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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 7; 27(41): 7005-7209

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 27 Number 41 November 7, 2021

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The *WJG* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for *WJG* as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Ze-Mao Gong*.

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski, Subrata Ghosh

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

PUBLICATION DATE
November 7, 2021

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World J Gastroenterol 2021 November 7; 27(41): 7005-7013

DOI: 10.3748/wjg.v27.i41.7005

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

EDITORIAL

Nucleic acid vaccines: A taboo broken and prospect for a hepatitis B virus cure

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Author contributions: Tsounis PE was responsible for the literature search and analysis, drafting of the manuscript, and interpretation of the data; Mouzaki A and Triantos C were responsible for revising the manuscript for important intellectual content; All authors gave their final approval of the submitted version.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Country/Territory of origin: Greece

Specialty type: Infectious diseases

Peer-review report's scientific quality classification

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

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Abstract

Although a prophylactic vaccine is available, hepatitis B virus (HBV) remains a major cause of liver-related morbidity and mortality. Current treatment options are improving clinical outcomes in chronic hepatitis B; however, true functional cure is currently the exception rather than the rule. Nucleic acid vaccines are among the emerging immunotherapies that aim to restore weakened immune function in chronically infected hosts. DNA vaccines in particular have shown promising results *in vivo* by reducing viral replication, breaking immune tolerance in a sustained manner, or even decimating the intranuclear covalently closed circular DNA reservoir, the hallmark of HBV treatment. Although DNA vaccines encoding surface antigens administered by conventional injection elicit HBV-specific T cell responses in humans, initial clinical trials failed to demonstrate additional therapeutic benefit when administered with nucleos(t)ide analogs. In an attempt to improve vaccine immunogenicity, several techniques have been used, including codon/promoter optimization, coadministration of cytokine adjuvants, plasmids engineered to express multiple HBV epitopes, or combinations with other immunomodulators. DNA vaccine delivery by electroporation is among the most efficient strategies to enhance the production of plasmid-derived antigens to stimulate a potent cellular and humoral anti-HBV response. Preliminary results suggest that DNA vaccination *via* electroporation efficiently invigorates both arms of adaptive immunity and suppresses serum HBV DNA. In contrast, the study of mRNA-based vaccines is limited to a few *in vitro* experiments in this area. Further studies are needed to clarify the prospects of nucleic acid vaccines for HBV cure.

Key Words: Chronic hepatitis B; Therapeutic vaccination; Nucleic acid vaccines; DNA vaccines; Electroporation; Immunotherapy

Received: March 18, 2021
Peer-review started: March 18, 2021
First decision: July 3, 2021
Revised: July 7, 2021
Accepted: October 14, 2021
Article in press: October 14, 2021
Published online: November 7, 2021

P-Reviewer: Martin Mateos RM
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Ma YJ

Core Tip: A nucleic acid vaccine could be of particular value in the field of hepatitis B virus therapies. DNA vaccines have been studied more extensively over the past two decades and have been shown to overcome immune exhaustion in preclinical models of chronic infection. Although vaccination elicited robust humoral and cellular immune responses, it had negligible effects on clinical endpoints. Therefore, the scientific community has focused on optimizing vaccine design and delivery to improve immunogenicity. Electroporation-mediated delivery of multivalent plasmids in combination with molecular adjuvants could efficiently restore adaptive immunity in virally suppressed patients and be part of future combination therapy.

Citation: Tsounis EP, Mouzaki A, Triantos C. Nucleic acid vaccines: A taboo broken and prospect for a hepatitis B virus cure. *World J Gastroenterol* 2021; 27(41): 7005-7013
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7005.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7005

INTRODUCTION

Hepatitis B virus (HBV) is a relatively small (3.2 kb) DNA virus that causes acute or chronic liver infection leading to cirrhosis or hepatocellular carcinoma. Although an effective preventive vaccine containing hepatitis B surface antigen (HBsAg) produced from recombinant DNA has been available for more than two decades[1], chronic hepatitis B (CHB) remains a major burden of liver-related morbidity and mortality, with more than 292000000 infections worldwide[2]. Current treatment strategies, *i.e.* nucleoside or nucleotide (NA) drugs and interferon-α (IFN-α), effectively suppress viral replication, prevent progression of liver disease to end-stage, and improve patient quality of life. However, a truly functional cure, defined as undetectable HBV DNA with concomitant elimination of HBsAg over a limited therapeutic period, is rarely achieved with existing therapies[3]. HBV has evolved several mechanisms to become chronic, and the failure to eradicate HBV is largely due to the inability to address the following issues: (1) Organization of the viral genome into a highly stable, drug-resistant, chromosomal covalently closed circular DNA (cccDNA) conformation in the nuclei of infected cells; (2) Integration of HBV DNA into the host genome; (3) Disruption of innate immunity signaling; and (4) Depletion of HBV-specific T and B cells[4].

There is a growing consensus that elimination of HBV requires a combination of treatments that include traditional therapeutics, novel direct-acting antivirals that target different steps of the HBV life cycle, and immunotherapies to restore the host immune response. A number of immunomodulators are in clinical trials, including pattern recognition receptor agonists, immune checkpoint inhibitors, adaptive transfer of engineered T cells, and therapeutic vaccines[5]. Various vaccine delivery platforms are being explored to optimize immunogenicity and elicit robust responses to rejuvenate the exhausted immune system. In particular, viral vector technology, immune complex platforms, virus-like particle-based vaccines, and nucleic acid vaccines are some of the categories of therapeutic vaccines under development[6].

Although the world is still struggling with a global health crisis, coronavirus disease 2019 has been at the forefront of scientific breakthroughs in the field of vaccinology, bringing nucleic acid vaccine technology to the forefront. Recent technological innovations have improved the delivery, tolerability, and efficacy of DNA- and mRNA-based therapeutics. Two prophylactic mRNA vaccines against severe acute respiratory syndrome coronavirus 2 were the first drugs in this category to be approved for human use and represent the pinnacle of progress[7,8]. Clearly, the role of nucleic acid-based vaccines in restoring immune dysfunction in CHB and their prospects as part of future combination therapy need to be re-evaluated.

HBV-INDUCED IMMUNE DYSREGULATION

Resolution of acute HBV infection requires an alert innate immune system and polyclonal and multispecific cellular and humoral responses. The human innate immune system is equipped with several pattern recognition receptors that recognize pathogen- or damage-associated patterns and initiate intracellular signal transduction leading to the production of antiviral IFNs and proinflammatory cytokines. Previous studies in humans and chimpanzees reported limited IFN type I production during the initial phase of the logarithmic rise in viremia, supporting the view that HBV is a "stealth virus"[9,10]. However, recent data show that hepatocytes express pattern recognition receptors that recognize HBV components, highlighting the role of nucleic acid signaling and the associated activation of NF-κB-dependent pathways[11]. Therefore, it seems more likely that HBV impairs intrinsic immunity to establish chronic infection. Indeed, HBV alters the functional phenotype of monocytes/macrophages by inducing the secretion of anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor-β and suppressing the production of tumor necrosis factor-α and IL-12 by inhibiting the toll-like receptor-2 downstream signaling pathway[12,13]. In parallel, myeloid-derived suppressive cells are recruited to CHB and contribute to the immunosuppressive cascade by secreting IL-10 and arginase and downregulating IFN-γ expression by T cells[14]. In addition, dendritic cells, which are critical for generating effective adaptive immune responses, exhibit decreased antigen presentation capacity, cell migration capacity, phagocytic activity, and cytokine production, possibly due to inhibition of costimulatory molecule expression[15]. Therefore, HBV creates a tolerogenic microenvironment in the liver infiltrated with IFN-γ-deficient natural killer cells and T regulatory cell populations[16]. This dysregulated milieu has a significant impact on T and B cell maturation and differentiation, resulting in impaired adaptive immune responses. Moreover, prolonged exposure to high concentrations of viral antigens contributes to T cell exhaustion, characterized by reduced cytotoxic capacity, impaired proliferative capacity, and upregulation of inhibitory molecules (programmed death-1 (PD-1), CTLA-4, T cell immunoglobulin and mucin-domain containin-3). Clearly, approaches aimed at restoring or stimulating sustained HBV-specific cellular and humoral responses may be central to the treatment of chronically infected patients.

DNA VACCINES

DNA vaccine technology is based on genetically engineered plasmids containing a potent promoter that triggers increased transcriptional activity *in vivo*, followed by a sequence encoding the preselected immunogenic antigen(s). The corresponding plasmid is administered either systemically or primarily topically *via* intramuscular injection. Taking advantage of the host cellular apparatus, the exogenous DNA is delivered to the nuclei of transfected cells, including resident antigen-presenting cells (APCs).

The expression of plasmid-encoded antigens and their presentation by major histocompatibility complex (MHC) class I and MHC class II molecules are key elements of adaptive immunity. In particular, transfected myocytes or keratinocytes express the plasmid-derived proteins, which are later released into the bloodstream *via* exosomes or apoptotic bodies[17]. APCs play an important role in this process by mediating the presentation of vaccine peptides both on MHC-I molecules, either by direct transfection or cross-presentation, and on MHC-II molecules after uptake of circulating antigens. Subsequently, afferent lymphatic vessels transport APCs to lymph nodes, where they present the antigenic epitopes to naïve T and B cells and provide essential costimulatory signals. This interaction leads to clonal expansion of CD8+ T cells, which elicit a strong cytotoxic response, and CD4+ T cells, which regulate the differentiation of antigen-presenting B cells[18].

DNA vaccine platforms have many advantages: they are fast to develop, easy to replicate, and very stable at room temperature, resulting in low manufacturing and storage costs[19]. Theoretically, they are safer than conventional live attenuated vaccines because they do not elicit potential anti-vector immune responses and by continuously expressing antigens elicit a long-lasting response without being infectious[20]. Moreover, the intracellular synthesis of the encoded antigens enables the endogenous post-translational modifications that generate proteins in their native conformation[18]. In addition, the expression of a broader repertoire of antigenic epitopes is possible by combining two or more plasmids or by constructing polycis-

tronic carriers[19]. Indeed, DNA vaccination has shown promising results in preclinical models of chronic hepatitis virus infection. In HBV transgenic mice, vaccination broke immune tolerance and caused a significant decrease in viral replication [21], while in a duck HBV model it was able to reduce the intranuclear cccDNA pool [22]. Moreover, immunization with a DNA prime adenovirus boost vaccine in transgenic mice showed strong synergistic effects with NAs, eliciting sustained suppression of viral replication and strong and specific CD8+ T cell responses[23].

The initial clinical trials investigating therapeutic vaccination in CHB patients showed moderate efficacy in restoring host T cell responses but dampened enthusiasm by demonstrating moderate immunogenicity. In a phase I clinical trial, administration of a DNA vaccine expressing envelope proteins (S and preS2/S) resulted in transient activation of IFN-γ-producing T cells and a reduction in HBV DNA in 10 viremic patients[24]. Repeated immunization doses were well tolerated, resulted in proliferative responses against HBsAg, and achieved hepatitis B e antigen (HBeAg) seroconversion in 2 participants[24,25]. In another study, DNA-based vaccination resulted in changes in peripheral NK cell populations, with a relative increase in $CD56^{bright}$ NK cells correlating with HBV-specific T cell activity, indicating the importance of $CD56^{bright}$ NK cells in shaping the adaptive immune response[26].

To determine whether therapeutic vaccines are unable to reactivate pre-existing HBV-specific T cells due to persistent antigenemia, DNA vaccines were administered in combination with conventional therapeutics. The efficacy of a preS2/S-expressing DNA vaccine was evaluated in a phase I/II clinical trial in virus-suppressed patients on stable NA therapy. Addition of the vaccine to NA treatment elicited multispecific, polyfunctional, and far more sustained CD4+ T cell responses compared with monotherapy. Nevertheless, the immunostimulatory effect was not strong enough to influence relapse rates after discontinuation of NA[27]. Accordingly, five intramuscular injections of an envelope-expressing DNA vaccine failed to sufficiently restore immune function or reduce relapse risk after treatment discontinuation in a prospective multicenter study of 70 virus-suppressed CHB patients[28]. These results have underscored the need to optimize vaccine immunogenicity, use new delivery systems, and re-evaluate vaccination regimens.

Recently, a new generation of DNA-based vaccines has demonstrated significant immunostimulatory activity with a favorable safety profile. Their preventive or therapeutic value has already been investigated in clinical trials on infectious diseases (Ebola virus, ZIKA virus, HIV, influenza virus) or malignancies (prostate cancer, cervical cancer, and human papillomavirus-related head and neck tumors)[29]. This progress is mainly due to the breakthroughs in biomedical engineering and nanotechnology, which have introduced novel delivery platforms to improve DNA uptake compared to previous needle approaches, *e.g.*, gene gun, jet injection, advanced electroporation (EP), and chemical or biological adjuvant systems[19]. In addition, various molecular tools have been used to improve the immunogenicity of DNA vaccines, *e.g.*, codon optimization, plasmid vector backbone optimization, attachment of virus-derived nuclear localization signals to facilitate nuclear entry, or DNA-complexing nanocarriers to prevent extracellular DNA degradation[17,30]. The development of target-specific plasmid-encoded chimeric molecules and polycistronic vectors that contain genetic loci encoding cytokines or other signaling molecules in addition to immunogenic epitopes are alternative strategies to further stimulate immune responses[19,29].

In this regard, Wang *et al*[31] demonstrated the superior protective effect of a bicistronic DNA vaccine encoding the core protein plus IFN-γ gene sequences compared to a monovalent vaccine expressing the core antigen in a model of HBV infection in marmosets. Experimental *in vivo* models have also shown that coadministration of plasmids encoding cytokine sequences (IL-2, IFN-γ) with core-expressing DNA vaccines increases the production of HBV-specific neutralizing antibodies[32] and that the cccDNA reservoir was depleted in the majority of subjects receiving IFN-γ as an adjuvant[33]. In an attempt to optimize antigen delivery to APCs and thereby increase vaccine efficacy, plasmids were combined with a protein that targets dendritic cells *via* electrostatic coupling (pSVK-HBVA vaccine). In HBV transgenic mice, the pSVK-HBVA vaccine significantly reduced HBV DNA copy number as well as circulating HBsAg[34].

These encouraging preclinical data formed the basis for transferring these techniques from the laboratory bench to clinical trials. Yang *et al*[35] showed that the DNA-based vaccine HB-100, which consists of five different plasmids expressing most HBV antigens as well as a human IL-12 mutant (hIL-12N222L) induced durable CD4+ memory T cell responses when administered to 12 chronically infected Caucasian patients receiving regular lamivudine therapy. Moreover, the DNA vaccine in

combination with an oral antiviral resulted in HBeAg seroconversion in 4 of 6 participants, whereas HBsAg clearance was achieved in only 1 patient, who had the highest concentration of HBV-specific IFN-γ-secreting memory T cells[35]. Long-term control of viremia also correlated with an increase in plasma IL-12 and IL-12/p40 ratio [36].

These positive observations promoted the development of HB-110, a second generation multivalent HBV DNA vaccine comprising three plasmids encoding envelope proteins, core protein/polymerase, and the human IL-12 mutant. HB-110 was administered in a randomized, dose-escalated phase I clinical trial to Korean patients with CHB treated with adefovir dipivoxil. Although HB-110 elicited robust humoral and T cell responses in the HBV mouse model, vaccination in the Korean cohort resulted in weaker HBV-specific T cell responses and lower HBeAg seroconversion rates than HB-100 immunization in Caucasian patients[37]. These unexpected results may be due in part to the fact that HB-110 was tested in Asian patients, most of whom acquire HBV by vertical transmission and have high immune tolerance. It is clear that in addition to improving the immunogenicity of the vaccine, a combination of therapies is required to address compromised immune function and effectively eradicate chronic HBV infection.

In this context, the advent of immune checkpoint inhibitors has paved the way for alternative strategies to overcome T cell exhaustion or anergy. Remarkably, the addition of a PD-1 receptor inhibitor to treatment with NA plus DNA vaccine resulted in significant expansion and activation of virus-specific CD8+ T cells and prolonged suppression of viral replication in marmosets[38]. A phase I clinical trial of the PD-1 blocker nivolumab alone or in combination with the yeast-derived vaccine GS-4774 in 24 HBeAg-negative virally suppressed patients demonstrated not only high-affinity binding of nivolumab to its ligand but also sustained occupancy of the receptor. In addition, significant HBsAg responses were observed without serious adverse events, and HBsAg levels decreased below the limit of detection in 1 participant[39].

ELECTROPORATION-MEDIATED DELIVERY

One of the most efficient methods of DNA vaccine delivery is EP, which uses an electrical pulse to create a potential difference across the cell membrane that creates transient pores, thereby increasing membrane permeability[40]. Compared to conventional injection of plasmid DNA with a syringe, a pulsed electric field dramatically increases cellular uptake, approximately by a factor of 500, resulting in profound immune responses[29]. At the same time, low inflammation and altered vascular permeability cause a local influx of APCs and other immune cells, suggesting robust antigen processing and presentation at the injection site[41]. Cova[42] gave a concise overview of the progress of EP-mediated therapeutic vaccination from experimental models to clinical reality. Initial studies of EP-based DNA vaccination showed that it is capable of eliciting *in vivo* multispecific cytotoxic T cell responses and more effective immune stimulation, resulting in a dose-sparing effect of the vaccine[43]. EP-mediated administration of HB-110 accelerated antigen expression, increased anti-HBs antibody production, and elicited a broader repertoire of multispecific cellular responses in mice against all antigens, including subdominant epitopes[44]. Comparable conclusions emerged from high-pressure injection in combination with EP, to deliver codon-optimized HBc and IL-12 expressing plasmids that elicit polyfunctional T cell responses[45].

In the duck hepatitis B virus model, EP-mediated vaccination increased the production of neutralizing HBV-specific antibodies, expanded the spectrum of targeted epitopes[46] and resulted in T helper type 1 polarization[47]. Short non-coding DNA fragments appear to increase the immunopotency of EP-mediated HBV DNA vaccination[48]. DNA vaccine platforms based on EP show an enhanced ability to activate both arms of adaptive immunity and thus represent an important tool to overcome immunological exhaustion in CHB.

Regarding human studies, a dual plasmid-HBV vaccine consisting of a therapeutic plasmid encoding the preS2/S antigen and an adjuvant plasmid containing the fused sequence of IL-12 and IFN-γ was the first to be administered *via* electroporation against CHB. In total, 6 of 39 HBeAg-positive participants received vaccine monotherapy, while the remaining patients were randomized 1:2 to receive either lamivudine plus placebo or the experimental treatment, lamivudine plus vaccination. The EP-mediated immunization stimulated HBV-addressing IFN-γ-producing T cells, and the combination therapy produced a more profound suppression of viral replication

and a lower risk of virologic breakthrough compared with NA monotherapy[49].

The satisfactory immunostimulatory efficacy followed by a very consistent safety record as demonstrated in the pilot study has led to a phase IIb trial of the EP-mediated dual plasmid DNA vaccine. In this study, 225 previously untreated HBeAg-positive patients were divided 1:1 into groups treated with either lamivudine plus vaccine or lamivudine plus placebo. Although the primary endpoint of undetectable HBV DNA rate or HBeAg seroconversion was not met, four intramuscular doses of the vaccine showed a modest therapeutic effect, with more vaccinated patients achieving a > 2 log10 IU/mL decrease in HBV DNA compared with the control group[50].

Other HBV DNA vaccines administered *via* EP are currently being investigated in clinical trials with different eligibility criteria, combination regimens, or dosing schedules. An open-label phase I study (NCT02431312) evaluating the safety and reactogenicity of dose combinations of INO-1800 (DNA plasmid expressing core and surface antigens) and INO-9112 (DNA plasmid containing IL-12 sequences) in patients who have received stable NA therapy for at least 1 year has been completed, and final results are pending. In addition, a phase I study (NCT03463369) is underway to evaluate the efficacy of the DNA vaccine JNJ-64300535 administered by EP-mediated intramuscular injection in virally suppressed CHB patients on NA treatment. Overall, administration of the DNA vaccine by EP resulted in multispecific humoral and long-lasting memory T cell responses, although efficacy on clinical endpoints was subpar. These results are important for future studies in which careful patient selection is performed and EP-mediated vaccination realizes its full potential to efficiently restore adaptive immune responses in CHB.

MRNA VACCINES

mRNA vaccines are an attractive alternative to traditional vaccine platforms because they allow a rapid and scalable manufacturing process without being infectious or carrying the risk of integration. Compared to plasmid vaccines, mRNA vaccines have a stronger immunostimulatory effect on innate immunity and result in the desired vaccine responses[51]. Optimization of mRNA stability and translation as well as advances in vaccine delivery have led to increased use of mRNA therapeutics in basic research and clinical trials for the treatment of infectious diseases and cancer[52]. Nevertheless, research on mRNA-based vaccines in the field of anti-HBV prevention or control strategies has been sparse.

In an attempt to develop an anti-HBV mRNA vaccine for prophylactic or immunotherapeutic purposes, Lamb[53] developed and implemented an mRNA production process containing all critical HBsAg epitopes. The mRNA lipoplex nanoparticles were designed to protect against exonuclease degradation, promote endocytosis-mediated cellular uptake, and facilitate endosomal escape of the entrapped mRNA. Upon release into the cytoplasm, the mRNA is used by the host translational machinery as a template for the production of S-HBsAg or L-HBsAg, which are amenable to intrinsic post-translational modifications and are either secreted or degraded in a proteasome-dependent manner. *In situ* processing and presentation of the mRNA-derived antigen products would then resemble that of DNA vaccines and elicit robust pathogen-specific humoral and cell-mediated immune responses[53,54]. Interestingly, an mRNA-based vaccine formulation that was efficiently translated into detectable L-HBs and S-HBs in cultured hepatoma cells was produced using a highly reproducible method. However, L-HBs was expressed at lower levels than expected, and its secretion was modest, possibly reducing its immunostimulatory effect[53]. Clearly, in later stages of development, optimization of downstream processes is required to fully exploit the immunogenic benefits of the vaccine.

CONCLUSION

Recently, numerous nucleic acid vaccines have been used in clinical trials to prevent or treat infectious diseases as innovations in biotechnology have improved their immunogenicity and tolerability. Compared to mRNA-based formulations, plasmid vaccines have been studied more intensively in the field of HBV therapeutics. Pioneering preclinical studies have shown that DNA vaccination could suppress HBV transcriptional activity and even affect the cccDNA pool and was able to break CHB immune tolerance. In parallel, the introduction of *in vivo* EP as a delivery platform has dramatically improved the immunostimulatory effect of DNA vaccines without

serious adverse events.

Given these data and despite the moderate results of the initial clinical trials, the design of new studies to clarify the role of DNA vaccination in this field is essential. An ideal vaccine candidate would contain multiple dominant and subdominant HBV epitopes in combination with appropriate adjuvants to elicit potent and multispecific responses and should be administered *via* EP to enhance immunogenicity. Add-on or sequential treatment regimens could be applied to progressively repair the dysregulated adaptive immune responses and eventually achieve HBV eradication. Identifying patients who benefit most from immunization strategies should be a priority of future studies. Preferably, vaccination should be studied in virus-suppressed CHB patients on stable therapy with second-generation NAs that are less susceptible to drug resistance, such as entecavir or tenofovir. In summary, nucleic acid-based immunotherapies still have a long way to go before market approval but may retain a place in the context of combination therapy aimed at a functional cure for HBV.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7014-7024

DOI: 10.3748/wjg.v27.i41.7014

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

FRONTIER

Recent insights into the characteristics and role of peritoneal macrophages from ascites of cirrhotic patients

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Author contributions: García-Peñ arrubia P designed the overall concept outline of this manuscript and was responsible for writing, editing, reviewing the bibliography and approving the final version of the article; Ruiz-Alcaraz AJ wrote the manuscript, compiled, reviewed and edited the bibliography, adapted the figures and approved the final version of the article; Martínez-Esparza M wrote the manuscript, compiled and reviewed the bibliography and approved the final version of the article; Ruiz-Ballester M and Rami rez-Pávez TN meet the criteria for authorship established by the International Committee of Medical Journal Editors and approved the final version of the article.

Conflict-of-interest statement: The authors declare no conflict of interest.

Country/Territory of origin: Spain

Specialty type: Gastroenterology

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Abstract

Macrophages are a diverse myeloid cell population involved in innate and adaptive immune responses, embryonic development, wound repair, and regulation of tissue homeostasis. These cells link the innate and adaptive immunities and are crucial in the development and sustainment of various inflammatory diseases. Macrophages are tissue-resident cells in steady-state conditions; however, they are also recruited from blood monocytes after local pathogen invasion or tissue injury. Peritoneal macrophages vary based on their cell complexity, phenotype, and functional capabilities. These cells regulate inflammation and control bacterial infections in the ascites of decompensated cirrhotic patients. Our recent work reported several phenotypic and functional characteristics of these cells under both healthy and pathological conditions. A direct association between cell size, CD14/CD16 expression, intracellular level of GATA-6, and expression of CD206 and HLA-DR activation/maturation markers, indicate that the large peritoneal macrophage $CD14^{high}CD16^{high}$ subset constitutes the mature phenotype of human resident peritoneal macrophages during homeostasis. Moreover, elevated expression of CD14/CD16 is related to the phagocytic capacity. The novel large $CD14^{high}CD16^{high}$ peritoneal subpopulation is increased in the ascites of cirrhotic patients and is highly sensitive to lipopolysaccharide (LPS)-induced activation, thereby exhibiting features of inflammatory priming. Thus, phosphorylation of ERK1/2, PKB/Akt, and c-Jun is remarkably increased in response to LPS *in vitro*, whereas that of p38 MAPK is reduced compared with the monocyte-derived macrophages from the blood of healthy controls. Furthermore, *in vitro* activated monocyte-derived macrophages from ascites of cirrhotic patients secreted significantly higher levels of IL-6, IL-10, and TNF-α and lower amounts of IL-1β and IL-12 than the corresponding cells from healthy donor's blood. Based on these results, other authors have recently reported that the surface expression level of CD206 can be used to identify

mature, resident, inflammatory peritoneal macrophages in patients with cirrhosis. Soluble CD206 is released from activated large peritoneal macrophages, and increased concentrations in patients with cirrhosis and spontaneous bacterial peritonitis (SBP) indicate reduced odds of survival for 90 d. Hence, the level of soluble CD206 in ascites might be used to identify patients with SBP at risk of death. In conclusion, peritoneal macrophages present in ascites of cirrhotic patients display multiple phenotypic modifications characterized by reduced ratio of cells expressing several membrane markers, together with an increase in the ratios of complex and intermediate subpopulations and a decrease in the classic-like subset. These modifications may lead to the identification of novel pharmaceutical targets for prevention and treatment of hepatic damage.

Key Words: Cirrhosis; Inflammation; Peritoneal macrophages; Phenotypic markers; Activation routes

Core Tip: This frontier article is based on a summary of recent relevant publications on the biology of mouse, as the main animal model used, and human peritoneal macrophages under the perspective of its future clinical translation to the role that these cells can play on several human liver diseases. Concretely, we have reviewed recent findings on several characteristics of human peritoneal macrophages obtained from the ascites of cirrhotic patients compared with those obtained from healthy donors. Featured article: Role of MAP kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from the ascites of cirrhotic patients.

