fistula. The patients with fistula in the radiotherapy plus pembrolizumab group were all T4 and the tumor was closely related to the trachea, which was suspected to have invasion. Furthermore, the radiotherapy dose in this group was 63 Gy, and the high radiotherapy dose was also the main cause of fistula. Studies have shown that higher 60 Gy did not improve long-term survival and simultaneously increased AEs[31]. Therefore, in the era of ICIs, for locally advanced patients with T3-T4, radiotherapy dose should be carefully selected and safety should be taken into account in the absence of clear evidence of benefit. The data and results of randomized phase III studies on the combination therapy of radiotherapy and ICIs are lacking at present. There are still some problems to be solved such as the timing of combination therapy, selection of the combination chemotherapy regimen, clinical target volume and so on. The results of KEYNOTE-975, ESCORT-CRT, RATIONALE-311 and other randomized phase III studies are expected.

A total of 14 patients in our study received chemotherapy combined with pembrolizumab. The 12-mo OS rate was 75% and the 18-mo OS rate was 66.7%. The 12-mo PFS rate and 18-mo PFS rate were 67.7%. In the randomized phase III JUPITER-06 study, toripalimab combined with chemotherapy significantly prolonged PFS in patients with a 42% reduction in the risk of disease progression and resulted in a significant benefit in median OS (17 mo *vs* 11 mo) compared with placebo plus chemotherapy. The 1-year PFS rate was 27.8% and the 1-year OS rate was 66% in the toripalimab-based



DOI: 10.3748/wjg.v29.i41.5641

Figure 4 Kaplan-Meier plot of overall survival at baseline. A: Kaplan-Meier plot of overall survival (OS) at baseline (neutrophil-to-lymphocyte ratio < 2.43 *vs* > 2.43); B: Kaplan-Meier plot of OS at baseline (platelet-to-lymphocyte ratio < 139.7 *vs* > 139.7); C: Kaplan-Meier plot of OS at baseline [neutrophil-to-(leukocyte-

neutrophil) ratio < 1.72 *vs* > 1.72]. OS: Overall survival; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; dNLR: Neutrophil-to-(leukocyte-neutrophil) ratio; CI: Confidence interval; HR: Hazard ratio.

group[11]. The long-term survival results in our study were better than those in the JUPITER-06 study, and even better than those in the radiotherapy plus pembrolizumab group. There are several possible reasons for this result: (1) The tumor burden in the chemotherapy combined with pembrolizumab group was relatively low. Some patients with stage III refused radiotherapy due to toxicity, while others had single liver metastasis or small nodules in lung metastasis; (2) In the radiotherapy plus pembrolizumab group, 4 patients died due to esophageal fistula after radiotherapy and survival outcomes were negatively affected; and (3) Small sample size and short follow-up time may have led to deviations in the results.

As indicators of systemic inflammation, the NLR, PLR and dNLR can reflect the microenvironment of inflammation. Neutrophils can promote tumor invasion and progression by secreting cytokines, vascular endothelial cell growth factors and chemokines[32]. However, lymphocytes play an important role in the immune system and can inhibit tumor proliferation[33]. Studies have reported that in patients with non-small cell lung cancer and a higher baseline NLR, ICIs had poor efficacy, which had a negative predictive value on PFS and OS[34]. Our study found that low baseline NLR, dNLR and PLR showed a trend for OS benefit, but a statistically significant difference was not observed. This result may have been limited by the small sample size. Thus, a larger sample size is needed to examine this issue in the future.

This study also had some limitations: (1) This was a single-arm, single-center retrospective clinical study, with a small number of patients and did not include a control group; (2) The follow-up period should have been longer, as there is a lack of 3-year and 5-year long-term survival outcomes; and (3) Prospective randomized controlled studies with long-term follow-up data are needed.

CONCLUSION

Our real-world results revealed that pembrolizumab combined with chemotherapy or radiotherapy resulted in a favorable long-term survival outcome in patients with locally advanced and metastatic esophageal cancer. Long-term toxicities associated with these regimens were manageable.

ARTICLE HIGHLIGHTS

Research background

Although pembrolizumab combined with chemotherapy has been proven effective as first-line therapy in patients with advanced esophageal cancer, few trials have assessed the safety and efficacy of this treatment in patients with locally advanced disease.

Research motivation

Progress has been made in the immune checkpoint inhibitors combined with chemotherapy as the first-line treatment of advanced esophageal cancer. The efficacy and safety of pembrolizumab in locally advanced or metastatic esophageal squamous cell carcinoma (ESCC) in the real world were worth studying.

Research objectives

To analyze the long-term outcomes of pembrolizumab in locally advanced or metastatic ESCC in the real world.

Research methods

This was a single-arm, single-center, retrospective clinical study. Patients who were initially diagnosed with locally advanced or metastatic esophageal cancer from October 1, 2019 to October 1, 2021 were included. According to the different clinical stages and treatment modalities, the patients were divided into different subgroups. Long-term survival outcomes were evaluated.

Research results

A total of 55 patients with ESCC were enrolled in this study from October 1, 2019 to October 1, 2021. The median overall survival (OS) in all patients was not reached. The 12-mo OS rate was 78.8% and the 18-mo OS rate was 72.7%. Nine patients died due to tumor progression and 7 patients died due to treatment-related complications.

Research conclusions

Pembrolizumab combined with chemotherapy or radiotherapy resulted in favorable long-term survival in patients with locally advanced or metastatic ESCC, with safe and manageable long-term adverse effects.

Research perspectives

It is necessary to explore the efficacy of pembrolizumab combined with chemotherapy or radiotherapy in patients with locally advanced or metastatic ESCC. Randomized phase III trials should be carried out for further verification of the efficacy.

ACKNOWLEDGEMENTS

We thank Dr. Run-Kun Yang from Merck Sharp and Dohme Medical Affairs for his scientific comments on the manuscript.

FOOTNOTES

Author contributions: Liu F, Wang HC, and Huang X were involved in the study conception and design; Wang HC, Huang X, and Feng SQ drafted the article and interpreted the data; Chen J, Li Y, and Cong Y collected and analyzed the data; Qu BL supervised the report.

Institutional review board statement: The study was reviewed and approved by the Medical Ethics Committee of the General Hospital of the Chinese People's Liberation Army, No. S2021-265-01.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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

Data sharing statement: No additional data are available.

Country/Territory of origin: China

ORCID number: Hong-Chi Wang 0009-0000-7669-8757; Xiang Huang 0000-0001-9319-9909; Jing Chen 0009-0003-2379-2231; Ye Li 0000-0002-8150-5056; Yang Cong 0000-0001-6186-4759; Bao-Lin Qu 0000-0002-8911-3460; Sheng-Qiang Feng 0000-0002-3996-0872; Fang Liu 0000-0002-8563-4664.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Cai YX

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5657-5667

DOI: 10.3748/wjg.v29.i41.5657

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Colorectal motility patterns and psychiatric traits in functional constipation and constipation-predominant irritable bowel syndrome: A study from China

Chao-Lan Lv, Geng-Qing Song, Jie Liu, Wei Wang, Yi-Zhou Huang, Bo Wang, Jia-Shuang Tian, Meng-Qing Yin, Yue Yu

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Chakrabarti S, India; Kumar A, India; Setiawati Y, Indonesia

Received: July 10, 2023
Peer-review started: July 10, 2023
First decision: September 1, 2023
Revised: September 14, 2023
Accepted: October 11, 2023
Article in press: October 11, 2023
Published online: November 7, 2023

Chao-Lan Lv, Jie Liu, Yue Yu, Department of Gastroenterology, The First Affiliated Hospital of University of Science and Technology of China, Hefei 230001, Anhui Province, China

Chao-Lan Lv, Jie Liu, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui Province, China

Geng-Qing Song, Department of Gastroenterology and Hepatology, Metrohealth Medical Center, Case Western Reserve University, Cleveland, OH 44109, United States

Wei Wang, Jia-Shuang Tian, Meng-Qing Yin, Department of Gastroenterology, Affiliated Anhui Provincial Hospital, Anhui Medical University, Hefei 230001, Anhui Province, China

Yi-Zhou Huang, Bo Wang, Department of Gastroenterology, Graduate School of Bengbu Medical College, Bengbu 233000, Anhui Province, China

Corresponding author: Yue Yu, MD, PhD, Professor, Department of Gastroenterology, The First Affiliated Hospital of University of Science and Technology of China, No. 9 Lujiang Road, Hefei 230001, Anhui Province, China. yuyuemd@ustc.edu.cn

Abstract

BACKGROUND

Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) represent a spectrum of constipation disorders. However, the majority of previous clinical investigations have focused on Western populations, with limited data originating from China.

AIM

To determine and compare the colorectal motility and psychiatric features of FC and IBS-C in an Eastern Chinese population.

METHODS

Consecutive chronic constipation patients referred to our motility clinic from December 2019 to February 2023 were enrolled. FC and IBS-C diagnoses were established using ROME IV criteria, and patients underwent high-resolution anorectal manometry (ARM) and a colonic transmit test using the Sitz marker

study. Constipation-related symptoms were obtained through questionnaires. Anxiety and depression were assessed by the Hamilton anxiety rating scale and the Hamilton Depression Rating Scale-21. The clinical characteristics and colorectal motility patterns of FC and IBS-C patients were compared.

RESULTS

No significant differences in sex, age or abdominal discomfort symptoms were observed between IBS-C and FC patients (all $P > 0.05$). The proportion of IBS-C patients with delayed colonic transit was higher than that of patients with FC (36.63% *vs* 15.91%, $P < 0.05$), while rectosigmoid accumulation of radiopaque markers was more common in the FC group than in the IBS-C group (50% *vs* 26.73%, $P < 0.05$). Diverse proportions of these dyssynergic patterns were noted within both the FC and IBS-C groups by ARM. IBS-C patients were found to have a higher prevalence of depression than FC patients (66.30% *vs* 42.42%, $P < 0.05$). The scores for feelings of guilt, suicide, psychomotor agitation, diurnal variation, obsessive/compulsive disorder, hopelessness, self-abasedment and gastrointestinal symptoms were significantly higher in IBS-C patients than that in FC patients ($P < 0.05$). For IBS-C ($\chi^2 = 5.438$, $P < 0.05$) but not FC, patients with normal colon transit time were significantly more likely to have anxiety than those with slow colon transit time. For IBS-C patients but not FC patients, the threshold of first constant sensation, desire to defecate and sustained urgency were all weakly correlated with the degree of anxiety ($r = 0.414$, $r = 0.404$, and $r = 0.418$, respectively, $P < 0.05$). The proportion of patients with a low threshold of desire to defecate among IBS-C patients with depression was lower than that in those without depression (69.6% *vs* 41.9%, $\chi^2 = 4.054$, $P < 0.05$).

CONCLUSION

Our findings highlight both overlapping and distinctive patterns of colon transit, dyssynergic patterns, anorectal sensation, psychological distress, and associations of psychiatric and colorectal motility characteristics in FC and IBS-C patients in an Eastern Chinese population, providing valuable insights into the pathophysiological underpinnings of these disorders.

Key Words: Functional constipation; Constipation-predominant irritable bowel syndrome; High-resolution anorectal manometry; Colonic transmit test; Anxiety; Depression

Core Tip: Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) are the two primary subtypes of constipation. Previous clinical studies that attempted to illuminate distinctive physiological mechanisms between FC and IBS-C patients were predominantly from Western countries, with limited data originating from China. Our study has revealed distinctive elements of FC and IBS-C across multifaceted parameters, namely colonic transmit time, psychological distress, and dyssynergic patterns, and the relationship among these parameters. These findings extend our comprehension of the intricate pathophysiological mechanisms underlying FC and IBS-C. These findings could provide guidance for constipation patients to choose appropriate colorectal tests.

Citation: Lv CL, Song GQ, Liu J, Wang W, Huang YZ, Wang B, Tian JS, Yin MQ, Yu Y. Colorectal motility patterns and psychiatric traits in functional constipation and constipation-predominant irritable bowel syndrome: A study from China. *World J Gastroenterol* 2023; 29(41): 5657-5667

URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5657.htm

DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5657

INTRODUCTION

Chronic constipation (CC) represents a prevalent health challenge globally, impacting approximately 4% to 10% of the population in China[1,2]. The process of fecal evacuation is governed by a complex interplay of brain-gut axis interactions, intestinal peristalsis, and the function of the pelvic floor muscles and anal sphincter. The multifaceted pathogenesis of CC involves a dynamic interplay between biological and psychosocial factors. Two principal contributors to the pathophysiology of CC are colonic sensorimotor disturbances and pelvic floor dysfunction[3,4]. A multitude of diagnostic tools, including high-resolution anorectal manometry (HR-ARM), the colonic transmit test (CTT), barium and magnetic resonance defecography, and the balloon expulsion test (BET) can provide valuable insights into the underlying mechanisms of CC. In a clinical setting, the assessment of colorectal motility and psychiatric evaluations are often essential when patients with CC do not respond to conventional laxative treatments[5].

Functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) are the two primary subtypes of CC. As per the Rome IV criteria, an IBS-C diagnosis requires the presence of abdominal pain or discomfort, a criterion that is not required for FC. The Rome IV guidelines also suggest that IBS-C and FC are not distinct disorders but

rather exist on a continuum of constipation disorders. Despite overlaps in symptoms, pathophysiological mechanisms and treatment responses, distinctions between IBS-C and FC exist[6]. A thorough understanding of these physiological mechanisms could enhance our ability to differentiate between IBS-C and FC more effectively than relying solely on symptoms. While several clinical studies, predominantly from Western countries, have attempted to illuminate this issue [7-9], data from Chinese populations remain scarce.

In China, the first choice of treatment for patients with treatment-resistant constipation is often ARM, the only minimally invasive tool available to measure anorectal pressures. Due to its affordability, ease of execution, and ready availability, the radiopaque marker technique is widely utilized to assess colonic transit[10]. Therefore, in our study, we selected these two modalities to examine the physiological mechanisms of CC. However, controversy persists regarding the correlation between these two tests, and it remains unclear which test provides more meaningful data for IBS-C or FC [7-11]. Thus, our study aimed to compare the psychiatric (depression and anxiety) and colorectal motility (colonic transit and anorectal motility and sensation) characteristics between FC and IBS-C patients in an Eastern Chinese population. We also sought to investigate the correlations between psychiatric and colorectal motility characteristics in both FC and IBS-C patients.

MATERIALS AND METHODS

Patients

For this study, we recruited patients diagnosed with FC and IBS-C from the Anhui Provincial Hospital's motility clinic between December 2019 and February 2023. The diagnoses of FC and IBS-C were made according to the Rome IV criteria [6]. The exclusion criteria included pregnancy or lactation; diabetes; thyroid dysfunction; and cardiovascular, hepatic or renal disease. All patients underwent standardized HR-ARM and CTT. The study received approval from the Ethics Committee of Anhui Provincial Hospital.

HR-ARM

HR-ARM was conducted using a water-perfusion HR-ARM device (GAP-08A, Maida Instruments, Ningbo, China) according to the London consensus protocol[12]. Patients remained in the left lateral decubitus position during simulated evacuation. Four patterns of dyssynergia were classified according to the Rao classification, along with a normal and an unclassified pattern. Type I dyssynergia showed an adequate increase in rectal pressure (≥ 45 mmHg) accompanied by a paradoxical simultaneous increase in anal pressure; type II dyssynergia showed an inadequate increase in rectal pressure of (< 45 mmHg) (poor propulsive force) accompanied by a paradoxical simultaneous increase in anal pressure; type III dyssynergia showed an adequate increase in rectal pressure (≥ 40 mmHg) accompanied by failure of reduction in anal pressure (≤ 20% baseline pressure); and type IV dyssynergia showed an inadequate increase in rectal pressure of (< 45 mmHg) (poor propulsive force) accompanied by failure of reduction in anal pressure (≤ 20% baseline pressure). The normal pattern showed an adequate increase in rectal pressure (≥ 45 mmHg) accompanied by a simultaneous reduction in anal pressure. The unclassified pattern showed anorectal pressure changes not consistent with any patterns mentioned above[13]. The rectal sensory test was subsequently performed, recording sensory thresholds based on balloon volumes at first constant sensation, desire to defecate, maximum tolerance, and sustained urgency[10].

CTT

Colonic transit time was assessed using radiopaque marker techniques (Sitzmarks; Konsyl Pharmaceuticals, TX, United States). Medications that might affect gastrointestinal transmission were discontinued for 1 wk before and during the CTT study. Patients were instructed to adhere to their regular diet and avoid laxatives throughout the study. The patients ingested a single capsule containing 24 radiopaque markers on day 1, and an abdominal X-ray was obtained 48 h later. The X-ray analysis determined the number and distribution of the markers as per the protocol described by Metcalf *et al* [14]. Spinal processes and imaginary lines from the fifth lumbar vertebra to the pelvic outlets served as landmarks by which the right colon (RC), left colon (LC) and rectosigmoid (RS) colon were defined. Patients were classified as positive for evidence of normal transit constipation (NTC) when less than 10% of the markers were visible throughout the colon at 48 h. Slow transit constipation (STC) was defined as retention of more than 50% of the markers in the RC and LC on imaging. RS accumulation of radiopaque markers (RSARM), defined as retention of more than 50% of the markers in the RS region, suggested the possibility of functional defecation disorders[10,14,15].

Questionnaires

All patients were asked to complete the clinical symptoms questionnaires capturing data such as age, sex, stool frequency, Bristol stool form scale score, abdominal pain, abdominal bloating, relationship between abdominal discomfort and defecation, straining during a bowel movement, feeling of incomplete emptying, sensation that stool cannot be passed and feeling of defecation urgency. The Hamilton anxiety rating scale (HAMA) and the Hamilton Depression Rating Scale (HAMD)-21 were used to evaluate patients' mental health, with higher scores signifying more severe anxiety and depression. A score ranging from 7 to 13 was indicative of possible anxiety, a score ranging from 14 to 20 was indicative of anxiety, and a score ≥ 21 was indicative of severe anxiety. A score ranging from 8 to 19 was indicative of mild depression, a score ranging from 20 to 34 was indicative of moderate depression, and a score ≥ 35 was indicative of severe depression[16,17].

Table 1 Comparison of baseline characteristics between constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C	FC	*P* value
Age (yr)	42.32 ± 14.41	43.95 ± 18.04	> 0.05
Sex			> 0.05
Male	17	8	
Female	84	33	
Stool frequency			> 0.05
> 6 d	23	6	
< 6 d	72	27	
Feeling of incomplete emptying			> 0.05
Yes	76	26	
No	18	13	
Sensation that stool cannot be passed			> 0.05
Yes	54	16	
No	16	11	
Feeling of defecation urgency			> 0.05
Yes	39	7	
No	56	24	
Straining during a bowel movement			> 0.05
Yes	91	28	
No	5	5	

The values are expressed as numbers (*n*) or mean ± SD. IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.

Statistical methods

Baseline demographic, clinical, ARM, and CTT variables were compared between IBS-C patients and FC patients. The proportions of each variable were compared using the chi-square test, while means and medians were evaluated using the Student's *t* test and the Wilcoxon rank-sum test, respectively. The association between variables was determined using the Pearson correlation coefficient. All data were analyzed using SPSS 19.0. $P < 0.05$ was considered statistically significant.