Citation: García-Peñarrubia P, Ruiz-Alcaraz AJ, Ruiz-Ballester M, Ramírez-Pávez TN, Martínez-Esparza M. Recent insights into the characteristics and role of peritoneal macrophages from ascites of cirrhotic patients. *World J Gastroenterol* 2021; 27(41): 7014-7024
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7014.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7014

BIOGRAPHY

Pilar García-Peñarrubia (Figure 1), MD, PhD is a Professor in the Departments of Biochemistry and Molecular Biology B and Immunology in the School of Medicine, at the University of Murcia, Spain. She received her MD degree from the School of Medicine at the University of Murcia, Spain in 1975. She certified as MD specialist in Microbiology and Parasitology in 1980. She received her PhD degree from the School of Medicine at the University of Murcia, Spain in 1979 with honor "cum laude".

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Her subjects of interest include the immunopathology of hepatic cirrhosis and endometriosis, the physiology of human NK cells and peritoneal macrophages, and theoretical models of biological systems, especially the immune system.

INTRODUCTION

Liver cirrhosis is the end stage of various different chronic hepatic diseases, characterized by a gradual substitution of the liver structure by fibrotic tissue[1]. The role of monocytes and macrophages in the physiopathology of liver cirrhosis has been extensively reported[2-5]. Rapid mobilization of these cells to peritoneum or hepatic tissue is an important mechanism of defense against incidental bacterial infection translocated from the gut[6]. Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, mannan, glucans, bactDNA, and many

and hepatology

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

Received: April 14, 2021
Peer-review started: April 14, 2021
First decision: June 23, 2021
Revised: July 2, 2021
Accepted: October 11, 2021
Article in press: October 11, 2021
Published online: November 7, 2021

P-Reviewer: Ferrarese A, Zhang L
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

Figure 1 Pilar García-Peñarrubia, MD, PhD is a Professor in the Departments of Biochemistry and Molecular Biology B and Immunology in the School of Medicine, at the University of Murcia, Spain. She received her MD degree from the School of Medicine in the University of Murcia, Spain in 1975. She certified as MD specialist in Microbiology and Parasitology in 1980. She received her PhD degree from the School of Medicine in the University of Murcia, Spain in 1979 with honor "cum laude". From 1986 to 1989 she was a Research Associate with the Department of Medicine, School of Medicine, University of New Mexico, Albuquerque, United States, and a Fellow of the Fulbright Foundation (1986-1987). She was Vice Dean of the School of Medicine (University of Murcia) since 1999 to 2002. Her subjects of interests include the immunopathology of hepatic cirrhosis and endometriosis, the physiology of human NK cells and peritoneal macrophages, and theoretical models of biological systems, especially the immune system.

others, induce the secretion of cytokines from myeloid-derived monocytes and macrophages[7-10]. Chronic inflammation and fibrosis are crucial features associated with macrophage accumulation in the liver[2,10]. Moreover, marked hepatic and systemic damage in cirrhotic patients is associated with high secretion of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as anti-inflammatory cytokines such as IL-10 and TGF-β[11]. In this sense, recent large-scale observational studies have pointed to systemic inflammation as a hallmark of acute decompensation of cirrhosis. Hence, a recent hypothesis proposes that systemic inflammation is the key mechanism in the progression from compensated to decompensated cirrhosis, as well as in the development of acute episodes of decompensation, which are associated with generalized dysfunction or even with extrahepatic multiorgan failure[4].

The study of human tissue-resident macrophages presents several challenges. First, it is necessary to carry out surgical interventions to obtain these macrophages; second, the cell count obtained is low; and third, these cells are difficult to grow *in vitro*. Thus, accumulated research on resident macrophages in both homeostasis and pathology has been performed in animal models, particularly in mice. Nevertheless, it is not always possible to extrapolate from mice to man, in particular in this topic[12,13]. Thus, it is essential to verify whether the tissue factors and macrophage transcription molecules identified in murine models play similar roles in the origin and biology of human tissue-resident macrophages. Additionally, research on the role of the immune system in human inflammatory diseases is principally carried out with peripheral blood leukocytes; however, the study of macrophages from an inflammatory setting can provide relevant information for a better understanding of the physiopathology of numerous human diseases[8,14]. Hence, human peritoneal macrophages (pMϕ) are a potential option for studying the biological characteristics of this cell type[15]. Peritoneal leukocytes play a crucial role in the defense against microbial infections within the peritoneal cavity, and they also contribute to endometriosis and cancer pathologies. Thus, while knowledge about the inflammatory status in peripheral blood and liver of cirrhotic patients has increased dramatically in the last few years, less is known about its correlation with the inflammatory status in ascites fluid (AF), and limited information is available on differences related to cirrhotic etiology[16]. Recent studies have demonstrated that pMϕ are crucial in regulating inflammation and controlling peritoneal infections in decompensated cirrhotic patients. The accurate phenotypic characterization of macrophages obtained from AF of cirrhotic patients helps us to understand and presumably prognosticate the risk of experiencing episodes of spontaneous bacterial peritonitis (SBP), which impairs the outcome in this clinical condition[17,18]. Furthermore, as macrophages are implicated in the physiopathology associated with hepatic cirrhosis, these cells are also studied as targets of new therapies expected to avert the progression of hepatic damage[2,3,14].

Very recently, novel findings on the ontogeny, phenotype, function, specific transcription factors, migratory activity[19-21] and sex differences[22], of mouse resident pMϕ have been reported. These findings open new avenues for the study of human pMϕ in both health and disease conditions.

ORIGIN OF HUMAN PERITONEAL MACROPHAGES

Macrophages are a heterogeneous myeloid cell population involved in innate and adaptive immune responses, as well as in embryonic development, regulation of tissue homeostasis, and wound repair. These immune cells link innate and adaptive immunities by acting as antigen-presenting cells and are crucial in the development and persistence of various inflammatory conditions.

Macrophages are found in all tissues as resident cells in steady-state conditions, and also as immigrant foreign cells derived from peripheral blood monocytes in response to microbial invasion, tissue injury, or inflammation[7,8]. The contribution of monocytes to resident macrophages is highly tissue-dependent, and until recently it was admitted that most of the homeostatic murine pMϕ are terminally differentiated and replenished by blood monocytes[8]; however, groundbreaking findings revealed that the majority of tissue-resident macrophages do not arise from hematopoietic progenitors, as they directly originate from embryonic precursors (yolk sac and fetal liver) and are able to proliferate and self-renew[19]. Thus, local proliferation reestablishes the normal macrophage number after inflammation-induced loss of resident pMϕ[20]. Moreover, it has been reported that the expression of transcription factor GATA-binding protein 6 (GATA-6) is mostly limited to the long-lived murine F4/80^{hi}CD11b^{hi} large peritoneal macrophages (LPMs) of embryonic origin, whereas the subpopulation of F4/80^{low}MHC-II^{hi}, namely small peritoneal macrophages (SPMs), arise from inflammatory monocytes[21]. Recently, Louwe *et al*[23] reported that the fate and function of inflammation-activated pMϕ seem to be regulated by environmental changes. Thus, moderate inflammation-elicited murine pMϕ survive for 5 mo, although they do not acquire the GATA-6^{hi} resident signature. In contrast, high inflammation results in depletion of resident macrophages for a sustained period, although ultimately, stimulated cells achieve a mature GATA-6^{hi} expression.

Bain *et al*[22] reported that murine pMϕ exhibit sexual dimorphism, determined by different microenvironmental cues and a differential replenishment rate from the bone marrow. After sexual maturity, the time of residency and local tissue factors seem to result in increased expression of immune function-related genes in F4/80^{hi}CD102+ macrophages, particularly CD209b, in female mice, which more efficiently control the peritoneal infection with *Streptococcus pneumoniae*. In contrast, the rate of replenishment from the bone marrow is higher in male animals. In this regard, Oh *et al*[24] identified the mTORC2-FOXO1 axis as crucial for integrating microenvironmental signs to regulate metabolic reprogramming, differentiation, and activity of peritoneal tissue-resident macrophages.

PERITONEAL MACROPHAGES CAN MIGRATE VIA A NONVASCULAR ROUTE TO THE INJURED LIVER

Studies conducted in mice have revealed the crucial role played by pMϕ in homeostasis as well as in the physiopathology of multiple systemic or abdominal diseases[25,26]. In this regard, Wang and Kubes[27] reported that murine mature pMϕ F4/80^{hi}GATA-6^{+} rapidly (within 12 h post-injury) infiltrate the injured liver through a non-vascular route across the mesothelium layer, thereby adopting an alternatively activated phenotype with an increased expression of arginase 1, and protect against acute liver damage. The recruitment of pMϕ toward the sites of liver injury was dependent neither on chemokine receptor signaling nor on β1 or β2 integrins, which indicates that this mechanism differs from that of the recruitment of immune cells *via* an intravascular route. Recruitment guidance was dependent on ATP and hyaluronan in the injured tissues, as well as on macrophage expression of CD44, which is a known receptor for the last molecule.

These findings challenge the present assumption that tissue-resident macrophages are stationary cells, and suggest that rapid mobilization of pMϕ, with ability to induce tissue repair, into the damaged liver, can be an important defense mechanism against infections, trauma, metabolic diseases, fibrosis, and tumor diseases.

CHARACTERISTICS OF HUMAN PERITONEAL MACROPHAGES IN HOMEOSTASIS

Human pMϕ are the best choice for carrying out studies on the biological properties of tissue-resident macrophages under homeostatic conditions; moreover, these macrophages can be used as a healthy control group to compare data from individuals suffering from various pathologies affecting the peritoneal cavity, such as cirrhotic or cancer ascites. For this purpose, peritoneal fluid (PF) samples must be obtained either from healthy people or from individuals whose disease does not affect the peritoneal compartment. The most frequent control samples referred to in the corresponding literature were collected from patients on continuous ambulatory peritoneal dialysis (CAPD), not affected by SBP, as well as from exploratory gynecological laparoscopies/laparotomies performed in healthy women[28-31].

Nevertheless, CAPD patients are not healthy people; moreover, it has been reported that the fluid flow through the rat omentum increases with peritoneal dialysis, thereby leading to a dramatic enlargement of the leukocyte aggregates called "milky spots", which are rich in macrophages, lymphoid B and T cells, mast cells, and stromal cells [32,33], and promoting omental fibrosis[34]. Thus, due to these objections, peritoneal cells from CAPD do not really qualify as representative of homeostatic peritoneal cells to be used as healthy control.

We have recently described an optimized method for obtaining human pMϕ from the PF of healthy women[35], and studied several characteristics of healthy human pM ϕ compared with the well-known CD14/CD16 blood monocyte subsets, in order to analyze common properties or tissue-specific differences[36]. Hence, PF from 79 healthy women was acquired from the Gynecological Unit of the HCUVA, Murcia, Spain. Cell samples from blood and PF were obtained during exploratory or therapeutic laparoscopies for benign gynecological pathology (simple ovarian cysts or uterine fibroids) or tubal ligation. Under physiological conditions, a small amount of 5-20 mL PF is present in the peritoneal cavity. It is produced by mesothelial cells and contains a mix of plasma transudate, ovarian exudates, tubal fluid, and macrophages' secretions[37-39]. The physiological functions of PF include lubricating the friction of the intestinal loops and other organs contained in the peritoneal cavity, allowing the exchange of nutrients, repairing injured tissues, and eliminating detritus and microorganisms. In our experience, the first PF obtained by the endoscopic aspirator is quite scarce; with a mean of 6.85 ± 2.6 mL (range 5-8.7 mL). Moreover, this fluid has practically no polymorphonuclear (PMN) leukocytes, which is indicative of the absence of local inflammatory signals. Among human peritoneal leukocytes, macrophages are the predominant cell type (45-90%) followed by T lymphocytes (predominantly T effector/memory cells, CD45RO) (45%), NK cells (8%), dendritic cells (2-6%), B lymphocytes (2%) and less than 5% of PMN cells[31,35,40-43].

Our results revealed that primary human pMϕ have phagocytic and oxidative activities, and they respond to activation of the main proinflammatory routes such as Toll-like receptors and inflammasomes, which further results in the secretion of different proinflammatory cytokines[35]. Furthermore, we demonstrated that pMϕ are heterogeneous with respect to their morphology and CD14/CD16 cell expression. This peritoneal population is made up of akin proportions (approximately 42%) of classic ($CD14^{++}CD16^{-}$) and intermediate ($CD14^{++}CD16^{+}$) small cells, and a novel subset of complex $CD14^{high}CD16^{high}$ cells (approximately 16%), which are not found in the peripheral blood. In contrast, nonclassical blood monocyte-like cells are not detected in the peritoneal cavity[36]. Moreover, pMϕ reveal higher expression of CD14 and CD16 than blood monocytes, which makes them more competent or available for phagocytosis in the presence of LPS or microorganisms. Notably, the percentages of these cell subpopulations are modulated under inflammatory processes. Thus, besides describing the presence of a novel human $CD14^{high}CD16^{high}$ LPM subpopulation (33% ± 2.4%) in the ascites of decompensated cirrhotic patients for the first time, we also found that the percentage of intermediate $CD14^{++}CD16^{+}$ subset was predominant (49% ± 2.0%), whereas the classic $CD14^{++}CD16^{-}$ subset revealed lowest values (18% ± 1.3%) [44]. These modifications in pathological versus steady-state conditions strengthen the importance of these results.

We also analyzed the expression of several monocyte/macrophage-associated membrane receptors implicated in phagocytosis of IgG-opsonized (CD64, high affinity FcγRI) and complement-opsonized microorganisms (CD11b and CD11c, the α chains of Complement receptors, CR3 and CR4); adhesion to activated endothelial cells and tissue recruitment (CR3, CR4, CD62L, and 6-sulfo LacNAc (Slan)), antigen presentation (MHC class II molecule HLA-DR), costimulatory markers (CD80, CD86, CD40),

cytokines receptors (CD116, GM-CSFR and CD119, IFNγR1 or IFNγ chain α receptor), and the mannose receptor (CD206), reported as a M2 polarized marker and denotative of activation/maturation[45]. In comparison with the complete population of blood monocytes, CD86, CD64, and CD11b revealed similar expression on pMϕ; whereas, small significant differences were observed for a higher expression of HLA-DR, CD116 and CD119 on pMϕ. The most compelling differences were found for CD40, CD80, CD11c, CD206, Slan, and CD62L, of which CD62L was the only receptor expressing higher levels of blood monocytes. These findings suggest that human pMϕ could exert remarkable antimicrobial (also high phagocytic and oxidative capacity), antigen-presenting, and T-cell costimulatory capacities; however, this remains to be further explored. Conversely, the steady increase in the percentages and density of CD206 expression from 28.2% in $CD14^{++}CD16^{-}$ to 60.3% in $CD14^{++}CD16^{+}$ and 92.8% in $CD14^{high}$ $CD16^{high}$ suggested that human pMϕ may also exhibit features and functional characteristics of M2 macrophages, as previously described in CAPD[46,47] and endometriosis patients[48]. Nevertheless, the most remarkable differences between blood and pMϕ subsets were detected on selectin CD62L expression, that is, percentages of pMϕ expressing CD62L in each subpopulation increase in parallel with the expression of CD16, whereas the corresponding expression of CD62L in blood monocytes diminishes as CD16 increases. Moreover, it was observed that the percentages of cells expressing Slan were statistically higher in the peritoneal subset[49]. These differences in adhesion molecules could be associated with a differential pattern of cell-tissue recruitment (endothelium/mesothelium)[27]. Expression of GATA-6 in the three subsets of pMϕ was similar, whereas it was absent in blood monocytes. Nevertheless, we found a high correlation between the increment of GATA-6 and the cell membrane expression of CD14 and CD16; suggesting that monocyte migration to the peritoneal compartment in steady-state is scarce, or that the GATA-6 expression in recently arrived peritoneal monocytes is rapid. The homeostatic state of this cell population was confirmed by the low percentages of cells exhibiting intracellular IL-6, TNF-α, and IL-10 cytokines. Notably, the intermediate subset revealed the highest level of intracellular cytokines, whereas the $CD14^{high}CD16^{high}$ LPM subset presented a higher number of IL-10 positive cells related to the named proinflammatory cytokines, supporting the hypothesis related to its M2 polarization tendency. Eventually, we found a linear relationship between CD14/CD16 cell expression and activation/maturation markers, such as CD206 and HLA-DR, intracellular level of GATA-6, phagocytic/oxidative capacity, and intracellular level of IL-6, TNF-α, and IL-10. These data suggest that the population of LPM $CD14^{high}CD16^{high}$ could act as the phenotypic marker of mature differentiated human-resident pMϕ in homeostasis, whereas the intermediate $CD14^{++}CD16^{+}$ subset could be a transitional cell type also integrated by newly recruited blood monocytes.

CHARACTERISTICS OF HUMAN PERITONEAL MACROPHAGES FROM THE ASCITES OF CIRRHOTIC PATIENTS

In the last decade, our group has also focused on the study of pMϕ characteristics in patients with decompensated cirrhosis and culture-negative ascites. We found that these pMϕ display a preactivated status at baseline, with elevated expression of HLA-DR, CD86 and CD54 membrane markers, increased phosphorylated levels of PKB (Akt), ERK1/2 and c-Jun intracellular signaling molecules, and high secretion of IL-6 [50]. These findings presumably indicate that repeated events of bacterial translocation (BT) promote a sustained immune response, even in the temporary absence of PAMPs. This primed state could enhance an IL-6-regulated fast response to intermittent BT events[50]. Further studies performed *in vitro* with pMϕ from ascites of cirrhotic patients revealed that the secretion of proinflammatory cytokines TNF-α, IL-1β, and IL-6 are regulated by the MAPK signaling intracellular cascades, whereas the PI3K-Akt pathway plays an important role in regulating the anti-inflammatory activity of IL-10[51,52].

The inhibitors of MEK1 and c-Jun N-terminal kinases (JNK) decreased the synthesis of TNF-α, IL-1β and IL-6, and could thus be assayed as therapeutic compounds to reduce hepatic damage associated with liver failure[16,35,52]. Conversely, inhibitors of PI3K-Akt blocked the secretion of IL-10 and augmented the production of IL-1β, mainly by inducing the secretion of intracellular IL-1β and caspase-1 to the extracellular compartment (Figure 2). Based on these results, PI3K-Akt inhibitors are excluded as potential drugs for the treatment of hepatic fibrosis, since these agents may enhance the inflammatory status[51].

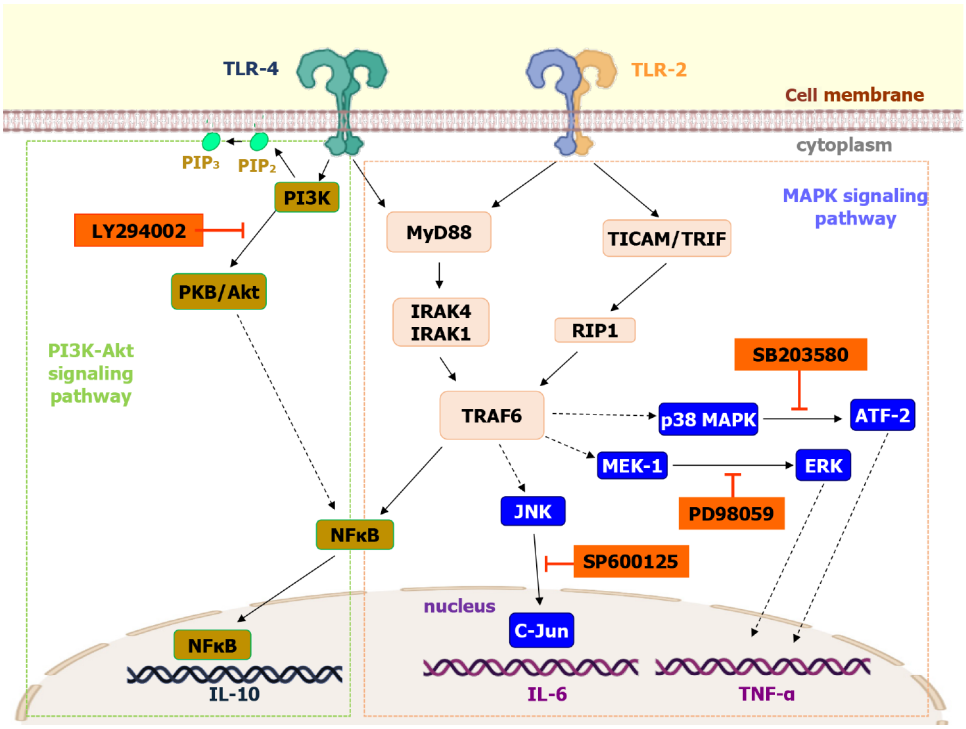

Figure 2 TLR4 and TLR2 cell signaling pathways in normal subjects. TLR2 and TLR4 engagement induce activation of PKB-Akt and MAPK intracellular signaling pathways leading to the phosphorylation of several molecules, which control the expression levels of pro- and anti-inflammatory cytokines. The targets of PD98059, SB203580, SP600125, and LY294002 inhibitors (orange boxes) are indicated by dashed arrows. Adapted from Tapia-Abellán *et al*[51] with permission from John Wiley and Sons, Inc. Citation: Tapia-Abellán A, Ruiz-Alcaraz AJ, Hernández-Caselles T, Such J, Francés R, García-Peñarrubia P, Martínez-Esparza M. Role of MAP kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from the ascites of cirrhotic patients. *Liver Int* 2013; 33: 552-560. Copyright© John Wiley and Sons, Inc.

Peritoneal macrophages from non-infected AF present basal activation of caspase-1 and an increased expression of IL-1β, IL-18, and AIM2 compared to peripheral blood macrophages. The inflammasome activation *in vitro* did not need a priming signal, which supports the preactivated status of these pMϕ[52,53]. As mentioned above, our group reported that a novel $CD14^{high}CD16^{high}$ LPM subpopulation in the AF of cirrhotic subjects is highly sensitive to stimulation with LPS. The $CD14^{++}CD16^{+}$ intermediate subpopulation is augmented in the blood of decompensated cirrhotic patients (from 4% to 11%) and is prevalent in ascites (49%). Baseline hyperactivation of ERK and JNK/c-Jun routes found in ascites pMϕ was associated with cell subsets expressing high levels of CD14/CD16, whereas PI3K/PKB was correlated with CD16 low expressing cells. *In vitro* stimulated pMϕ from ascites of cirrhotic individuals generated statistically higher levels of TNF-α, IL-6, and IL-10, and lower amounts of IL-1β and IL-12 than monocyte-derived macrophages (M-DM) from the blood of controls[44] (Figure 3).

Moreover, Irvine *et al*[54] reported two subsets of pMϕ in AF from decompensated cirrhotic patients: that is, a more phagocytic subset expressing high levels of *VSIG4* (encoding CRIg) and Tim4, and a second less phagocytic subset exhibiting low levels of *VSIG4*, high levels of CCR2a, and responsiveness to retinoic acid. Our unpublished data revealed that these subsets are equivalent to our $CD14^{high}CD16^{high}$ LPMs and $CD14^{++}CD16^{+/-}$, respectively.

More recently, Stengel *et al*[18] have reported that LPMs from the AF of cirrhotic patients present a proinflammatory signature based on the expression of $CD14^{+}$, $CD16^{+}$, $CD206^{+}$, $CD163^{+}$, $MERTK^{+}$, $CD40^{+}$, $CCR2^{-}$, and on *in vitro* transcriptomic analysis and cytokine secretion in the presence and absence of LPS or viable *Escherichia coli* stimulation, respectively. Meanwhile, the corresponding subset of SPMs from AF expresses $CD14^{+}$, $CD16^{+}$, $CD206^{-}$, $CD163^{+}$, $MERTK^{+}$, $CCR2^{+}$. As normal control group, they used macrophages from effluents of CAPD patients with end-stage renal disease, not affected by SBP. These control LPMs displayed a similar phenotype to that of the corresponding subset from cirrhotic patients AF. Interestingly, during SBP episodes, LPMs change to a more inflammatory phenotype characterized by low CD206, low MERTK, and normal CD163 cell surface expression. In particular, LPMs shed surface-bound CD206 as soluble CD206 (sCD206) in response to bacterial peritonitis as well as

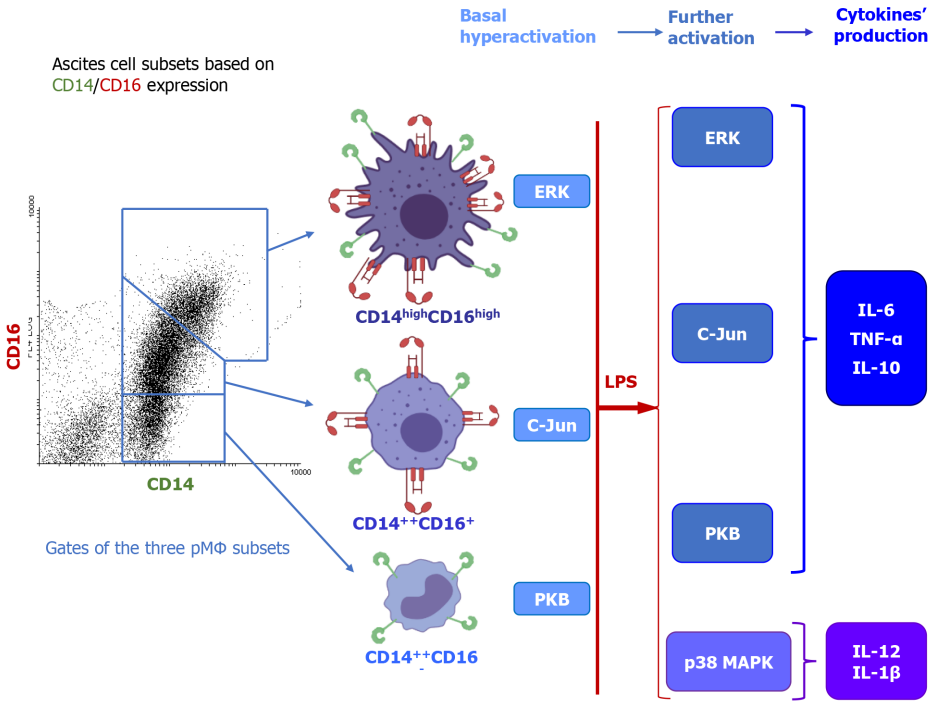

Figure 3 Peritoneal macrophage subsets from cirrhotic patients. The ascitic fluid of cirrhotic patients presents three different subpopulations of peritoneal macrophages based on their cell morphology and CD14/CD16 expression. Baseline hyperactivation of ERK and JNK/c-Jun signaling routes detected in ascites peritoneal macrophage (pMϕ) correlates with CD14/CD16 high expressing subsets, whereas PI3K/PKB correlated with the CD16 low expressing cells. *In vitro* treatment with LPS drastically increases PKB/Akt, ERK1/2, and c-Jun activation, whereas the corresponding p38 MAPK is lowered in pMϕ from ascites cells compared to monocyte-derived macrophages (M-DM) from the control blood. *In vitro* LPS-activated macrophages from cirrhotic ascites also produce statistically higher levels of TNF-α, IL-6, and IL-10, as well as lower levels of IL-1β and IL-12 than the control blood M-DM. Adapted from Ruiz-Alcaraz *et al*[44] with permission from Elsevier. Citation: Ruiz-Alcaraz AJ, Tapia-Abellán A, Fernández-Fernández MD, Tristán-Manzano M, Hernández-Caselles T, Sánchez-Velasco E, Miras-López M, Martínez-Esparza M, García-Peñarrubia P. A novel CD14(high) CD16(high) subset of peritoneal macrophages from cirrhotic patients is associated to an increased response to LPS. *Mol Immunol* 2016; 72: 28-36. Copyright© Elsevier.

in vitro response to LPS and *E. coli*. AF sCD206 is an independent predictor of death in patients with SBP. Concentrations of AF sCD206 of > 0.53 mg/L prognosticate a lower 90-day survival rate. In contrast, the rapid loss of CD206+ LPMs in cirrhotic patients in response to SBP is consistent with a process of macrophage depletion, which could allow for blood monocyte settlement.