RESULTS

Patient demographics

Our study comprised 230 patients with CC, of whom 149 were diagnosed with IBS-C and 81 with FC. Females represented a larger portion of both groups (82.39%) compared to males (17.61%). No statistically significant differences were observed in terms of sex, age, or abdominal discomfort symptoms between the IBS-C and FC cohorts (Table 1; $P > 0.05$).

Colonic transit test results

IBS-C patients demonstrated a higher prevalence of delayed colonic transit than FC patients (36.63% *vs* 15.91%, $P < 0.05$). Conversely, RSARM was more common among FC patients (50% *vs* 26.73%, $P < 0.05$). No significant differences were observed in the proportions of patients with a normal colon transit time between the two groups (36.63% *vs* 34.09%, $P > 0.05$) (Table 2).

Anorectal motility and sensation

There were no significant differences in the prevalence of dyssynergic patterns (I-IV) observed on HR-ARM between the FC (81%) and IBS-C (85.2%) groups. Moreover, diverse proportions of these dyssynergic patterns were noted within both groups. In FC patients, the type I pattern was most prevalent (41.94%), while the type II pattern was most commonly observed in IBS-C patients (34.55%). The type III pattern was found to be the least common in both groups (3.23% in FC

Table 2 Comparison of psychiatric and colorectal motility characteristics between constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C	FC	*P* value
ARM			
Dyssynergic patterns			> 0.05
I	19	12	
II	18	8	
III	5	1	
IV	14	10	
Normal	6	1	
Unclassified	7	1	
Anorectal sensation thresholds			
First constant sensation			> 0.05
Low	2	1	
High	38	14	
Normal	25	10	
Desire to defecate			> 0.05
Low	30	10	
High	4	1	
Normal	22	10	
Sustained urgency			> 0.05
Low	30	12	
High	4	1	
Normal	29	14	
CTT			< 0.05
STC	37	7	
RS accumulation	27	22	
Normal	37	15	
Psychiatric characteristics			
Scores			> 0.05
HAMA	11.71 ± 9.48	12.00 ± 9.15	
HAMD	11.51 ± 8.61	9.41 ± 0.33	
Anxiety			> 0.05
No	34	13	
Possible	24	7	
Yes	35	13	
Depression			< 0.05
No	31	19	
Possible	46	9	
Yes	15	5	

The values are expressed as numbers (*n*) or mean ± SD. ARM: Anorectal manometry; CTT: Colonic transmit test; IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation; HAMA: Hamilton anxiety rating scale; HAMD: Hamilton Depression Rating Scale.

Table 3 Comparison between psychiatric and colorectal motility characteristics in constipation-predominant irritable bowel syndrome and functional constipation patients

Variable	IBS-C				FC			
	Anxiety	Without anxiety	Depression	Without depression	Anxiety	Without anxiety	Depression	Without depression
STC	21	16	25	12	4	1	3	2
NTC	24	6	21	9	8	3	5	6
First sensation								
High	26	8	21	11	10	1	5	6
Normal	14	9	11	12	4	5	4	5
Desire to[a]								
Low	19	11	13	16	6	4	6	4
Normal	16	6	15	6	8	2	4	6
High	4	0	3	1	1	0	0	1
Urgency								
Low	15	12	14	12	4	4	5	3
Normal	21	5	15	10	10	2	5	7
High	3	0	2	1	1	0	0	1

[a]$\chi^2 = 4.054$, $P < 0.05$ for the association of the threshold of desire to defecate and depression in constipation-predominant irritable bowel syndrome patients. The values are expressed as numbers (*n*). STC: Slow transmit constipation; NTC: Normal transmit constipation; IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.

and 10.91% in IBS-C). Although a marginal increase in the prevalence of type II and type IV patterns was noticed in FC patients compared to IBS-C patients, the difference was not statistically significant (56.3% *vs* 55.9%, $P > 0.05$).

In the assessment of sensory thresholds, a majority of both IBS-C (58.46%) and FC (56%) patients exhibited high thresholds for the first constant sensation. Low thresholds for the desire to defecate were more commonly observed in IBS-C patients (53.57%) and in approximately half of the FC patients (47.62%). Slightly higher proportions of high thresholds for the first constant sensation, low thresholds for the desire to defecate, and anxiety or potential anxiety (63.44% *vs* 60.61%) were observed in IBS-C patients compared to FC patients, although these differences lacked statistical significance ($P > 0.05$) (Table 3).

Psychiatric characteristics

A significantly higher prevalence of depression was observed among IBS-C patients compared to their FC counterparts (66.30% *vs* 42.42%, $P < 0.05$). Although the incidence of anxiety was slightly higher among IBS-C patients, this difference was not statistically significant (63.44% *vs* 60.61%, $P > 0.05$). The mean scores for HAMA and HAMD were similar for both groups (Table 1; $P > 0.05$). However, IBS-C patients reported significantly higher scores for specific symptoms, such as feelings of guilt, suicidal ideation, psychomotor agitation, diurnal variation, obsessive-compulsive tendencies, hopelessness, self-abasement, and gastrointestinal symptoms (Figure 1; $P < 0.05$).

Correlation between psychiatric and colorectal motility characteristics

In both the IBS-C and FC patient cohorts, a significant percentage of individuals with NTC or STC demonstrated symptoms of anxiety or depression. However, the incidence of depression was comparable among patients with NTC and STC (70% *vs* 67.57% for IBS-C, 45.45% *vs* 60% for FC, $P > 0.05$). Interestingly, in the IBS-C group (but not the FC group), patients with NTC exhibited a notably higher prevalence of anxiety than those with STC (80% *vs* 56.76%, $\chi^2 = 5.438$, $P < 0.05$).

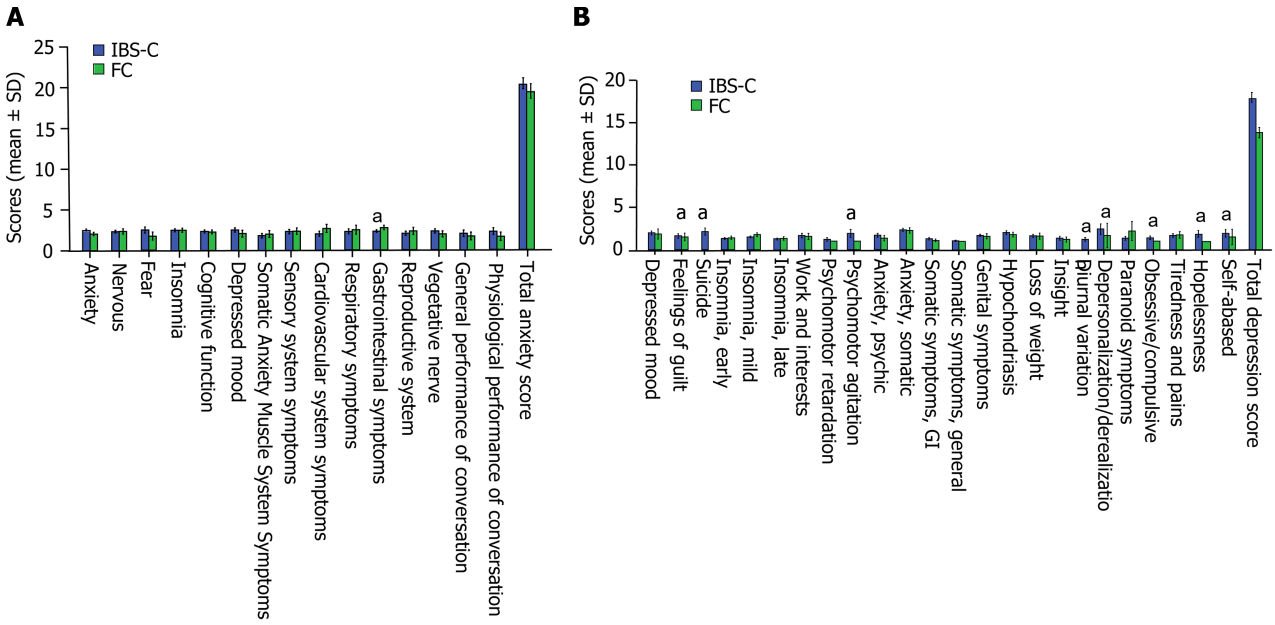
In the IBS-C group, again not observed in the FC group, weak correlations were identified between the degrees of anxiety and the thresholds for first constant sensation, desire to defecate, and sustained urgency ($r = 0.414$, $r = 0.404$, and $r = 0.418$, respectively, $P < 0.05$). Furthermore, IBS-C patients suffering from depression exhibited a lower prevalence of low thresholds for the desire to defecate than those without depression (69.6% *vs* 41.9%, $\chi^2 = 4.054$, $P < 0.05$). No substantial correlation was observed between depression and other anorectal sensations ($P > 0.05$) (Table 3 and 4).

The probability of normal patterns in ARM was 9.68% for patients who exhibited RSARM during CTT tests. Conversely, a 16.67% probability of RSARM was found in patients devoid of type I-IV patterns. RSARM was prominent in 77.78% of FC patients with a type IV pattern, while only 16.67% of IBS-C patients with a type I pattern exhibited RSARM. Notably, all patients with a type III pattern demonstrated RSARM in both the IBS-C and FC cohorts.

Table 4 Correlations of psychiatric characteristics and anorectal sensation thresholds in constipation-predominant irritable bowel syndrome and functional constipation patients

		Thresholds of first sensation				Thresholds of desire to defecate					Thresholds of urgency				
		High	Normal	*r*	*P* value	Low	Normal	High	*r*	*P* value	Low	Normal	High	*r*	*P* value
IBS-C	Anxiety degree			0.414	< 0.05				0.404	< 0.05				0.407	< 0.05
	Depression degrees			0.803	> 0.05				0.019	> 0.05				-0.018	> 0.05
FC	Anxiety degree			0.241	> 0.05				0.111	> 0.05				0.242	> 0.05
	Depression degrees			-0.098	> 0.05				-0.166	> 0.05				-0.233	> 0.05

IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation.



DOI: 10.3748/wjg.v29.i41.5657

Figure 1 Comparison of psychological symptom scores between constipation-predominant irritable bowel syndrome and functional constipation patients. A: Hamilton anxiety rating scale; B: Hamilton Depression Rating Scale-21. $^{a}P < 0.05$ for comparison between the functional constipation and constipation-predominant irritable bowel syndrome groups. IBS-C: Constipation-predominant irritable bowel syndrome; FC: Functional constipation; GI: Gastrointestinal.

DISCUSSION

We observed high proportions of dyssynergic patterns in both IBS-C and FC patients. Interestingly, these patterns also occurred in a substantial proportion of asymptomatic individuals, potentially due to the unnatural posture adopted during simulated defecation[18]. A previous study by Grossi *et al*[19] suggested that the type IV pattern could be helpful for differentiating FC patients from healthy volunteers, with rectal pressure measurements proving more indicative than anal pressure measurements. Furthermore, a study conducted in India highlighted a higher prevalence of insufficient rectal force in FC patients than in those with IBS-C[9]. Consistent with these findings, our study detected a marginal increase in the incidence of insufficient rectal force and type IV dyssynergia among FC patients compared with their IBS-C counterparts, although these differences were not statistically significant. The potential for abnormal rectal pressure shifts during a push maneuver to serve as an effective discriminator between IBS-C or FC and a healthy state within the Chinese population requires further investigation.

Beyond the evaluation of anorectal pressure, ARM also offers the opportunity to gather additional physiological data relating to anorectal sensation[20]. Abnormal visceral sensitivity has been linked with intestinal dysfunction. It is common for constipation patients to exhibit rectal hyposensitivity, whereas rectal hypersensitivity often accompanies IBS [3,4]. In our study, we observed a substantial proportion of both IBS-C and FC patients displaying high thresholds for

initial sensation, potentially contributing to fecal retention. This occurrence was slightly more prevalent in IBS-C patients than in FC patients; however, the difference between the two patient cohorts was not statistically significant.

Psychiatric conditions have been suggested to influence constipation[5]. In our study, we observed a higher incidence of depression among IBS-C patients than among FC patients, a finding consistent with prior studies conducted in China [21]. Moreover, our results showed a positive correlation between anxiety levels and anorectal sensory thresholds in IBS-C patients, suggesting that elevated anxiety may decrease rectal sensitivity, potentially leading to constipation in this patient group.

Impaired peristaltic motility within the intestine is another potential factor contributing to CC. Utilizing radiopaque markers, we evaluated colonic transit in our study population. Our results showed comparable proportions of NTC in IBS-C and FC patients and an increased prevalence of STC among IBS-C patients. Previous studies reported varied findings regarding the prevalence of STC and NTC in IBS-C and FC patients. For example, research by Lam *et al*[22] indicated that STC was more common in FC patients than in IBS-C patients, a finding corroborated by Patcharatrakul and Gonlachanvit[23]. Conversely, Shekhar *et al*[24] did not observe a significant difference in STC prevalence between the two patient groups. The discrepancies in these findings may be attributable to variations in diet, ethnicity, or methodology across studies. Psychological stress may also affect colonic motor activity[25]. We further explored the relationship between CTT and psychological stress. Our data showed that IBS-C patients with NTC were more likely to experience anxiety compared to those with STC. However, we found no significant correlations between psychological stress and colonic motility in FC patients. This difference indicates that emotional factors may have varying effects on colonic motility between these two patient groups.

Dyssynergic defecation (DD) is a condition characterized by a patient's inability to effectively coordinate abdominal and pelvic floor muscles to eliminate stool, commonly seen in functional defecatory disorders. According to the ROME IV classification, DD can be diagnosed in both FC and IBS-C patients. Given the high diagnostic sensitivity of ARM for DD, a normal ARM result can be quite effective in excluding DD[26]. Radiopaque transit studies not only assess colonic transit but can also indicate outlet obstruction based on marker accumulation in the RS region. RSARM has been proven to distinguish DD from STC and NTC, with specificity ranging between 81.2% and 88.2%[27-29]. In our study, most CC patients with normal ARM showed no RS marker accumulation during CTT, with two IBS-C patients being exceptions. We hypothesize that these two patients might have structural anorectal abnormalities causing difficulty in eliminating the radiopaque markers. Alternatively, overlapping colon segments might have made marker separation challenging, potentially leading to classification errors. The relationship between DD and IBS-C and FC is currently a subject of debate. Our study indicated a higher prevalence of RSARM and elevated anal resting pressure in FC patients than in IBS-C patients. High resting pressures associated with anal dyssynergia have been proposed as a useful diagnostic tool to distinguish DD patients from healthy individuals[19,30]. Hence, our findings suggest that DD might be more prevalent among Chinese FC patients. Furthermore, we observed that nearly half of the FC patients with RSARM exhibited type IV dyssynergia, nearly double the prevalence of IBS-C patients, implying distinct pathogeneses of DD in FC and IBS-C patients.

Recognizing DD is crucial because it is a substantial indicator that patients may benefit from biofeedback therapy[13]. The Rome IV criteria define DD according to CC symptoms and at least two abnormal anorectal tests, such as BET, ARM, or defecography. However, no single method is sufficient to diagnose DD[20,31-33]. It is important to note that many institutions may not have access to these diagnostic tools. In our study, we found that most FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia displayed RSARM. Given that RSARM indicates a possibility of DD, we hypothesize that FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia are more likely to have DD. Consequently, further BET or defecography might not be necessary for these patients. However, the exact mechanisms underlying the absence of RSARM in most IBS-C patients with type I or II dyssynergia remain elusive. Although RSARM shows a fair correlation with DD, the absence of markers in the RS region does not conclusively rule out DD due to high false-negative rates[31]. Therefore, type I or II dyssynergia cannot rule out the need for CTT in CC patients. On the other hand, it might not be necessary for FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia to undergo CTT. Although RSARM can somewhat differentiate STC from DD, it cannot distinguish NTC from DD[29,34]. Therefore, further BET or defecography may still be necessary in all aforementioned scenarios.

Our study has several limitations. The clinical features of hospital-based constipation patients are somewhat different from those of community-based patients[35]. As all patients were from one tertiary hospital, our results may represent more severe cases of constipation, and these psychosocial profiles and colorectal motility patterns may not be generalizable to a broader population. Future studies adding community-based patients will be more informative. Deeper and wider psychosocial studies should also be performed in the future. Additionally, our sample size was relatively small, which may have limited our ability to detect significant differences.

CONCLUSION

In conclusion, our study revealed elements that can distinguish between FC and IBS-C across multifaceted parameters, namely colonic transmit time, psychological distress, the association between colonic transit and psychiatric conditions, variations in dyssynergic patterns, and the relationship between RSARM and these patterns. Clinical manifestations, manometric evidence of dyssynergia, and elevated thresholds for first sensation, although valuable, have limited discriminatory power in distinguishing FC from IBS-C. These findings extend our comprehension of the intricate pathophysiological mechanisms underlying FC and IBS-C and could potentially guide the formulation of more precise diagnostic protocols and personalized treatment methodologies.

ARTICLE HIGHLIGHTS

Research background

The comparation of colorectal motility, psychiatric features, and the association of colorectal motility patterns and psychiatric traits between functional constipation (FC) and constipation-predominant irritable bowel syndrome (IBS-C) groups, especially in the Chinese population has not been fully studied.

Research motivation

Controversy persists regarding the correlation between high-resolution anorectal manometry (HR-ARM) and the colonic transmit test (CTT), and it remains unclear which test provides more meaningful data for IBS-C or FC.

Research objectives

We aimed to compare the psychiatric and colorectal motility characteristics between FC and IBS-C patients in an Eastern Chinese population. We also sought to investigate the correlations between psychiatric and colorectal motility characteristics in both FC and IBS-C patients.

Research methods

Colorectal motility patterns were obtained by HR-ARM and CTT. Anxiety and depression were assessed by the Hamilton anxiety rating scale (HAMA) and the Hamilton Depression Rating Scale (HAMD)-21.

Research results

Our study indicated a higher prevalence of rectosigmoid accumulation of radiopaque markers (RSARM) and elevated anal resting pressure in FC patients compared to IBS-C patients. Furthermore, we observed that nearly half of the FC patients with RSARM exhibited type IV dyssynergia, a prevalence nearly double that of IBS-C patients. Our data also showed that IBS-C patients with normal transit time were more likely to experience anxiety compared to those with slow transit time. However, we found no significant correlations between psychological stress and colonic motility in FC patients. FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia are more likely to have dyssynergic defecation. Type I or II dyssynergia cannot rule out the need for CTT in chronic constipation patients, while it might not be necessary for FC patients with type IV dyssynergia and IBS-C patients with type III dyssynergia to undergo CTT, but further balloon expulsion test or defecography might still be necessary.

Research conclusions

The associations of psychological stress and colonic motility in our study are discrepant from results of Western studies, indicating that emotional factors may have varying effects on colonic motility between these two patient groups. The associations we found between CTT results and dyssynergia patterns by ARM could provide guidance for different constipation groups to choose appropriate colorectal tests.