Previous studies from episodes of SBP have shown that the ascites from negative-culture SBP and positive-culture SBP patients had significantly more macrophages than those from patients with sterile ascites. Furthermore, pMϕ from positive-culture SBP showed poor bactericidal capacity[55,56] and a tolerant state[57], which is consistent with the hypothesis of systemic inflammation. Moreover, high ascites bacterial burden was associated with reduced pMϕ HLA-DR expression. The presence of pMϕ (CD14+/HLA-DR+) in ascites was associated with a lower number of neutrophils and a tendency towards a lower bacterial burden[58]. Given the scarcity of studies on the role of pMϕ in SBP, new lines of research may be opened in this regard to provide new knowledge about the pathophysiology and potential treatments of liver cirrhosis.

CONCLUSION

These new findings can pave way for several important questions: (1) Are human resident peritoneal macrophages able to migrate through the new described nonvascular route as those cells in mice; (2) Are resident peritoneal macrophages able to migrate to virus or bacterial infected liver; (3) Are human resident peritoneal macrophages able to migrate toward other abdominal organs, such as pancreas, spleen, ovary, or gut; (4) Could omentum comprise a reservoir of mature peritoneal macrophages, ready to move toward other peritoneal organs by detecting danger signs in order to repair tissue damage and maintain health; (5) Are there any differences in GATA-6 expression depending on the type or stage of distinct liver pathologies; (6)

Are there differences in the pattern of cytokine secretion between the three CD14/CD16 cell populations identified in ascites of decompensated cirrhotic patients; (7) Could data of proinflammatory potential of CD206+ LPM in the AF of cirrhotic patients be reproduced in cohorts of cirrhosis from other etiologies; (7) Is AF sCD206 a useful marker to prognosticate mortality risk from decompensated cirrhotic patients; and (8) Could AF sCD206 be used as a useful marker to prognosticate evolution of other peritoneal diseases, such as endometriosis, ovarian cancer, or others?

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7025-7040

DOI: 10.3748/wjg.v27.i41.7025

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

FRONTIER

Involvement of parathyroid hormone-related peptide in the aggressive phenotype of colorectal cancer cells

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Conflict-of-interest statement: Authors declare no conflict of interest for this article.

Supported by Agencia Nacional de Promoción Científica y Tecnoló gica, No. PICT-2013-1441; Consejo Nacional de Investigaciones Científicas y Técnicas, No. PIP11220150100350; Instituto Nacional del Cáncer, Asistencia financiera II, No. 12002-4395-14-1; Instituto Nacional del Cáncer, Asistencia Financiera III, No. RESOL-2016-1006-E-APN-MS; and Universidad Nacional del Sur, Argentina, No. PGI: 24/B230; PGI: 24/B303.

Country/Territory of origin:

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Abstract

Colorectal cancer (CRC) remains one of the leading causes of mortality from malignant diseases worldwide. In general terms, CRC presents high heterogeneity due to the influence of different genetic and environmental factors; also, the neoplastic cells are strongly influenced by the extracellular matrix and several surrounding cells, known together as the tumor microenvironment (TME). Bidirectional communication takes place between the tumor and the TME through the release of autocrine and paracrine factors. Parathyroid hormone-related peptide (PTHrP) is a cytokine secreted by a wide variety of tissues and is able to regulate several cellular functions both in physiological as well as in pathological processes. It exerts its effects as a paracrine/autocrine factor, although its mode of action is mainly paracrine. It has been shown that this peptide is expressed by several tumors and that the tumor secretion of PTHrP is responsible for the malignant humoral hypercalcemia. Eight years ago, when our research group started studying PTHrP effects in the experimental models derived from intestinal tumors, the literature available at the time addressing the effects of PTHrP on colorectal tumors was limited, and no articles had been published regarding to the paracrine action of PTHrP in CRC cells. Based on this and on our previous findings regarding the role of PTH in CRC cells, our purpose in recent years has been to explore the role of PTHrP in CRC. We analyzed the behavior of CRC cells treated with exogenous PTHrP, focalizing in the study of the following events: Survival, cell cycle progression and proliferation, migration, chemoresistance, tumor-associated angiogenesis, epithelial to mesenchymal transition program and other events also associated with invasion, such us the induction of cancer stem cells features. This work summarizes the major findings obtained by our investigation group using *in vitro* and *in vivo* CRC models that evidence the parti-

Argentina

Specialty type: Gastroenterology and hepatology

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

Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: June 14, 2021
Accepted: August 23, 2021
Article in press: August 23, 2021
Published online: November 7, 2021

P-Reviewer: Rojas A, Zhu Y
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Liu JH

cipation of PTHrP in the acquisition of an aggressive phenotype of CRC cells and the molecular mechanisms involved in these processes. Recently, we found that this cytokine induces this malignant behavior not only by its direct action on these intestinal cells but also through its influence on cells derived from TME, promoting a communication between CRC cells and surrounding cells that contributes to the molecular and morphological changes observed in CRC cells. These investigations establish the basis for our next studies in order to address the clinical applicability of our findings. Recognizing the factors and mechanisms that promote invasion in CRC cells, evasion to the cytotoxic effects of current CRC therapies and thus metastasis is decisive for the identification of new markers with the potential to improve early diagnosis and/or to predict prognosis, to predetermine drug resistance and to provide treatment guidelines that include targeted therapies for this disease.

Key Words: Parathyroid hormone-related protein; Colorectal cancer; Tumor biomarkers; Neoplastic processes; Drug resistance; Tumor microenvironment

Core Tip: In colorectal cancer (CRC) cells, we found that parathyroid hormone-related peptide (PTHrP) promotes survival, cell cycle progression, proliferation, migration and chemoresistance and also modulates markers expression associated with invasion, angiogenesis, epithelial to mesenchymal transition and cancer stem cells features. *In vivo* tests indicate that PTHrP administration increases the expression of several markers related to tumorigenic events. Recently, we observed that PTHrP influences on tumor microenvironment cells, inducing events associated with the progression of CRC. Our project focuses in understanding the biology of CRC and the underlying mechanisms related to the aggressive behavior of CRC cells with the aim to identify new markers that improve the diagnosis, prognosis and therapy of CRC.

Citation: Novoa Díaz MB, Carriere PM, Martín MJ, Calvo N, Gentili C. Involvement of parathyroid hormone-related peptide in the aggressive phenotype of colorectal cancer cells. *World J Gastroenterol* 2021; 27(41): 7025-7040
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7025.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7025

INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of mortality from malignant diseases worldwide[1]. In general terms, CRC presents high heterogeneity due to the influence of different genetic and environmental factors[2,3]. Among the factors associated with colorectal tumorigenesis are the damage to intestinal tissue, the presence of harmful microorganisms and the persistence of inflammatory reactions, which can lead to pre-malignant lesions that progress towards a neoplasm[4]. These conditions result in the deregulation of several signaling pathways, a fact that directly alters the cell survival and induces the transformation of the normal epithelium into a hyperproliferative mucosa, causing the development of adenomatous polyps[3,5]. These processes can result in tumor progression, metastasis and resistance to drug therapy and are accompanied by alterations in the DNA repair mechanism, epigenetic changes, genomic instability and several mutations[6,7]. In this context, it is important to highlight that the neoplastic cells are strongly influenced by the extracellular matrix and several surrounding cells, known together as the tumor microenvironment (TME)[8,9]. Bidirectional communication takes place between the tumor and the TME through the release of autocrine and paracrine factors. As a consequence, in the neoplastic cells, multiple molecular mechanisms are triggered to promote their aggressive capacities[10,11]. Simultaneously, the extracellular matrix undergoes modifications that facilitate invasiveness and migration of tumor cells to other tissues where they metastasize[12]. In this instance, the tumor cells show changes in their cell polarity and acquire a mesenchymal-like phenotype, a process known as epithelial to

mesenchymal transition (EMT)[13]. Cumulative evidence associates the EMT process with the acquisition of cancer stem cell (CSC) features[13,14].

CSCs are a fraction of cells in the tumor with the ability of self-renewal, differentiation and drug resistance[14,15]. Another important parameter in tumor development is angiogenesis. This process is stimulated by the production and secretion of pro-angiogenic molecules from tumor cells and the TME[10,11]. In consequence, fibroblasts, mesenchymal cells and even tumor cells can differentiate into cells with an endothelial phenotype and form new blood vessels[16]. The above mentioned events are implicated in the development of a more aggressive phenotype of CRC cells.

Parathyroid hormone (PTH)-related peptide (PTHrP) is a cytokine described for the first time by Fuller Albright in 1941. He suggested that some tumors might cause hypercalcemia by ectopic PTH production or by secreting a very similar molecule[17]. Then, it was discovered that although both molecules present biochemical similarities, PTH and PTHrP have distinct roles and modes of action. PTH is a hormone secreted by the parathyroid gland and is an important mediator of bone remodeling; it acts together with calcitriol and calcitonin as an essential regulator of calcium and phosphate homeostasis[18]. In contrast, PTHrP can be secreted by a wide variety of tissues and can regulate several cellular functions both in physiological as well as in pathological processes[19]. This cytokine exerts its effects as a paracrine/autocrine factor, although its mode of action is mainly paracrine[17]. It has been shown that this peptide is expressed by several tumors such as breast, prostate, lung, kidney, skin and stomach[20] and that the tumor secretion of PTHrP is responsible for the malignant humoral hypercalcemia[21]. Moreover, bone resorption and tumor establishment and expansion are effects closely related to the over-expression of PTHrP by the tumor[19,22,23].

Regarding its production by normal or tumor cells, PTHrP messenger RNA is translated into a long peptide that undergoes an extensive post-translational process from which several functional fragments are derived (Figure 1). Each of these peptide fragments acts through one or more receptors on the cell surface. However, only those fragments that contain the N-terminal region (1-34) show homology with PTH and share the type 1 PTH receptor (PTHR1)[20,24,25].

Eight years ago, when our research group started studying PTHrP effects in experimental models derived from intestinal tumors, the literature at the time described how the behavior of various types of malignant cells is affected by this factor[20,22,23,26]. However, only limited research had addressed the effects of PTHrP on colorectal tumors. The fact that PTHrP and PTHR1 were detected in the normal rectal and colonic epithelium[27] clearly indicated that PTHrP is a cytokine that acts as a local regulator through a paracrine/autocrine pathway[26]. According to this finding, clinical data revealed that PTHrP over-expressed in the tumors of CRC patients correlated with a poor prognosis[20]. Moreover, *in vitro* investigations showed that the proliferation and migration of LoVo cells derived from CRC were increased when these cells over-expressed PTHrP[28,29]. These *in vitro* assays together with others performed in that decade[30,31] provided knowledge about how this cytokine acts through the autocrine/intracrine modes of action, but until that date, no articles had been published regarding to the paracrine action of PTHrP in CRC cells.

Before we began to evaluate the effects of PTHrP in CRC models, we had demonstrated an anti-tumor role of PTH in the Caco-2 cell line derived from human CRC that expresses PTHR1; through much evidence we had found that the hormone exerts its effects by the modulation of the well-known pathways involved in CRC[32]. These previous investigations led us to consider the need to expand the knowledge about the biology of cells derived from this disease. Since several factors regulate the events involved in the aggressiveness of CRC cells, we inquired if PTHrP could be one of those involved in the malignant behavior. Therefore, the purpose of our next investigations was to explore the role of PTHrP in CRC and to accomplish this broad objective, it was necessary to incorporate new experimental models by including more CRC-derived cell lines and an *in vivo* study model.

This work summarizes the major findings obtained in recent years by our investigation group using *in vitro* and *in vivo* CRC models that evidence the role of the cytokine PTHrP in the acquisition of an aggressive phenotype of CRC cells and the molecular mechanisms involved in these processes. We analyzed the behavior of CRC cells under PTHrP action, focalizing in the study of the following events: Cell cycle progression and proliferation, migration, chemoresistance, tumor-associated angiogenesis and morphological changes related to EMT, a key program associated with invasion. Due to this, the readers will find our results described and discussed in the next four sections. These investigations establish the basis for our next studies to address the clinical applicability of our findings. Recognizing the factors and

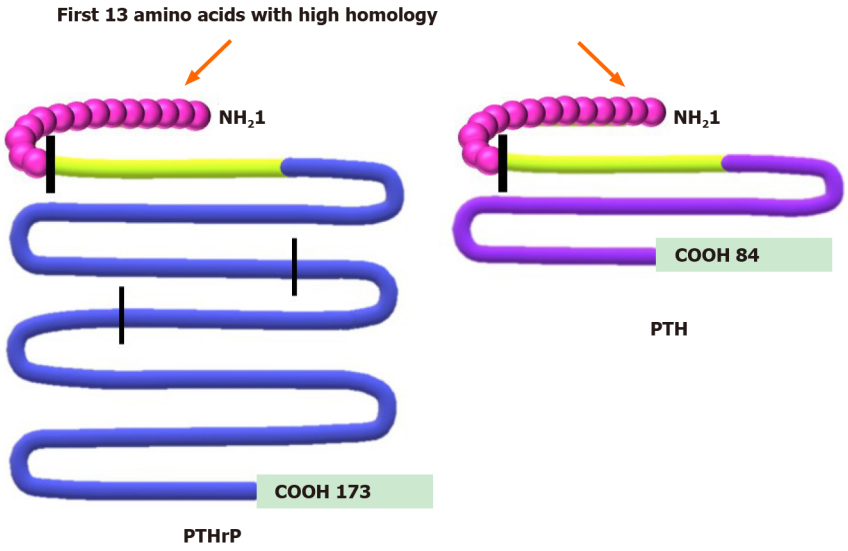

Figure 1 Comparison between protein structure of parathyroid hormone-related peptide and parathyroid hormone. Parathyroid hormone-related peptide (PTHrP) peptide (left side) undergoes a complex post-translational process, obtaining several secreted forms. The N-terminal region 1-34 (green) shows high homology with parathyroid hormone (PTH) and shares more than 60% of the first 13 amino acids (first vertical line). This region allows PTHrP to interact with the type 1 PTH receptor. The PTHrP 36-86, region between N-terminal domain and the second vertical line, is related to placental calcium transport. The 87-107 domain contains a nuclear localization signal (domain between the second and third vertical lines), and the remaining COOH region corresponds to the osteostatin domain[20,24,25]. This figure is original for this work and is based on data published in Soki *et al*[20], Wysolmerski JJ[24] and Goltzman D[25].

mechanisms that promote in CRC cells the invasion, evasion to the cytotoxic effects of current CRC therapies and thus metastasis is decisive for the identification of new markers with the potential to improve early diagnosis and/or to predict prognosis, to predetermine drug resistance and to provide treatment guidelines that include targeted therapies for this disease.

PTHrP PROMOTES CELL CYCLE PROGRESSION, PROLIFERATION AND MIGRATION OF CRC CELLS

As mentioned in the Introduction section, we previously observed that PTH exerts anti-tumor effects in Caco-2 cells through PTHR1[32]. Given that PTHrP (1-34) also binds to the same receptor on the plasma membrane[19,21,33], our first objective was to analyze the actions of this fragment in cell lines derived from colorectal tumors and the associated molecular mechanisms. As previously stated, PTHrP is a factor whose mode of action is mainly paracrine, and, for this reason, all our *in vitro* experiments were performed with the addition of exogenous PTHrP to cells in culture. Regarding the selection of the concentration of this cytokine, we decided to start our investigations employing doses similar to those used with PTH[32] and considering studies carried out in other experimental models[34]. Since we observed that PTH (1-34) (10^{-8} M) induced apoptosis in Caco-2 cells[32], we investigated whether PTHrP employed at the same dose (10^{-8} M) is able or not to induce this response in this cell line. Surprisingly, PTHrP exerts the opposite effect to PTH, since we obtained evidence that PTHrP through a paracrine pathway increases the survival of Caco-2 cells under apoptotic conditions[35]. According to our findings, it was observed in other tumor cells such as breast, renal and prostate cancer cells that PTHrP also increases the resistance of death through the inhibition of apoptosis[20] It is known that the malignant cell transformation involves enhanced cell proliferation, enhanced cell survival by evasion of apoptosis or a combination of both processes[36]. Considering this important concept and based on our initial and interesting result concerning the PTHrP effect on Caco-2 cells, the following goal was to study further the role of this cytokine employing the same concentration in these tumor cells.

By the implementation of multiple assays, we found that PTHrP (1-34) stimulates the cell cycle progression and proliferation of Caco-2 cells[37,38]. In line with these results, we reported similar effects induced by the cytokine in HCT116 cells, a CRC-derived cell line more undifferentiated and aggressive concerning Caco-2 cells, which also expresses PTHR1[39]. Despite the notable differences between Caco-2 cell and

HCT116 cell phenotypes, the molecular mechanisms leading to these responses to the peptide in both CRC cells were similar and implied the activation of well-known deregulated pathways in CRC. Specifically, we found the activation by PTHrP of the non-receptor tyrosine kinase Src, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or Akt), extracellular signal-regulated kinase (ERK) 1/2 and p38, both members of the mitogen-activated protein kinases family (MAPK), p90 ribosomal S6 kinase (RSK) and β-catenin signaling pathways in CRC cells[37-40]. Figure 2 shows the molecular mechanisms modulated by PTHrP and the complex cross-talk among the pathways when this cytokine acts on CRC cells. The upstream/downstream relations between the proteins of these cascades were analyzed employing specific inhibitors that block the protein activity. So, using Ro-318220, PP1 and LY294002, which are the inhibitors of PKC, Src and PI3K, respectively, we found that the activity of these three kinases converges in the phosphorylation/activation of Akt in CRC cells exposed to PTHrP. Furthermore, the specific inhibitor of Akt, GSK690693, suppressed the phosphorylation/activation of ERK1/2 MAPK induced by PTHrP, suggesting the role of Akt in the activation of this MAPK[38,39].

Although the upstream cascades that regulate the activation of p38 MAPK by PTHrP are still unknown, we think that the mechanisms involved are triggered immediately after the activation of PTHrP receptor. We suppose this hypothesis because, in CRC cells exposed to PTHrP, we observed that p38 MAPK is phosphorylated and subsequently activated faster than Src, PKC and Akt. More studies are needed to confirm our idea. RSK is a serine/threonine kinase associated with several types of cancer, including CRC. Its activation is complex and involves various signaling pathways such as MAPKs[41]. We found that RSK is activated by PTHrP and to investigate the involvement of ERK1/2 MAPK and p38 MAPK in this activation, we used PD98059 (a specific inhibitor of MEK1/2, which are the upstream kinases of ERK1/2 MAPK) and SB203580 (a p38 MAPK inhibitor). Experimental data revealed that ERK1/2 inhibitor totally blocked the phosphorylation of RSK induced by PTHrP, whereas the inhibition of p38 MAPK did not reverse the effect of PTHrP over RSK phosphorylation. These results indicate that PTHrP activates RSK *via* ERK1/2 MAPK signaling pathway but not through p38 MAPK[40].

Additionally, as shown in Figure 2, the signaling pathways regulated by PTHrP are responsible for modulating several cell cycle regulators. Our data demonstrated that PTHrP increases the protein expression of cyclin D1, cyclin D-dependent kinase 6 and c-Myc. As PTHrP treatment in CRC cells increases β-catenin protein expression and its subsequent nuclear translocation[38] and as it is known that c-Myc is a target gene of β-catenin[42,43], we suggest that the positive modulation of c-Myc by PTHrP in CRC cells may be *via* the β-catenin pathway. More experiments are needed to confirm this hypothesis. On the other hand, PTHrP paracrine action diminishes the expression of the following negative cell cycle regulators: p27Kip1, p15INK4B and p53. The inhibition of ERK1/2 MAPK, p38 MAPK, PI3K, Akt and RSK pathways suppressed the changes in the protein expression of all the mentioned molecular markers[37-40]. Other transcription factors related to cell proliferation were also activated by exogenous PTHrP, such as cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF-1). The pre-incubation of CRC cells with the specific MAPK inhibitors suppressed the activation of these transcription factors induced by PTHrP[38]. Taken together, our results demonstrated that in CRC cells, PTHrP positively modulates cell cycle progression and proliferation through the modulation of several mitogenic pathways such as PI3K, Akt, ERK1/2 MAPK, p38 MAPK and RSK.

In order to assess PTHrP effects in a more complex CRC model, we also performed *in vivo* investigations. The studies employing subcutaneous murine xenografts of HCT116 cells revealed that the intratumor administration of PTHrP also stimulates ERK 1/2 MAPK pathway among other mitogenic markers. These data validated part of the results that we observed *in vitro*[39,40]. One difficulty encountered when implementing the study of *in vivo* models was that xenografts of HCT116 cells grew rapidly in the subcutaneous area of the mice, and this situation led to insufficient blood irrigation in the center of the tumors. This caused some of the tumors to show internal areas of necrosis that were detrimental from the experimental point of view, since we were unable to observe differences in tumor volume growth due to treatment with PTHrP. Due to this, and in order to preserve the welfare of the animals, all of the *in vivo* assays were forced to finish at 20 d of the initial administration of PTHrP or vehicle solution. Although at the end of the trials the differences between the volumes and weights of tumors from untreated and treated animals were not significant, we are sure that if we had continued the assays the size of the tumors in the mice treated with PTHrP would be higher than that observed in control mice. We support this idea

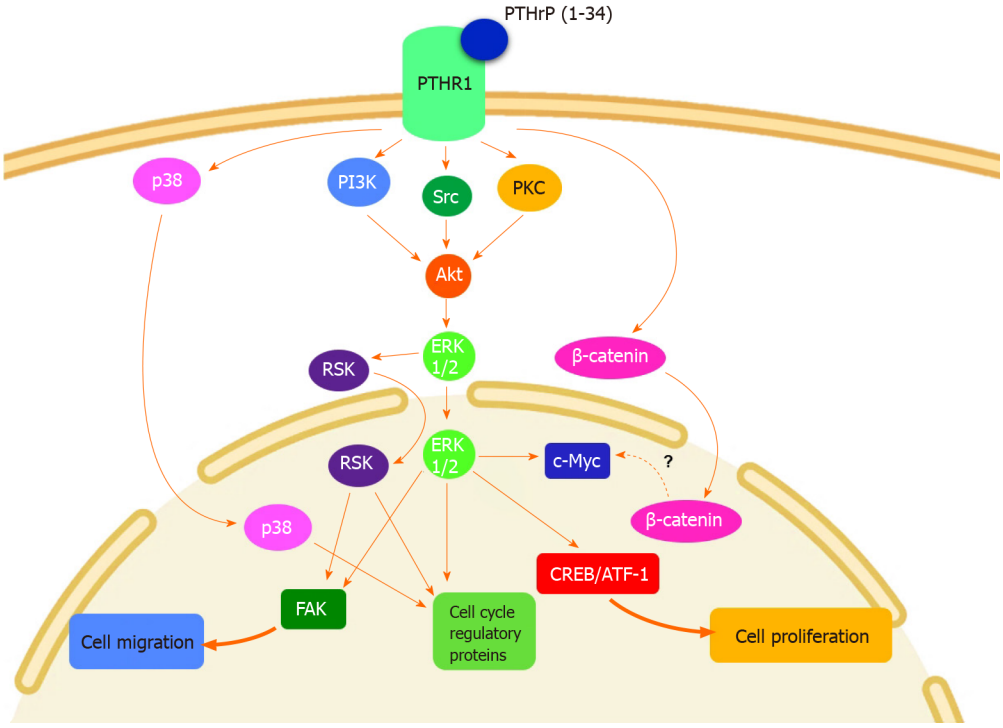

Figure 2 Molecular mechanisms involved in parathyroid hormone-related peptide effects on colorectal cancer cells. Parathyroid hormone-related peptide (PTHrP) induces cell cycle progression and proliferation of colorectal cancer (CRC) cells through non-receptor tyrosine kinase Src (Src), extracellular signal-regulated kinase (ERK) 1/2 and p38, both members of the mitogen activated protein kinases family (MAPK), PI3K/protein kinase B (Akt), p90 ribosomal S6 kinase (RSK) and β-catenin pathways. This cytokine also promotes CRC cell migration and focal adhesion kinase (FAK) protein expression through ERK/RSK signaling pathway[37-40]. This figure is original for this work and shows the results published in Calvo *et al*[37], Martín *et al*[38], Martín *et al*[39], and Calvo *et al*[40]. ATF-1: Activating transcription factor 1; CREB: cAMP response element binding protein; PI3K: Phosphoinositide 3-kinase;PKC: Protein kinase C; PTHR1: Parathyroid hormone receptor 1.

because we observed that the continued administration of PTHrP in nude mice xenografts increased the protein levels not only of the mentioned ERK 1/2 MAPK but also of Ki67, which is a marker of CRC cell proliferation, and the following markers: Cyclin D1, CREB/ATF-1, RSK[39,40] and others[44,45], which we will mention in the next sections due to their relation with the tumor-associated angiogenesis and invasion.

As several of these studied signaling pathways are also involved in cell migration [46], we then decided to study this process. As shown in Figure 2, we observed that PTHrP enhances the motility of CRC-derived cell lines[40]. However, contrary to the results obtained studying tumor proliferation, the effect of this cytokine on migration was higher in the more aggressive cell line, HCT116, than in Caco-2 cells. Furthermore, our investigations revealed that under PTHrP action, ERK 1/2 MAPK and RSK pathways have a relevant role in the increased expression of the focal adhesion kinase and in the migration of CRC-derived cells (Figure 2)[40].

An aspect that we want to comment is about the experiments we performed at that moment to confirm that our findings were exclusively mediated by PTHrP (1-34) and involved only the activation of PTHR1. We used an antibody against PTHR1 to block the PTHrP/PTHR1 interaction and then we evaluated the status of the active ERK1/2 under these conditions since this is a kinase that is involved in most of the processes induced by PTHrP and evaluated by us. We found that the antibody against PTHR1 totally suppressed the response of both Caco-2 cells and HCT116 cells to PTHrP, indicating that ERK activation in cells derived from CRC is due PTHrP/PTHR1 interaction.