Research perspectives

We compared not only colorectal motility and psychiatric features, but also the correlations between psychiatric and colorectal motility characteristics in FC and IBS-C patients. What we found could provide guidance for constipation patients to choose appropriate colorectal tests.

FOOTNOTES

Author contributions: Yu Y designed the study and critically revised the article for important intellectual content; Lv CL participated in the analysis and interpretation of the data, and drafted the initial manuscript; Song GQ participated in project supervision and editing the manuscript; Liu J, Wang W, Huang YZ, Wang B, Tian JS, and Yin MQ participated in acquisition of the data.

Supported by the External Science and Technology Cooperation Planning Projects of Anhui Province of China, No. 1604b060202.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Anhui Provincial Hospital.

Informed consent statement: As approved by the Ethics Board, informed consent was not required for this study.

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

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: China

ORCID number: Chao-Lan Lv 0000-0002-8698-9344; Geng-Qing Song 0000-0001-5551-1818; Jie Liu 0000-0001-6079-7566; Wei Wang 0000-0003-3063-3557; Yi-Zhou Huang 0000-0002-6125-195X; Bo Wang 0009-0009-3858-3540; Jia-Shuang Tian 0009-0000-6066-7665; Yue Yu 0000-0002-3617-2037.

S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Chen YX

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5668-5682

DOI: 10.3748/wjg.v29.i41.5668

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Inflammatory bowel diseases patients suffer from significant low levels and barriers to physical activity: The "BE-FIT-IBD" study

Antonietta Gerarda Gravina, Raffaele Pellegrino, Tommaso Durante, Giovanna Palladino, Rossella D'Onofrio, Simone Mammone, Giusi Arboretto, Salvatore Auletta, Giuseppe Imperio, Andrea Ventura, Mario Romeo, Alessandro Federico

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Sipos F, Hungary; Wang LH, China; Ferenc Sipos, Hungary

Received: August 16, 2023
Peer-review started: August 16, 2023
First decision: October 8, 2023
Revised: October 9, 2023
Accepted: October 23, 2023
Article in press: October 23, 2023
Published online: November 7, 2023

Antonietta Gerarda Gravina, Raffaele Pellegrino, Giovanna Palladino, Rossella D'Onofrio, Simone Mammone, Giusi Arboretto, Salvatore Auletta, Giuseppe Imperio, Andrea Ventura, Mario Romeo, Alessandro Federico, Department of Precision Medicine, Hepatogastroenterology Unit, University of Campania Luigi Vanvitelli, Naples 80138, Italy

Tommaso Durante, Mental Health Department, S. Pio Hospital, Benevento 82100, Italy

Corresponding author: Raffaele Pellegrino, MD, Department of Precision Medicine, Hepatogastroenterology Unit, University of Campania Luigi Vanvitelli, Via L. de Crecchio, Naples 80138, Italy. raffaele.pellegrino@unicampania.it

Abstract

BACKGROUND

The place regular physical activity (PA) should occupy in managing patients with inflammatory bowel diseases (IBD) is unclear.

AIM

To assess PA levels and barriers in a southern Italian IBD population.

METHODS

IBD patients with non-severe disease activity [assessed with partial Mayo score for ulcerative colitis (UC) and Harvey-Bradshaw index for Crohn's disease] were approached to receive an anonymous online questionnaire to assess PA levels using the International Physical Activity Questionnaire (IPAQ) and to assess disease activity as patient-reported outcomes 2 (PRO-2) and finally to assess habits, beliefs and barriers in conducting regular PA. Clinical, anthropometric and demographic data of patients were also collected. PA was expressed as continuous units of resting metabolic rate (Met) in min/wk. Three PA groups were identified: Inactive (< 700 Met min/wk), sufficiently active (700-2500 Met min/wk) and health enhancing PA (HEPA) (*i.e.*, HEPA active, > 2500 Met min/wk) patients.

RESULTS

Included patients (219) showed overall PA levels of 834.5 Met min/wk, with a large proportion (94, 42.9%) classified as inactive while only a minority (9, 4.1%)

as health-enhancing PA. Patients without dyslipidaemia ($P < 0.0001$) or on biologics therapy ($P = 0.022$) showed better IPAQ scores in moderate activities. UC PRO-2 correlated negatively with IPAQ intense activities scores ($\tau = -0.156$, $P = 0.038$). PRO-2 did not show notable sensitivity/specificity in predicting IPAQ inactivity (AUC < 0.6). IBD activity did not differ between active and inactive patients ($P > 0.05$). Active patients expressed the need to discuss PA with their gastroenterologist. Some barriers (*e.g.*, diagnosis of IBD and fear of flare-ups after PA) are significantly more reported by inactive patients.

CONCLUSION

A significant rate of physical inactivity was recorded in this setting. IPAQ showed good feasibility. PA should be an element of discussion in IBD visits assessed quickly with non-invasive questionnaires.

Key Words: Crohn's disease; Inflammatory bowel disease; International Physical Activity Questionnaire; Physical activity; Ulcerative colitis

Core Tip: The place regular physical activity (PA) should occupy in managing patients with inflammatory bowel diseases (IBD) is unclear. IBD patients were approached to receive an anonymous questionnaire to assess PA levels using the International Physical Activity Questionnaire (IPAQ), assess disease activity as patient-reported outcomes 2, and assess habits, beliefs and barriers in conducting regular PA. A large proportion of included IBD patients were classified as inactive. Patients on biologics therapy showed better IPAQ scores in moderate activities. Inactive patients report some barriers. PA should be an element of discussion in IBD gastroenterology visits.

Citation: Gravina AG, Pellegrino R, Durante T, Palladino G, D'Onofrio R, Mammone S, Arboretto G, Auletta S, Imperio G, Ventura A, Romeo M, Federico A. Inflammatory bowel diseases patients suffer from significant low levels and barriers to physical activity: The "BE-FIT-IBD" study. *World J Gastroenterol* 2023; 29(41): 5668-5682
URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5668.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5668

INTRODUCTION

Inflammatory bowel diseases (IBD), mainly comprising Crohn's disease (CD) and ulcerative colitis (UC), underlie sustained and chronic gastrointestinal inflammation[1] associated with varying disabilities, including those in the psychological sphere[2,3], impacting patients' quality of life (QoL)[4]. Physical activity (PA) is part of and positively affects QoL [5]. A recent consensus encouraged regular PA (consistent with individual tolerance level) to ameliorate the IBD course [6]. PA is, by definition, the use of skeletal muscles with energy expenditure and can promote an anti-inflammatory phenotype in various tissues (such as muscle, adipose tissue, and heart)[7]. It is also widely framed as a modifiable risk factor for several chronic diseases, such as cardiovascular and metabolic as well as neoplastic ones and, in some, such as rheumatoid arthritis, has even shown the ability to associate with a milder disease course[8].

Moreover, low-intensity PA can positively impact mild/remission CD patients' QoL[9], and no change in CD activity nor predisposition to flare-ups had been observed[10]. Finally, a further study on the IBD population also showed how higher PA associates with better QoL, excluding sweat-inducing exercises[11].

In addition, IBD patients seem less likely to perform PA, and, despite the potential benefits of it, precise recommendations and guidelines on how to approach this subject in IBD have not yet been codified[12]. IBD patients often suffer from sarcopenia, which is, among other things, also a predictive factor for the need to incur surgery and worsening the risk of postoperative complications[13]. PA improves muscle mass and poses as an ameliorative measure of sarcopenia [14,15]. In addition, the World Health Organization recommends, generally for every age group as well as for adults with disabilities, regular PA stigmatizing its multidimensional benefits (from cardioprotective effects to beneficial psychological effects)[16].

Ultimately, the epidemiologic data regarding PA levels in IBD is severely lacking. The barriers that block patients from practising regular PA and what factors instead are facilitators of this are unknown. Even less evidence is definitive on what impact PA (splitting the data even by intensity) may have on IBD activity. Therefore, this study aimed to assess self-reported PA levels in an IBD population and to examine whether there are differences in that setting concerning clinical/demographic, patient-reported IBD activity and reported barriers to regular PA.

MATERIALS AND METHODS

Study design

This cross-sectional observational study was conducted at the Hepatogastroenterology Unit of the University of Campania Luigi Vanvitelli in the first half of 2023. IBD participants included were given an online questionnaire to fill out anonymously. This study was written following the "strengthening the reporting of observational studies in epidemiology" (*i.e.*, STROBE) checklist. The study was conducted in compliance with the Declaration of Helsinki and received approval from the Ethics Committee of the University of Campania Luigi Vanvitelli (protocol number 7892, 15 March 2023).

Inclusion and exclusion criteria

Patients with an established histologic diagnosis of IBD (*i.e.*, CD or UC) were included. Patients with known psychiatric conditions and severe comorbidities, recent surgery, clinically significant infection (*e.g.*, *Clostridioides difficile*), hospitalised or who had received contraindications to performing any form and degree of PA were, instead, excluded. In addition, patients with severe disease activity assessed (within one month before the inclusion) by partial Mayo score[17] for UC patients *(i.e.*, score > 7) and by Harvey-Bradshaw index for CD patients (*i.e.*, score > 16)[18] were also excluded.

Collected variables

Through the questionnaire, several variables were collected. First, demographic and anthropometric data were, in detail, collected, such as sex, age, weight (in Kg), height (in cm), body mass index (BMI) (in Kg/m^2), level of education, employment, smoking status, alcohol consumption (the patient was defined as an alcohol user if daily consumption was ≥ 20 g in female or ≥ 30 g in the male)[19] as well as, finally, having or not having a partner. Concerning IBD, the type (*i.e.*, CD or UC), age at diagnosis of IBD, the current treatments, and previous biologic failure were collected. IBD disease activity was assessed with patient-reported outcomes 2 (PRO-2) for both CD[20] and UC[21]. Therefore, the sub-score on stool frequency (SF) and abdominal pain (AP) for patients with CD was evaluated. On the other hand, the subscore, SF and that on rectal bleeding (RB) were examined for UC patients.

Finally, patients were also asked whether they had comorbidities (such as diabetes, hypertension, nephropathies, dyslipidaemia, or pneumopathies) or extra-intestinal manifestations.

PA Assessment

The international PA questionnaire (IPAQ) showed good validity and reliability characteristics[22,23] and was used to assess PA in this study. IPAQ evaluate the PA type and amount performed by the compiler by referring to the past 7 d. IPAQ contemplates intense activities (such as aerobic activities like running), moderate activities (such as carrying light weights) and mild activities (walking for at least 10 min). IPAQ identifies three categories of respondents based on PA levels: Type 1 (*i.e.*, inactive), type 2 (*i.e.*, minimally active), and, finally, type 3, also defined as health enhancing PA (HEPA) (*i.e.*, HEPA active).

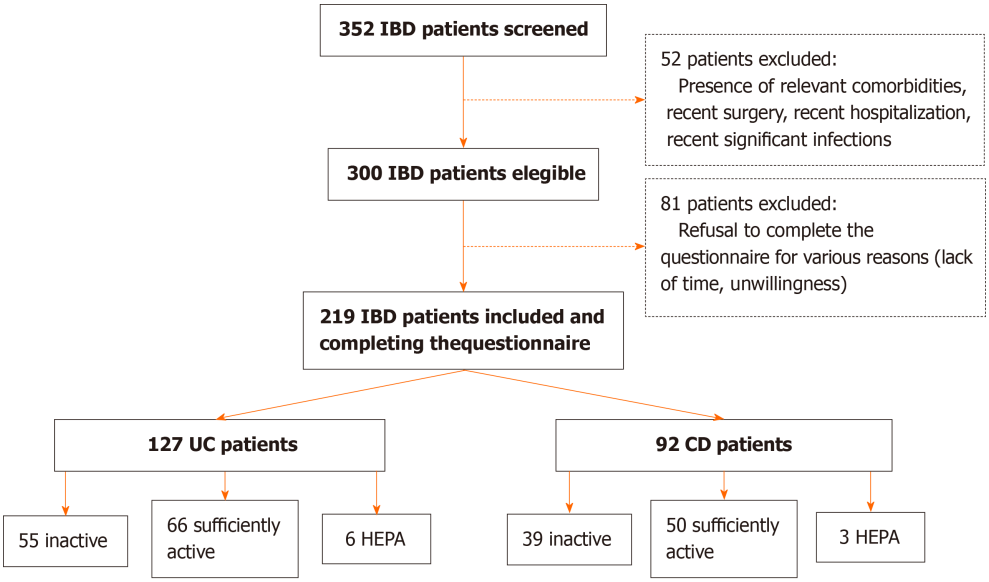
The IPAQ score was made continuous using multiples of the resting metabolic rate [*i.e.*, metabolic rate (Met)] as units. Therefore, the Met of PA was calculated by level and specifically for intense (minutes × days × 8 Met), moderate (minutes × days × 4 Met) and, finally, for mild/walking (minutes × days × X Met) activities. The value of X for the last activities is a function of a multiplier based on the steep grade. Specifically, for an intense stride that gave the compiler a marked perceived increase in respiratory rate relative to normal, the multiplier is 3.3; for a moderate stride that increased respiratory rate at a rate only moderately higher than usual, the multiplier is 3 while, finally, for a slow stride with no change in respiratory rate the multiplier is 2.5. PA levels were, therefore, ultimately expressed as Met min/wk. IPAQ has, moreover, already been employed in IBD[24]. IPAQ was scored according to the available guidelines (http://www.ipaq.ki.se/) using the Italian-validated IPAQ version[25]. Patients were finally considered inactive (< 700 Met min/wk), sufficiently active (700-2500 Met min/wk), or active/HEPA (> 2500 Met min/wk)[23,26].

In addition, the link provided to patients included additional questions to weigh the possible presence of barriers/facilitators to performing regular PA. These questions associate with five levels of agreement (with extremes from completely agree to disagree) responses. Finally, an 11-point Likert scale question was administered to understand how important the patient thought it was from 0 to 10 to discuss PA with their gastroenterologist during outpatient visits.

Statistical analysis

Descriptive statistics were used for data presentation. Continuous variables are presented as a median and relative interquartile range, while categorical and ordinal variables as a percentage of the total (%) for each degree of freedom. The Kolmogorov-Smirnov test preliminarily evaluated variables distribution to choose between parametric and nonparametric analyses for data analysis according to study outcomes. The Chi-square and Fisher's exact test were used for the relationship between categorical variables. The Mann-Whitney U-test compared ordinal continuous variables with two-level categorical independent variables. In the case of ordinal variables with multiple degrees of freedom, Kruskal-Wallis's test was used instead. The strength of correlations between the variables of interest was probed with Kendall's tau-b test. If it was necessary to categorize PA levels dichotomously (active/inactive), that of 699 Met wk/min (according to IPAQ scoring) was chosen as the threshold, defining active as those who had a PA > of this threshold.

To evaluate the predictors of physical inactivity, the independent variables of the implemented logistic regression model coincided with the other continuous and/or categorical variables deemed relevant. The regression model was evaluated according to the goodness of fit according to Hosmer-Lemeshow (as well as according to Cox and Snell R^2 and Nagelkerke R^2 values) by expressing the data as an exponential value of B, *i.e.*, exp (B). The latter was presented as the



DOI: 10.3748/wjg.v29.i41.5668

Figure 1 Flow chart summarizing the inclusion in the study patients, divided by type of inflammatory bowel disease and resulting physical activity levels. HEPA: Health enhancing physical activity; IBD: Inflammatory bowel disease; CD: Crohn's disease.

odds ratio, and the risk measure was expressed as the OR and its 95% confidence interval (95%CI).

The receiver operating characteristic (ROC) method was used to weigh the specificity/sensitivity of any variables regarding PA levels. These assessments were performed after checking for the existence of an adequate area under the ROC curve (AUC) > 0.699, which was calculated along with its 95%CI.

To evaluate the internal reliability of the questions in our survey to assess patient barriers to PA, we analysed Cronbach's alpha coefficient, recording a value of 0.7. In addition, a statistical significance value was accepted for $P < 0.05$ (two-tailed) values, placing an alpha error of 0.05. Statistical analyses were performed with IBM® SPSS® software, graphs with GraphPad PRISM®, and sample size calculation with G*Power software.

RESULTS

Sample characteristics

Figure 1 describes the steps for the enrolment of all patients. Two hundred nineteen patients were finally included, and Table 1 summarizes their characteristics by stratifying by type of IBD. Most of the sample, 127 (58%) patients, had UC.

Some differences emerged between IBD subgroups. CD patients had a higher rate of biologics use than the UC ones (*i.e.*, 82.6% *vs* 61.4%, $P = 0.001$) and unemployment (60.9% *vs* 40.2%, $P = 0.009$). In addition, females had a significantly lower age than males [38 (28-50) *vs* 45 (31-56), $P = 0.017$].

Most of the sample was on subcutaneous biologic drug therapy (113, 51.6%), while a minority were taking intravenous (24, 11%) or oral (17, 7.8%) biological treatment, while the remainder (65, 29.7%) were not taking biologics. The overall failure rate of a previous biologic was 21.5% (47/219).

PA levels

Most of the sample (116, 53%) met the IPAQ criteria for sufficiently active, while only a minority (9, 4.1%) met the criteria for HEPA activity. On the contrary, a large sample portion was classified as inactive (94, 42.9%). The overall IPAQ total score was 834.5 (384.5 – 1424) Met min/wk. Gender did not seem particularly impactful concerning PA (Figure 2A). The other variables in Table 1 showed no variations when stratified by PA grade (Table 2). IBD type did not result in variations in PA levels (see Table 3 and Figure 2B) since both the type of PA (*i.e.*, intense, moderate, or mild) and the class of PA (*i.e.*, inactive, sufficiently or HEPA active) did not vary particularly differentially between CD and UC patients. In addition, the comorbidities most represented in our sample, hypertension ($P = 0.095$), arthritis ($P = 0.101$), or Hashimoto's thyroiditis ($P = 0.540$), did not particularly impact IPAQ total score levels. In contrast, PA levels differed according to dyslipidaemia ($P < 0.0001$). In detail, dyslipidaemia patients presented higher [956 (325 - 1622)] levels of PA than those without dyslipidaemia [811 (393.75 - 1358.77)]. However, in contrast, the moderate activity score was higher in healthy patients than in those with dyslipidaemia [176 (0-567) *vs* 160 (0-480), $P < 0.0001$].

Patients on biological therapy showed some advantage over those on standard therapy [246 (0-642) *vs* 56 (0-394), $P = 0.022$], as shown in Figure 2C. At bivariate analysis, neither age ($\tau = -0.27$, $P = 0.550$) nor BMI ($\tau = 0.75$, $P = 0.100$) showed correlations with IPAQ total score.