Together, these results confirmed that PTHrP is not only involved in the humoral hypercalcemia syndrome but also participates in other responses that may contribute to the progression of CRC. Besides, although PTH and PTHrP interact with the same receptor, these ligands exert opposite effects on intestinal tumor cells. This fact is today explained by the ability of PTHR1 to adopt two different active conformations in response to PTH or PTHrP binding, which will differ markedly in the signaling pathway triggered[47].

PTHrP IS INVOLVED IN THE CHEMORESISTANCE OF CRC CELLS

The incidence and mortality rates related to CRC have decreased in the last decades thanks to the implementation of prevention programs, the early diagnoses by regular testing starting at age 55 and the development of new therapies[48]. Despite all these efforts, late diagnoses are very frequent, and 20% of cases present severe symptoms and a poor or non-existent response to therapy regimens, implying poor survival[15,49-52]. In addition to this situation, in the last decade, there has been an increase in the detection of CRC in patients younger than 55 of age and the appearance of tumor subtypes with poor response to the currently employed treatments[53]. The processes that we have mentioned and analyzed in the previous section suggested that PTHrP can participate in other events associated with the malignant behavior of CRC cells. Since the tumor progress to the most advanced stages mostly implicates the acquisition of resistance to chemotherapy, our next investigations focused to elucidate whether PTHrP also participates in the chemoresistance to drugs commonly used in CRC therapy. One of the drugs most used as first and second-line chemotherapy for advanced or recurrent CRC is Irinotecan (also denominated CPT-11). The combination of CPT-11 with other drugs has shown to increase significantly the survival of patients who have not responded to the initial treatment[54,55]. However, resistance to these improved chemotherapeutic schemes has also been registered[55,56]. As we mentioned earlier, several mechanisms are involved in the development of drug resistance by tumor cells. Regarding recent evidence, the EMT program, the induction of CSC properties and angiogenesis stand out as key events in this process[57]. In a process known as EMT, tumor cells change their morphology acquiring a mesenchymal phenotype to evade the cytotoxic effects of the therapy[58]. In addition to these morphological changes, neoplastic cells acquire CSC properties. These cells are capable of maintaining their population and differentiate into several types of tumor cells, favoring tumorigenesis, the metastatic process, drug resistance and disease recurrence[59,60]. Furthermore, the formation of new vasculature from pre-existing ones, a process known as angiogenesis, is essential not only for tumor growth but also to drug delivery. Factors associated with this neovascularization, such as hypoxia-inducible factor 1-alpha (HIF-1α), may participate in drug resistance[61]. The main processes that participate in CRC chemoresistance are summarized in Figure 3.

The concept of PTHrP decreasing the sensitivity to therapeutic drugs in CRC-derived cells had not been described at the time that we started our studies related to chemoresistance. Also, there was only limited information about the effect of this cytokine on the induction of chemoresistance in other tumor cells[26,62]. Considering this background, we decided to study whether PTHrP may be an underlying factor in the observed chemoresistance to CPT-11. To achieve this objective, Caco-2 and HCT116 cell lines were treated with PTHrP followed by exposure to CPT-11 (10 μM). We found that the exogenous addition of the peptide attenuated the cytotoxic effect of the cytostatic agent in both cell lines. These results suggest that PTHrP favors the chemoresistance of CRC cells to CPT-11. Furthermore, ERK 1/2 MAPK, an enzyme that we mentioned in the previous section due to its involvement in the proliferation and migration of CRC cells, also participates in this pro-tumor response[39]. In line with our results, other researchers reported that the activation of ERK 1/2 MAPK in the HCT116 cell line can generate resistance to other antitumor agents, such as oxaliplatin[63].

So far, we could demonstrate the PTHrP-induced resistance to topoisomerase inhibitors such as CPT-11. The question that immediately emerged after this analysis was whether this cytokine also exerts resistance to other kinds of anti-tumor drugs or a combination of them. Investigations involving the participation of other signaling pathways and the resistance to other drugs in the treatment of CRC, such as oxaliplatin and 5-fluorouracil, are actually in study in our laboratory. This information will be relevant in determining to what extent PTHrP is able to promote drug resistance.

EFFECTS OF PTHrP ON TUMOR-ASSOCIATED ANGIOGENESIS

As previously mentioned, tumor angiogenesis is one of the main mechanisms by which tumors can generate blood vessels and is an essential process for cancer growth and metastasis that can influence therapeutic effectiveness. It is highly regulated by a fine balance between pro-angiogenic and anti-angiogenic factors and is modulated by different signaling pathways. In cancer this balance is lost due to an increased release

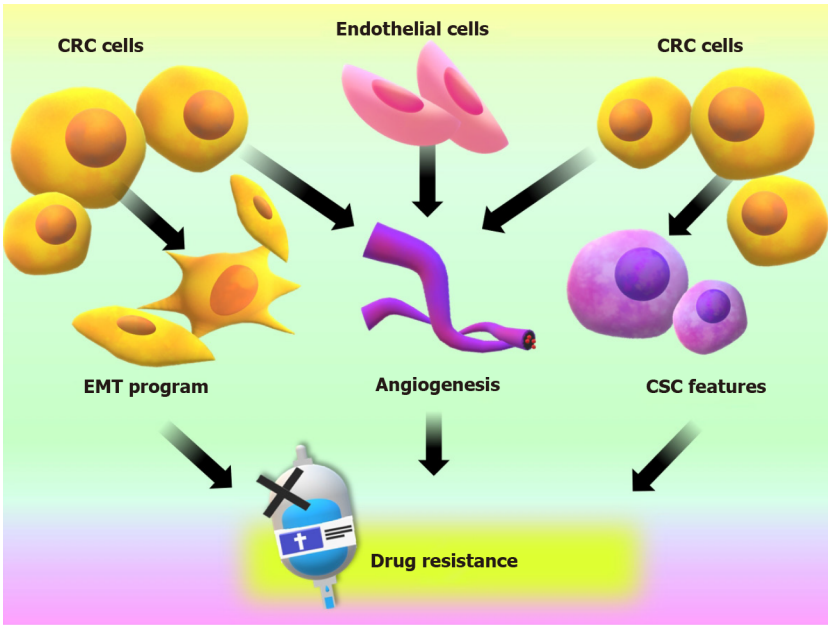

Figure 3 Events that promote an aggressive phenotype on colorectal cancer cells and are related to chemotherapeutic drug resistance and treatment failure. CRC: Colorectal cancer; CSC: Cancer stem cell; EMT: Epithelial to mesenchymal transition.

of pro-angiogenic factors, such as the vascular endothelial growth factor (VEGF), that are produced by tumor cells and the tumor microenvironment, stimulating endothelial cells and promoting tumor angiogenesis[64]. Because of this imbalance, the tumor vessels do not form completely, are abnormal, tortuous, irregular, dilated and permeable, have weak unions, few pericytes and incomplete basal membrane and do not differ in venules, capillaries or arterioles. In addition, blood lakes are formed, and thus the flow becomes irregular, slow and oscillating, a fact that leads to a decrease in oxygen levels and can contribute to the difficulty of successful therapy[65].

Based on this background and our previous results obtained during the years 2013 to 2018, we initially set out to explore whether PTHrP regulates the expression of pro-angiogenic factors in Caco-2 and HCT 116 cell lines to evaluate the effect of this cytokine in the angiogenesis associated to tumor progression. We observed that PTHrP increases messenger RNA levels of VEGF, HIF-1α and matrix metalloproteinase 9 *via* ERK1/2 and PI3K/Akt pathways in both cell lines. Moreover, and as we mentioned in the previous section, we evidenced increased levels of VEGF in HCT116 xenograft tumors treated with PTHrP concerning to control tumors. These results were complemented with the presence of cells forming structures with characteristics of neoformed vessels and stained positively for the vascular endothelial marker cluster of differentiation 31[44]. The ability to distinguish quantitatively between tumor neovascularization and pre-existing vessels is important because these data provide more accurate information in the assessment of tumor angiogenesis. Altogether, these results suggested a pro-angiogenic role of PTHrP in CRC. In line with our results, other authors had found that PTHrP can modulate the expression of factors involved in angiogenesis in tumor cells, resulting in the stimulation of this process. This cytokine stimulates VEGF expression in MDA-MB-231 cells from breast cancer *via* protein kinase A, PKC, ERK 1/2 MAPK and p38 MAPK signaling pathways, and this tumor cell response increased the proliferation and migration of endothelial HUVECs cells [66]. PTHrP modulates the expression of other factors involved in the angiogenesis associated with breast tumors such as the connective tissue growth factor (CTGF/CCN2)[67] and the factor VIII[22]. Moreover, PTHrP can induce the expression of the angiogenic factor interleukin-8 in PCa prostate cancer cells[68].

Our findings suggested an interaction between tumor cells and their microenvironment through pro-angiogenic factors. So, we decided to continue these investigations to evaluate further the molecular crosstalk between tumor cells and endothelial cells. From this goal, we employed conditioned media from CRC tumor cells (TCMs) and indirect co-culture with transwell inserts, and we incorporated the HMEC-1 cell line as an endothelial cell model. We found that the TCMs from colorectal cancer cells exposed to PTHrP exhibit increased proliferation, migratory capacity and formation of tube-like structures of these endothelial cells[44]. Besides, TCMs that were pre-

incubated with the anti-VEGF antibody decreased the stimulating effects of TCMs on endothelial cells[44]. This finding indicates that PTHrP increases the expression of VEGF in Caco-2 cells and HCT116 cells with its subsequent release into the culture medium. This factor in turn exerts its pro-angiogenic effects on endothelial cells. These data broadened the view concerning to the mechanism of action of PTHrP since this cytokine not only acts directly on CRC cells but also exerts its effects acting as a mediator between the tumor and its microenvironment (Figure 4).

Until that moment the progress of our research had demonstrated that PTHrP exerts its effects on the endothelial cells in a tumor cell-dependent manner. Despite the available literature mentioned earlier in this work[22,66,67] that demonstrated, like our research, a pro-angiogenic role of PTHrP, other authors had suggested an inhibitory role for this cytokine in this process[69]. In view of this controversial information, our next challenge was to evaluate if PTHrP acting directly on the endothelial cells also promotes angiogenesis. First, we evidenced the presence of PTHR1 in HMEC-1 cells. Then, we observed that PTHrP increases the phosphorylation of ERK 1/2 MAPK and the proliferation of these endothelial cells. Nevertheless, this cytokine did not stimulate the migration or tube formation of HMEC-1 cells[44]. Assays performed to evaluate cell motility and tube formation on a matrix are widely used to study angiogenesis *in vitro*[70]. However, it would be necessary to carry out *ex vivo* or *in vivo* assays to study further if PTHrP has or not a direct action on endothelial cells with the aim to promote angiogenesis. Using xenografts of HCT116, we cannot discern whether the effect observed in vessel formation is due to the direct or indirect role of PTHrP. We plan to use in the future other experimental models to continue the study of this part of our project.

EFFECTS OF PTHrP ON THE MODULATION OF EMT PROGRAM AND OTHER EVENTS ASSOCIATED WITH INVASION

The fact that PTHrP promotes the chemoresistance of CRC cells and the angiogenesis associated with these tumor cells led us to explore if this cytokine is also involved in other events associated with tumor progression. The process of invasion requires the acquisition of characteristics by tumor cells and the presence of various environmental factors that participate in the remodeling of extracellular matrix, such as matrix metalloproteinases (MMPs)[71]. Therefore, we focalized in the study of MMP-7 because it is overexpressed in 80% of patients with CRC[71], and we found that the treatment with PTHrP induces an increase in MMP-7 transcription in CRC cells. In view of this, our next goal was to investigate if the CRC cells undergo morphological changes under PTHrP action that are related to events associated with tumor progression. EMT is a cellular program that is observed in embryonic development, tissue remodeling and wound healing, and it has also been established as a crucial step in the progression of different tumors. As mentioned, during EMT, epithelial cells reduce intercellular adhesion and acquire mesenchymal properties that increase their migration and invasion capacity, recognized characteristics of tumor cells[58]. The results from the study of the EMT program were incorporated in our work recently accepted for publication[45]. In this manuscript, we reported that PTHrP modulates the expression of factors and favored morphological changes associated with EMT in the CRC-derived HCT116 cell line (Figure 5); we also show that the key molecular mechanisms associated with EMT observed in this cell line in response to PTHrP were not found in the more differentiated and less aggressive Caco-2 cells. The difference in the response of both CRC-derived cell lines raises an interesting new scenario for the action of PTHrP where its effect would depend on the different aggressiveness of the cell line.

Our findings described herein showed that PTHrP through a paracrine manner is involved in events related to the aggressive behavior of CRC cells. The fact that this cytokine establishes a communication between CRC cells and endothelial HMEC cells through molecular factors promoting tumor-associated angiogenesis (see Figure 4), motivated us in this last time to continue evaluating how PTHrP promotes the interaction between the tumor cell and cells from its microenvironment. In our recent work[45] we demonstrated that this cytokine acts on these endothelial cells promoting the release of factors that contribute to EMT program in CRC-derived cells (Figure 5).

After investigating the effects of PTHrP on the EMT program, we began to inquire about other programs strictly associated with malignant progression and chemoresistance, such as CSC. The evidence suggests that the EMT program is tightly associated with the CSC phenotype. This process is recognized for altering not only the

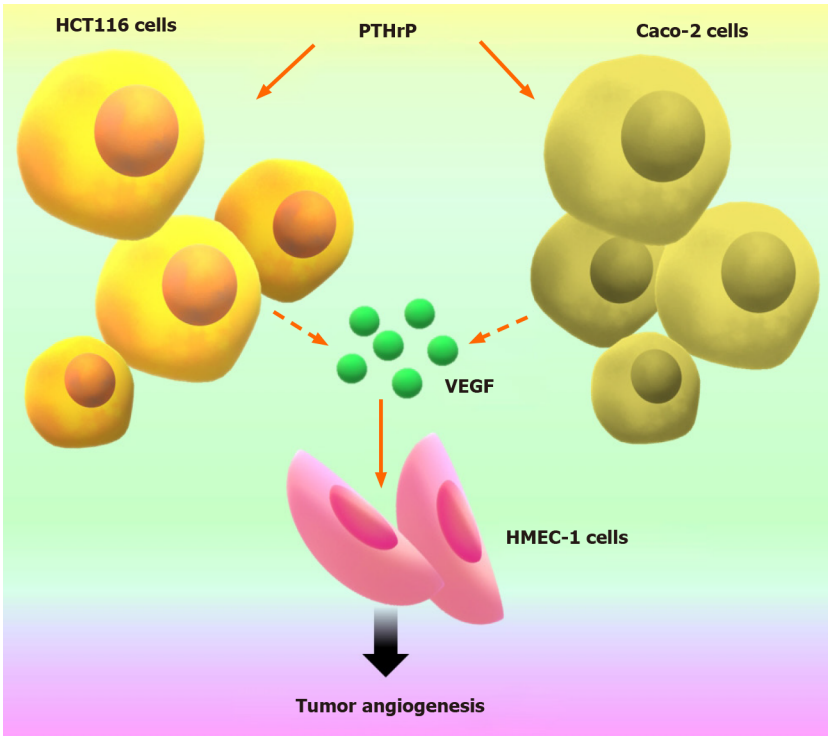

Figure 4 Parathyroid hormone-related peptide induces tumor-associated angiogenesis through the pro-angiogenic factor vascular endothelial growth factor released from colorectal cancer cells. This figure is original for this work and shows results published in Calvo *et al*[44]. PTHrP: Parathyroid hormone-related peptide; VEGF: Vascular endothelial growth factor.

phenotype of the tumor cells but its microenvironment, inducing the initiation of CSC and regulating their features[13,14]. Recent findings from our research group suggest that, in CRC-derived cells, PTHrP modulates the protein expression of cell surface markers widely linked to colon CSC, possibly participating in the initiation and reprogramming of this cell subpopulation. Accumulating evidence associates these cells with resistance to cytotoxic drugs through several mechanisms[15].

Given all these results, we postulate that PTHrP participates in the modulation of several events related to an aggressive phenotype of colorectal tumor cells. The action of autocrine and paracrine factors derived from the tumor and their stroma can promote several events contributing to the phenotypic and genetic heterogeneity of tumor cells and affecting the response to currently used treatments.

Together, these investigations made it possible to project PTHrP as a mediator in the tumor microenvironment and delineate new lines of research.

FUTURE APPROACHES OF PTHrP

Despite the contributions from our research group regarding the role of PTHrP in the modulation of events associated with the aggressive phenotype of CRC cells, we consider that other key aspects of the action of this cytokine are necessary to evaluate. In fact, we are now designing new experiments to analyze if PTHrP also confers chemoresistance to other drugs for CRC treatment and to elucidate how this cytokine contributes to the aggressive behavior of CRC cells through its action on the TME. Another challenge in our project is to analyze the clinical relevance of our observations.

The TME is a factor that is acquiring more and more evidence regarding its relevant role in the progression of CRC and drug resistance[72]. Solid tumors consist of tumor cells that are surrounded by a stroma composed of a variety of cells such as fibroblasts, myofibroblasts, endothelial cells, lymphocytes, mast cells and macrophages. The stroma interacts with tumor cells through cytokines, integrins and proteases to influence functions such as proliferation, apoptosis, migration and angiogenesis[73]. Although it is well defined that PTHrP is expressed by tumor cells in CRC[74-76], it is still unknown the sources from the TME where PTHrP is produced and secreted. The fact that PTHrP is expressed in the stromal cells of other types of tumors contributing

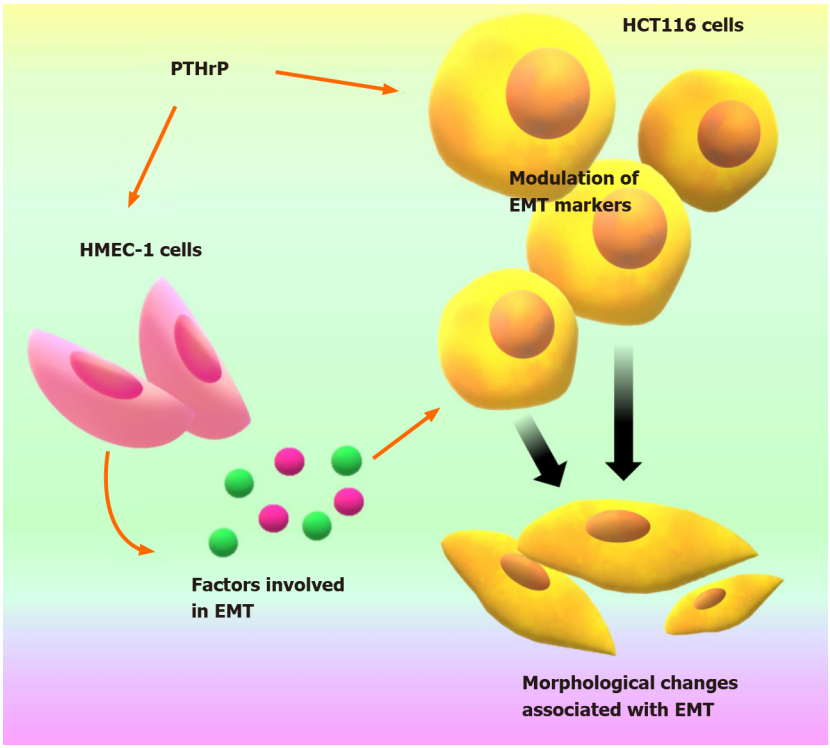

Figure 5 Parathyroid hormone-related peptide establishes a communication between colorectal cancer cells and endothelial HMEC cells through molecular factors modulating markers expression and morphological changes associated with cellular programs that promote the invasive phenotype in HCT116 cells. This figure is original for this work and shows results published in Carriere *et al*[45]. EMT: Epithelial to mesenchymal transition; PTHrP: Parathyroid hormone-related peptide.

to their aggressive behavior[77,78] supports the hypothesis that the cytokine derived from the TME may play a role in the pathogenesis and progression of CRC. Perhaps PTHrP from TME through a paracrine manner also induces its own expression in tumor cells. The influence of the TME in the expression of PTHrP in tumor cells was shown in the work of Yan and collaborators[79]; they reported that the cytokine TGF-β derived from the TME stimulated the secretion of PTHrP from prostate cancer cells promoting the progression of the disease.

As we mentioned in previous sections of this work, our approaches recently revealed that PTHrP, through molecular factors, establishes a communication between CRC cells and endothelial cells derived from TME that leads to the promotion of events related to the aggressive behavior of tumor cells[44,45]. Despite the advances by our research group highlighting the impact of PTHrP on TME cells, it still remains to study key aspects of the action of this peptide, especially regarding the origin of PTHrP in the tumor niche and its effect on CRC cells through its influence on other types of TME cells and also on the ECM.

According to this, we are now planning to study the chemoresistance and other events associated with the aggressive behavior of CRC cells with the focus on the role of TME to understand further the implication of PTHrP in this complex process.

All the experimental models that we used heretofore allowed us to evaluate the signaling pathways triggered as well as the molecular and phenotypic changes in response to PTHrP. Despite this, to evaluate functional aspects regarding TME and drug therapy, we consider it relevant to implement new techniques and models to extrapolate reliably these results. We recognize that two-dimensional *in vitro* cultures present several limitations when studying the interaction networks of TME and drug resistance since they do not represent the three-dimensional character of the tissues. Considering this aspect and following the National Institutes of Health standards for the use and care of laboratory animals that seek to reduce the use of experimental animals, we will perform our next experiments incorporating cell co-cultures in our experimental models. We planned to do new experiments using several tumor cell lines derived from CRC co-cultured with stromal cells (such as fibroblasts, endothelial cells and macrophages) in a three-dimensional model of spheroids to evaluate the complex interaction between neoplastic cells and TME cells under PTHrP action. To validate the results obtained in these experiments, it will be necessary to implement

new *in vivo* methods that allow us to evaluate the drug resistance and the progression of angiogenesis, invasion and malignancy programs considering the impact of tumor stroma. Athymic nude mice have been very useful so far since they have allowed us to extrapolate the results observed in the cell lines to a complete organism. Nevertheless, this model represents a challenge since the deficient immune response of these animals makes it difficult to assess the interactions between the tumor and the stroma; the subcutaneous xenograft models are limited because tumors cells interact poorly with the surrounding stroma and also, they do not metastasize. An alternative model is the co-injection of tumor and stromal cells with matrigel or the orthotopic model.

Another aspect that we decided to evaluate is the clinical relevance of our *in vitro* and *in vivo* results by a retrospective study that is currently in process. To this end, we are using diagnostic biopsies preserved in paraffin plugs from patients who received the diagnosis of CRC and subsequently adjuvant chemotherapy. The objective of this part of our project is to correlate the expression of PTHrP and other markers (that were relevant in the cell and animal models studied by us) with the characteristics of the tumor, the tumor response to the established treatment, the progression-free time and the overall survival of the CRC patient in order to identify PTHrP and its effectors as possible prognostic markers and/or predictive of CRC. In these retrospective studies, we will analyze 300 to 400 samples to consider the data as statistically significant. These samples are from the Hospital Provincial de Neuquén (Province of Neuquén, Argentina) and from the Hospital Dr. José Penna, Bahia Blanca (Province of Buenos Aires, Argentina), which are two hospitals with a high attendance of patients, and therefore we consider that our work will have a regional scope because we will evaluate biopsies of CRC patients from two different provinces of our country.

Following our proposed studies in the clinical context, we plan to resort to strategies such as *in silico* modeling methods because they are tools that allow a comprehensive approach of published genomic and proteomic data related to CRC progression and can predict the clinical efficacy of treatments. It is clear that innovative models are required to translate data involving biological and genomic/proteomic networks into suitable therapeutic schemes. With this approach, we plan to evaluate our experimental system against available databases to contrast our findings and predict the effect of PTHrP in the response of patients to the chemotherapy employed.

CONCLUSION

Recent publications denote the importance of the tumor microenvironment in the response in different stages of CRC[80]. It is known that cytokines in the tumor stroma critically influence the development and progression of CRC by direct stimulation of neoplastic cells or by altering the function and activity of cells in the microenvironment[81].

These antecedents, together with the promising results obtained by our research group throughout these years, allow us to hypothesize that PTHrP is a cytokine that acts through a paracrine manner to play an important role in the acquisition of an aggressive phenotype of intestinal tumor cells. By its action on CRC cells and on its microenvironment, this peptide promotes an interaction between cells from the tumor niche favoring the tumor aggressive behavior. Our findings suggest that PTHrP and its effectors could be involved in the tumorigenesis and/or progress of CRC disease and also could influence the success of chemotherapeutic treatment.

ACKNOWLEDGEMENTS

The authors would like to thank Silvana Gigola for her helpful advice. Being an English Professor graduated from Universidad Nacional de Quilmes (Argentina) and currently working at Instituto Superior Juan XXIII (from Bahia Blanca city, Argentina), she revised the paper and made useful comments regarding spelling, grammar and punctuation of this manuscript. Novoa Díaz MB, Martín MJ and Carriere PM appreciate CONICET for the fellowships.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7041-7064

DOI: 10.3748/wjg.v27.i41.7041

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

REVIEW

Over-feeding the gut microbiome: A scoping review on health implications and therapeutic perspectives

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Conflict-of-interest statement: The authors declare no conflict of interest.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

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Abstract

The human gut microbiome has gained increasing attention over the past two decades. Several findings have shown that this complex and dynamic microbial ecosystem can contribute to the maintenance of host health or, when subject to imbalances, to the pathogenesis of various enteric and non-enteric diseases. This scoping review summarizes the current knowledge on how the gut microbiota and microbially-derived compounds affect host metabolism, especially in the context of obesity and related disorders. Examples of microbiome-based targeted intervention strategies that aim to restore and maintain an eubiotic layout are then discussed. Adjuvant therapeutic interventions to alleviate obesity and associated comorbidities are traditionally based on diet modulation and the supplementation of prebiotics, probiotics and synbiotics. However, these approaches have shown only moderate ability to induce sustained changes in the gut microbial ecosystem, making the development of innovative and tailored microbiome-based intervention strategies of utmost importance in clinical practice. In this regard, the administration of next-generation probiotics and engineered microbiomes has shown promising results, together with more radical intervention strategies based on the replacement of the dysbiotic ecosystem by means of fecal microbiota transplantation from healthy donors or with the introduction of synthetic communities specifically designed to achieve the desired therapeutic outcome. Finally, we provide a perspective for future translational investigations through the implementation of bioinformatics approaches, including machine and deep learning, to predict health risks and therapeutic outcomes.

Key Words: Gut microbiome; Microbial metabolites; Obesity; Next-generation probiotics; Fecal microbiota transplantation; Deep learning

Received: April 29, 2021
Peer-review started: April 29, 2021
First decision: June 17, 2021
Revised: July 2, 2021
Accepted: October 14, 2021
Article in press: October 14, 2021
Published online: November 7, 2021

P-Reviewer: Cheungpasitporn W
S-Editor: Fan JR
L-Editor: Webster JR
P-Editor: Fan JR

Core Tip: The gut microbiome (GM) has gained increasing attention in recent years due to its key role in contributing to host health, potentially serving as a target for personalized precision medicine. This review summarizes the current evidence for the involvement of the GM in the regulation of various pathophysiological aspects, particularly in obesity and related comorbidities. The influence of diet and the molecules produced by commensal microorganisms is discussed, together with traditional and innovative microbiome-based strategies in the prevention and treatment of obesity, up to the development of machine and deep learning bioinformatics tools for the prediction of health risks and therapeutic outcomes.