Table 1 Sample characteristics concerning the type of inflammatory bowel disease

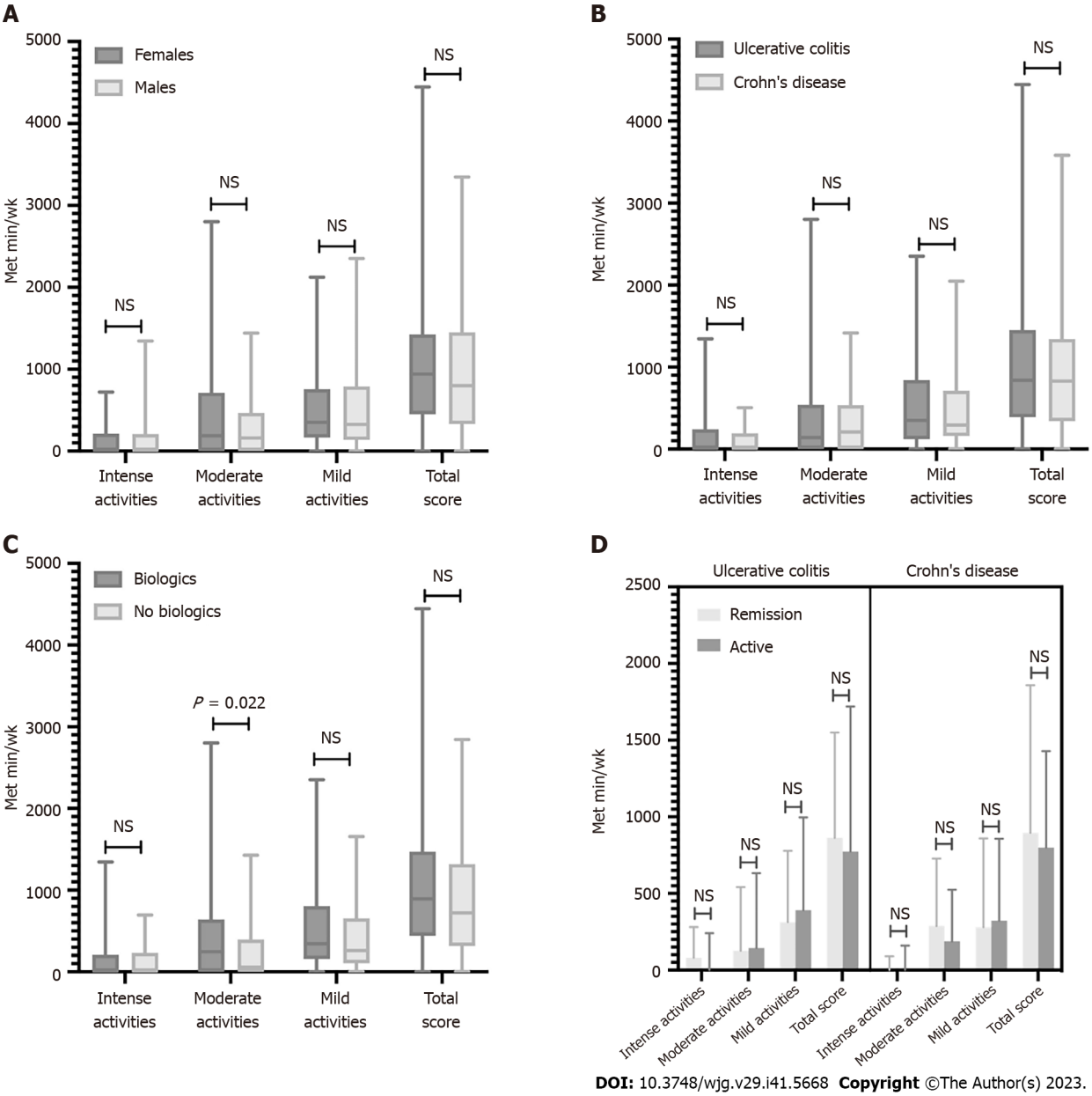
Variable	Crohn's disease (*n* = 92)	Ulcerative colitis (*n* = 127)	*P* value[1]
Age	43.5 (29-53)	41 (30-55)	0.871
BMI	23.8 (20.8-26.4)	23.8 (21.4-25.9)	0.787
Gender			0.157[2]
Male	44 (47.8%)	73 (57.5%)	
Female	48 (52.5%)	54 (42.5%)	
Education			0.192
Primary school	32 (24.8%)	27 (21.3%)	
Secondary school	52 (56.5%)	78 (61.4%)	
Degree	8 (8.7%)	22 (17.3%)	
Job			**0.009**
Unemployed	56 (60.9%)	51 (40.2%)	
Employee	16 (17.4%)	38 (29.9%)	
Entrepreneur	8 (8.7%)	16 (12.6%)	
Worker	4 (4.3%)	7 (5.5%)	
Student	8 (8.7%)	15 (11.8%)	
Smoking status			**0.003**
Active smoker	16 (17.4%)	20 (15.7%)	
Past smoker	32 (34.8%)	16 (12.6%)	
Non-smoker	44 (47.8%)	91 (71.7%)	
Alcohol consumer			0.827[2]
Yes	8 (8.7%)	10 (7.9%)	
No	84 (91.4%)	117 (92.1%)	
Comorbidity			0.256
Diabetes	8 (8.7%)	4 (3.1%)	
Hypertension	20 (21.7%)	10 (7.9%)	
Recurrent UTI	2 (2.2%)	4 (3.1%)	
Chronic renal failure	1 (1.1%)	2 (1.6%)	
Nephrolithiasis	3 (3.3%)	1 (0.8%)	
Asthma	3 (3.3%)	-	
COPD	2 (2.2%)	1 (0.8%)	
Previous pneumonia	1 (1.1%)	1 (0.8%)	
Dyslipidaemia	12 (13%)	35 (27.6%)	
Arthritis	32 (34.8%)	34 (26.8%)	
Hashimoto's thyroiditis	7 (7.6%)	15 (11.8%)	
Partner			0.322[2]
Yes	68 (73.9%)	86 (67.7%)	
No	24 (26.1%)	41 (32.3%)	
Biologics (yes)	76 (82.6%)	78 (61.4%)	**0.001**[2]
Steroids (yes)	4 (4.3%)	8 (6.3%)	0.531[2]

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, Crohn's disease and

ulcerative colitis).

[2]The Chi-square test or Fisher's exact test was employed for evaluation.

BMI: Body mass index; COPD: Chronic obstructive pulmonary disease. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%). Significant *P* values are indicated in bold.



DOI: 10.3748/wjg.v29.i41.5668

Figure 2 Physical activity levels in the main subgroups examined. A-D: Physical activity levels observed in males and females (A), patients with ulcerative colitis and Crohn's disease (B), patients on treatment and not on biologics (C), and, finally, patients physically active or inactive concerning baseline disease activity (D). Met: Metabolic rate; NS: Not significant.

In this work, we detected a significantly higher unemployment rate in patients with CD on bivariate analysis. This finding led us to consider whether work occupation could impact PA levels. By processing the specific variable categorically over the entire sample (employed/unemployed), PA levels (as IPAQ total score) were not found to be impacted by employment rate (P = 0.851). This trend was also preserved when filtering by IBD type in both UC (P = 0.654) and CD (P = 0.481). Furthermore, the result was also maintained by comparing the employment and PA rates using the Chi-square test (χ^2 = 0.321, P = 0.588).

Disease activity and PA levels

PA levels (*i.e.*, as IPAQ total score in Met min/wk) were not different concerning PRO-2 measured IBD activity. In detail, this trend was confirmed by stratifying by PA intensity (*i.e.*, intense, moderate and mild/walking) and total score (Figure 2D). PRO-2 data are summarized in Table 4 and related to the PA intensity.

Table 2 Sample characteristics concerning the levels of physical activity

Variable	Physically active (*n* = 125)	Physically inactive (*n* = 94)	*P* value[1]
IBD			0.892[2]
Crohn's disease	53 (42.4%)	39 (41.5%)	
Ulcerative colitis	72 (57.6%)	55 (58.5%)	
Age	39 (29.5-52)	44 (29-55.25)	0.506
BMI	24.25 (21.47-26.28)	22.72 (20.95-25.71)	0.185
Gender			0.952[2]
Male	67 (53.6%)	50 (53.2%)	
Female	58 (46.4%)	44 (46.8%)	
Education			0.903
Primary school	34 (27.2%)	25 (26.6%)	
Secondary school	75 (60%)	55 (58.5%)	
Degree	16 (12.8%)	14 (14.9%)	
Job			0.432
Unemployed	59 (47.2%)	48 (51.1%)	
Employee	30 (24%)	24 (25.5%)	
Entrepreneur	15 (12%)	9 (9.6%)	
Worker	6 (4.8%)	5 (5.3%)	
Student	15 (12%)	8 (8.5%)	
Smoking status			0.607
Active smoker	21 (16.8%)	15 (16%)	
Past smoker	29 (23.2%)	19 (20.2%)	
Non-smoker	75 (60%)	60 (63.8%)	
Alcohol consumer			0.892[2]
Yes	10 (8%)	4 (4.3%)	
No	115 (92%)	90 (95.7%)	
Comorbidity			0.899
Diabetes	8 (6.4%)	4 (4.3%)	
Hypertension	20 (16%)	10 (10.6%)	
Recurrent UTI	3 (3.2%)	2 (2.1%)	
Chronic renal failure	-	1 (1.1%)	
Nephrolithiasis	3 (2.4%)	2 (2.1%)	
Asthma	2 (1.6%)	2 (2.1%)	
COPD	1 (0.8%)	1 (1.1%)	
Previous pneumonia	2 (1.6%)	-	
Dyslipidaemia	28 (22.4%)	19 (20.2%)	
Arthritis	32 (25.6%)	34 (36.2%)	
Hashimoto's Thyroiditis	12 (9.6%)	10 (10.6%)	
Partner			0.570[2]
Yes No	86 (68.8%) 39 (31.2%)	68 (72.3%) 26 (27.7%)	
Biologics *(yes)*	91 (72.8%)	63 (67%)	0.354[2]

Steroids *(yes)*	10 (8%)	2 (2.1%)	0.059[2]

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, physically active or inactive).
[2]The Chi-square test or Fisher's exact test was employed for evaluation.
BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; UTI: Urinary tract infections. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Table 3 Physical activity concerning the type of inflammatory bowel disease

PA variable	Crohn's disease (*n* = 92)	Ulcerative colitis (*n* = 127)	*P* value[1]
Intense activities (Met min/wk)	0 (0-192)	0 (0-240)	0.099
Moderate activities (Met min/wk)	208 (0-536)	140 (0-540)	0.590
Mild activities (Met min/wk)	293.75 (158.12-711.6)	350 (120-840)	0.940
Sitting time at work (min)	210 (113-292.5)	215 (125-292)	0.719
Sitting time at home (min)	174 (118.75-221.75)	177 (115-229)	0.855
Total score (Met min/wk)	828.25 (339.37-1343.5)	839 (390-1451)	0.678
PA level			0.995
Inactive	39 (42.4%)	55 (43.3%)	
Sufficiently active	50 (54.3%)	66 (52%)	
HEPA active	3 (3.3%)	6 (4.7%)	

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, Crohn's disease and ulcerative colitis).
HEPA: Health enhancing physical activity; PA: Physical activity. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Table 4 Disease activity and physical activity levels expressed as total International Physical Activity Questionnaire score

PA variable	*n* (%)	PA active (Met min/wk)	PA inactive (Met min/wk)	*P* value[1]
PRO-2 CD	***n* = 92**	***n* = 53**	***n* = 39**	
Remission	27 (29.3%)	1353 (1026.5-2064)	210.75 (101.25-313.75)	0.303
Mild	24 (26.1%)	1213.65 (1039.52-1534.75)	346.25 (229.62-566.37)	
Moderate	41 (44.6%)	1240.75 (879.67-1950)	352.5 (45-468.75)	
Overall	**92 (100%)**	**1234 (981.75-1769.25)**	**280 (157.5-465)**	
PRO-2 UC	***n* = 127**	***n* = 72**	***n* = 55**	
Remission	74 (58.3%)	1345.35 (1057.5-1766.75)	321.25 (204.25-535.87)	0.994
Active	53 (41.7%)	1457 (952-1964.75)	350 (0-574)	
Overall	**127 (100%)**	**1373.35 (990-1792.62)**	**325 (111.5-538.5)**	

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, active and inactive patients).
PRO: Patient reported outcome; CD: Crohn's disease; UC: Ulcerative Colitis; IPAQ: International Physical Activity Questionnaire; PA: Physical activity. Data are expressed for continuous variables as median (interquartile range) and, for categorical and ordinal variables, as numerosity (%).

Considering the whole sample, the SF of CD patients showed a median of 4.5 (2 - 8) bowel movements, while AP was reported as absent in 56 (60.9%) patients, as mild in 24 (26.1%) and finally, as moderate in 12 (13%) patients. UC patients, on the other hand, reported normal SF in most cases (92, 72.4%), increased 1-2 times in 17 (13.4%) and increased 3-4 times in 18 (14.2%) cases. Moreover, UC patients concerning RB reported no visible blood in most cases (91, 71.7%), traces in less than half of bowel movements in 27 (21.3%) cases and, finally, visible blood in most bowel movements in 9 (7.1%) cases. In general, as shown in Table 4, even in the absence of significance, CD patients in remission with regular PA had better disease activity scores than those with mild and moderate activity, while this trend was not superimposable in the

Table 5 Beliefs about physical activity of patients included

Question (*n* = 219)	Completely agree	I think it is irrelevant	Partially agree	Partially disagree	Completely disagree	*P* value[1]
I think my IBD is a block to doing regular PA	12 (5.5%)	65 (29.7%)	58 (26.5%)	28 (12.8%)	56 (25.6%)	0.957
The treatment I am taking for my IBD is a block to performing regular PA	5 (2.3%)	78 (35.6%)	15 (6.8%)	16 (7.3%)	105 (47.9%)	0.520
I believe that engaging in regular PA may reactivate my IBD or, if already active, make it worse	61 (27.9%)	43 (19.6%)	18 (8.2%)	19 (8.7%)	78 (35.6%)	**< 0.001**
I believe that performing regular PA may result in complications in my IBD (*e.g.*, fistula formation, abscesses or other)	13 (5.9%)	45 (20.5%)	21 (9.6%)	26 (11.9%)	114 (52.1%)	0.527
I believe that performing regular PA can improve my IBD	28 (12.8%)	55 (25.1%)	87 (39.7%)	6 (2.7%)	43 (19.6%)	0.942
I believe that performing regular PA can protect me from new IBD recurrence	25 (11.4%)	74 (33.8%)	87 (39.7%)	18 (8.2%)	15 (6.8%)	0.538
My family doctor adequately informed me regarding the possibility of performing regular PA	52 (23.7%)	45 (20.5%)	51 (23.3%)	18 (8.2%)	53 (24.2%)	0.936
My gastroenterologist adequately informed me regarding the possibility of performing regular PA	76 (34.7%)	26 (11.9%)	80 (36.5%)	10 (4.6%)	27 (12.3%)	0.871
People close to me (*e.g.*, relatives and friends) have repeatedly urged me to conduct a regular PA	91 (41.6%)	22 (10%)	69 (31.5%)	18 (8.2%)	19 (8.7%)	0.795
People close to me (relatives, friends) have repeatedly advised/banned me from conducting regular PA	0 (%)	35 (16%)	29 (13.2%)	24 (11%)	131 (59.8%)	0.291
Before receiving the diagnosis of IBD, I was more inclined to perform regular PA, but now, upon receiving the diagnosis, I feel less convinced to perform PA	41 (18.7%)	45 (20.5%)	45 (20.5%)	13 (5.9%)	75 (34.2%)	**< 0.001**

[1]The *P* value was calculated by checking the difference in the distribution of different variables between the two identified groups (*i.e.*, active and inactive patients).

IBD: Inflammatory bowel disease; PA: Physical activity. Data are expressed as numerosity (%). Significant *P* values are indicated in bold.

case of UC. At bivariate analysis, the UC PRO-2 score negatively correlated with the IPAQ intense activity subscore (τ = -0.156, P = 0.038). This correlation was not met by CD patients (τ = 0.114, P = 0.160). PRO-2 showed no other relationships with other IPAQ parameters (P > 0.05). Finally, on ROC analysis, neither PRO-2 in the UC (AUC = 0.512, 95%CI 0.409-0.614) nor CD (AUC = 0.431, 95%CI 0.311-0.551) showed notable AUCs.

IBD patient's beliefs and barriers toward PA

The sample felt, on average, essential to discuss PA with their gastroenterologist during outpatient visits, as evidenced by a median of 6 (4-8) on the 11-point Likert scale administered to patients (Figure 3A and B) and active patients tended to respond more frequently with scores at the positive extreme (*i.e.*, 9, 10, P = 0.044). Figure 3C resumes sports practised by patients, and differences in the chosen sport between active and inactive in terms of PA were not found (P = 0.445). In addition, several IBD-related barriers to PA were reported (Figure 3D), with some reported more frequently by inactive patients, specifically diarrhoea and evacuation urgency (P = 0.004). Table 5 reports the central beliefs of our patients about several PA aspects. In this context, 63.8% (60/94) were wholly convinced that PA could reactivate/worsen the clinical activity of their IBD. Sixty percent (75/125) of PA active patients thoroughly reported the opposite (P < 0.001). In addition, 46.8% (44/94) of PA inactive believed (entirely or partially) that the diagnosis of IBD was the starting point of their distrust of PA. The trend was predictably opposite in PA active patients (P < 0.001).

A traceable element, in general, is how the patients' social network in majority urged the patient to practice regular PA. In contrast, less than half of the patients felt adequately informed by their family doctor or gastroenterologist about the possibility of performing regular PA.

Finally, a binary logistic regression analysis was conducted to investigate physical inactivity predictors among all the study variables, not recording any significant predictor (Figure 4 and Table 6).

DISCUSSION

This study weighed patient-reported PA in a group of IBD European patients. In this study, IBD adults showed a particularly worrying rate of physical inactivity (*i.e.*, 42.9%), with only 4.1% of the sample meeting the HEPA criteria. Median overall PA levels (*i.e.*, 834.5 Met min/wk) were just above the IPAQ threshold for inactivity (*i.e.*, 700 Met min/wk).

In this experience, PA showed no relationship with IBD activity (employing the PRO-2 tool), except for a negative relationship between UC PRO-2 and moderate PA levels. Clinical PROs, as moreover measured by PRO-2, have been

Table 6 Predictors of physical inactivity analysis among clinical and demographic variables evaluated by binary logistic regression

Variable	Exp (B)/Odds ratio	95%CI	*P* value
Age (yr)	0.996	0.976-1.018	0.743
Weight (Kg)	1.017	0.992-1.041	0.180
Height (cm)	1.018	0.989-1.048	0.231
BMI (Kg/m^2)	0.999	0.998-1.001	0.867
IBD (CD)	0.931	0.503-1.721	0.819
IBD (UC)	1.074	0.581-1.987	
Gender (Male)	1.196	0.642-2.225	0.573
Gender (Female)	0.836	0.449-1.556	
Partner (Yes)	1.343	0.681-2.652	0.395
Partner (No)	0.744	0.377-1.469	
Biologics (Yes)	0.816	0.430-1.549	0.534
Biologics (No)	1.225	0.646-2.325	
Arthritis (Yes)	1.710	0.892-3.278	0.106
Arthritis (No)	0.585	0.305-1.121	
Dyslipidaemia (Yes)	0.747	0.343-1.627	0.463
Dyslipidaemia (No)	1.338	0.615-2.913	
Diabetes (Yes)	1.074	0.209-5.517	0.932
Diabetes (No)	0.931	0.181-4.786	
Hypertension (Yes)	0.622	0.204-1.893	0.403
Hypertension (No)	1.608	0.528-4.893	
Hashimoto's thyroiditis (Yes)	1.121	0.462-2.717	0.800
Hashimoto's thyroiditis (No)	0.892	0.368-2.162	
Importance PA discussion (Likert 10-point scale)	0.911	0.823-1.008	0.072
Employed	0.857	0.501-1.464	0.571
Unemployed	1.167	0.683-1.994	

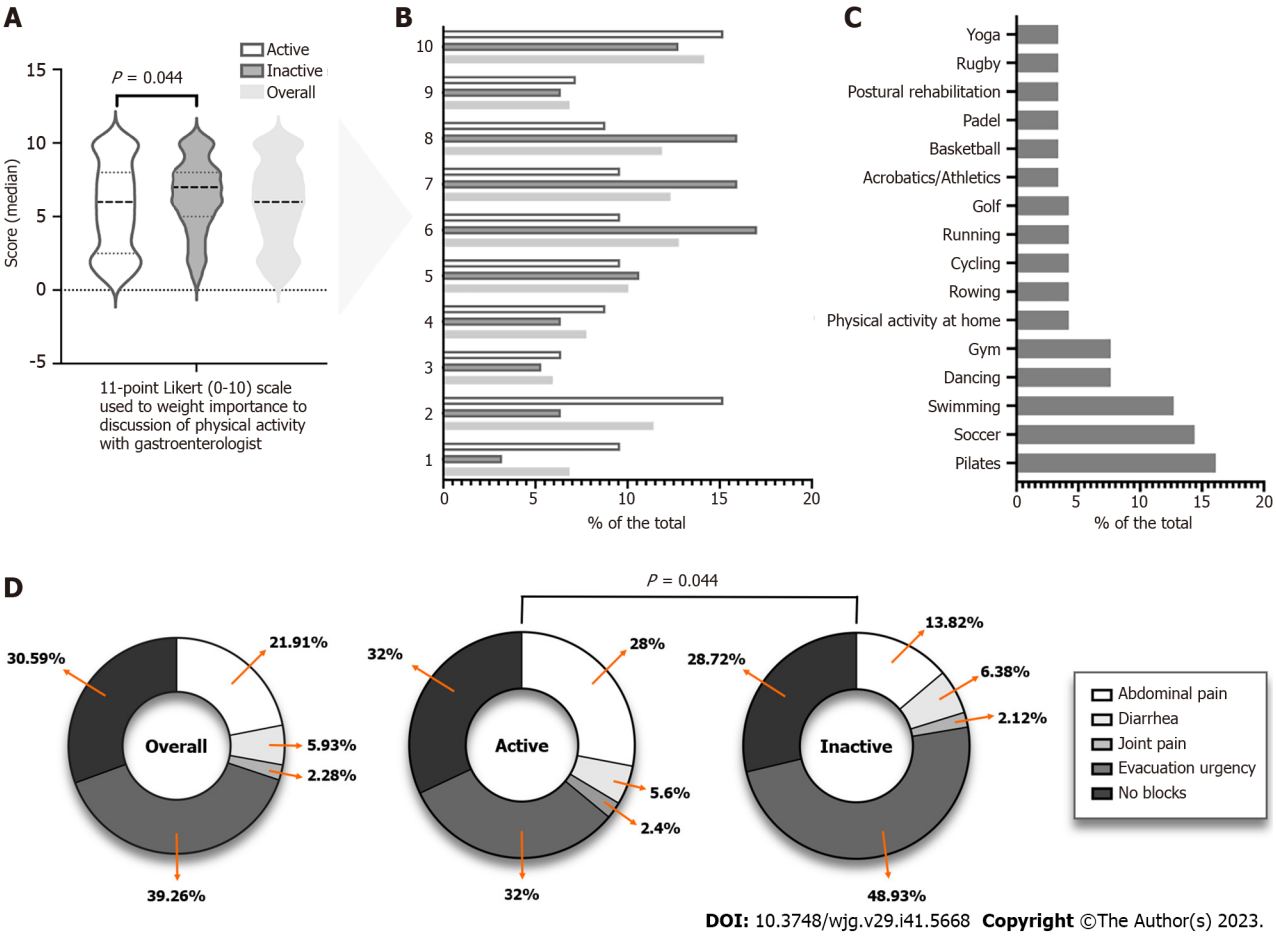
Risk is expressed as the exponential value of B, or Exp(B), presented in odds ratio with a relative 95% confidence interval and relative *P* value. 95%CI: 95% confidence interval; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative Colitis; PA: Physical activity.

shown in a recent cross-sectional study to associate with daily activities impairment compared with physician-reported ones[27]. PA and IBD relationship is still highly controversial and under study. Khalili *et al*[28], in a cohort study, showed an inverse association between PA and the risk of CD but not UC.

In contrast, another Japanese study showed an inverse association between intense-type PA and mucosal healing but not with clinical remission[29]. Much of the available evidence, albeit little, seems to suggest in IBD a moderate and, in a significant minority, mild PA intensity, and it appears that this increased PA is associated with better management of symptoms (including fatigue) as well as better psychological outcomes and QoL[30]. Despite this, there are still no detailed recommendations on the best sport to suggest, at what intensity and for how long for IBD patients.

Complicating the picture, there are vast geographical differences in conceptions of PA[31]. A previous New Zealand survey (which examined a smaller sample of 77 patients) found a higher rate of PA (*i.e.*, 66%) and PA levels (1613 *vs* 834.5 Met min/wk) than ours[32]. In each case, however, this study also found similar barriers reported by patients with IBD to PA (*i.e.*, evacuation urgency). These data were also similar to that of Tew *et al*[24].

Whether regular PA can give tangible benefits to IBD activity is still not completely clear. However, it appears that PA may increase the T-regulatory lymphocytes expression, reduce the immunoglobulins secretion by negatively regulating T helper 1 Lymphocytes, and increase the anti-inflammatory cytokine IL-10 production[8]. However, as in our study, all in all, not a strong relationship between PA and IBD activity was also obtained from another American sample of about 250 patients with an average age similar to ours (*i.e.*, 39.6 years)[33]. In contrast, in another study, more marked differences in PA had emerged between patients with active disease and in remission, postulating a negative role of disease activity [24]. These differences may be partially explained by the fact that a higher rate of patients in our study was on biologics, which have a pronounced impact on the course of the disease[34]. Not surprisingly, as written before, we observed how



DOI: 10.3748/wjg.v29.i41.5668

Figure 3 Importance given by patients to discuss physical activity with their gastroenterologist, major sports played by them, and barriers to physical activity related to inflammatory bowel disease. A and B: The importance given by patients to discuss with their gastroenterologist physical activity stratified by physical activity level (A) and detailed by individual Likert scale score (B); C: Main sports stated by participants; D: Factors related to inflammatory bowel disease hinder regular physical activity.

being on biological therapy provided an advantage toward moderate PA activities ($P = 0.022$, Figure 2C).

This study also examined the potential impact of work employment on PA levels by leaning toward the little impact of the former on the latter. Although not detailed in IBD, this finding contrasts with what is already reported in the general population[35]. This matter is difficult to interpret in a population (*i.e.*, IBD) already heavily impacted by unemployment [36]. Not coincidentally, our unemployment rate was high (*i.e.*, 48.9%, 107/219), so studies including a larger sample of employed people probably need to verify a real difference in PA levels.

The comorbidities we noted did not have much effect on PA levels. However, to assess arthritis, we did not evaluate clinical activity because of the study design and purpose. Therefore, although having or not having this comorbidity did not impact PA levels in our setting, the limitation of not grading joint disease/disability activity must be considered. We also found better PA levels in dyslipidaemia-affected patients. However, we believe the small number of dyslipidaemia-affected patients must weigh this result compared to healthy ones, so a subgroup analysis should be considered merely exploratory. In any case, IBD patients without dyslipidaemia tolerated higher activity levels better than those with dyslipidaemia, as reported in a non-IBD setting[37].

The problem of physical inactivity in IBD is relevant because an inactive patient risks losing the potential benefits that PA can provide in several aspects already undergoing impairment in such patients (such as mood disorders[38], metabolic syndrome[39], and sarcopenia[14]).

To recover inactive patients (in terms of PA), our study offers several insights. The first point is probably, to discuss with the patient of PA during the gastroenterology visit and identify the patient's fears. Secondly, providing the patient with a cognitive intervention is necessary by discussing possible solutions to the barriers for PA (*i.e.*, evacuation urgency, thinking that there may be disease reactivation and the like).

In view also that our inactive patients significantly identified the core of their PA-related fears at the diagnosis of IBD (Table 5, $P < 0.001$) compared with active patients, this suggests that these complementary aspects should be discussed at diagnosis before patients integrate misconceptions into the management of their IBD and PA.

In addition, as exhibited in Table 5 (questions 7, 8), a not insignificant percentage of patients feel uninformed about the IBD-PA relationship by their family physician and gastroenterologist. Therefore, training courses that aim to provide general knowledge about the possibilities of practising PA in patients with chronic digestive diseases should solve this unmet patient need.

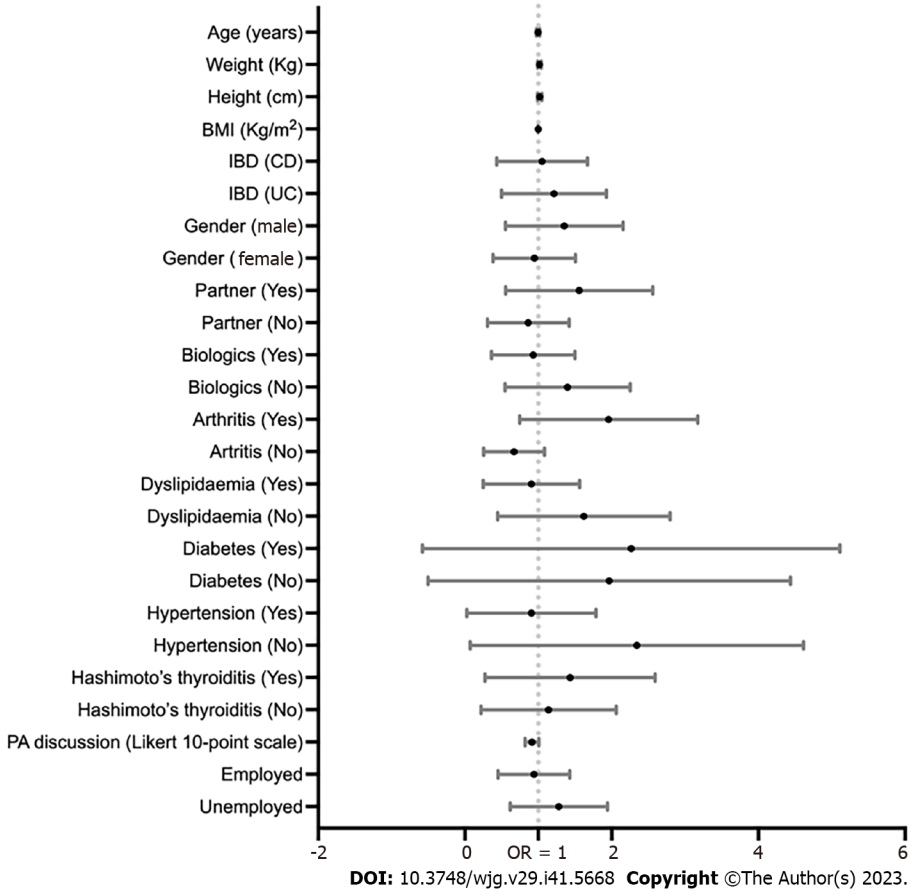


Figure 4 Forest plot showing predictors analysis of physical inactivity analysis among clinical and demographic variables evaluated by binary logistic regression. CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds ratio.

Moreover, regular PA can pose a valuable strategy for reducing inflammatory burden, especially in diseases with inflammatory pathogenesis, such as IBD. Exercise can contribute to the promotion of an anti-inflammatory phenotype in several ways.

In fact, at the level of fatty tissue, it can downregulate several pro-inflammatory cytokines such as IL-1, IL-16 and tumour necrosis factor (TNF), and, in addition, it can promote the M2 cytotype of macrophages (*i.e.*, their anti-inflammatory cytotype) and act against oxidative stress[7]. In the context of muscle tissue, moreover, these actions are, to a large extent, repeated[7] with an increase also in peroxisome proliferator-activated receptor γ co-activator 1α, a molecule that in knockout mice for the same, results in the promotion of IL-6 and TNF[40]. Repeated exercise also appears to induce adaptive changes in the immune system by predisposing to lower neutrophil recruitment[41]. For these reasons, exercise has been repeatedly proposed to counter chronic inflammation[42].

In addition to the above, regular PA can improve vascular endothelial balance by ameliorating oxidative stress and nitric oxide availability[43].

Although set in a research context severely lacking solid evidence already available, this study has several limitations. The data are from a single-centre experience, and future multicentre evidence would be desirable; our subgroup analyses are, by definition, exploratory; therefore, studies of larger sample sizes are desirable to confirm them. In addition, it will be preferable to confirm and strengthen our data even more a multicentre, prospective study design to bring out more differences in population subgroups.

CONCLUSION

IBD southern Italian patients seem physically inactive and may be exposed to all the complications of not practising regular PA. This does not seem totally dependent on disease activity but is affected by patients' beliefs about PA's impact on underlining IBD. Using validated and feasible questionnaires (*e.g.*, IPAQ) could be a strategy to weigh patient-reported PA levels and get an initial idea about which patients have insufficient PA levels.

ARTICLE HIGHLIGHTS

Research background

Patients with inflammatory bowel diseases (IBD) often experience reduced quality of life (QoL) and disability. Regular physical activity (PA) determines QoL. Initial studies have shown that mild PA seems safe in IBD and is not associated with an increased risk of flare-ups.

Research motivation

There are no precise guidelines on what type of PA and the intensity to recommend for patients with IBD. Epidemiological levels of PA in the IBD population are not yet fully known, nor are the barriers that block patients from practising regular PA.

Research objectives

This study aimed to weigh PA levels with standardised instruments in an Italian IBD population to examine PA's relationship with IBD disease activity and identify barriers to PA.

Research methods

This cross-sectional study employed the standardised International Physical Activity Questionnaire (IPAQ) to weigh PA and the patient-reported outcome 2 (PRO-2) to assess IBD disease activity. PA was expressed as multiples of resting metabolic rate (Met) in Met min/wk. This study included only patients with confirmed, excluding patients with severe or hospitalised activity.

Research results

Two hundred nineteen patients were included. Fifty-three per cent were found to be sufficiently active, 42.9% as inactive, and only 4.1% as health-enhancing PA active. Median overall PA levels were 834.5 Met min/wk, just above the threshold for inactivity (*i.e.*, 700 Met min/wk). Ulcerative colitis PRO-2 showed a negative correlation with intense PA activities. Several barriers to PA were identified (*e.g.*, fear of IBD flare-up, fears initiated as early as IBD diagnosis).

Research conclusions

Patients with IBD were found in this setting to be burdened by a significant rate of physical inactivity. Barriers persist on which to act to regain adherence to regular PA. As measured by the PRO-2, disease activity did not drastically affect PA. The IPAQ questionnaire showed excellent feasibility and ease of completion and interpretation.

Research perspectives

Regular PA has multiple benefits (from cardiovascular health to psychological health), and it is necessary to make sure that patients with IBD practice it so that these benefits are not lost. It is appropriate for gastroenterologists to pay more attention to this aspect during medical visits. IPAQ can be a potential tool for recognising and monitoring physically inactive patients.

FOOTNOTES

Author contributions: Gravina AG, Pellegrino R, and Federico A designed the study; all authors participated in the acquisition and interpretation of the data and drafted the initial manuscript; Pellegrino R performed the analysis; all the authors revised the article critically for important intellectual content.

Institutional review board statement: The study was conducted in compliance with the Declaration of Helsinki and received approval from the Ethics Committee of the University of Campania Luigi Vanvitelli (protocol number 7892, 15 March 2023).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: Italy

ORCID number: Antonietta Gerarda Gravina 0000-0001-8049-0115; Raffaele Pellegrino 0000-0001-5074-230X; Giovanna Palladino 0000-0002-7367-4175; Rossella D'Onofrio 0009-0002-4761-0028; Giusi Arboretto 0009-0000-7938-8949; Salvatore Auletta 0009-0008-8565-0120; Giuseppe Imperio 0000-0002-4182-2858; Andrea Ventura 0009-0005-5735-7195; Mario Romeo 0000-0002-2970-9019; Alessandro Federico 0000-0002-0885-0793.

Corresponding Author's Membership in Professional Societies: United European Gastroenterology.

S-Editor: Lin C
L-Editor: A
P-Editor: Yu HG

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2023 November 7; 29(41): 5683-5698

DOI: 10.3748/wjg.v29.i41.5683

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

First report on establishment and characterization of the extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2

Ning-Zu Jiang, Ming-Zhen Bai, Chong-Fei Huang, Ze-Long Ma, Ru-Yang Zhong, Wen-Kang Fu, Long Gao, Liang Tian, Ning-Ning Mi, Hai-Dong Ma, Ya-Wen Lu, Zi-Ang Zhang, Jin-Yu Zhao, Hai-Ying Yu, Bao-Ping Zhang, Xian-Zhuo Zhang, Yan-Xian Ren, Chao Zhang, Yong Zhang, Ping Yue, Yan-Yan Lin, Wen-Bo Meng

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Wang GX, China; Yildiz K, Turkey; Zharikov YO, Russia

Received: August 3, 2023
Peer-review started: August 3, 2023
First decision: September 18, 2023
Revised: September 20, 2023
Accepted: October 11, 2023
Article in press: October 11, 2023
Published online: November 7, 2023

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Ning-Zu Jiang, Ming-Zhen Bai, Chong-Fei Huang, Ze-Long Ma, Ru-Yang Zhong, Wen-Kang Fu, Long Gao, Liang Tian, Ning-Ning Mi, Hai-Dong Ma, Ya-Wen Lu, Zi-Ang Zhang, Jin-Yu Zhao, Hai-Ying Yu, Bao-Ping Zhang, Xian-Zhuo Zhang, Yan-Xian Ren, Chao Zhang, Yong Zhang, Ping Yue, Yan-Yan Lin, Department of General Surgery, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Ning-Zu Jiang, Ming-Zhen Bai, Chong-Fei Huang, Ze-Long Ma, Ru-Yang Zhong, Wen-Kang Fu, Long Gao, Liang Tian, Ning-Ning Mi, Hai-Dong Ma, Ya-Wen Lu, Zi-Ang Zhang, Jin-Yu Zhao, Hai-Ying Yu, Bao-Ping Zhang, Xian-Zhuo Zhang, Yan-Xian Ren, Chao Zhang, Yong Zhang, Ping Yue, Yan-Yan Lin, Laboratory of Biological Therapy and Regenerative Medicine Transformation Gansu Province, The First Hospital of Lanzhou University, Lanzhou 730000, Gansu Province, China

Wen-Bo Meng, Department of General Surgery, The First Hospital of Lanzhou University and Laboratory of Biological Therapy and Regenerative Medicine Transformation Gansu Province, Lanzhou 730000, Gansu Province, China

Corresponding author: Wen-Bo Meng, MD, PhD, Doctor, Professor, Department of General Surgery, The First Hospital of Lanzhou University and Laboratory of Biological Therapy and Regenerative Medicine Transformation Gansu Province, No. 1 Donggang West Road, Chengguan District, Lanzhou 730000, Gansu Province, China. mengwb@lzu.edu.cn

Abstract

BACKGROUND

Extrahepatic cholangiocarcinoma sarcoma is extremely rare in clinical practice. These cells consist of both epithelial and mesenchymal cells. Patient-derived cell lines that maintain tumor characteristics are valuable tools for studying the molecular mechanisms associated with carcinosarcoma. However, cholangiocarcinoma sarcoma cell lines are not available in cell banks.