Citation: Barone M, D'Amico F, Fabbrini M, Rampelli S, Brigidi P, Turroni S. Over-feeding the gut microbiome: A scoping review on health implications and therapeutic perspectives. *World J Gastroenterol* 2021; 27(41): 7041-7064
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7041.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7041

INTRODUCTION

Over the past two decades, the trillion-member community that resides in the human gastrointestinal tract (*i.e.*, the gut microbiome-GM) has emerged as a key regulator of host physiology, supporting overall host health or, vice versa, contributing to triggering and sustaining pathological conditions when altered. The wide range of metabolic activities and the multiple levels of bidirectional interaction with the host strongly support GM as a strategic therapeutic target, laying the foundations for the development of innovative microbiome-tailored intervention strategies aimed at restoring an eubiotic layout. In parallel, advances in sequencing techniques and bioinformatics tools are proving crucial to deepen our understanding of the complex interactions established by the GM with the host, as well as to rationally fine-tune and successfully translate personalized microbiome-based interventions into clinical practice.

Our scoping review aims to discuss the state of the art on research and application aspects related to the role of GM in obesity, a complex and multifactorial disease that represents a major health risk factor. In particular, we first discuss the influence of GM on human physiology and its contribution to the pathogenesis of numerous enteric and non-enteric diseases when imbalances occur. Next, we focus on evidence for a link between GM and obesity and discuss the growing literature on the impact of diet on GM structure, and the key role played by GM-produced or derived bioactive compounds [*e.g.*, fatty acids, amines, bile acids (BAs) and neuroactive metabolites] in affecting host physiology and metabolism, at both local and systemic level. We then review the adjuvant interventions currently available to manipulate GM and alleviate the obesity phenotype, which are based on diet modulation and the supplementation of prebiotics, probiotics and synbiotics. Since these traditional approaches have shown only moderate ability to induce sustained change in the complex and dynamic GM ecosystem, we stress the need to develop innovative and tailored microbiome-based intervention strategies, such as the administration of next-generation probiotics (NGPs) and engineered microbiomes, to be organically integrated into clinical practice. More radical approaches involving replacement of the dysbiotic microbial ecosystem through fecal microbiota transplantation (FMT) or the infusion of synthetic microbial communities, rationally designed to meet the desired therapeutic outcome and patient needs, are also discussed as promising alternatives for personalized clinical applications. Finally, we provide a perspective for future translational investigations through the implementation of bioinformatics approaches, including machine and deep learning, to predict health risks and therapeutic outcomes. Current clinical practice is increasingly leveraging new artificial intelligence technologies and refined bioinformatics approaches, but still little has been done in relation to GM. Therefore, we conclude by discussing the possibilities offered by machine and deep learning for the development of microbiome-based strategies, especially in the context of obesity and related morbidities. All articles included in this review were identified through

the PubMed platform, the full-text archive of biomedical and life sciences journal literature at the United States National Institutes of Health's National Library of Medicine. The most pertinent and relevant articles for each aforementioned topic were selected and commented on.

HUMAN GM: WHERE DO WE STAND?

From the development of next-generation sequencing approaches and their application to the microbiome field, the analysis of the intestinal microbial community has had a strong burst, as evidenced by > 3 million hits returned by typing "gut microbiota" on the NCBI database (as accessed on April 26, 2021). Of course, this is not surprising when one considers the plethora of actors and functions involved in the whole GM field[1,2]. Indeed, GM is composed of a multitude (over trillions) of microbial entities from all domains of life (*i.e.*, bacteria, archaeabacteria and micro-eukaryotes), which encode a number of genes probably more than 500 times greater than the human genome. This genetic heritage, still largely underestimated, allows them to perform various functions recognized as instrumental to maintaining host homeostasis[3,4]. With specific regard to the bacterial counterpart, indisputably the most explored to date, despite the thousands of different species identified so far, most of them belong to 2 phyla, Firmicutes and Bacteroidetes, which together account for approximately 90% of an adult-like community, with Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia representing the most commonly found subdominant taxa[3]. This ecosystem is characterized by essential ecological features, such as stability, resistance and resilience, associated with high diversity and functional redundancy, the loss of which can lead to unhealthy microbe-microbe and microbe-host interactions[5]. Established at birth, GM develops structurally and functionally over time, in relation to the personal exposome (*i.e.*, the totality of exposures that individuals experience in their lives), while always providing the host with a series of fundamental immunological and metabolic ecosystem services for a mutualistic GM-host relationship[6,7]. In particular, GM is known to act as a protective barrier against infectious threats (the so-called "colonization resistance" by occupying niches, taking up resources and producing antimicrobials) and play an active role in the development and modulation of immune responses[8,9]. On the other hand, GM is also called "metabolic organ" as it provides numerous bioactive molecules from the degradation of dietary compounds, which are the main actors of the well-known local and systemic functions attributed to GM, just to name a few: (1) Nutritional support for the intestinal epithelium; (2) Synthesis of vitamins and balance of energy intake; (3) Lipid and carbohydrate metabolism-related effects; (4) Immune system modulation; and (5) Enteric and central nervous system regulation through the gut-brain axis[10]. Moreover, recent findings on GM-drug interactions have indicated its role in influencing the response to treatments, including the occurrence of side effects, with potentially groundbreaking implications in precision medicine[11].

That said, it is not hard to imagine how strategic it is for our health to maintain this intricate and complex balance with our microbial inhabitants. Indeed, when this balance fails, the so-called dysbiosis is established, *i.e.*, a disease-promoting or associated GM alteration[12]. Several endogenous (*e.g.*, immune dysregulation and inflammation) and exogenous (unbalanced diet, antibiotic intake, pathogen infection, *etc*.) factors are capable of promoting GM dysbiosis, and several studies have tried to explain the mechanisms underlying these events[13-15]. Generally speaking, dysbiosis may present with one or more of the following characteristics: (1) Loss of biodiversity (a recognized hallmark of healthy gut); (2) Depletion of beneficial, health-associated taxa, typically short-chain fatty acid (SCFA) producers from the *Lachnospiraceae* and *Ruminococcaceae* families; and (3) Enrichment in pathogens or pathobionts[16]. To date, dysbiotic profiles have been associated with a plethora of enteric and non-enteric disorders, including metabolic, hepatic, respiratory, cardiovascular, immunological and oncological disorders, and are supposed to cause some of these[12]. However, it is of the utmost importance to corroborate claims on GM-related causality in human diseases, as only a few of them have been validated[17]. Regardless, a very hot topic in the field now is the development of GM modulatory strategies, to increase the resilience of healthy states (prevention) or overcome that of unhealthy states (treatment) to alleviate the disease phenotype and restore eubiosis.

EVIDENCE FOR A LINK BETWEEN GUT MICROBIOTA AND OBESITY

The prevalence of overweight and obesity has dramatically risen over the past four decades[18]. Combined with polygenic host susceptibility, increased food consumption and sedentary lifestyles lay the foundation for a widespread obesity epidemic[19]. Recognized as a multifactorial disease, obesity represents a major risk factor for health, with dramatic consequences on quality of life and healthcare costs [20-22]. Comorbidities linked to obesity (*e.g.*, diabetes, cardiovascular disease, cancer) are indeed among the leading causes of premature death, and researchers are striving to find more effective treatments for these conditions[23]. Over the past 15 years, pioneering studies have proposed GM as a key factor involved in energy storage and fat mass gain. Among the first, Bäckhed *et al*[24,25] demonstrated that germ-free mice gained less body weight and fat mass than conventional mice harboring a GM[24], as well as providing proof of concept by showing that the lack of a microbial ecosystem confers resistance to obesity induced by a high-fat diet[25]. In addition, a pivotal study by Turnbaugh *et al*[26] showed that the obese phenotype can be induced in lean mice by transferring the GM of obese animals[26], thus suggesting a causality between unbalanced GM and the development of obesity. Subsequently, the GM of obese individuals was shown to have reduced microbial richness and biodiversity, combined with an altered representation of the two main phyla, namely Bacteroidetes and Firmicutes, compared to lean subjects[27]. These findings have paved the way for several epidemiological studies focused on showing differences in the GM of obese and lean individuals, which have led to accumulating evidence of the GM role in mediating the impact of environmental factors on obesity pathogenesis[28-31]. As expected, given the high taxonomic level, not all studies have been successful in validating the association between the Firmicutes/Bacteroidetes ratio and obesity, and the biological relevance of this ratio is now highly controversial. On the other hand, greater resolution was gradually provided in the description of the bacterial composition, as well as in the understanding of the underlying mechanisms through which a dysbiotic layout might contribute to triggering host metabolic imbalances. For instance, species-level characterization of GM in twins highlighted a positive association of SCFA producers *Eubacterium ventriosum* and *Roseburia intestinalis* with obesity[32]. Similar correlations were observed for *Collinsella* spp.[33], which were also found to be overrepresented in type 2 diabetes (T2D) and atherosclerosis[34,35]. The hypothesized mechanisms include reduced expression of tight junction proteins, possibly leading to gut leakage and metabolic endotoxemia, as well as impaired cholesterol absorption, decreased hepatic glycogenesis and increased triglyceride synthesis[36,37]. It is therefore not surprising that *Collinsella* has been proposed as a target in future GM intervention studies for the improvement of metabolic parameters [38]. On the other hand, the most common methanogenic archaeon found in GM, *Methanobrevibacter smithii*, as well as butyrate producers, such as potentially heritable *Oscillospira* spp., were found to be more represented in lean individuals[39]. *Bacteroides thetaiotaomicron* was depleted as well in obesity-related GM configurations and inversely correlated with serum concentration of glutamate, a common food additive able to induce obesity and insulin resistance[40]. *In vivo* studies have highlighted the ability of this glutamate-fermenting commensal to protect against adiposity[40], suggesting its potential application in probiotic-based intervention strategies in obese individuals. Similarly, *Parabacteroides goldsteinii*, whose levels are reduced in high-fat diet-fed mice, has been proposed as an anti-obesogenic probiotic, capable of promoting adipose tissue thermogenesis and intestinal integrity, and reducing inflammation and insulin resistance[41]. Finally yet importantly, the mucin-degrading bacterium *Akkermansia muciniphila* has been repeatedly and consistently found to be inversely correlated with body fat mass, fasting blood glucose levels and subcutaneous adiposity in mice and humans[42,43], potentially representing a NGP or live biotherapeutic candidate for obesity treatment. In human studies, *A. muciniphila* has shown protective effects against gut permeability and endotoxemia, as well as improving glucose homeostasis and promoting better overall health[43]. Very recently, in a proof-of-concept exploratory study, Depommier *et al*[44] demonstrated that its daily oral administration, as live or pasteurized bacteria, for three months to overweight/obese insulin-resistant volunteers, was safe and well tolerated, and led to the improvement of multiple metabolic parameters[44]. However, it should also be remembered that microorganisms interact with each other in complex syntrophic networks, the understanding of which could help guide more rational preventive and therapeutic strategies. In an attempt to take a step forward in this direction, Tavella *et al*[45] recently identified a distinct GM compositional structure (with elevated proportions of *Christensenellaceae*, *Porphyromonadaceae* and *Rikenellaceae*) associated with reduced

visceral adipose tissue and healthier metabolic profile in elderly Italians[45]. It has also been hypothesized that peculiar "steady states" of GM, combined with long-term dietary habits, may predict the development of childhood obesity[31].

INFLUENCE OF DIET ON THE GUT MICROBIOTA

Diet is a major driver of GM variation and undoubtedly plays a central role in promoting and maintaining GM diversity, a strategic element to ensure eubiosis and resilience. Indeed, GM can rapidly shift its composition and functionality in response to dietary changes, contributing to the generation of health-relevant metabolic outputs [46]. In recent years, interest in understanding the relationship between dietary habits, GM and host physiology has grown remarkably. Different dietary patterns, such as Western-type diets, vegetarian or vegan diets and Mediterranean-style diets have been explored and each has been found to be associated with quite distinct GM profiles, which obviously affect host metabolism in a distinct way[47]. In particular, the advent of a Westernized lifestyle, with the fast-paced globalization of food, excessive sanitation and modern medicines, has led to the introduction of a dietary pattern mostly based on saturated fat, sugar and salt, and dramatically low in fiber, otherwise known as "microbiota-accessible carbohydrates"[13]. Several studies have shown that this type of diet is associated with alterations in the GM structure and functionality, in particular enrichment in mucus degraders, bile-tolerant and antibiotic-resistant species ("BloSSUM", *i.e.*, bloom or selected in societies of urbanization/modernization) and the loss of diversity, ancestral fiber-degrading taxa and related functions ("VANISH", *i.e.*, volatile and/or associated negatively with industrialized societies of humans)[48]. This has collectively been referred to as the "microbiota insufficiency syndrome" and is supposed to result in broad dysfunctions, including obesity and chronic inflammation, thus contributing to the emergence of non-communicable chronic diseases. In contrast, high-fiber dietary patterns are typically associated with highly diverse GMs, enriched in fibrolytic SCFA-producing bacteria, *e.g.*, *Ruminococcus*, *Faecalibacterium*, *Eubacterium* and *Roseburia*, along with high fecal levels of SCFAs[49,50], to which multiple beneficial effects are attributed, as detailed below. Moreover, a microbial footprint of this dietary habit is the greater abundance of *Prevotella*, as also found in hunting-gathering and rural populations who consume a plant-based diet with unprocessed foods[51-53]. It is also worth noting that these dietary habits involve increased intake of polyphenols, which are known to have important GM-mediated health benefits[54], In a recent study in Italian individuals habitually following omnivore, vegetarian or vegan diets[55], we found that high-level adherence to a Mediterranean diet (rich in fruit, legumes and vegetables) was associated with beneficial GM and metabolome profiles, *i.e.*, increased proportions of fiber-degrading bacteria, higher levels of fecal SCFAs and lower urinary levels of trimethylamine N-oxide (TMAO), a risk factor for cardiovascular disease, potentially helping to explain the effectiveness of this diet against obesity, T2D and other inflammatory disorders. Consistently, a 1-year Mediterranean diet intervention was found to positively modulate GM and metabolome (including lower production of secondary BAs and p-cresols) and reduce frailty in elderly subjects, thus paving the way for novel intervention strategies possibly based on Mediterranean diet-responsive taxa and/or metabolites[56]. All this considered, it is not surprising that in recent years, the Paleolithic diet, with a high intake of plant foods while totally excluding industrially processed products and refined sugars, has received a lot attention. Despite some concerns about its long-term adherence, especially due to the consumption of fat and meat[57], it appears to lead to improved metabolic parameters in obese and T2D patients[58] and high levels of GM diversity, similar to those found in traditional rural populations[59].

MICROBIOME-DERIVED COMPOUNDS

SCFAs, protein metabolites, TMAO and BAs

SCFAs, mainly acetate, propionate and butyrate, are the best-known examples of diet-derived microbial metabolites with several local and systemic functions. Indeed, the human genome encodes only a limited number of carbohydrate-active enzymes (CAZymes), thus requiring complementation by the GM for the degradation of otherwise indigestible dietary fibers (*e.g.*, glycans, xylans, *etc.*)[60]. SCFAs are the end-

products of fermentation of these complex polysaccharides. These metabolites can be variously beneficial to health, as local (butyrate) and peripheral (acetate and propionate) energy sources, inflammation modulators, regulators of gut motility, vasodilators and even wound healing promoters[4]. SCFAs also affect the proliferation and differentiation of colonic epithelial cells, modulate their gene expression, reinforce the epithelial barrier (through increased mucus production and strengthening tight junctions), and influence the expansion and function of other cell lineages, including hematopoietic lineages[61]. With specific regard to metabolic health, they control the expression and secretion of appetite and glucose regulatory peptides, such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), by enteroendocrine L-cells, and activate intestinal gluconeogenesis mainly by the regulation of gene expression[10,61]. They have also been attributed functions involved in lipid metabolism, with acetate exerting an anti-lipolytic effect, which could be beneficial in the long term by reducing systemic lipid spillover[62]. Their immunomodulatory and anti-inflammatory activity is also extremely important, for maintaining the delicate balance between tolerogenic and immunogenic signals[61].

On the other hand, branched-chain fatty acids, such as isobutyrate, 2-methylbutyrate and isovalerate, resulting from protein fermentation, have been associated with insulin resistance[63], probably through activation of mammalian target of rapamycin complex 1 (mTORC1)[64]. Protein metabolism by GM may also lead to other potentially harmful compounds, including: (1) P-cresyl and indoxyl sulfate, both of which are associated with cardiovascular morbidity and mortality; (2) 4-ethylphenylsulfate, implicated in promoting autism-like behavior in animal models; and (3) Phenylacetate, which has been shown to contribute to the development of fibrosis and non-alcoholic fatty liver disease (NAFLD)[65].

An admirable but unfortunate example of GM-host co-metabolism of dietary compounds is TMAO. Several gut microbes, *e.g.*, *Campylobacter*, *Shigella* and *Ruminococcus gnavus*, can in fact convert choline and L-carnitine present in seafood, cheese, eggs and red meat, into trimethylamine (TMA) that, once absorbed, circulates to the liver where it is oxidized by host enzymes of the flavin monooxygenase family to TMAO[66-68]. TMAO has recently emerged as a candidate risk factor for cardiovascular disease, as it is proatherogenic, increases platelet hyperreactivity and therefore the risk of thrombosis[69]. In particular, TMAO can reduce reverse cholesterol transport and BA synthesis, interfering with the normal pathway of cholesterol metabolism and elimination[70]. However, it is worth noting that some condiments, such as cold-pressed extra virgin olive oil, grape seed oil and balsamic vinegar, along with some red wines contain a structural analogue of choline, 3,3-dimethyl-1-butanol, which inhibits TMA lyase (*i.e.*, the microbial enzyme involved in TMA formation), thus paving the way for the use of selective enzyme inhibitors for the prevention and treatment of cardiometabolic diseases.

The metabolism of BAs is another example of GM-host co-metabolism, through which the GM can modify the composition of the pool of primary and secondary BAs available to the host and therefore modulate their signaling, meaning not only the traditional role in fat absorption but various effects related to glucose, lipid and energy homeostasis, thermogenesis, insulin signaling, immune responses and inflammation [71].

Finally, it is worth mentioning that GM is increasingly recognized as a major, still largely underestimated, player in determining the toxicity of environmental pollutants [72]. With specific regard to diet, for example it can mediate the adverse metabolic effects of non-calorie artificial sweeteners, whose chronic consumption increases the risk of glucose intolerance[73]. In contrast, some GM components have been shown to be involved in the bioremediation of common food processing products, including Maillard reaction products and advanced glycation end-products, which have been implicated in a wide variety of civilization disorders, *e.g.*, atherosclerosis and diabetes [74].

Neuroactive metabolites

GM is well known to interact with the enteric and central nervous systems *via* the bidirectional gut-brain axis[75]. On the one hand, GM can be directly influenced by mental health, through the luminal secretion of endocrine mediators capable of interacting with microbial receptors, thus having direct effects on microbial gene expression and signaling mechanisms. At the same time, the microbial community can be indirectly modulated as a result of induced changes in the gut environment. On the other hand, as anticipated above, the GM is capable of producing neuroactive metabolites in a diet-dependent manner, *e.g.*, SCFAs and conjugated fatty acids, which besides exerting peripheral effects can modulate the central nervous system through

direct or indirect mechanisms, involving a complex network of neuroendocrine factors and their receptors. Interestingly, these interactions have been shown to affect central appetite, food reward signaling and energy balance[76-79]. GM has also been found to influence eating behaviors through vagal nerve stimulation and immune activation [80]. Perturbations of the gut-microbiome-brain axis could therefore compromise the inhibitory mechanisms normally involved in the regulation of food intake, resulting in unbalanced eating patterns towards cravings, overeating and hedonic-driven eating behavior[79,81]. Regarding neuroactive GM metabolites, it is worth noting that propionate modulates reward pathways by reducing anticipatory reward responses to high-energy foods *via* striatal pathways[82]. Moreover, tryptophan metabolites have been closely implicated in the modulation of gut-microbiome-brain interactions, and indole propionate has recently been associated with increased food addiction behaviors in obese individuals[83,84]. GM metabolites may also interact with the endocannabinoid system, affecting the homeostatic and hedonic control of appetite and food intake[85], while the dopaminergic mesolimbic system, involved in reward mechanisms and hypothesized to play an important role in the development of obesity, is influenced by GM through the modulation of gut hormone secretion[86]. Not least, it should be remembered that microbes can even produce neurotransmitters, *e.g.*, serotonin and GABA, potentially affecting our mood and feeding[87,88].

In conclusion, particular GM layouts with distinct metabolic activities might have pleiotropic effects on host physiology, including eating behaviors, thus strongly contributing to the development of obesity and eating disorders. In this context, GM modulation or its replacement could be valuable tools to implement and increase the success of current preventive or therapeutic interventions against obesity and related comorbidities, as detailed below.

MICROBIOME-BASED STRATEGIES FOR PREVENTION AND TREATMENT OF OBESITY

The main microbiome-based strategies that are or could be effective for the prevention and treatment of obesity are discussed below and summarized in Figure 1. In short, traditional (prebiotics, probiotics and synbiotics) and innovative (NGPs and engineered microbes) interventions were considered, along with microbiome replacement strategies, based on FMT and synthetic ecology approaches. Finally, in the next paragraph, the potential of bioinformatics tools, such as machine learning and deep learning, for health risk or outcome prediction is discussed.

Prebiotics, probiotics and synbiotics

Prebiotics are typically referred to as "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[89]. Prebiotic supplementation has been proposed as a means of driving changes in GM while benefitting the host in the context of various disorders, including obesity, for improved glucose homeostasis and enteroendocrine L-cell activity. For instance, dietary supplementation with whole-grain barley and brown rice improved GM diversity, increasing the Firmicutes/Bacteroidetes ratio and the relative abundance of *Blautia* (an acetate producer from the *Lachnospiraceae* family), as well as attenuating postprandial blood glucose levels and decreasing plasma interleukin (IL)-6 levels in healthy individuals[90]. Oligosaccharide supplementation has shown promising anti-obesity effects *via* the SCFA and BA pathways. In particular, several animal studies have confirmed that SCFAs produced following oligosaccharide fermentation stimulate the secretion of PYY and GLP-1 *via* the G-protein-coupled receptors (GPRs) GPR-41 and GPR-43 expressed on enteroendocrine L cells[91-93]. These appetite-decreasing intestinal hormones help reduce food intake[94], increase satiety and energy expenditure[95] and improve glucose metabolism and insulin secretion[96]. Microbial fiber metabolism has additional, SCFA-independent effects, mediated by ferulic acid, a plant cell wall component with antioxidant, anti-inflammatory and anti-diabetic effects, and by alteration of the intestinal BA pool, with downstream implications in terms of energy and glucose homeostasis[97]. Not least, adding fermentable fiber to a high-fat diet in mice has been shown to result in IL-22 induction, increased enterocyte proliferation, reduced microbiota encroachment into the mucosa and pro-inflammatory gene expression, and increased antimicrobial gene expression, thereby protecting against high-fat diet-induced metabolic syndrome[97]. Although current prebiotics are mainly carbohydrate-based, other substances, such as polyphenols and polyunsaturated fatty acids (PUFAs), could exert prebiotic effects as well, as tested in both mice and humans

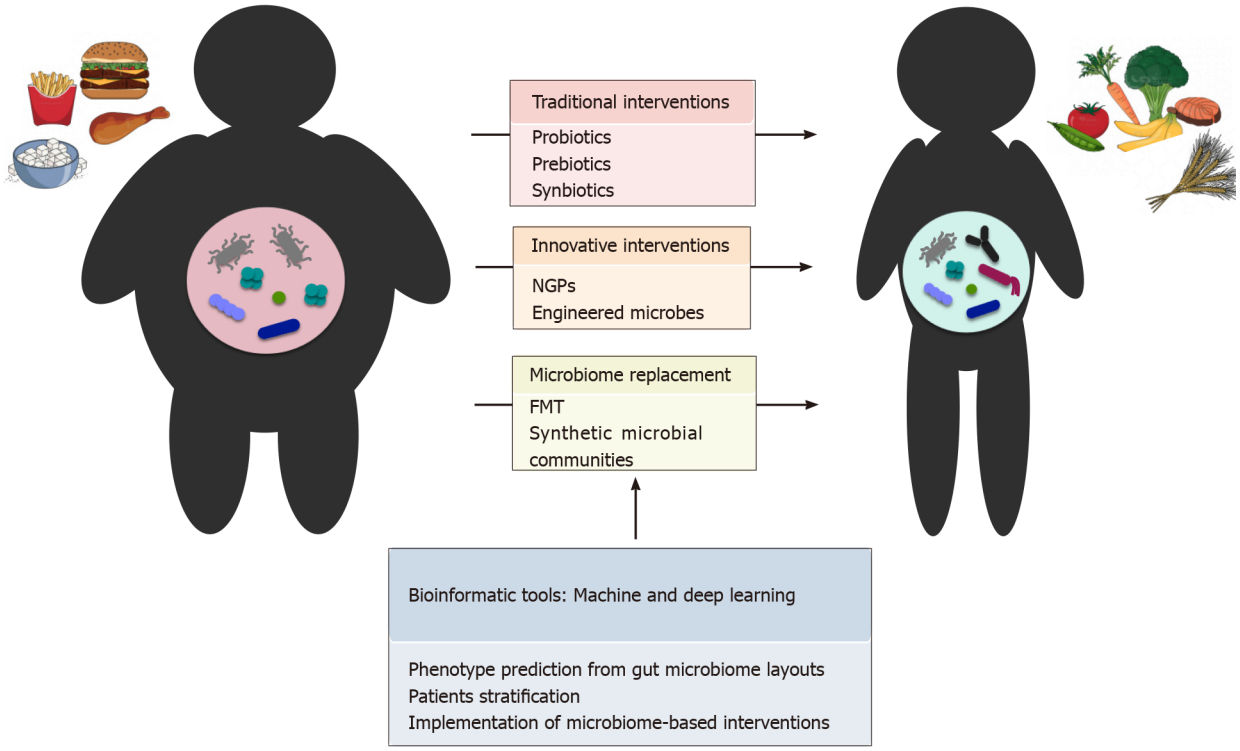

Figure 1 Overview of the main microbiome-based strategies currently in use or potentially effective in the prevention and treatment of obesity. Traditional intervention strategies include dietary supplementation with prebiotics, probiotics and synbiotics, which have generally been shown to be moderately effective for the prevention and amelioration of obesity. Innovative strategies have therefore been implemented for improved treatment efficacy, using next-generation probiotics and non-pathogenic engineered microorganisms designed for the *in-situ* delivery of specific modulators. More recently, more direct modulation strategies based on gut microbiome replacement by means of fecal microbiota transplantation or synthetic communities, are being considered. In this scenario, bioinformatic tools, including machine and deep learning, could be crucial not only for the rational design of synthetic communities, but also for stratifying patients based on disease-associated phenotypes and thus predicting their health risks and outcomes. In the near future, all the accumulating knowledge about the gut microbiome and technological advances should lead to a rational implementation of innovative microbiome-based interventions geared towards personalized precision medicine. Food items were obtained from the Mind the Graph platform (https://mindthegraph.com/). NGPs: Next-generation probiotics; FMT: Fecal microbiota transplantation.

[89]. Polyphenols, *i.e.*, secondary metabolites derived from plant sources, are extensively metabolized in the intestine and converted into phytoestrogens with multiple beneficial effects[50,98]. Following regular consumption of polyphenols, a reduced risk of cardiometabolic diseases has been observed, together with antioxidant, anti-inflammatory and anti-obesogenic effects[99,100]. However, the prebiotic effects of polyphenols can be affected by the food source and although these compounds are generally recognized as GM compositional modulators, further research is needed to validate their prebiotic potential[101]. Multiple health benefits have also been reported for nutritional supplementation with PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid, including anticancer activity[102,103], secondary prevention of ischemic heart disease[104] and prevention of cardiovascular diseases[105], as well as a reduction of mucosal inflammation and modulation of the GM composition in patients with ulcerative colitis[106]. Dietary supplementation with PUFAs may therefore be useful not only for obesity mitigation but also for the treatment of obesity-associated comorbidities[107,108]. In addition, PUFA-derived mediators, such as resolvins and protectins, have shown the potential to counteract inflammation in the context of obesity[109]. In this regard, a recent study in obese diabetic *db/db* mice demonstrated a marked improvement in insulin sensitivity following the administration of protectin D1[110]. In light of these findings, prebiotic supplementation should be considered a potential integrative therapy for the prevention and treatment of obesity.

Probiotics, *i.e.*, "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host"[111], represent one of the most widely used GM manipulation tools, for which an increasing number of clinical studies have been carried out in subjects with various pathological conditions, including obesity[112, 113]. However, it should be noted that conflicting results have emerged in relation to the ability of probiotics to counterbalance weight loss and obesity-related features. In particular, two meta-analyses of randomized controlled clinical trials[114,115] found

almost no efficiency in terms of weight and body mass index (BMI) reduction in obese individuals, especially in short-term interventions. On the other hand, the meta-analysis carried out by Zhang *et al*[116] on a large number of clinical trials reported substantially different results and a significant reduction in body weight and BMI, with consistent maximum outcomes with multi-strain product supplementation for at least 8 wk. Similar conclusions were drawn by John *et al*[117] in a similar meta-analysis, showing that probiotic administration was associated with a reduction in all considered parameters (*i.e.*, body weight, BMI and fat mass). As expected, several studies have found strain-specific probiotic effects on body weight and metabolism, with only a few species belonging to *Lactobacillus* (*e.g.*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. reuteri*) and *Bifidobacterium* (*e.g.*, *B. bifidum*, *B. lactis*, *B. longum*) proven effective in overweight/obese individuals[104,118,119]. The greatest decreasing effect on BMI and body weight was reported with high doses of single-strain probiotics[117, 120], although John *et al*[117] observed a considerable reduction in both parameters even at lower doses when interventions continued for more than 12 wk[117]. More recently, a study by our group showed that administering a multi-strain probiotic mixture along with a hypocaloric Mediterranean diet led to weight loss, improvement in oxidative stress markers and an increase in *Akkermansia* in elderly obese women, even in 15 d[33]. Regardless of the strain, the beneficial effects of probiotics in the treatment of obesity are also attributable to the following general mechanisms of action: (1) Antimicrobial activity, by inhibiting the growth of pathogenic microorganisms and exerting antagonistic effects against colonization of the intestinal mucosa and epithelium adherence; (2) Improvement of the barrier function, reducing intestinal permeability and increasing mucus production; and (3) Immunomodulation, through interaction with innate and adaptive components of the immune system[120]. Taken together, these mechanisms contribute to positively modulate the GM composition towards restoring a health-associated layout, which in turn can help alleviate host metabolic imbalances.

Synbiotics refer to "a mixture comprising live microorganisms and substrates selectively used by host microorganisms that confers a health benefit on the host" [121]. Accumulating evidence has reported the stronger effect of synbiotics in terms of GM modulation than either probiotics or prebiotics alone[122,123], with an overall improvement in lipid metabolism, glycemic status and inflammatory mediators[124, 125]. Although the appropriate dose, duration of administration and the composition of a synbiotic product necessary to confer a health benefit are influenced by several factors (*e.g.*, baseline GM layout, medications, habitual diet and lifestyle), the randomized clinical trial conducted by Dao *et al*[42] on 225 overweight and obese adults resulted in a 4.5% reduction in body fat mass when administering a combination of *B. animalis* subsp. *Lactis* 420 and polydextrose[42]. The control of body fat mass in overweight or obese individuals by the aforementioned synbiotic was also confirmed in a second clinical trial, along with a peculiar rearrangement in the GM composition that included an increased abundance of *Akkermansia*, *Christensenellaceae* and *Methanobrevibacter*, all taxa related to improved metabolic health and leanness [126]. GM modulation with increased proportions of potentially beneficial microbial groups (*e.g.*, *Lactobacillus*) was also observed in a clinical trial in obese individuals following administration of a synbiotic consisting of a multi-strain probiotic formulation (*i.e.*, *B. lactis*, *B. longum*, *B. bifidum* and *L. acidophilus*) and galactooligosaccharides as a prebiotic component[127].

In conclusion, prebiotics, probiotics and synbiotics are promising dietary agents for the modulation of human GM also in the context of obesity and metabolic disorders. However, given the still conflicting data in the scientific literature on probiotics, further studies are needed to rationally include their prescription as a preventive or therapeutic supplement for obesity.

NGPs and engineered microbes

Moving from the "one-size-fits-all" concept related to traditional probiotics, researchers are striving to thoroughly elucidate the role of each commensal member within the complex ecosystem of the human gastrointestinal tract in influencing host health. Compared to the modest ameliorative effects generally shown by traditional probiotics in obese individuals, emerging NGPs are beginning to reveal their great potential as novel preventive and therapeutic tools[128]. In this perspective, several studies have shown differences in the GM composition between obese and lean individuals, pointing out that an increased abundance of *A. muciniphila* could lead to an improvement in obesity and metabolic disorders[129,130]. Subsequently, the mechanism underlying the beneficial effect of *A. muciniphila* was investigated and the involvement of an immunomodulatory membrane protein "Amuc_1100" was

proposed, which showed the same beneficial effects as live bacteria[131]. Cani *et al* [132] demonstrated the ability of this promising NGP to modulate the endocannabinoid system[132], a crucial regulatory system involved in controlling glucose metabolism in obesity, T2D and inflammatory conditions[132]. More recently, as anticipated above, the daily oral administration of live or pasteurized *A. muciniphila*, for three months to overweight/obese insulin-resistant volunteers, has been shown to be safe and well tolerated, while leading to improved metabolic parameters[44]. However, since some animal studies have reported an increase in *A. muciniphila* in multiple sclerosis[133] and Parkinson's disease[134], further studies are needed to fully unravel its effects on host health.

Christensenella minuta also showed potential probiotic effects by ameliorating obesity and associated metabolic disorders through modulation of dysbiotic GM layouts[135], and its abundance was greater in lean individuals with low BMI[136]. However, Yang *et al*[137] recently highlighted potential pathogenic features (*e.g.*, LPS-mediated triggering of a mild inflammatory response *via* NF-kB pathway) of *C. minuta*, suggesting that its application should be limited to therapeutic interventions focused on obesity control[137]. As mentioned above, *P. goldsteinii* is also a promising anti-obesity and anti-inflammatory NGP candidate. Being selectively enriched in the GM of mice fed a high-fat diet supplemented with oriental medicinal fungi, this commensal bacterium has been associated with increased adipose tissue thermogenesis, reduced levels of inflammation, enhanced intestinal integrity and amelioration of insulin resistance[41]. While promising, these *in vivo* results have not yet been translated into clinical trials. On the other hand, *Faecalibacterium prausnitzii*, one of the most promising NGPs due to its well characterized anti-inflammatory activity[138,139], showed a lower clade diversity in intestinal diseases and obese individuals[140]. In light of this finding, caution must be taken in selecting the most suitable strain for the development of therapeutic interventions. Despite the growing amount of NGP candidates so far isolated and characterized, further strain-level functional analyses are required to fully assess the underlying mechanisms by which they could confer health benefits to the host, before pushing them for clinical application.

Synthetic biology approaches have recently been exploited to address disease-specific mechanisms and meet medical needs by designing non-pathogenic and commensal bacteria to deliver therapeutic effectors[141,142]. Regarding the feasibility of using engineered probiotics to alleviate obesity-related characteristics, Long *et al* [143] demonstrated a decrease in body weight gain in overweight mice given an engineered *Bifidobacterium* strain secreting oxyntomodulin, an anorexigenic hormone that reduces appetite and food intake[143]. In a similar study, Chen *et al*[144] developed an engineered probiotic strain of *Escherichia coli* with increased secretion of N-acyl-phosphatidylethanolamine, the immediate precursor of the anorexigenic metabolite N-acylethanolamide, resulting in reduced weight gain and less accumulation of fat mass in mice fed a high-fat diet[144]. Another potential strategy to alleviate obesity and metabolic disorders has recently been proposed by Bai *et al*[145]. Administration of *Bacillus subtilis* SCK6 strain BsS-RS066550 engineered to increase butyric acid production resulted in decreased body weight and food intake in high-fat diet-fed mice, along with beneficial effects on insulin resistance, blood glucose and hepatic biochemistry[145]. In addition, Wang *et al*[146] showed that administration of genetically engineered *Lactococcus lactis* expressing GLP-1 significantly reduced body weight and blood glucose of obese mice fed a high-fat diet[146]. Anti-obesity mechanisms included the promotion of fatty acid oxidation and the restoration of GM biodiversity[146]. To date, numerous therapeutic interventions based on engineered live bacteria have entered the early or mid-stage of clinical development[141]. If successfully completed, such studies will be crucial in providing the missing proof of concept required to pave the way for a new class of precision therapeutics. Once their efficacy and risk ratio have been verified and approved for use in humans, engineered bacterial therapeutics will enable specific disease mechanisms and unmet medical needs to be addressed, even in obese patients.

FMT

Consisting of the transfer of microbes from healthy individuals to recipients hosting a dysbiotic GM layout with the aim of restoring eubiosis[147,148], FMT has attracted considerable attention in clinical practice, particularly for the treatment of recurrent infections by antibiotic-refractory *Clostridioides difficile*[149,150]. Recently, the potential of FMT in treating a large number of conditions, including obesity, has been explored. In a pivotal study, Ridaura *et al*[63] showed that mice receiving GM from obese individuals developed obesity, while those receiving GM from healthy individuals remained lean[63]. Sequencing analysis of post-treatment stools confirmed the

successful engraftment of the donor microbiota, along with the transfer of functions associated with either "lean" or "obese" microbial communities. Shortly before, a clinical study on diabetic and obese adult males by Vrieze *et al*[151] demonstrated improved microbial diversity and insulin sensitivity, along with the expansion of Bacteroidetes and butyrate-producing taxa, following GM transplantation from lean donors[151].

As of April 2021, there were 15 registered clinical trials (ClinicalTrials.gov search terms: "gut microbiota", "obesity" and "fecal microbiota transplantation") on obese individuals undergoing FMT for the replacement of the obesogenic microbial community (Table 1). Of these, four have been completed and only two have made the results publicly available. Yu *et al*[152] performed FMT on 24 obese individuals with the aim of improving metabolic outcomes[152]. Administration of FMT capsules ensured engraftment, persisting for at least 12 wk after treatment, although no clinically significantly metabolic effects were observed. Allegretti *et al*[153] performed FMT on 22 obese, metabolically uncompromised patients, and although no significant changes in BMI occurred, a sustained shift of obesity-associated microbiomes towards the donor microbiome layout was observed, along with improved BA profiles and decreased fecal levels of taurocholic acid[153]. In a secondary analysis focused on the prevention of metabolic syndrome within the same patient group, the authors found a significant change in glucose and insulin levels after FMT[154]. Similar to other microbiome-based therapeutics, while promising, FMT is still at an early stage for the treatment of obesity and associated comorbidities. Therefore, it should be recognized as a separate pharmacological category, consisting of an entirely novel class of agents and requiring systematic research to fill knowledge gaps, thereby facilitating the development of standardized next-generation microbiota therapeutic interventions with improved safety and efficacy.

Microbial replacement therapy through synthetic ecology approaches

It is generally believed that the administration of multi-species microbial consortia is more useful than a single probiotic organism[155], as it probably retains some properties of the community structure, with community members continuously interacting and communicating with each other. Furthermore, a consortium of bacteria possesses a larger gene pool than monocultures, resulting in greater diversity in metabolic pathways. This richness is reflected in a greater ability of the consortium to perform more complex tasks than single organisms, thus exploiting the resources available in the surroundings more efficiently and better adapting to the environment, also through self-organization to form spatial patterns in response to substrates and metabolite gradients[156-160].

Synthetic ecology indicates the rational design of ecosystems, where two or more defined microbial populations are assembled in a well-characterized and controlled environment[161]. This approach requires *in vitro* controlled environments, biologically relevant bacterial strains and mathematical models of ecological interactions [162] to simulate community behavior in response to several factors. The idea is to shape a complex microbial community in order to obtain a desired compositional and functional profile that meets the needs of specific industrial production processes or pharmacological interventions.

Synthetic microbial communities are systems of known and trimmed-down complexity that can undergo experimental treatments and mathematical modelling, enabling a system-level understanding of the consortium[163,164]. These communities are not only a way to study how the microbial consortium structure emerges and the conditions necessary to generate specific interaction networks among its components, but they can also elucidate the overall function, resistance and resilience of microbial systems[10]. By the so-called microbial resource management[165,166], *i.e.*, the management of consortium parameters such as richness, evenness, predation, abiotic factors, quorum sensing and spatial disposition, the community can be steered to the desired functionality, in order to obtain novel products and processes or potentially improve human health by transplanting the desired synthetic community into a recipient patient.

To date, many studies[167-169] have reported the *in vitro* assembly of synthetic GM communities with at least two bacterial strains, but clinical applications are still limited. Petrof *et al*[170] obtained a synthetic consortium consisting of 33 individual microbial species derived from a stool sample from a healthy donor and demonstrated the potential of such synthetic microbiota in the treatment of *C. difficile* infection. Furthermore, they reported that some of the administered bacteria that were forming the synthetic community stably colonized the recipient's colon, as opposed to most commercially available probiotics, which only transiently colonize the intestine.

Table 1 Registered clinical trials on ClinicalTrials.gov (as accessed on April 2021) focused on fecal microbiota transplantation for the replacement of obesogenic microbial communities. Search terms included "gut microbiota", "obesity" and "fecal microbiota transplantation"

Rank	Title	Status	Results	Condition	Intervention	Location	URL
1	Fecal Microbiota Transplantation for the Treatment of Obesity	Completed	Available	Obesity	FMT *vs* placebo	United States	https://ClinicalTrials.gov/show/NCT02741518
2	Faecal Microbiota Transplantation in Obesity	Recruiting	Not available	Obesity	FMT *vs* placebo	Finland	https://ClinicalTrials.gov/show/NCT03391817
3	Randomized Controlled Trial of Fecal Microbiota Transplantation in Severe Obesity	Enrolling by invitation	Not available	Obesity	FMT *vs* placebo	Norway	https://ClinicalTrials.gov/show/NCT03273855
4	Fecal Microbiota Transplant (FMT) to Induce Weight Loss in Obese Subjects	Active, not recruiting	Not available	Obesity	FMT and mucosal microbiota assessment	China	https://ClinicalTrials.gov/show/NCT03789461
5	FMT and Fiber in Patients With Metabolic Syndrome	Completed	Not available	Obesity, Metabolic Syndrome	FMT and dietary supplement with fiber (cellulose) *vs* placebo	Canada	https://ClinicalTrials.gov/show/NCT03727321
6	Assessment of the Health Improvement of Obese Patients After Fecal Microbiota Transplantation (FMT)	Completed	Not available	Obesity, Type 1 and 2 Diabetes	FMT	Russian Federation	https://ClinicalTrials.gov/show/NCT04579263
7	Fecal Microbiota Transplantation for Diabetes Mellitus Type II in Obese Patients	Unknown status	Not available	T2DM, Obesity	FMT and dietary intervention (high-fat low-fiber diet, sham diet or low-fat high-fiber diet)	Israel	https://ClinicalTrials.gov/show/NCT02346669
8	Fecal Microbial Transplantation and Fiber Supplementation in Participants With Obesity and Metabolic Syndrome	Active, not recruiting	Not available	Obesity, Metabolic Syndrome	FMT and dietary supplement with fiber (cellulose) or FMT only *vs* placebo	Canada	https://ClinicalTrials.gov/show/NCT03477916
9	Randomised Placebo-controlled Study of FMT to Impact Body Weight and Glycemic Control in Obese Subjects With T2DM	Active, not recruiting	Not available	T2DM, Obesity	FMT and lifestyle modification program or FMT only *vs* placebo	China	https://ClinicalTrials.gov/show/NCT03127696
10	Fecal Microbiota Transplant for Improvement of Metabolism	Completed	Available	Obesity	FMT *vs* placebo	United States	https://ClinicalTrials.gov/show/NCT02530385
11	The Role of Microbiome in Recurrent Obesity	Not yet recruiting	Not available	Obesity	FMT *vs* placebo	Israel	https://ClinicalTrials.gov/show/NCT04697550
12	Effects of Fecal Microbiota Transplantation on Weight in Obese Patients With Non-alcoholic Fatty Liver Disease	Recruiting	Not available	NAFLD	FMT, dietary intervention and physical activity *vs* placebo	India	https://ClinicalTrials.gov/show/NCT04594954
13	Proposal to Examine the Effect of Fecal Transplantation on Obesity	Unknown status	Not available	Obesity	FMT *vs* placebo	Israel	https://ClinicalTrials.gov/show/NCT02336789
14	Safety and Efficacy of Fecal Microbiota	Recruiting	Not available	IBD, IBS, Obesity,	FMT	China	https://ClinicalTrials.gov/show/NCT04014413

	Transplantation			Metabolic Syndrome, Infections, Others			
15	Transplantation of Microbes for Treatment of Metabolic Syndrome & NAFLD	Completed	Not available	Type 1 and 2 Diabetes, NAFLD, Obesity	FMT	Canada	https://ClinicalTrials.gov/show/NCT02496390

FMT: Fecal Microbiota transplantation; IBD: Inflammatory bowel disease; IBS: Inflammatory bowel syndrome; NAFLD: Non-alcoholic fatty liver disease; T2DM: Type 2 diabetes mellitus.

Despite its proven efficacy for the treatment of *C. difficile* infection, FMT hides some uncertainties that may be resolved by a synthetic stool replacement strategy. Indeed, the synthetic ecology approach has multiple advances over the canonical use of fecal matter from a donor: (1) The exact composition of the administered bacteria is known and can be reproduced; (2) The bacterial composition can be virtually tailored to the specific patient's needs; (3) The absence of pathogens and viruses can be more reliably guaranteed, improving safety; and (4) The administered microorganisms forming the consortia can be selected based on their sensitivity to antimicrobials, resulting in further improvement of the safety profile.

Applications of synthetic communities have been reported in other fields, such as bioremediation[157] and chemical production[171,172]. Notwithstanding the potential of the synthetic ecology approach in GM replacement therapy, there is still some way to go before synthetic ecology can be translated into the clinic. One major limitation is the lack of a truly representative *in vitro* system to mimic the *in vivo* microbiota, but steps in this direction have been achieved by the gut-on-a-chip model[173] and the HuMiX system[173]. In addition, a large and well-documented collection of cultures of human microbiota members will be extremely useful for improving ecology experiments and building mathematical models. In this regard, since 2015, culturomics has led to the discovery of 232 novel human gut species[174] and it is expected that this number will increase in the coming years. The technique is based on the multiplexing of bacterial isolation conditions through serial addition of specific growth promoters and/or inhibitors, coupled with high-throughput identification with MALDI-TOF mass spectrometry, and is capable of overcoming the limitations of conventional single-medium strategies[175-177]. The resulting knowledge could then be used to improve our understanding of the complex gut ecosystem and rationally design efficient and sophisticated synthetic communities, tailored for the treatment of the disease of interest.

THE FUTURE OF MICROBIOME-BASED PRECISION MEDICINE: HEALTH RISK OR OUTCOME PREDICTION THROUGH DEEP LEARNING

Machine learning and deep learning application to microbiome data

Machine learning and, in particular, deep learning have been the subject of intense media hype in recent years. Thanks to the explosion of available data and the rapid growth in the number and size of databases, they have accomplished nothing short of a revolution in the field of modern artificial intelligence, with notable progress in perceptual problems, such as facial and speech recognition[178,179], and have the potential to do the same in medical disciplines[180,181]. However, we are still exploring the full extent of what machine learning and deep learning can do and have just begun to apply them to a wide variety of problems outside of classical algorithms.

In light of the rapid increase in data from microbiome studies induced by the concomitant decrease in sequencing costs (< 10000 times in the past 10 years)[182], there are requirements to apply machine learning and deep learning algorithms to host-microbiome data, exploiting their associations in various diseases. Improved data analytical tools are needed to explore all the information contained in those datasets and identify key features that represent different aspects of the microbiome and that can be linked to host phenotypes. In particular, the possibility of predicting the patient's phenotype from one's GM is an integral part of personalized medicine, as it represents not only a way to overcome individual variability, but also a potential therapeutic target for pharmacological interventions. In this field, machine learning

might provide new insights, by developing models capable of stratifying individual patients into therapeutic classes, thus paving the way for the fine-tuning of interventions based on the GM structure. An example of machine learning algorithms are Support Vector Machines (SVMs), which were implemented by Cui and Zhang [183] to classify metagenomic samples into inflammatory bowel disease (IBD) and non-IBD classes. More recently, Pasolli *et al*[184] used SVMs to predict diseases such as liver cirrhosis, colorectal cancer and IBD from fecal metagenomes. To date, deep learning is the most advanced machine learning technique for a variety of applications [185] and has already achieved several results in the microbiome field[186-189], including predicting the microbiome structure in terms of bacterial relative abundance and metabolic layout. Other machine learning methods include Ensemble Methods, which combine multiple classifiers for better performance. The best known ensemble method is Random Forest[190], which has been widely used in microbiome studies for patient stratification[191,192] and biomarker search[193,194].

Machine learning, GM and obesity

In a prospective study on obesity in European children, Rampelli *et al*[31] underscored the importance of the microbiome–host–diet configuration as a possible predictor of obesity. In particular, GM was found to mediate dietary impact on individual metabolic and immunological homeostasis, which, in the context of other individual lifestyle and genetic variables, may be involved in the development of the multifactorial obese phenotype. Such results were based on experimental observations and no machine learning/deep learning algorithms were applied or developed. However, the study suggested that applying a machine learning algorithm to microbiome data could be a feasible method of predicting obesity, when other variables concerning host physiology, diet and lifestyle (*e.g.*, physical activity) are included.

Some attempts have been made in recent years, with poor results, mainly due to a lack of data (often lifestyle and dietary information is not collected or collected in a non-systematic and non-standardized way). Specifically, Pasolli *et al*[184] developed a Random Forest-based approach that exclusively utilized metagenome data without success in predicting obesity and T2D. A few years later, Fernández-Navarro *et al*[195] have implemented several machine learning methods, such as decision tree-based methods, ensemble methods and SVMs, to identify predictors of obesity. Starting from serum free fatty acid levels, microbial quantitative polymerase chain reaction information and dietary intake interviews, their model revealed a non-obese profile related to serum eicosapentaenoic acid levels and *Bacteroides* amount in feces.

Nevertheless, this represents just the tip of the iceberg, as the full deployment of machine learning methods in the human GM sphere for full integration into the field of personalized precision medicine requires additional efforts. Indeed, machine learning often runs like black boxes, which makes it difficult to conduct feature selection. In addition, a large amount of data and computational power are required to train powerful and reliable machine learning-based algorithms. In general, novel technologies have dramatically increased our ability to characterize the human GM, but the way to effectively harness that information is uncertain and presents several key challenges. For example, there is a high need for dimensionality reduction to handle the information of hundreds of thousands of gene markers for just a few hundred samples. In this regard, neural encoder-decoder networks[196] based on a deep learning architecture have proved effective[187], but further efforts are still needed to fully exploit microbiome data. What is certain is that machine learning has already shown its great potential when applied to the microbiome field. In the next years, cutting-edge machine learning-based models might enable a further step towards the microbiome implementation in personalized precision medicine.

LIMITATIONS OF CURRENT EVIDENCE AND NEXT STEPS

As discussed in the respective paragraphs, there are several limitations to the design and application of microbiome-based strategies in clinical routine, with particular reference to the prevention/treatment of obesity and related comorbidities. Although promising data come from the field of NGPs/engineered microbes, synthetic ecology and even FMT, it should be remembered that the evidence is still too little to support the clinical benefit of these novel microbiome modulation tools. Added to this are the sometimes inconsistent results on GM compositional and functional changes in the pathological context, and the lack of a full understanding of the underlying mecha-

nisms. Of course, several steps forward have been made, especially methodological ones, but there is still no standardization of study designs and the way of reporting the results, which makes it difficult to compare different studies. In parallel with the implementation of internationally recognized standard operating procedures for GM analysis, it is expected that in the future: (1) -Omics approaches, including metagenomics, metatranscriptomics, metaproteomics and metabolomics are combined to provide mechanistic insights; (2) The mechanisms are possibly validated in animal models; (3) Culturomics approaches are increasingly exploited to unravel the dynamics and ecological rules that govern the establishment of microbial networks; (4) The accumulating microbiome knowledge allows to fine-tune the design of precision strategies to achieve specific objectives, whether based on traditional or next-generation tools; and (5) Progressively generated datasets, including host and microbiome data, enable machine and deep learning technologies to maximize translational impact, through accurate prediction of health outcomes and thus provision of high-quality personalized care. As expected, the same limitations and implications also apply in other pathological contexts, where the modulation of GM can be impactful as well.

CONCLUSION

The emerging role of GM as a contributor to various pathological conditions is fascinating even if not yet easy to untangle. Diseases resulting from multiple factors, such as obesity and metabolic diseases in general, may be difficult to prevent or treat effectively solely relying on currently available therapies. In this scenario, gut microbes and their influence on the host constitute a piece of an intricate puzzle, to be exploited for novel integrated intervention strategies. In a systems biology approach, the advent of –omics technologies and the development of bioinformatics tools are pushing microbiome research to the next level, allowing to extrapolate general principles on community structure and translate the results into rationally designed, personalized microbiome-based interventions aimed at restructuring dysbiotic layouts, thereby contributing to the restoration and maintenance of host health.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7065-7079

DOI: 10.3748/wjg.v27.i41.7065

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

REVIEW

Gut microbiota in a population highly affected by obesity and type 2 diabetes and susceptibility to COVID-19

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Author contributions: García-Mena J, Corona-Cervantes K, Cuervo-Zanatta D, Benitez-Guerrero T, Vélez-Ixta JM, Zavala-Torres NG, Villalobos-Flores LE, Hernández-Quiroz F, Pérez-Cruz C, Murugesan S, Bastida-González FG, Zárate-Segura PB, contributed equally to conceptualization and design, paper writing, critical review, and approved the final version of the paper.