AIM
To establish and characterize a new extrahepatic cholangiocarcinoma sarcoma cell line, namely CBC2T-2.

METHODS
We conducted a short tandem repeat (STR) test to confirm the identity of the CBC2T-2 cell line. Furthermore, we assessed the migratory and invasive properties of the cells and performed clonogenicity assay to evaluate the ability of individual cells to form colonies. The tumorigenic potential of CBC2T-2 cells was tested *in vivo* using non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The cells were injected subcutaneously and tumor formation was observed. In addition, immunohistochemical analysis was carried out to examine the expression of epithelial marker CK19 and mesenchymal marker vimentin in both CBC2T-2 cells and xenografts. The CBC2T-2 cell line was used to screen the potential therapeutic effects of various clinical agents in patients with cholangiocarcinoma sarcoma. Lastly, whole-exome sequencing was performed to identify genetic alterations and screen for somatic mutations in the CBC2T-2 cell line.

RESULTS
The STR test showed that there was no cross-contamination and the results were identical to those of the original tissue. The cells showed round or oval-shaped epithelioid cells and mesenchymal cells with spindle-shaped or elongated morphology. The cells exhibited a high proliferation ratio with a doubling time of 47.11 h. This cell line has migratory, invasive, and clonogenic abilities. The chromosomes in the CBC2T-2 cells were polyploidy, with numbers ranging from 69 to 79. The subcutaneous tumorigenic assay confirmed the *in vivo* tumorigenic ability of CBC2T-2 cells in NOD/SCID mice. CBC2T-2 cells and xenografts were positive for both the epithelial marker, CK19, and the mesenchymal marker, vimentin. These results suggest that CBC2T-2 cells may have both epithelial and mesenchymal characteristics. The cells were also used to screen clinical agents in patients with cholangiocarcinoma sarcoma, and a combination of paclitaxel and gemcitabine was found to be the most effective treatment option.

CONCLUSION
We established the first human cholangiocarcinoma sarcoma cell line, CBC2T-2, with stable biogenetic traits. This cell line, as a research model, has a high clinical value and would facilitate the understanding of the pathogenesis of cholangiocarcinoma sarcoma.

Key Words: Carcinosarcoma; Drug resistance; Xenograft; Cell line; Establishment

Core Tip: The study established and characterized the CBC2T-2 cell line as a potential model for studying human extrahepatic cholangiocarcinoma sarcomas. The cells exhibited both epithelial and mesenchymal characteristics and demonstrated high proliferation, migration, invasion, and clonogenic abilities. Chromosomal analysis revealed polyploidy with varying chromosome numbers and *in vivo* tumorigenicity was confirmed in non-obese diabetic/severe combined immunodeficient mice. Immunohistochemistry indicated positive expression for both epithelial marker CK19 and mesenchymal marker vimentin. The cell line was also used to screen clinical agents, and paclitaxel and gemcitabine exhibited optimal effects. Whole-exome sequencing further revealed genetic insights.

Citation: Jiang NZ, Bai MZ, Huang CF, Ma ZL, Zhong RY, Fu WK, Gao L, Tian L, Mi NN, Ma HD, Lu YW, Zhang ZA, Zhao JY, Yu HY, Zhang BP, Zhang XZ, Ren YX, Zhang C, Zhang Y, Yue P, Lin YY, Meng WB. First report on establishment and characterization of the extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2. *World J Gastroenterol* 2023; 29(41): 5683-5698
URL: https://www.wjgnet.com/1007-9327/full/v29/i41/5683.htm
DOI: https://dx.doi.org/10.3748/wjg.v29.i41.5683

INTRODUCTION

Extrahepatic cholangiocarcinoma sarcomas are extremely rare clinically because they represent a mixture of carcinomas and sarcomas, accounting for < 0.1% of cholangiocarcinoma[1]. Polypoid growth and ossification within the carcinoma are representative features of extrahepatic cholangiocarcinoma sarcoma[2]. It is composed of epithelial and stromal cells, with a sarcomatous component that differentiates from the spindle or pleomorphic cells[3]. Carcinosarcoma is commonly observed in the elderly, and it is mostly diagnosed in the lungs, bladder, pancreas, ovary, esophagus, thyroid gland, and breast and rarely occurs in the liver or biliary system[4,5]. Extrahepatic cholangiocarcinoma sarcoma with chondrogenic differentiation is a poorly differentiated and aggressive cancer with poor prognosis. The effectiveness of radiotherapy and chemotherapy for cholangiocarcinoma sarcoma is limited; hence, complete surgical resection of the tumor is the ultimate

treatment option for cholangiocarcinoma sarcoma[6-8]. However, most patients experience local recurrence even after extensive local excision. Many patients receive treatment for jaundice or abnormal liver function without significant increases in the levels of tumor markers, and their prognosis is worse than that of patients with common bile duct cancer, with a low survival rate of 1 mo to 5 years[9-11]. Therefore, the development of novel therapeutic strategies for cholangiocarcinoma sarcoma is crucial.

Patient-derived cell lines can maintain tumor characteristics and are valuable tools for studying the molecular mechanisms of carcinosarcoma, disease progression, and biological features[12,13]. Owing to the rarity of cholangiocarcinoma sarcoma and its incompletely elucidated oncogenicity, its clinical and therapeutic significance remain uncertain. Basic research models are essential for thorough analysis of the developmental processes of cholangiocarcinoma sarcoma. Therefore, we established CBC2T-2, the first histologically confirmed cell line for cholangiocarcinoma sarcoma and comprehensively described its application in research.

MATERIALS AND METHODS

Patient's background

A 62-year-old female, was admitted to the First Hospital of Lanzhou University in July 2022 with intermittent pain in her right upper abdomen and lower back. The results of physical examination were unremarkable, except for slight pressure pain in the upper right abdomen. Preoperative computed tomography and magnetic resonance imaging revealed the dilatation of the common bile duct and intrahepatic bile duct. This was accompanied by a soft tissue shadow in the upper part of the common bile duct showing arterial phase enhancement, indicative of bile duct cancer (Figures 1A and B; arrow). Radical surgery was performed under general anesthesia without any treatment prior to surgery. Intraoperative frozen section confirms negative margins. However, the postoperative pathological diagnosis revealed cholangiocarcinoma sarcoma of the porta hepatis, comprising 90% adenocarcinoma and 10% chondrosarcoma. The American Joint Committee on Cancer staging system of the tumor was T2aN2Mx (Figures 1C and G; arrow). The tumor markers were within the normal range, with alpha-fetoprotein at 2.1 U/mL, carbohydrate antigen 19-9 (CA19-9) at 9.4 U/mL, and carcinoembryonic antigen (CEA) at 0.7 U/mL (Figure 1G). Hematoxylin and eosin (HE) staining revealed microscopic epithelial heterogeneous hyperplasia with striated, nested, and glandular arrangements; increased cell volume; large deep-stained nuclei with pathological nuclear abnormalities; localized mucus and mucous cartilage; and infiltrative growth of cancer cells (Figures 1D and E; arrow).

This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL-2022-489) and informed consent was obtained from all the patients. This study adhered to the 1964 Declaration of Helsinki and its subsequent amendments and similar ethical standards[14].

Cell culture

The specimens were collected under the guidance of a pathologist to ensure that the diagnosis in the pathology report was unaffected. The tumor specimens were rinsed thrice with phosphate buffer saline (PBS; BI), cut into 1-2 mm^3 pieces using a sterile blade, and then digested with collagenase type IV (0.1 mg/mL; Gibco) for 10 min in a 37 °C incubator. The cell pellet was maintained in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). It was cultured at 37 °C under humidified air containing 5% CO_2. For continuous culture, the cells were periodically passaged and frozen in liquid nitrogen at regular intervals. No contamination with other cells or foreign microorganisms was observed during the culture process. No external growth factors or stimulatory cytokines were added during the establishment of the cell lines.

Short tandem repeat analysis for cell line validation

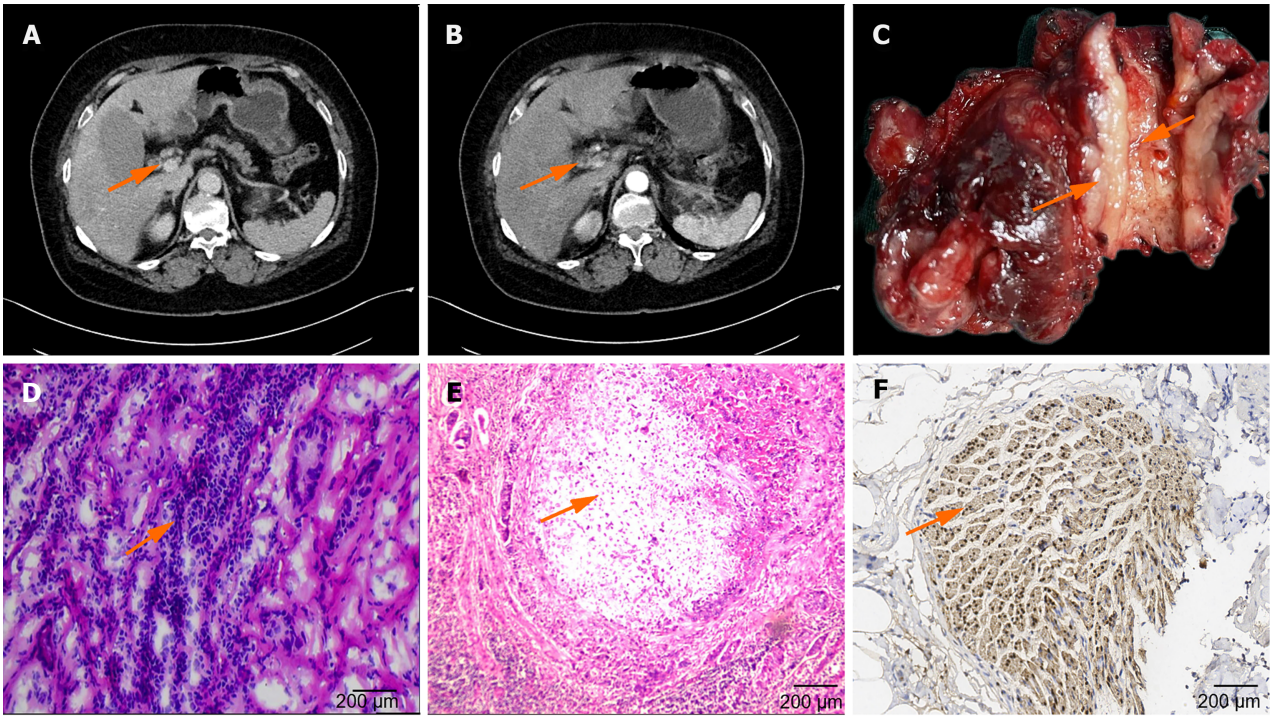
Freshly cultured human cholangiocarcinoma sarcoma cell line CBC2T-2 (p25) and frozen tumor tissues were collected following the manufacturer's instructions. The genomic DNA of the cells was extracted using an Animal Genome Extraction Kit and then subjected to polymerase chain reaction with fluorescently labeled primers at the 5′ end. The resulting products were sequenced and analyzed for sequence repeats of 21 short tandem repeat (STR) loci, including AMEL, D19S433, D5S818, D21S11, D18S51, D6S1043, D3S1358, D13S317, D7S820, D16S539, CSF1PO, Penta D, D2S441, vWA, D8S1179, TPOX, Penta E, TH01, D12S391, D2 S1338, and FGA. The obtained STR profiles were compared with reference STRs from public cell repositories, including the American Type Culture Collection (ATCC), Deutsche Sammlung Mikroorganismenund Zelkulturen (DSMZ), and the CELLOSAURUS cell database.

Chromosome analysis

Cells in the logarithmic growth phase (p25) were incubated with 10 μg/mL of colchicine for 2 h in a cell culture incubator. Chromosomes were prepared using standard methods and their numbers were mostly distributed across the G-dominant band. Representative images of chromosomes were obtained for karyotype analysis. Karyotype interpretation was based on the International System for Human Cytogenetic Nomenclature (1995)[15].

Spheroid formation assay

In total, 1 × 10^5 cells in the logarithmic growth phase (p25) were digested and inoculated into ultra-low-attachment 96-well plates (Corning). Sphere formation was monitored on days 3, 7, 10, and 14 after inoculation to assess the ability of the cells to form spheres.



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Cell line	Patient age/ethicity	Gender	Current Status (days)	Edmondson -Steiner (grade)	Tumor size (cm)	Prior therapy	Culture date	Serum		
								AFP (IU/mL)	CEA (ng/mL)	CA199 (U/mL)
CBC2T-2	62/Asian	Female	NA	Ⅲ	8×4	None	2022-6-20	2.1	4.0	9.4

DOI: 10.3748/wjg.v29.i41.5683

Figure 1 Clinical and pathological profile of CBC2T-2. A and B: Contrast-enhanced computed tomography of the abdomen showing a high-grade occupying lesion of the common bile duct with high level obstruction of the common bile duct (orange arrows); C: Specimen of postoperative carcinosarcoma of the extrahepatic bile duct with thickened duct wall and polyp-like protrusion into the lumen; D and E: Hematoxylin and eosin and immunohistochemistry staining of primary tumor tissue. Scale bars, 200 μm; F: Immunohistochemistry staining of primary tumor tissue. Antibodies against Vimentin, Scale bars, 200 μm; G: Clinicopathological characteristics of patients. AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CA199: Carbohydrate antigen199.

Cell counting kit-8 cell growth assay

CBC2T-2 cells were inoculated into 96-well plates at a density of 5000 cells per 100 μL. Cell counting kit 8 (CCK-8) (APEx BIO) was added at time points of 0, 24, 48, 72, 96, and 120 h at a ratio of 1:10. The cell growth curve was plotted with time on the horizontal axis and absorbance on the vertical axis. The ploidy doubling time (PDT) software was used to calculate the cell doubling time (http://www.doubling-time.com).

Live cell imaging

CBC2T-2 cells were inoculated into 96-well plates at a density of 4000 cells per 100 μL. The plate was then placed in a Cytation 1 imaging system (Biotek) under a 4 × objective, and the field of view was selected for each well. Images were taken every 2 h for 120 h and processed using the Gen5 Software.

Flow cytometry

Tumor cells (p25) in the logarithmic growth phase were washed with PBS, digested with ethylenediaminetetraacetic acid-free trypsin, and prepared as a single-cell suspension. The CBC2T-2 cell precipitates were washed twice with PBS and the density was adjusted to 1×10^7/mL. The cells were fixed by adding pre-cooled 75% ice-cold ethanol to each tube for 1-2 h. After incubating away from light for 15-30 min, the cells were stained with 0.5 mL of propidium iodide stain [consisting of 0.5 mL staining buffer, 25 μL propidium iodide staining solution, and 10 μL RNaseA (50 ×)] (Bioscience).

Migration and invasion abilities

To assess the migratory ability of the cells, a suspension of 200 μL of cells (1×10^5/mL) was evenly distributed in the upper chamber of a transwell without matrix gel (BD). To evaluate the invasive ability of the cells, a suspension of 200 μL of cells (1×10^5/mL) was evenly distributed in the upper chamber of a transwell with matrix gel (Corning). The lower chamber was filled with medium containing 15% FBS and incubated in a cell culture incubator. After 24 and 48 h of incubation, the contents of the upper lumen were removed. After staining the cells with 0.1% crystal violet solution (Beyotime) for 20 min, they were rinsed with PBS; next, they were observed under an inverted microscope and the images were captured. This procedure was repeated using TFK-1 cells from the extrahepatic bile duct as controls for the CBC2T-2

cells.

Wound healing assay

CBC2T-1 and TFK-1 cells were seeded into 6-well plates. When the cells reached 95% confluence, the cell monolayers were scraped into a cross-shape using a pipette tip and gently washed thrice with PBS. Medium containing 10% FBS was added to continue the culture. The images were captured at 0, 24, and 48 h, and the entire scratch area was measured using the ImageJ software.

Colony formation assay

The cells were digested with 0.25% trypsin, and the cell density was adjusted to 700 cells/well before inoculation into 6-well plates. Colony formation was monitored at 3, 7, 10, and 14 d after inoculation. On day 14, the colonies were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet for 20 min, photographed, and analyzed using ImageJ software.

Histology and immunostaining

Patient and mouse tumor tissues and crawling cells were fixed in formaldehyde solution. Subsequently, the specimens were subjected to baking, decolonization, hydration, antigen retrieval, peroxidase blocking, and goat serum closure. The slides were incubated with antibodies against CK19, Vimentin, S100, Desmin, CD56, CD117, and S100. Drops of DAB liquid were added, followed by reaction for 1-5 min before color development was promptly terminated.

Whole-exome sequencing

Sequencing and data analyses were conducted using the BGI software (Wuhan, China). The exome region DNA was captured using probe capture technology and the target region DNA was sequenced using high-throughput sequencing technology. Genomic DNA was extracted from CBC2T-2 cells and compared with that from normal tissues adjacent to the patient's resected tumor. Library construction and whole-exome capture of genomic DNA were performed using SureSelect Human All Exon V6 (Agilent Clara, Technologies, Santa CA, United States) and the captured DNA library was sequenced on the DNBSEQ platform. This procedure uses FACETS[16] software to detect somatic copy number variation (CNV) in tumor and normal paired samples, the depth distribution of reads to compare copy number variation with the reference genome, and Ensemble VEP[17] to annotate the CNV. We performed InDel detection using the results of the GATK[18] comparison, followed by the annotation of the detected InDels. We used the GATK MuTect2 tool to identify somatic single-nucleotide variant (SNV) loci and the GATK Funcotator tool to annotate these loci.

Tumor driver gene analysis

We compared somatic mutations with known driver genes from databases and the literature and screened for known driver genes in tumor samples. The reference data sources were Integrative OncoGenomics (IntOGen), the Cancer Gene Census (CGC), three highly cited articles, and pan-cancer data[19-22].