Conflict-of-interest statement: The authors report no conflicts of interest.

Supported by Secretaría de

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Abstract

Coronavirus disease 2019 (COVID-19) is a disease produced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and it is currently causing a catastrophic pandemic affecting humans worldwide. This disease has been lethal for approximately 3.12 million people around the world since January 2020. Globally, among the most affected countries, Mexico ranks third in deaths after the United States of America and Brazil. Although the high number of deceased people might also be explained by social aspects and lifestyle customs in Mexico, there is a relationship between this high proportion of deaths and comorbidities

Relaciones Exteriores México (SRE), No. SRE/027/2021; Agencia Mexicana de Cooperación Internacional para el Desarrollo (AMEXCID), No. AMEXCID 2020-5; CONACyT for Doctoral Fellowships, No. 777953 (KC-C), No. 635676 (TB-G), No. 291236 (FH-Q), and No. 336296 (LEV-F); CONACyT for Master Fellowships, No. 997494 (NGZ-T), and No. 997152 (JMV-I); Fellows from the Sistema Nacional de Investigadores, Mexico, No. 43142 (PZ-S), No. 225525 (FB-G), No. 47399 (CP-C), and No. 19815 (JG-M).

Country/Territory of origin: Mexico

Specialty type: Gastroenterology and hepatology

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

Received: May 1, 2021
Peer-review started: May 1, 2021
First decision: June 12, 2021
Revised: June 25, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Verma AK, Wang CY
S-Editor: Fan JR
L-Editor: A

such as high blood pressure (HBP), type 2 diabetes, obesity, and metabolic syndrome. The official epidemiological figures reported by the Mexican government have indicated that 18.4% of the population suffers from HBP, close to 10.3% of adults suffer from type 2 diabetes, and approximately 36.1% of the population suffers from obesity. Disbalances in the gut microbiota (GM) have been associated with these diseases and with COVID-19 severity, presumably due to inflammatory dysfunction. Recent data about the association between GM dysbiosis and metabolic diseases could suggest that the high levels of susceptibility to SARS-CoV-2 infection and COVID-19 morbidity in the Mexican population are primarily due to the prevalence of type 2 diabetes, obesity, and metabolic syndrome.

Key Words: SARS-CoV-2; COVID-19; High blood pressure; Hypertension; Type 2 diabetes; Obesity; Metabolic syndrome; Gut microbiota; Immunity

Core Tip: This work reviews recent data about gut microbiota (GM) diversity in Mexico, a country in which more than 18.4% of adults present high blood pressure, 39.1% are overweight, 36.1% are obese, and more than 10.3% suffer from type 2 diabetes. This review highlights the link between GM dysbiosis and severe acute respiratory syndrome coronavirus 2 prevalence, which ranks Mexico third in cumulative coronavirus disease 2019 deaths in the world.

Citation: García-Mena J, Corona-Cervantes K, Cuervo-Zanatta D, Benitez-Guerrero T, Vélez-Ixta JM, Zavala-Torres NG, Villalobos-Flores LE, Hernández-Quiroz F, Perez-Cruz C, Murugesan S, Bastida-González FG, Zárate-Segura PB. Gut microbiota in a population highly affected by obesity and type 2 diabetes and susceptibility to COVID-19. *World J Gastroenterol* 2021; 27(41): 7065-7079
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7065.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7065

INTRODUCTION

Bacteria maintain the immune response in the gut

The human body harbors approximately 100 trillion cells belonging to commensal microorganisms[1], and they are primarily concentrated in the intestine[2]. The term gut microbiota (GM) refers to the symbiotic intestinal collection of bacteria, archaea, and some eukaryotes with an important influence on health and disease[3]. Among the several functions in the host, the GM participates in the synthesis of water-soluble vitamins, the supply of quinones[4], the metabolism of xenobiotics[5], neurotransmitter modulation[6], the production of energy substrates from dietary fiber[7] and the regulation of immune homeostasis[8].

A functional microbiota promotes the host's immunity[9]. For example, the polysaccharide A in *Bacteroides fragilis*' directs lymphoid organogenesis and corrects systemic T lymphocyte (TL) deficiencies and TL-helper Th1/Th2 imbalances through mechanisms such as interleukin (IL)-12/Stat4-mediated Th1 differentiation. Moreover, *B. fragilis*' polysaccharide A presentation by intestinal dendritic cells (DCs) activates clusters of differentiation in CD4+ TLs, eliciting appropriate cytokine production[10]. Commensal GM is also required for Th17 cell differentiation in the small intestine by activating the transforming growth factor (TGF)-β[11] and influences gut immunoglobulin (Ig) repertories and B lymphocyte (BL) development in the intestinal mucosa [12]. Elevated serum levels of IgE through BL isotype switching at mucosal sites have been reported for germ-free (GF) mice in a CD4+ TL- and IL-4-dependent manner, suggesting that a healthy GM is required to inhibit high IgE induction[13].

The GM plays a vital role in the innate immune system[14]. A total lack of TL and DC under GF conditions in the jejunum of piglets was reverted by *Escherichia coli* colonization, favoring the recruitment of both cell types to the lamina propria[15]. GM

P-Editor: Fan JR

metabolites such as trimethylamine N-oxide and butyrate drive macrophage polarization using the NLRP3 inflammasome as a proteolytic activator[16], they promote monocyte to macrophage differentiation by inhibiting histone deacetylase (HDAC3), and they amplify antimicrobial host defense[17]. Furthermore, GF mice lack IL-22-producing natural killer (NKp46+) cells[18] and have lower levels of mast cell densities in the small intestine than conventional mice due to the absence of CD182 ligands from gut epithelial cells[19]. All this evidence illustrates the vital function of the GM in relation to innate immunomodulation.

The interactions of the host with the microbiota are complex, numerous, and bidirectional. The GM significantly regulates the development and function of the innate and adaptive immune systems[20]. Intestinal bacterial commensals secrete antimicrobial peptides and compete for nutrients and habitat sites, thereby aiding in the state of homeostasis[21]. The GM and immune homeostasis have a reciprocal relationship and are a topic of great interest and intense research investigation in the field of infectious diseases. Additionally, GM-derived signals modulate immune cells for pro- and anti-inflammatory responses, thereby affecting susceptibility to various diseases[22]. Immune gut homeostasis is orchestrated by fine adjustments in the regulatory balance of pro-inflammatory responses such as Th17 cells *vs* inflammatory regulatory T cells (Tregs), whose function is influenced by commensal microorganisms [23]. During the process of launching a response against pathogenic infections and etiological agents such as coronavirus, a healthy gut microbiome is pivotal to maintaining an optimal immune system to prevent an array of excessive immune reactions that eventually become detrimental to the lungs and vital organ systems. Under those circumstances, it becomes crucial to have a balanced immune response as opposed to an overreactive or an under reactive response that could aggravate the disease, causing clinical complications such as pneumonia and/or even acute res-piratory distress syndrome to occur in response to viral diseases such as coronavirus disease 2019 (COVID-19).

Several studies have linked the GM with adaptative immune system homeostasis [24]. For instance, *B. fragilis* induces CD4+ TL differentiation to Th1 and interferon (IFN)-γ production[10], whereas segmented filamentous bacteria favor this process of Th17 differentiation and IL-17 and IL-22 production[8]. However, bacteria such as indigenous *Clostridium* spp. promote this differentiation to CD4+ T regulatory cells and the production of IL-10 and IL-35 through the induction of the TGF-β cytokine and the FOXP3 transcription factor expression[8,25].

An essential role of the GM in the host's susceptibility to viral infection has been suggested by some reports[26]. For example, while *Bifidobacterium breve* and prebiotic oligosaccharides prevented rotavirus infection through IFN-γ, IL-4, tumor necrosis factor (TNF-α), and Toll-like receptor (TLR2) expression[27], human milk oligosaccharides (HMOs) increased *Enterobacter*/*Klebsiella* abundance and rotavirus infectivity, possibly through the viral structural stability conferred by HMOs[28] and lipopolysaccharides[29]. Moreover, there is an interesting report showing that short-chain fatty acids produced by GM protect against allergic inflammation in the lungs[30].

ASSOCIATION OF COVID-19 SEVERITY WITH HIGH BLOOD PRESSURE, TYPE 2 DIABETES, OBESITY, AND METABOLIC SYNDROME

Non-communicable diseases (NCDs) are the leading cause of mortality and premature disability worldwide, with over 36 million deaths *per* year[31]. Obesity (OB) is considered a major risk factor for NCDs, and it is associated with an estimated loss of 5–20 years of life expectancy[32]. OB also increases the risk of metabolic diseases such as fatty liver disease and type 2 diabetes mellitus (T2DM)[33]. From 2000 to 2019, there was an increase in global T2DM prevalence from 151 to 463 million, and this number is expected to grow to 700 million by 2045[34]. It is estimated that T2DM accounts for 87 to 91% of diabetes cases, while type 1 diabetes is only considered to be responsible for 7 to 12% of global diabetes cases[35]. Although there are some reports indicating that in general, the prevalence of diabetes is stabilizing in some populations, overall, it keeps increasing in non-Hispanic black and Hispanic populations[36].

The OB prevalence in Mexico is one of the highest in the world, corresponding to 36.1% of the Mexican population[37]. The number of cases of T2DM in Mexico is 12.8 million people, with 101257 deaths due to related complications, and T2DM is second in Latin America and sixth in the world[34,38].

The global epidemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has immediate implications for the therapy of common metabolic disorders such as T2DM, gestational diabetes, OB, metabolic syndrome (MetS), and high blood pressure (HBP). T2DM is associated with an increased risk of severe bacterial and viral respiratory tract infections, including H1N1 and influenza[39]. T2DM was also a comorbidity associated with adverse outcomes in hospitalized patients with SARS-CoV-2 in both China and Italy[40,41]. In the Italian cohort, hyperglycemic COVID-19 patients had a higher risk for mechanical ventilation, shock, and multiple organ failure requiring intensive care unit (ICU) assistance and showed higher mortality rates than normoglycemic COVID-19 patients. Hyperglycemic COVID-19 patients treated with insulin infusion had reduced inflammation and coagulation markers and a better prognosis[41]. In a series of 168 lethal cases of SARS-CoV-2 pneumonia collected from 21 hospitals between January 21 and 30, 2020 in Wuhan, China, 75% were men, with a median age of 70 years old, and T2DM was reported in 25% of cases[42].

In Mexico, the first case of COVID-19 was detected on February 27, 2020, and 64 d after this first diagnosis, the number of patients increased exponentially, reaching 19224 confirmed cases and 1859 (9.67%) deceased; currently, these figures amount to 2667769 confirmed cases and 244081 deaths[43]. An epidemiological study conducted in Mexico from February 27, 2020 to April 30, 2020 showed that most cases of COVID-19 were in Mexico City, and the average age of patients was 46 years old. Among the 12656 confirmed cases, the highest number of infected people occurred in the 30- to 59-year-old range (65.85%), with a higher incidence in men (58.18%) than in women (41.82%). Deceased patients had one or multiple comorbidities, primarily HBP or hypertension (45.53%), T2DM (39.39%), and OB (30.4%)[44]. One of the first reports from Wuhan, China indicated that most hospitalized COVID-19 patients presented underlying diseases, such as diabetes, hypertension, and cardiovascular disease (CVD). The occurrence of hypertension worsened the prognosis and was associated with a higher rate of death[40]. In another study in Mexicans conducted from February 27, 2020 to April 10, 2020, a total of 23593 patient samples were evaluated by a laboratory from the Mexican Institute of Epidemiological Diagnosis and Reference. Of these, 18443 were negative for COVID-19, and 3844 were positive for COVID-19. The results showed that patients diagnosed with COVID-19 who developed a severe condition upon admission had higher proportions of OB (17.4%), T2DM (14.5%), and HBP (18.9%) than those without a confirmed diagnosis[45]. Moreover, OB, T2DM, and HBP conditions were accompanied by an inflammatory status, and some molecular mechanisms induced by inflammation altered the microvasculature, resulting in endothelial dysfunction (ED) and lung damage. Thus, COVID-19 patients with these comorbidities have higher rates of ICU treatment[46].

The Mexican Ministry of Health reported that HBP (17.21%), T2DM (13.25%), OB (13.25%), and smoking (7.33%) were the top 4 risk factors associated with SARS-CoV-2 infection mortality[43]. Many OB cases in Mexico live in geographical areas of increased social vulnerability, which poses a fundamental inequality that might also increase mortality from COVID-19 associated with both T2DM and OB. In a study conducted in Mexico on 177133 subjects with COVID-19, the odds of SARS-CoV-2 positivity were higher in subjects affected by T2DM, HBP, OB, being more than 65 years old, and of male sex[47]. When assessing age, reduced odds of SARS-CoV-2 positivity in patients less than 40 years old were observed, but when exploring its interaction with T2DM, an increased probability of SARS-CoV-2 infection was noted [47].

Having a diagnosis of T2DM has been linked to increased susceptibility and adverse outcomes associated with bacterial, mycotic, parasitic, and viral infections, all of which are attributed to a combination of dysregulated innate immunity and defective inflammatory responses[48]. Pulmonary and systemic coronavirus infections, including SARS-CoV-2, may be complicated by secondary bacterial infection, denoting the importance of the epithelial barrier function in the lungs and gastrointestinal tract. T2DM alone or in combination with older age, HBP, and/or CVD characterized by pro-inflammatory states can contribute to SARS-CoV-2 infection and to a larger pro-inflammatory response, which would lead to a more severe and ultimately lethal form of the disease[49].

OB is also a risk factor for increased severity of SARS-CoV-2-related symptoms. An analysis of 124 consecutive ICU admissions in a single center in Lille, France, from February 27, 2020 to April 5, 2020 revealed a large frequency of OB among SARS-CoV-2 patients in comparison to non-SARS-CoV-2 controls. In this observational study, the frequency of OB was 47.5%, compared to 25.8% in a historical control group of ICU subjects with non-SARS-CoV-2 illness. In this study, the requirement for intubation and mechanical ventilation was higher in subjects with OB[50]. In another report

conducted in Shenzhen, China, with 383 patients with COVID-19, overweight (OW) was associated with 86% and OB with a 142% higher risk of developing severe pneumonia compared to patients of normal weight in a statistical model controlling for potential confounders[51]. In another study conducted in Mexico between April 1, 2020 and May 8, 2020, 167 hospitalized patients (67% male) with an average age of 54-years old were suspicious or confirmed for COVID-19; approximately 75.3% suffered from OW or OB, including 7.8% with grade III OB. An 11% mortality rate among patients with Grade I OB was observed, along with a high 33% mortality rate in underweight or Grade III OB patients[52].

Mexicans exhibiting comorbidities such as CVD, HBP, OB, and T2DM, which are also related to MetS, also show more severe disease and higher mortality related to COVID-19. An additional analytical study including 528651 cases for the period from February 25, 2020 to June 6, 2020, of which 202951 were confirmed for COVID-19, allowed the authors to conclude that the presence of one MetS factor doubles the risk of death from COVID-19, and it was higher among patients affected by HBP and T2DM[53].

With regards to SARS-CoV-2 infection and intestinal health, important enzymes such as dipeptidyl peptidase-4 (DPP-4), angiotensin-converting enzyme 2 (ACE2), and transmembrane serine protease 2 (TMPRRSS2) are substantially expressed outside the lungs in epithelial tissues, including small and large bowel enterocytes[54-56]. Acute hyperglycemia has been shown to upregulate ACE2 expression in cells, which might facilitate viral cell entry, but paradoxically, chronic hyperglycemia downregulates ACE2 expression, making the cells vulnerable to the inflammatory and damaging effects of the virus[57]. In addition, the expression of ACE2 on pancreatic β cells directly affects β cell function, suggesting that T2DM is not only a risk factor for a severe form of COVID-19 disease but also that viral infection could trigger diabetes [58]. A great proportion of insulin requirements in patients with a severe course of the infection has also been observed in different countries affected by COVID-19. Nevertheless, it is not clear whether SARS-CoV-2 has a direct role in insulin resistance. Another aspect to consider is the link between COVID-19 and T2DM involving the DPP-4 enzyme, which is commonly targeted pharmacologically in people with T2DM [59].

The gut plays an important role in metabolic homeostasis, producing metabolically active gut hormones, interacting with the microbiota, and by its potential capacity to contribute to gluconeogenesis[60]. It is crucial to have adequate gut health and microbiota to achieve the best absorption of medicinal drugs designed to lower blood glucose levels in patients with diabetes[61].

There is important evidence supporting the notion that intestinal dysbiosis due to HBP, T2DM, OB, and MetS predisposes a patient to greater clinical severity from COVID-19. However, it cannot go unnoticed that other social aspects and lifestyle customs in Mexico, including vulnerability and undernutrition, might substantially contribute to the probability of hospitalization among individuals with COVID-19 and associated comorbidities, as discussed previously[62].

THE DEATH TOLL FOR COVID-19 IN MEXICO IS NOW MORE THAN TWO HUNDRED THOUSAND CASES

During the last month of 2019, a respiratory-type infectious outbreak emerged in China, and despite the sanitary measures established in that country, the disease continued to expand around the world, becoming a critical health issue[63]. This SARS-CoV-2 outbreak has become more serious, becoming a pandemic with more than 150 million confirmed cases and more than 3 million deaths worldwide[64]. According to the Mexican government, there were more than 2.5 million reported estimated cases with 234178 confirmed deaths by April 2021[43], and an association of comorbidities such as HBP, OB, smoking, and T2DM with COVID-19 disease severity has been reported[45]. Among the most affected countries, Mexico ranks third in deaths worldwide after the United States of America, with more than 30 million estimated positive cases and more than half a million deaths, and Brazil, with more than 14 million positive cases and almost 400 thousand deaths due to COVID-19[65], Table 1).

Research in other countries of the world showed that the most common comorbidities are also HBP, T2DM, CVD, and respiratory disease[66], similar to the panorama in Mexico. In Mexico, the principal comorbidities are HBP, OB, T2DM, and smoking[43]. It notable that according to the Non-Communicable Disease Risk Factor

Table 1 Coronavirus disease 2019 reported cases and deaths

Location	Cases	*per* 100K people	Deaths	*per* 100K people
United States	32152531	9795	573044	175
Brazil	14369423	6809	391936	186
Mexico	2329534	1826	215113	169
India	17636307	1291	197894	14
United Kingdom	4409635	6598	127451	191

The figures are based on data from the Johns Hopkins University Center for Systems Science and Engineering, accessed 2021-04-27 (https://coronavirus.jhu.edu/map.html).

Collaboration (NCD RisC), the United States of America, Brazil, and Mexico rank among the top countries afflicted by some of these maladies. For HBP, there is a 19.9% prevalence in Brazil, 17.3% in Mexico, and 10.5% in the United States of America; as a reference, Nigeria has a prevalence of 35.5%[67]. Regarding T2DM, there is an 11.5% prevalence in Mexico, 8.7% in Brazil, and 6.4% in the United States of America; as a reference, there is a 19.8% prevalence in Egypt[68]. Lastly, for OB, there is 38.2% prevalence in the United States of America, 34.0% prevalence in Mexico, and 26.4% prevalence in Brazil, and as a reference, Qatar has a 44.6% prevalence[69].

UNDER PANDEMIC CONDITIONS, BREASTFEEDING PROVIDES THE BEST SEEDING OF THE GM FOR NEWBORNS

As has been discussed, the importance of the functional GM is critical to contribute to appropriate primary (innate) and secondary immune responses. In the context of the global COVID-19 pandemic, a particular concern about mother and infant health is related to the possibility of vertical transmission from infected mothers to neonates or infants. In the mother-neonate pair, transmission may occur primarily through breastfeeding or the consumption of human milk, which may carry the virus. However, although it is essential to consider the potential role of human milk in SARS-CoV-2 transmission, it is more important to consider the protective effects of targeted antibodies and other immunoprotective components present in human milk against the viral agent of COVID-19. Among the multiple benefits breastfeeding provides to neonates, human milk contains a complex community of bacteria that helps to seed the infant GM[70,71]. This event is extremely important since appropriate initial bacterial colonization is essential for adequate intestinal immune development[72,73]. Whether infective SARS-CoV-2 viruses are present in human milk, the data are still limited, and breastfeeding by women with COVID-19 remains a controversial issue. In a recent work reporting data from 30 COVID-19-positive mothers, only one human milk sample was positive for the SARS-CoV-2 *via* quantitative real-time polymerase chain reaction (RT-qPCR) test, even after repeating the analysis the next day. The authors did not find proof for the transmission of the SARS-CoV-2 virus from mother to child through breastfeeding in the Indian population[74].

Furthermore, there are 37 published studies in which the presence of SARS-CoV-2 RNA was assessed in 68 human milk samples from mothers with a positive COVID-19 diagnosis. Only 9 of the 68 samples (13.23%) had detectable levels of SARS-CoV-2 RNA[75]. However, a previous report analyzing milk from two nursing mothers infected with SARS-CoV-2 reported positive results for the presence of viral RNA in only one of the two sampled mothers. Viral RNA was detected in milk for 4 consecutive days, and its presence coincided with mild COVID-19 symptoms and a SARS-CoV-2-positive diagnostic test for the newborn. However, whether the newborn was infected by breastfeeding or by other modes of transmission remains unclear[76]. In another study performed on two participants, only 50% of human milk samples were positive for SARS-CoV-2 RNA, suggesting that the virus is shed intermittently in the milk[77]. Both works conclude that further studies on milk samples from lactating women are needed to propose recommendations on whether mothers with COVID-19 should breastfeed. In a recent review, the authors concluded that there was no evidence of SARS-CoV-2 transmission through breast milk[75]. Human milk contains antibodies, and a recent publication reports the presence of SARS-CoV-2-specific

Table 2 High abundance bacterial taxa characterizing the gut microbiota dysbiosis of selected diseases

Population	Disease	Relevant taxa	Analysis	Ref.
Mexico	ED	f_Veillonellaceae, f_ S24-7, g_*Ruminococcus*, g_*Bacteroides*, g_*Parvimonas*, g_*Oscillospira*.	MaAsLin	Nirmalkar *et al* [87], 2018
	T2DM	o_Bacteroidales, f_Koribacteraceae, g_*Suterella*, g_*Roseburia*, g_*Pelomonas*, g_*Oscillospira*.	LEfSe	Chávez-Carbajal *et al*[85], 2020
	OB	f_ S24-7, g_*Roseburia*, g_*Succinivibrio*.	LEfSe	Chávez-Carbajal *et al*[86], 2019
		g_*Lachnospira*, g_*Roseburia*, g_*Faecalibacterium*.	UPGMA	Murugesan *et al* [88], 2015
	MetS	g_*Lachnospira*, g_*Coprococus*, g_*Faecalibacterium*, g_*Ruminococcus*, g_*Megamonas*.	LEfSe	Chávez-Carbajal *et al*[86], 2019
United States	HBP	g_Dorea, s_Alistipes finegoldii, s_A. indistinctus.	LEfSe	Kim *et al*[89], 2020
	ED	g_*Bifidobacterium*, g_*Akkermansia*, g_*Oxalobacter*.	Pearson's correlation	Johnson *et al*[90], 2019
		o_Bacteroidales, f_ Prevotellaceae, g_ *Hungatella*, g_*Succiniclasticum*.	Mann Whitney U	Kummen *et al*[91], 2018
	T2DM	g_*Bifidobacterium*, g_*Prevotella*.	Mann-Whitney nonparametric test	Barengolts *et al* [92], 2018
	OB	c_Bacilli, f_Streptococcaceae, f_Lactobacillaceae, g_*Streptococcus*, g_*Blautia*.	Kruskal-Wallis	Peters *et al*[93], 2018
		f_Ruminococcacea, g_*Prevotella*, g_*Gardnerella*, g_*Turicibacter*, g_*Megasphera*.	LEfSe	Sergeev *et al*[94], 2020
	MetS	g_Ruminococcus, g_Haemophilus, g_Varibaculum, g_Veillonella, g_Sarcina, g_Lactobacillus, g_Turicibacter, g_Actinomyces, g_Bifidobacterium, g_Lachnobacterium.	Correlations	Tricò *et al*[95], 2019
		g_Clostridium, g_Ruminococcus, g_Faecalibacterium, g_Oscillospira, g_Coprococcus, g_Prevotella.	Compute core microbiome (95%)	Zupancic *et al*[96], 2012
Brazil	ED	f_Lachnospiraceae g_*Roseburia* g_*Coprococcus*	Mann–Whitney U	Silveira-Nunes *et al* [97], 2020
	T2DM	g_*Gemella* g_*Coprococcus* g_*Desulfovibrio*	Relative Abundance	Al Assal *et al*[98], 2020
	OB	g_*Fusobacterium* g_*Enterococcus* s_*Escherichia coli*	FISH	Sarmiento *et al* [99], 2019
	MetS	p_Firmicutes	RT-qPCR	Miranda *et al*[100], 2019

ED: Endothelial dysfunction; T2DM: Type 2 diabetes; OB: Obesity; MetS: Metabolic syndrome; UPGMA: Unweighted pair group method with arithmetic mean; FISH: Fluorescence *in situ* hybridization.

antibodies in human milk after a COVID-19 vaccination scheme in 84 breastfeeding Israeli mothers[78].

Regarding the human milk that is handled and distributed by human milk banks (HMBs), when this review was written, there was no basis for imposing restrictions on the consumption of human milk by neonates in need. It should be mentioned that a requirement for the use of milk from HMBs is heat treatment aimed at reducing the bacterial load, which might include potential pathogens[79]. The standard heat treatment procedure used is Holder pasteurization, which is reported to inactivate the SARS-CoV-2 virus efficiently[80].