Tumorigenesis in mice

To study the *in vivo* tumorigenicity of CBC2T-2 cells, cells at a concentration of 1×10^7/mL were injected into the axillae of three 4-wk-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The mice were housed in a specific pathogen-free laminar flow animal facility and observed for 8 wk. Tumor diameters were measured every 3 d during this period. After 8 wk, the mice were euthanized, the tumors were excised and photographed, and the samples portions were immersed in a 10 % formalin solution for routine processing.

Screening of anticancer drugs

CBC2T-2 cells (p30) at the logarithmic growth phase were washed with PBS and digested with 0.25% trypsin. The concentration of the cell suspension was adjusted to 10000 cells/100 μL. After culturing the cells for 24 h, different concentrations of chemotherapeutic drugs, including gemcitabine, oxaliplatin, cisplatin, paclitaxel, and 5-fluorouracil were added. Following a drug exposure period of 72 h, the viability of each group was measured by incubating the cells with CCK-8 reagent for 2 h.

Statistical analysis

The results are presented as the mean ± standard error of at least three independent experiments. Graphs were created using GraphPad Prism 9.0, and a one-way analysis of variance was used to compare the two groups. Statistical significance was set at $P < 0.05$.

RESULTS

Establishment and identification of the CBC2T-2 cell line

We successfully established the cell line, namely CBC2T-2, from a patient with cholangiocarcinoma sarcoma. The cells were cultured in monolayers for > 13 mo and subjected to > 100 generations. The cell line was established from the original tumor tissue by comparing the 21 STR loci of both the tissue and cell lines using the assay currently

Table 1 Short tandem repeat profile of tumor tissue and cell line

STR loci	CBC2T-2	Tumor tissue
AMEL	X, X	X, X
D19S433	13	13
D5S818	11, 13	11, 13
D21S11	30, 31.2	30, 31.2
D18S51	13, 13	13, 13
D6S1043	12, 18	12, 18
D3S1358	16, 16	16, 16
D13S317	8, 11	8, 11
D7S820	9, 10	9, 10
D16S539	9	9
CSF1PO	10, 12	10, 12
Penta D	9, 11	9, 11
D2S441	11, 12	11, 12
vWA	17, 18	17, 18
D8S1179	14, 15	14, 15
TPOX	10, 11	10, 11
Penta E	12, 14	12, 14
TH01	9	9
D12S391	19, 20	19, 20
D2S1338	19, 22	19, 22
FGA	23, 23.2	23, 23.2

STR: Short tandem repeat.

recommended by the ATCC. The genomic characteristics of the CBC2T-2 cell line and the originating tumor tissue are presented in Table 1. The assay measurements showed that the cholangiocarcinoma sarcoma cell line was of human origin, and its genetic information was comparable with those from three prestigious culture collections (ATCC, DSMZ, and CELLOSAURUS). No sequences matching other cells were found, indicating that the cells were not contaminated. The cells were maintained at the China Center for Type Culture Collection (CCTCC No. C2022273).

Phenotypic characterization, doubling time, and cell cycle

CBC2T-2 cells (P1, P10, P20, and P50) were observed microscopically as they actively proliferate and grow in a monolayer against the wall. Two types of cell morphology were observed: Typical polygonal cholangiocarcinoma cell morphology and long spindle-shaped chondrosarcoma cell morphology (Figure 2A). Transmission electron microscopy revealed the presence of numerous mitochondria, rough endoplasmic reticulum, ribosomes, and irregular nuclei with deep indentations in the nuclear membrane. Scanning electron microscopy demonstrated microvilli-like protrusions on the cell surface as well as tight junctions and intercellular bridges (Figure 2B).

Flow cytometry was used to detect cell cycle progression in CBC2T-2 cells. Diploid cells were observed in G0/G1 phase, tetraploid cells in G2/M phase, and hyperdiploid cells in S phase (Figure 2C). The PDT of CBC2T-2 cells was calculated to be approximately 47.11 h using both live cell imaging and CCK-8 with the PDT software (PDT) (Figures 2D and E). A short PDT indicated active cell proliferation.

Sphere formation, wound healing assay, and migration, invasion, and clonogenic abilities

The novel cell line CBC2T-2 was inoculated into an ultra-low-attachment 96-well plate and observed for 14 d. The cells demonstrated their ability to form spheroids (Figure 3A). The results of the wound healing assay showed that CBC2T-2 cells showed higher levels of wound repair at both 24 and 48 h (Figures 3B and C). Furthermore, compared with TFK-1 cells, CBC2T-2 cells exhibited greater migratory and invasive capacities (Figure 3D). Moreover, CBC2T-2 cells exhibited significantly stronger clonogenic ability than TFK-1 cells (Figures 3E and F). These findings indicate that the cell line CBC2T-2 has a high capacity for healing, invasion, migration, and sphere formation, making it a valuable tool for studying the pathogenesis of cholangiocarcinoma sarcoma.

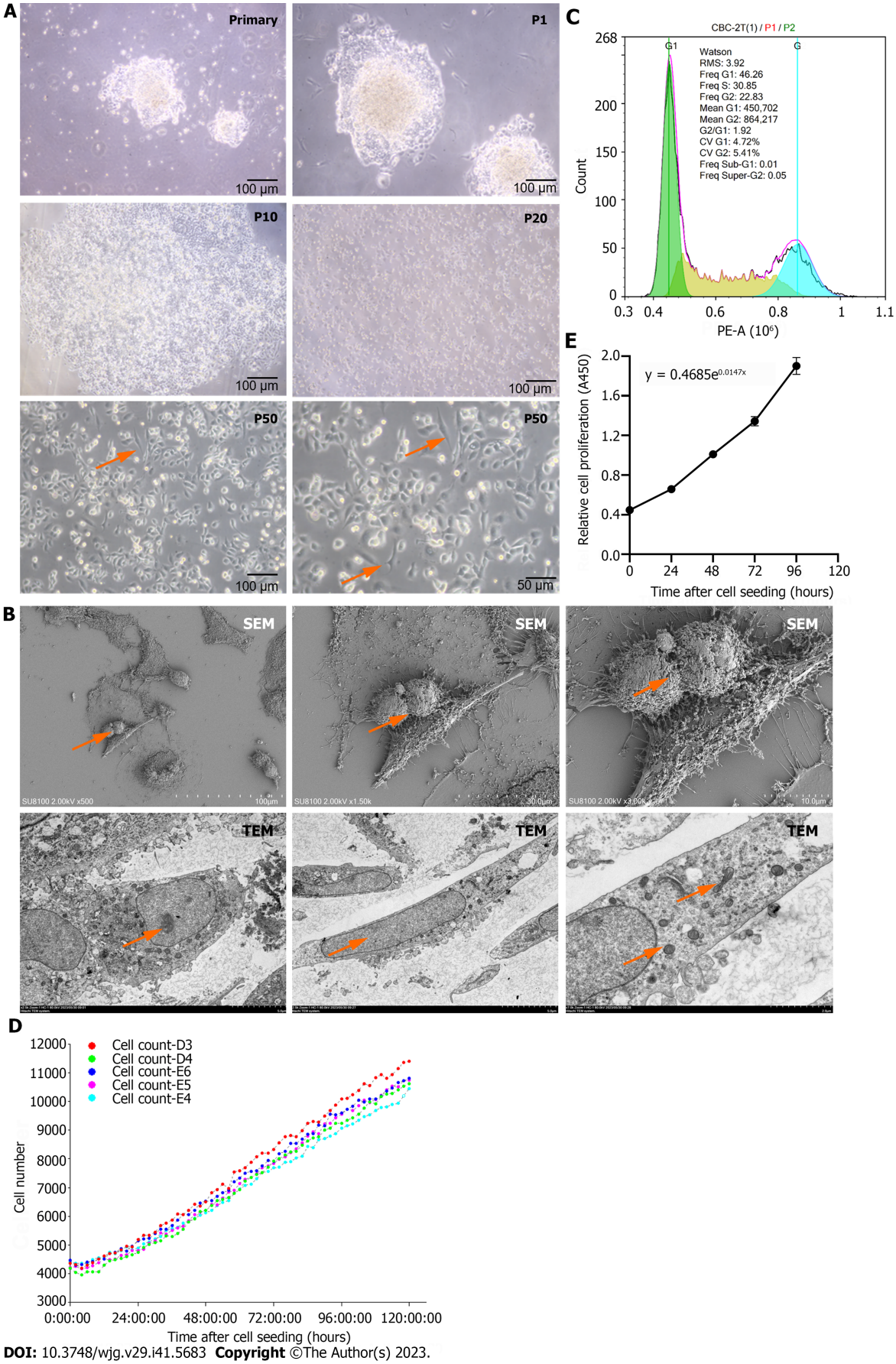

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Figure 2 Morphological features, doubling time and cell cycle of CBC2T-1 cells. A: Bright field morphology of CBC2T-2 cells in the primary,1st, 10th, 20th and 50th passages (scale bars, 100 μm); B: SEM and TEM electron microscopy were used to observe the cellular ultrastructure (scale bars, 100, 30, 10 μm); C: Flow cytometry of CBC2T-2 cells; D and E: Cumulative growth curve of CBC2T-2 cells.

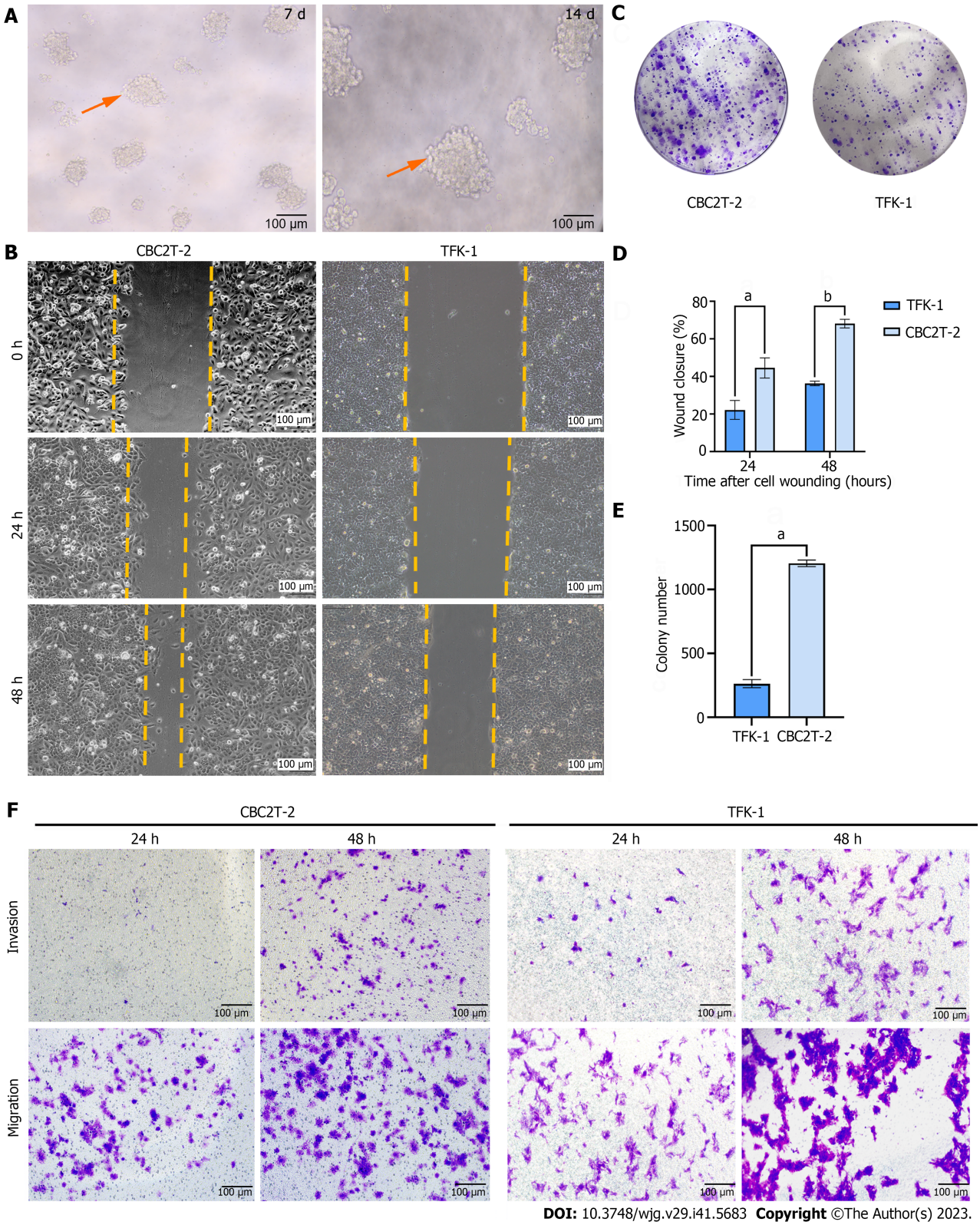

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Figure 3 Characterization of CBC2T-2 cell behavior. A: Representative images of the ability of CBC2T-2 cells to form spheres at days 7 and 14; B and C: Wound healing assay of CBC2T-2 and TFK-1 cells after 24 and 48 h; D: The migratory and invasive abilities of CBC2T-1 and TFK-1 cells were detected by transwell assay; E and F: Representative images showing the colony formation of CBC2T-1 and TFK-1 cells. Scale bars, 100 μm. [a]*P* < 0.01; [b]*P* < 0.001.

Cytogenetic analysis and genomic characteristics

Karyotype analysis of representative single cells from the CBC2T-2 cell line revealed abnormalities in both chromosome number and structure, with polyploidy chromosomes ranging from 69 to 79 and structural aberrations, including gain, deletion, and translocation (Figure 4A). When the value of the black line exceeds 2, it indicates an increase in the copy number, whereas a value below 2 suggests a decrease. Copy number alterations occurred throughout almost the entire chromosomal section of the cells and tumor tissues (Figure 4B). Single-nucleotide polymorphism analysis showed that the

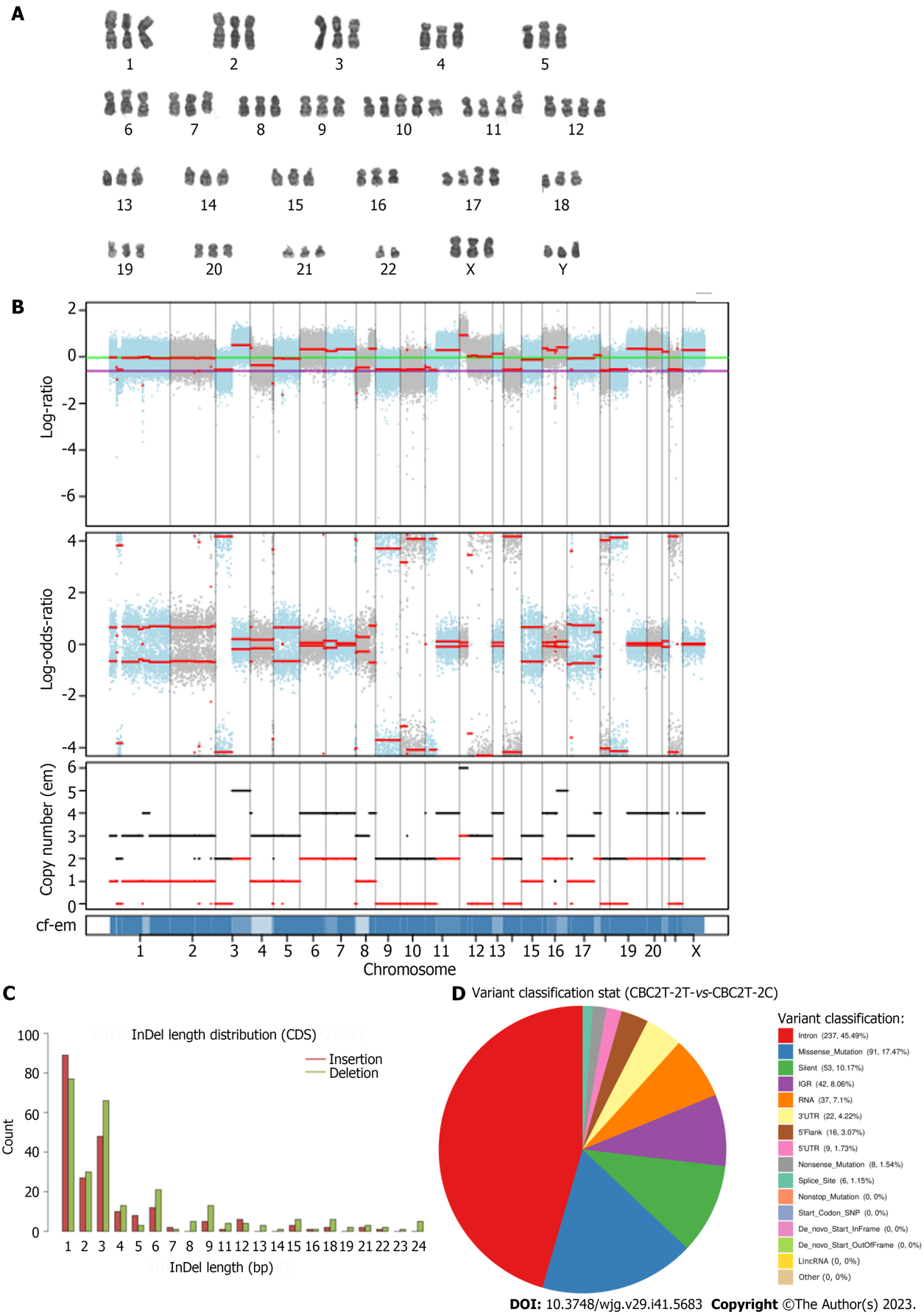

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Figure 4 Cytogenetic analysis and genomic characteristics. A: Chromosome karyotype analysis shows that the cells are triploid and there is variation in the number of structures; B: Cells and tumor tissues have copy number increases and decreases in almost the entire chromosome section; C: Histogram of the distribution of somatic cell InDel annotation results; D: Somatic somatic single-nucleotide variant annotation result distribution map.

Table 2 Comparison of somatic mutant genes detected with the Cancer Gene Census database

Gene name	Chrom	Pos	Ref	Alt	CGC-cancers
ALK	Chr2	29221210	G	T	Neuroblastoma
BARD1	Chr2	214809500	G	A	Ovariancancer; breastcancer; endometrioid cancer
KDR	Chr4	55106807	T	A	Melanoma
FAT1	Chr4	186621601	T	C	Pancreatic
WRN	Chr8	31167138	T	C	Osteosarcoma; meningioma; other tumour types
HNF1A	Chr12	120999418	T	C	Hepatic adenoma; hepatocellular carcinoma
BRCA1	Chr17	43071077	T	C	Breast; ovarian
BRCA1	Chr17	43092418	T	C	Breast; ovarian
AXIN2	Chr17	65535650	G	A	Colorectal carcinoma
SETBP1	Chr18	44876688	G	A	Neuroepithelial tumours

Chrom: Chromosome; Pos: Position on chromosome; Ref: Reference base on the genome; Alt: Mutated base; CGC-Cancers: Tumour types with driver genes included in the Cancer Gene Census database.

cell line CBC2T-2 had extensive insertions and deletions in almost the entire chromosomal fraction (Figure 4C). Somatic SNVs are single nucleotide variants of somatic mutations. On average, 521 SNVs were found in all samples, with an average of 91 missense mutations, eight nonsense mutations, and zero SNV invalidating the termination codon (Figure 4D).

A comparison of the detected mutated genes with the CGC database was used to screen for possible cancer susceptibility genes (Table 2). The major tumor susceptibility genes in CBC2T-2 cells included BALK, BARD1, KDR, FAT1, WRN, HNF1A, BRCA1, AXIN2, and SETBP1. We compared somatic mutations in CBC2T-2 with known driver genes from databases and the literature and identified known driver genes (Table 3). TP53 and ARID1A are the most frequently reported genetic alterations in extrahepatic cholangiocarcinoma[23].

Subcutaneous tumorigenesis assay and immunophenotyping

Xenograft tumor formation assays were performed and tumor growth was monitored weekly to confirm the tumorigenic ability of CBC2T-2 cells in NOD/SCID mice. Within 3 wk, tumors developed in all the three mice, indicating that the established cell lines exhibited good tumorigenicity and could be used to establish an *in vivo* model (Figures 5A-C).

After 8 wk, the mice were euthanized, and their tumors were removed for measurement and photography. HE and immunohistochemical staining were performed on the tumor xenografts. We observed tumors in mouse tumor grafts, CBC2T-2 (P30) cells, and the patient's primary tumor tissue using HE staining. The histological consistency was confirmed by the staining procedure (Figure 5D, first row). In addition, CK19, S-100, Vimentin, Desmin, CD117, and CD68 were positively expressed. These findings demonstrated that the primary tumor tissue had both an epithelial component (carcinoma) and a mesenchymal component (chondrosarcoma). Furthermore, *in vivo* and *in vitro* experiments were conducted to demonstrate histological concordance through immunohistochemistry (Figure 5D).

Sensitivity to anticancer drugs

The sensitivity of CBC2T-2 cells to the first-line anticancer drugs used for the treatment of cholangiocarcinoma was assessed. Among the chemotherapeutic agents tested, oxaliplatin (IC_{50} = 77.51 μM), paclitaxel (IC_{50} = 0.002 μM), 5-fluorouracil (IC_{50} = 7.516 μM), cisplatin (IC_{50} = 19.24 μM), and gemcitabine (IC_{50} = 0.009 μM) were evaluated. Our results demonstrated that among the five anticancer drugs, paclitaxel was the most sensitive, followed by gemcitabine. These findings provide clinical guidelines for the treatment of patients with cholangiocarcinoma sarcoma, suggesting a preference for combined paclitaxel and gemcitabine as the most effective treatment option (Figure 6).

DISCUSSION

Bile duct cancer is a highly aggressive malignant tumor that originates in the epithelial cells of the bile ducts. Its incidence and mortality rates are increasing worldwide and account for approximately 2% of all cancers, with autopsy detection rates ranging from 0.01% to 0.046%[24]. There are a few bile duct tumors with specific histological manifestations, among which carcinosarcomas are observed in the liver, gallbladder, pancreas, and jugular abdomen[25,26]. The pathological features of carcinosarcomas include tumor tissues with both epithelial and mesenchymal components[27,28]. The sarcomatous component typically comprises undifferentiated spindle cells and a variety of heterogeneous elements, including cartilage, bone, smooth muscle, and rhabdomyosarcoma cells[29]. The carcinoma component usually consists of adenocarcinoma and, rarely, squamous, small cell, and undifferentiated carcinomas. Moreover, the tissues are well

Table 3 Results of screening for known driver genes

Gene name	Chrom	Pos	Ref	Alt
ARID1A	Chr1	26780184	-	A
SFPQ	Chr1	35192164	G	C
DNMT3A	Chr2	25244384	G	A
CAD	Chr2	27237546	G	A
FBXO11	Chr2	47839654	C	T
LRP1B	Chr2	140683563	C	T
NEB	Chr2	151529162	G	A
NEB	Chr2	151540532	G	A
HOXD13	Chr2	176093701	C	T
MECOM	Chr3	169112718	C	A
MECOM	Chr3	169112734	C	G
ETV5	Chr3	186081304	G	C
NSD2	Chr4	1955726	C	T
NSD1	Chr5	177135451	G	C
AKAP9	Chr7	92097135	G	A
KMT2C	Chr7	152182489	G	A
BRD3	Chr9	134040128	C	T
FAS	Chr10	89014231	G	C
KIF20B	Chr10	89738070	C	G
NUP98	Chr11	3683422	G	T
NUP98	Chr11	3683472	C	T
TNKS1BP1	Chr11	57317951	T	C
KMT2D	Chr12	49040044	G	A
NAV3	Chr12	78159123	C	A
BTG1	Chr12	92145413	G	C
SRCAP	Chr16	30698165	G	A
CNOT1	Chr16	58538927	G	T
CNOT1	Chr16	58576526	C	T
TP53	Chr17	7661876	C	T
TP53	Chr17	7675217	T	C
PRKACA	Chr19	14092743	G	C
ASXL1	Chr20	32359040	C	G
CLTCL1	Chr22	19210202	G	A
MYH9	Chr22	36321935	G	C
DMD	ChrX	33077488	C	T
KDM5C	ChrX	53211367	C	T

Chrom: Chromosome; Pos: Position on chromosome; Ref: Reference base on the genome; Alt: Mutated base.

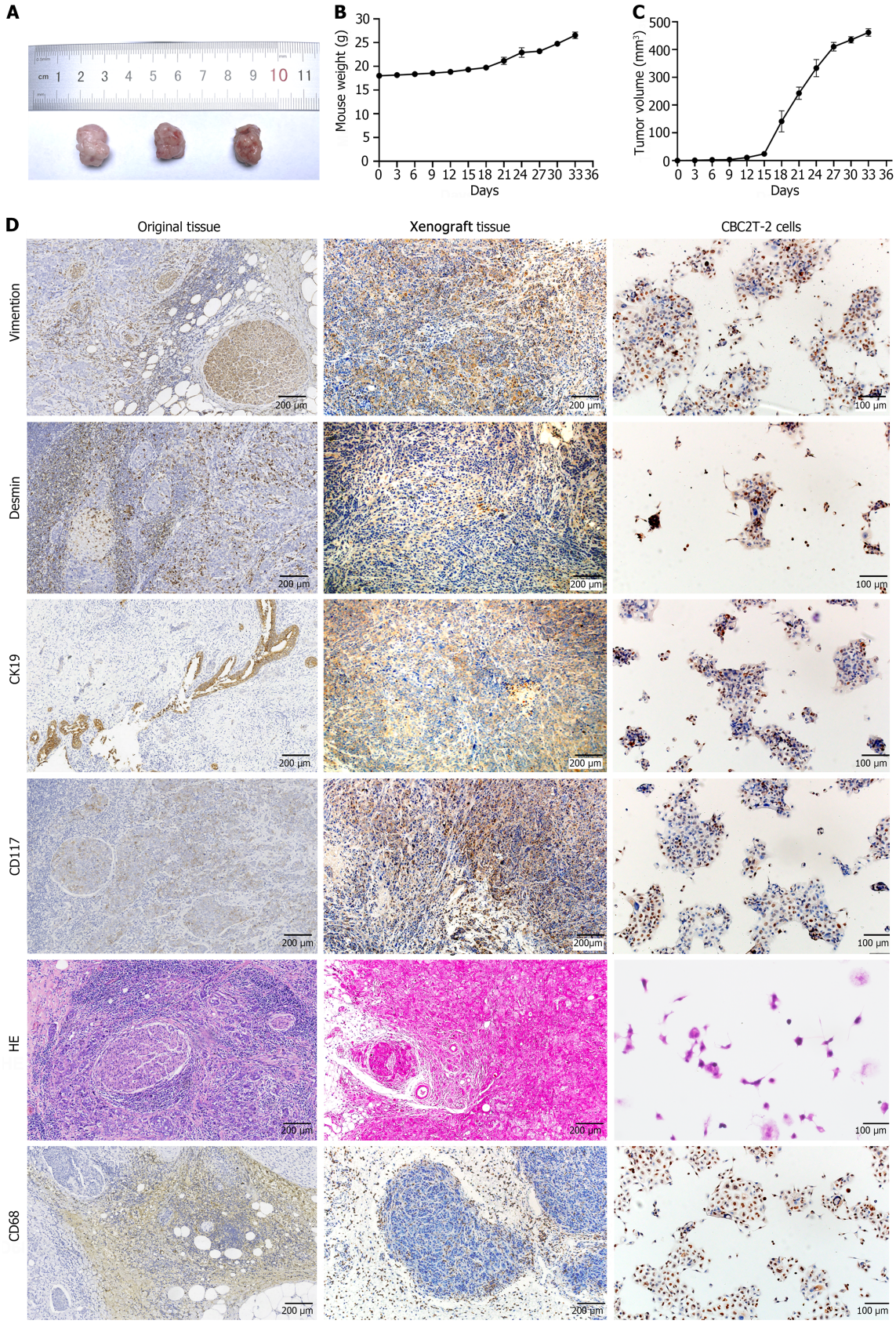

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Figure 5 Verification the tumorigenic ability of CBC2T-2 cells in non-obese diabetic/severe combined immunodeficient mice. A: Tumor formation in non-obese diabetic/severe combined immunodeficient mice; B: Body weight gain curves in xenograft mice; C: Tumor size over time in three xenograft mice; D: Hematoxylin and eosin staining and immunohistochemical (CK19, CD68, CD117, Vmention, Desmin) results of primary tumors, CBC2T-2 xenografts and CBC2T-2. Scale bars, 50 and 100 μm. HE: Hematoxylin and eosin.

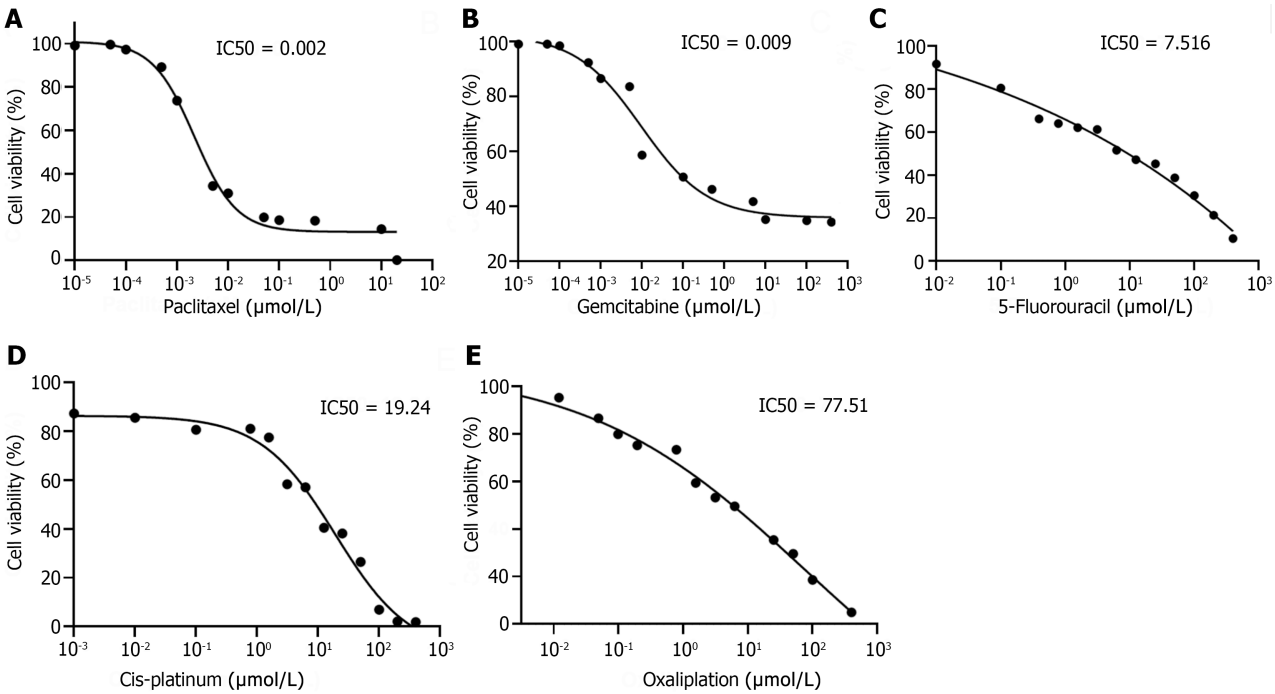

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Figure 6 Sensitivity of CBC2T-2 cell line to anticancer drugs. A-E: Sensitivity of CBC2T-2 cells to paclitaxel, gemcitabine, 5-fluorouracll, cisplatin, and oxaliplatin.

defined and not transformed[30]. Recently, a new hypothesis of epithelial-mesenchymal transition (EMT) suggested that during EMT, cancer cells lose their epithelial properties and intercellular adhesion, transform into mesenchymal cells, and acquire the ability to migrate[31,32]. In contrast, cholangiocarcinoma sarcoma with chondrocytes is a very rare malignancy that has rarely been reported in domestic and international literature[33].

The patient-derived cell lines were consistent with the principles of precision medicine and translational research[34]. Although cell lines derived from rare tumors have significant utility in research, they are difficult to obtain from public cell banks. The development and evaluation of new therapeutic approaches for cholangiocarcinoma sarcoma are limited by the lack of available models. A tumor model is necessary for gaining a deeper understanding and developing effective solutions to overcome the poor prognosis of patients diagnosed with this disease[35]. Herein, we report the successful establishment of a new cell line, CBC2T-2, derived from a patient with cholangiocarcinoma sarcoma, along with detailed clinical and pathological data from the donor patient. This newly established cell line is a breakthrough in the establishment of cholangiocarcinoma sarcoma cell lines and fills a gap in the sarcoma cell lines. This cell line may provide a valuable tool for studying the mechanisms of cholangiocarcinoma progression and developing new therapeutic approaches.

We confirmed that CBC2T-2 cells can proliferate, form spheroids and clones, and migrate for invasion. The short ploidy time and high migratory aggressiveness observed *in vitro* may have contributed to the poor clinical outcomes of cholangiocarcinoma sarcoma. It is biologically stable in culture and capable of undergoing stable passages, which now exceed 100 generations. To determine the tumorigenic potential of CBC2T-2 cells, they were xenografted into NOD/SCID mice. The resulting solid tumors were histologically identical to the original surgical specimens, indicating that CBC2T-2 may serve as a reliable tool for preclinical *in vivo* studies.

The cell line CBC2T-2 (P30) and the primary tumor tissue of the patient were subjected to immunohistochemistry and immunocytochemistry to verify histological consistency. Sarcoma cells are locally positive for keratin (CK19), an epithelial marker[36]. The sarcoma component markers, Vimentin and Desmin, and the mesenchymal cartilage differentiation marker, S-100, are positively expressed[37-41]. This confirmed that the tumor was composed of adenocarcinoma and sarcomatous components with chondroid differentiation[42]. The sarcomatous component was mixed with and was adjacent to the carcinomatous component. Based on the histological findings, it is possible that the sarcomatous component resulted from an invasive carcinomatous component[43]. However, determining the histogenesis of this tumor remains challenging, similarly to the histogenesis of carcinosarcomas in other organs[43].

We performed whole-exome sequencing (WES) on CBC2T-2 cells and normal tissues adjacent to primary tumors to screen for somatic mutations. In this study, we identified the cancer susceptibility genes associated with cholangiocarcinoma, including BALK, BARD1, KDR, FAT1, WRN, HNF1A, BRCA1, AXIN2, and SETBP1. Additionally, we identified the tumor driver genes, TP53 and ARID1A, in the CBC2T-2 cell line, which are common driver genes for extrahepatic cholangiocarcinoma and play important roles in the CBC2T-2 cell line. The identification of the most commonly mutated genes associated with cholangiocarcinoma using WES in the CBC2T-2 cell line makes the cells a potential model for studying the pathogenesis of this disease.

We characterized the response of CBC2T-2 cells to treatment with anticancer drugs. Furthermore, we calculated the semi-inhibitory concentration of the drugs on CBC2T-2 cells. Among the tested compounds, paclitaxel exhibited the lowest semi-inhibitory concentration, followed by gemcitabine. Although other anticancer drugs exhibited significant antiproliferative effects, their semi-inhibitory concentrations were lower than those of paclitaxel and gemcitabine. This drug sensitivity analysis can provide clinical treatment information and guide the clinical use of paclitaxel in combination with gemcitabine.

CONCLUSION

We established the first human cholangiocarcinoma sarcoma cell line, CBC2T-2, with stable biogenetic traits. This cell line, as a research model, has a high clinical value and would facilitate the understanding of the pathogenesis of cholangiocarcinoma sarcoma.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma sarcoma is a rare tumor composed of epithelial and mesenchymal cells, and the sarcomatous component in carcinosarcoma is differentiated from spindle cells or pleomorphic cells.

Research motivation

There is no extrahepatic cholangiocarcinoma sarcoma cell line.

Research objectives

To establish and characterize a new extrahepatic cholangiocarcinoma sarcoma cell line.

Research methods

Establish cell lines through primary culture and subculture, and identify their biological characteristics.

Research results

In this study, we successfully established and characterized of a extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2, from the primary tumor of a patient with cholangiocarcinoma sarcoma.

Research conclusions

To date, first report of the extrahepatic cholangiocarcinoma sarcoma cell line CBC2T-2.

Research perspectives

The establishment of extrahepatic cholangiocarcinoma sarcoma cell line provides suitable experimental models for further study of cholangiocarcinoma sarcoma.

ACKNOWLEDGEMENTS

We would like to thank Professor Hao Xu and Chun-Lu Dong for providing critical revisions to the manuscript.

FOOTNOTES

Author contributions: Jiang NZ and Ren YX interpreted the study design; Bai MZ and Gao L supervised our study; Yue P, Lin YY, and Meng WB obtained the research fund; Huang CF, Ma ZL, and Mi NN screened the publications, performed statistics, and drafted the manuscript; Ma HD and Lu YW helped perform statistics; Fu WK, Tian L, Zhao JY, Zhang BP, Zhang XZ, Zhang C, and Zhang Y revised the manuscript; and all authors contributed to the article and approved the submitted version.

Supported by the National Natural Science Foundation of China, No. 82060551; and Lanzhou Chengguan District Science and Technology Planning Project, No. 2019JSCX0092.

Institutional review board statement: This study was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL-2022-489) and informed consent was obtained from all the patients. This study adhered to the 1964 Declaration of Helsinki and its subsequent amendments and similar ethical standards.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the

Institutional Animal Care and Use Committee of the First Hospital of Lanzhou University.

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

Data sharing statement: The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

ORCID number: Wen-Kang Fu 0000-0001-9969-1603; Yan-Xian Ren 0000-0002-0433-613X; Chao Zhang 0000-0002-0228-7688; Yan-Yan Lin 0000-0001-7417-0190; Wen-Bo Meng 0000-0002-9355-0225.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Cai YX

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