GM DYSBIOSIS IS ASSOCIATED WITH ED, T2DM, AND OB IN MEXICANS AND OTHER POPULATIONS

During the pandemic, the ribonucleic acid of SARS-CoV-2 has been detected in different types of samples around the world, including feces[81]. There is evidence of gastrointestinal infection with the viral agent of COVID-19 under conditions in which

Table 3 Taxa associated with immunological or inflammatory diseases

Taxa	Immunological disease	Ref.
s_*Ruminococcus gnavus*	Crohn's disease	Henke *et al*[101], 2019
	Rheumatoid arthritis	Zhang *et al*[102], 2015
s_*Ruminococcus lactaris*	Inflammatory bowel disease	Forbes *et al*[103], 2016
g_*Faecalibacterium*	Multiple sclerosis	Cantarel *et al*[104], 2015
	Psoriasis	Zhang *et al*[105], 2021
	Inflammatory bowel disease	Gevers *et al*[106], 2014
f_Veillonellaceae	Multiple sclerosis	Cantarel *et al*[104], 2015
g_*Coprococus*	Anti-phospholipid syndrome	Ruff *et al*[107], 2015
g_*Roseburia intestinalis*	Increased risk of HIV infection	Libertucci and Young[108], 2019
g_*Parvimonas*	Acute Kawasaki disease	Chen *et al*[109], 2020
g_*Megamonas*	Psoriasis	Zhang *et al*[105], 2021
	Systemic lupus erythematosus	Hevia *et al*[110], 2014
g_*Bacteroides*	Arthritis susceptibility	Xu *et al*[111], 2019
f_S24-7	Reduction in antibody response	Yang *et al*[112], 2017
g_*Coprococcus*	Reduction in antibody response. Inflammatory bowel disease	Yang *et al*[112], 2017; Said *et al*[113], 2014
g_*Oscillospira*		
g_*Sutterella*		
g_*Gemella*	Asthma	Stiemsma *et al*[114], 2016
g_*Clostridium*	Colitis	Guerri *et al*[115], 2019
	Rheumatoid arthritis	Forbes *et al*[116], 2018
g_*Actinomyces*	Rheumatoid arthritis	Forbes *et al*[116], 2018
g_*Streptococcus*	Rheumatoid arthritis	Alpizar-Rodriguez[117], 2019
g_*Prevotella*	Rheumatoid arthritis. Allergic rhinitis, Asthma	Maeda and Takeda[118], 2019
		Chua *et al*[119], 2018
f_Lachnospiraceae	Rheumatoid arthritis	Forbes *et al*[116], 2018
	Asthma	Cherkasov *et al*[120], 2019
f_Veillonellaceae	Autoimmune hepatitis	Wei *et al*[121], 2020
g_*Veillonella*	Multiple sclerosis	Chen *et al*[122], 2016
g_*Blautia*	Multiple sclerosis	Chen *et al*[122], 2016
g_*Dorea*		
g_*Haemophilus*		
g_*Oscillospira*	Allergies	Hua *et al*[123], 2016
g_*Succinivibrio*		
g_*Suterella*		
o_Bacteroidales		

HIV: Human immunodeficiency virus.

more than 20% of the qPCR tests are positive in feces by the time the respiratory tract results are negative[82]. Based on this information, it is possible that an already established GM dysbiosis, such as that observed in some metabolic diseases, influences SARS-CoV-2 clinical manifestations and outcomes. The diversity of the fecal microbiota is reportedly affected during SARS-CoV-2 infection[83], and supported by additional results, an association between the GM dysbiosis seen in T2DM and OB

with the severity of COVID-19 is proposed[84].

Our group has found evidence of dysbiosis in the distal colon microbiota diversity in Mexican adults affected by T2DM, as characterized by an increased relative abundance of Bacteroidetes in relation to Firmicutes[85] and a decrease in the relative abundance of Bacteroidetes to Firmicutes in OB and MetS[86], three diseases of epidemic proportions among Mexicans. Additionally, the results by our group have also uncovered characteristic dysbiosis in ED among Mexican adolescents[87]. There are also reports of a high abundance of specific bacterial taxa depicting GM dysbiosis in epidemic diseases such as HBP, T2DM, and OB in the USA and Brazil, which, along with Mexico, are the top three countries with the highest COVID-19 mortality (Table 2).

The presence of distal GM dysbiosis, as supported by the bacterial profiles characterized in fecal samples of Mexican subjects affected by ED, T2DM, OB, and MetS, is enriched in different but common bacterial taxa. Moreover, the relative abundances of these taxa were augmented in several disorders associated with defective immune responses, allergies, and susceptibility to viral infections (Table 3).

CONCLUSION

As discussed in this review, there is a clear association between comorbidities such as type 2 diabetes, obesity, and MetS and COVID-19 severity in populations such as Mexicans, in which these diseases are a health problem. There is also a defined association of changes in the bacterial taxa of the GM associated with the same diseases. However, to complete the picture, a further characterization of these bacterial taxa should include their metabolic role in the GM function and the type of mutual interaction they maintain with the immune system of the host. This information should help to develop multidisciplinary strategies to manage the GM to improve the primary and secondary immune responses in the face of viruses such as SARS-CoV-2, the viral agent of COVID-19 disease.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7080-7099

DOI: 10.3748/wjg.v27.i41.7080

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

REVIEW

Role of cell-free network communication in alcohol-associated disorders and liver metastasis

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Author contributions: Kuracha MR and McVicker BL wrote the paper; Thomas P and Tobi M made critical revisions; all authors have read and approve the final manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Supported by Biomedical Laboratory Research and Development, VA Office of Research and Development, No. BX004127 (to McVicker BL and Tobi M).

Country/Territory of origin: United States

Specialty type: Biochemistry and Molecular Biology

Peer-review report's scientific quality classification

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

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Abstract

The aberrant use of alcohol is a major factor in cancer progression and metastasis. Contributing mechanisms include the systemic effects of alcohol and the exchange of bioactive molecules between cancerous and non-cancerous cells along the brain-gut-liver axis. Such interplay leads to changes in molecular, cellular, and biological functions resulting in cancer progression. Recent investigations have examined the role of extracellular vesicles (EVs) in cancer mechanisms in addition to their contribution as diagnostic biomarkers. Also, EVs are emerging as novel cell-free mediators in pathophysiological scenarios including alcohol-mediated gut microbiome dysbiosis and the release of nanosized EVs into the circulatory system. Interestingly, EVs in cancer patients are enriched with oncogenes, miRNA, lipids, and glycoproteins whose delivery into the hepatic microenvironment may be enhanced by the detrimental effects of alcohol. Proof-of-concept studies indicate that alcohol-associated liver disease is impacted by the effects of exosomes, including altered immune responses, reprogramming of stromal cells, and remodeling of the extracellular matrix. Moreover, the culmination of alcohol-related changes in the liver likely contributes to enhanced hepatic metastases and poor outcomes for cancer patients. This review summarizes the numerous aspects of exosome communications between organs with emphasis on the relationship of EVs in alcohol-associated diseases and cancer metastasis. The potential impact of

Received: May 15, 2021
Peer-review started: May 15, 2021
First decision: June 22, 2021
Revised: July 2, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Wang Z
S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

EV cargo and release along a multi-organ axis is highly relevant to the promotion of tumorigenic mechanisms and metastatic disease. It is hypothesized that EVs target recipient tissues to initiate the formation of prometastatic niches and cancer progression. The study of alcohol-associated mechanisms in metastatic cancers is expected to reveal a better understanding of factors involved in the growth of secondary malignancies as well as novel approaches for therapeutic interventions.

Key Words: Exosomes; Extracellular vesicles; Alcohol-associated liver disease; Colorectal cancer; Liver metastasis; Interorgan communication

Core Tip: Alcohol consumption is an independent risk factor for cancer development as well as the promotion of metastatic disease, a major cause of morbidity and mortality in cancer patients. The identification of mechanisms and potential therapeutic targets for metastases remains to be determined for many cancers. Interorgan communication involving extracellular vesicles (EVs) is considered a vital process in the promotion of tumorigenic pathways and the spread of disease. Understanding the role of EVs in organ-organ communication networks will likely contribute to the development of future opportunities to combat cancer metastasis.

Citation: Kuracha MR, Thomas P, Tobi M, McVicker BL. Role of cell-free network communication in alcohol-associated disorders and liver metastasis. *World J Gastroenterol* 2021; 27(41): 7080-7099
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7080.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7080

INTRODUCTION

The consumption of alcohol in chronic and/or aberrant drinking patterns correlates with a substantial burden of disease worldwide. A recent study conducted by the National Survey on Drug Use and Health stated that in the United States alone, 73.1% of adults regularly use alcohol and nearly 15 million people have an alcohol use disorder[1]. Based on World Health Organization reports, alcohol use has a negative impact on health and quality of life, creating more than 5% of global disease burden and premature deaths[2,3]. The processing of alcohol in the body significantly affects multiple organs including the liver, gut, lungs, heart and brain[4-7]. A prominent alcohol-related disorder is alcohol-associated liver disease (AALD) that is initially facilitated by ethanol metabolism in the liver[8]. However, AALD is a complex disease with factors from other organs also contributing to its development and progression. Notable contributing factors include cells of the innate immune system and bacteria of the alcohol-altered gut microbiota[9,10]. Overall, the interplay between alcohol-affected organs clearly plays a role in the outcomes of AALD as well as additional adverse consequences such as alcohol-related cancer development and metastatic disease.

Alcohol is an identified carcinogenic factor in several cancers including head and neck, esophageal, liver, breast, pancreatic, and colorectal[11,12]. Recent reports indicate that alcohol consumption is the third and fourth largest contributor of all primary cancers in women and men, respectively[3]. Further, studies have shown that alcohol associates with an increased risk of secondary cancers of the upper aerodigestive tract (*i.e.* oral cavity, pharynx and esophagus) as well as metastases of colorectal cancers[13,14]. Multiple mechanisms are attributed to alcohol-induced cancer risk including toxic products and reactive oxygen species generated by ethanol metabolism. Additionally, cellular factors produced in response to injury such as protein, lipids and microRNAs can be packaged and released in extracellular vesicles (EVs)[15]. The EVs can migrate to modulate neighboring cells and/or distant tissues, acting in many cases as tumorigenic signaling molecules. Multiple cell types including endothelial cells, epithelial cells, neuronal cells, immune cells, and cancer cells can secrete nanosized EVs as part of their normal physiology, as well as during the

pathophysiology of disease[16]. Recent studies have suggested that during pathophysiological conditions exosomes have multiple roles in disease progression. Interestingly, tumor-derived exosomes have been implicated as regulatory factors in cancer progression by promoting cancer cell proliferation, migration, and the establishment of a premetastatic niche for drug-resistant cells[17,18]. Overall, EVs have the capability to contribute to the progression of AALD as well as alcohol-related advanced or secondary cancers. A better understanding of the integrated cell-cell communication between cancer cells and normal cells is critical for the development of new therapeutic options. Research into the complex interactions of diverse organs by EVs is a focus of new and clinically relevant areas of study. Here, we review studies on exosome biology and EV communication networks associated with alcohol-related disorders and metastatic cancers.

EXOSOME CHARACTERISTICS

Exosome biogenesis

Exosomes were first identified in 1981 as cell-derived, membrane-bound enzymatic vesicles[19]. Subsequently, it was demonstrated that exosomes are nano-sized (30 to 150 nm) lumen vesicles that originate from the endosomal system[20]. Further, it was elucidated that EV biogenesis is a sequential process in which multivesicular bodies (MVBs) form following membrane invagination of intraluminal vesicles[18,20]. A small fraction of MVBs fuse with the plasma membrane and are released into the extracellular milieu[21]. The regulation of MVB fusion and release can involve cholesterol content as seen in B-lymphocytes where membrane fusion and exosome release were only observed for the high cholesterol pool of MVBs[22]. Additionally, several reports have shown that exosome release depends on the cell polarity and the contribution of specific components of apical or basolateral membranes[23-25]. Overall, existing evidence indicates that different MVB populations exist inside cells and that select pools are involved in extracellular release[25] as well as the scavenging of plasma membrane proteins[26] to maintain cellular homeostasis during the EV maturation process[27,28].

Endosome pathways identified in the regulation of exosome biogenesis include endosomal sorting complex required for transport (ESCRT)-dependent and independent pathways (Figure 1). Studies have eloquently described ESCRT pathways showing the direct control of ESCRT-mediated membrane machinery[29] and ESCRT-independent regulation of EV budding and release by factors such as sphingolipid ceramide[30,31]. It was also demonstrated that vesicle formation and trafficking involve functional proteins such as Rab GTPases, heat shock proteins (HSP70 or HSP90), tetraspanins (CD9, CD63, and CD81), and integrins[18,32]. Further, the role of sphingomyelin, phosphatidylcholine, diacylglycerol, and ceramide as exosome membrane lipids was described[33]. Altogether, these studies suggest that distinct exosome biogenesis pathways, in addition to specific sorting and cargo mechanisms, dictate diverse biological functions and effects of EVs on recipient cells.

Exosome sorting and cargo delivery

A significant feature of exosomes is the morphological and size profile of the vesicles. Based on size, EVs are classified into large exosome vesicles (90-120 nm), small exosome vesicles (60-80 nm), or non-membranous nanoparticles called exomeres (35 nm)[34]. While both large and small size exosomes can respond to signaling pathways such as IL-2/STAT5, density gradient centrifugation studies revealed differences in lipid compositions between various sized EVs[34]. Moreover, subpopulations of low-density and high-density exosomes can have differential effects on gene expression profiles.

In addition to EV size, the characterization of exosome cargo is important to the understanding of EV effects in healthy and pathophysiological scenarios. Exosomes contain distinct ratios of molecular constituents such as nucleic acids, proteins, lipids, and metabolites that vary depending upon their cellular conditions, cells of origin, epigenetic changes, and metabolomic stages[35]. Moreover, studies have described various RNA species that are components of exosome cargo including microRNAs (miRNAs), rRNAs, tRNAs, or long noncoding RNAs (lncRNAs)[36]. The role of miRNAs as EV cargo is an emerging area of study, especially in oncology. In cancer cells, exosomes are highly enriched in miRNAs compared to parent cells indicating that miRNAs are sorting into the exosome cargo[37-39]. Several studies have identified exosomal miRNAs as serum biomarkers for the prediction of cancer progression and

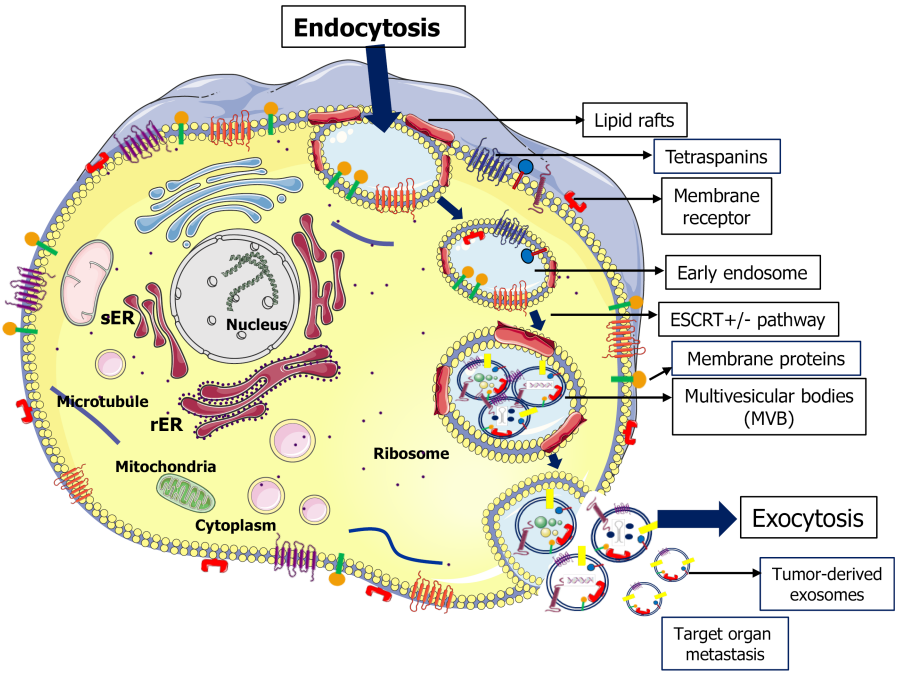

Figure 1 Extracellular vesicle biogenesis. Pathways involved in extracellular vesicle (EV) generation from the endocytosis of cargo components to release of targeting exosomes. EV biogenesis is achieved by endosomal sorting complex required for transport (ESCRT)-dependent or ESCRT-independent pathways. Several cytoplasmic and nuclear molecules can be sorted in the EVs such as ubiquitin-related proteins, heat shock proteins, miRNAs, and cytoskeleton proteins. ESCRT: Endosomal sorting complex required for transport; sER: Smooth endoplasmic reticulum; rER: Rough endoplasmic reticulum.

metastasis[40-42]. Significantly, the differential expression of exosomal miRNA was noted to have a role in the regulation of tumor progression and metastasis in various cancer models[43-45]. However, the mechanisms involved in the loading and sorting of molecules into exosome vesicles remain to be elucidated. Towards those efforts, Villarroya-Beltri *et al*[46], have identified a sequence motif that controls the miRNA loading into exosomes. In addition, Kirsten rat sarcoma (KRAS) oncogene-dependent miRNA sorting into exosomes was found to play a key role in colorectal cancer cell (CRC) since CRC cells expressing mutant KRAS have distinct miRNA profiles compared to wild-type cells[47]. In another study, it was shown that the hyper-activation of mutated KRAS inhibited the localization of the regulatory protein Argonaute 2 into exosomes[48]. The sorting of exosome mRNAs and enrichment of 3′ UTR fragments also demonstrates the importance of exosomal RNA effects in recipient cells[49,50]. Also, tumor-derived exosomes can carry double stranded DNA and genomic DNA fragments that reflect the mutational status of oncogene and tumor suppressor genes[51,52]. And finally, ubiquitination has been noted to have a role in the packaging of target proteins into exosomes[53-55].

Another important aspect of exosome cargo and sorting mechanisms is the lipid content of exosome membranes such as cholesterol, sphingomyelin, and glycosphingolipids that have specific roles in protein sorting into exosomes[33,56]. Data indicates that subdomains of the plasma membrane (lipid rafts) enriched with distinct proteins on exosome membranes mediate exosome signaling as well as molecule sorting into exosomes[57,58]. Further, mechanistic studies demonstrated the release of factors such as flotillin-1 and stomatin into the external medium *via* EVs associated with lipid microdomains[59]. Another study showed a positive regulation of sphingosine 1-phosphate (S1P) by sphingosine kinases that enabled S1P receptors to be continuously active on EVs[31]. The continuous activation of S1P has been shown to regulate CD63, CD81, and flotillin-mediated sorting into exosomes through inhibitory G protein-coupled S1P receptors located on MVBs[31]. This suggests that G protein receptor-mediated S1P signaling on MVEs is mainly involved in the ESCRT-independent exosome cargo. Collectively, these studies suggest that distinct molecular constituents such as proteins, lipids, and nucleic acids play an essential role in exosome maturation culminating in effective sorting and extracellular release of EV cargo. The molecular, cellular, and biological functions that result from the released EVs is a critical area of research, especially in the evolving era to understand the mechanisms of alcohol-associated diseases including cancer.

THE EFFECTS OF ALCOHOL ON EXOSOME COMMUNICATION

Alcohol and liver-associated EVs

Clinical manifestations of AALD include steatosis, steatohepatitis, fibrosis, and cirrhosis[8,60]. The liver is sensitized to triggers such as oxidative stress and endotoxins in early phases of AALD resulting in cellular damage and development of advanced disease. Further, consequences of ethanol metabolism lead to alterations in the function of hepatic cells as well as the recruitment of circulating cells and molecules that contribute to organ dysfunction. Previous reviews have comprehensively described the emerging role of EVs during the pathogenesis of alcohol-mediated diseases[61-63]. In brief, alcohol-mediated stresses result in elevated EV generation and release from hepatocytes as well as non-parenchymal cells. The released EVs can modulate gene expression and function of target cells contributing to the perpetuation of liver damage. Examples of the effect of EV cargo (*i.e.* miRNA, proteins, and lipids) include changes in macrophage phenotype and the activation status of hepatic stellate cells. Altogether, EVs generated in the liver are key players in alcohol-mediated liver inflammatory and profibrogenic mechanisms. In addition to EV-mediated intra-organ signaling, communication to extra-hepatic tissues can occur, as well as bidirectional exosome communication between organs such as liver, brain, gut, and lung.

Gut-Liver axis

Alcohol-induced impairments to the intestinal epithelial barrier result in increased gut permeability and release of bacterial products into the circulation[9,64]. The released products can perpetuate gut-barrier dysfunction, as well as contribute to hepatic injury, as the liver is the primary organ to receive and detoxify gut-derived factors. The translocation of intestinal products to the liver is involved in several diseases including obesity, metabolic syndrome, and non-alcoholic and alcoholic liver diseases. In the setting of alcohol, the gut-liver axis sustains bilateral communications between the intestine and the liver leading to gut-dysbiosis and progression of liver injury[65, 66]. Notably, the transfer of gut-derived toxins to the liver due to alcohol consumption is considered a pivotal event in the development and severity of AALD. Clinical data indicates that drinking patterns correlate with processes of the gut-liver axis as changes in intestinal permeability increase with the degree of alcohol consumption [64]. Next-generation sequencing data further confirmed the association between chronic alcohol consumption and altered gut microbiome functions in mice and humans[67,68]. Overall, alcohol consumption is linked to multiple changes in the gut including intestinal epithelial barrier dysfunction, alterations in gut epithelial and mucosal cells, and changes to the intestinal microbiota. As a result, bacterial products (*i.e.* endotoxin and other pathogen-associated molecular patterns) translocate to the liver and contribute to the production of proinflammatory pathways. Despite the current understanding of alcohol's effects on the gut microbiome, the role of EVs in the transfer of gut-derived products is not defined. However, emerging data indicates the EVs significantly contribute to alcohol-related liver inflammation.

The effects of alcohol on the intestinal microbiome and the translocation of injurious factors to the liver is an area of extensive research. It is well characterized that alcohol consumption results in the dysbiosis of bacterial and fungal intestinal species and the release of products including lipopolysaccharide (LPS) from the leaky gut[69,70]. In search of contributing mechanisms, studies have described alcohol-induced reductions in the expression of tight junction proteins as well as direct injury to gut epithelial cells [71,72]. The overexpression miRNA has been implicated in tight junction alterations as the knockdown of miRNA-21 prevented ethanol-induced disruption of tight junctions through the restoration of associated transmembrane proteins such as occludin and zonula occludens-1 (ZO-1)[71,73]. Additionally, the blockade of miRNA-122a was found to be protective against tight junction alterations in Caco-2 cells[74]. It is suggested that EVs generated during alcohol-induced changes to the intestinal barrier contain cargo such as miRNAs, LPS, and bacterial products that target the liver and contribute to AALD. Indeed, a recent study by Lamas-Paz *et al*[75] demonstrated that EVs derived from alcohol-affected intestinal epithelial cells contributed to hepatocellular injury. Further, it is likely that ethanol-mediated changes in intestinal barrier and microbiome composition result in the release of bacterial EVs. For example, in addition to its role as a soluble factor, LPS can also be packaged into EVs for transport from the injured gut. This is supported by a recent report indicating the presence and activity of bacterial EVs in patients with intestinal barrier dysfunction[76]. The role of bacterial EVs in alcohol consuming patients remains to be characterized along with the

therapeutic potential of targeting such EVs.

The mechanistic role of bacterial products in the progression of alcohol-associated diseases has led to the study of the gut microbiota as a therapeutic target in patients with alcohol use disorders[77]. Currently, probiotics (living bacterial cultures), prebiotics (promoters of beneficial or commensal bacteria), and antibiotics, all serve as potential therapies for alcohol-associated diseases[78]. For instance, *Lactobacillus rhamnosus* is protective against alcohol-induced liver injury in mice[79]. Further, the administration of prescribed probiotics is promising as a protective barrier against alcohol-induced gut permeability and AALD[80]. The use of prebiotics may also be beneficial as certain diets (*i.e.* oats, flaxseed) protect against alcohol-induced oxidative stress and hepatic inflammation[81,82]. Similarly, antibiotic treatment can attenuate alcohol-induced endotoxemia by preventing the overgrowth of harmful bacteria in the gut[83]. Overall, insight into the mechanistic utility of targeting exosomes generated by the alcohol-altered gut warrants investigation for the development of effective therapeutics against disease progression related to the gut-liver axis.

Liver–Brain axis

It is well described that manifestations of AALD lead to a spectrum of symptoms in the brain such as cerebral edema and hepatic encephalopathy. Of notable involvement, ammonia and other harmful substances produced by the alcohol-injured liver can reach the brain causing injury and neuroinflammation. However, mechanisms related to exosome communication networks of the liver-brain axis remain to be characterized. Reports to date indicate that the coadministration of alcohol and LPS result in altered profiles of cytokines such as TNF-α, MCP-1, IL-1β in the gut, liver, and brain[84]. Other studies demonstrate that the lack of the tumor necrosis factor receptor 1 results in the accumulation of TNF-α in mouse serum, gut, and liver; and that alcohol intake potentiates long-lasting levels of proinflammatory cytokines in the brain[85]. A recent study demonstrated that TNF-α inhibition reduced systemic inflammation and improved symptoms[86]. Additionally, chronic alcohol consumption not only influences brain inflammation but also interferes with stress-mediated psychiatric behavior through the disruption of the hypothalamic-pituitary-adrenal (HPA) axis [87]. The alcohol-mediated neutralization of the HPA axis could be a potential mechanism by which systemic inflammation continues in individuals who have an addiction to alcohol.

Besides chronic alcohol addiction, the loss of gut barrier integrity is a causative factor of endotoxin transport during sepsis and brain inflammation. Alcohol-induced gut dysbiosis is thought to not only play a role in alcohol dependency but also in the regulation of effects including neuro and endocrine signaling and immune system alterations[64,68]. However, a connective factor such as EVs in the gut-liver-brain axis has yet to be identified. Interestingly, the blood-brain barrier (BBB) serves as a defensive barrier against the extravasation of tumor cells and pathogens[88]. However, cancer cells can destruct the BBB structure to mediate migration during brain metastasis[89]. Overall, it is suggested that EVs facilitate cell network communications through the delivery of their cargo (*i.e.* proteins, mRNA, and miRNAs) to trigger the breakdown of the BBB through EV-induced changes in tight-junction proteins including ZO-1, N-cadherin, and actin.

Liver–Lung axis

Excessive alcohol use is a major factor in the enhanced risk of acute respiratory distress syndrome (ARDS)[90]. Chronic alcohol exposure in the liver-lung axis is linked to hepatopulmonary syndrome, bacterial infection, and increased mortality from ARDS [90,91]. Recently, Siore *et al*[92] reported that pulmonary edema and acute lung damage occur through the activation of inflammatory responses and oxidative stress involving liver-lung axis communications. It was shown that alcohol administration results in elevated levels of the TNF-α responsive chemokines, macrophage inflammatory protein, and keratinocyte chemoattractant. Further, the enhanced chemokine expression is associated with the recruitment of pulmonary neutrophils. Additional studies indicated that the liver-lung axis is bidirectional for the com-munication and effects involved in alcohol-enhanced hepatopulmonary injury. For instance, ventilator-induced lung injury in a mouse model resulted in significant inflammatory responses produced in cultured hepatic sinusoidal endothelial by perfusate from injured lungs [93]. In relationship to the role of the liver-lung axis in alcohol-related cancers, several studies have investigated the role of metastatic determinants[94,95]. In particular, tumor-derived exosomes may have a significant role in cancer cell metastasis that is mediated by cell adhesion molecules such as integrins, tenascin, and periostin[96-98]. In summary, the role of EVs in the interplay between pulmonary disease, AALD and

alcohol-associated cancers is a needed area of research for the identification of potential therapeutic targets.

EXOSOMES AND CANCER: ROLE OF ALCOHOL-MEDIATED EFFECTS

The interorgan communication mediated by EVs is clearly a factor of pathophysiology in various disease states. The role of alcohol-induced EV communication in the development and progression of cancer is not defined and is an area of clinical importance due to the prevalence of alcohol consumption and associated risk of cancers. Thus, investigations into the role of EVs in the initiation and severity of cancers aims to gain insight into the relationship of comorbid conditions related to the effects of alcohol consumption. Moreover, realization of the importance of EVs in cancer progression and metastasis has increased exponentially, as have their potential application in therapy and diagnosis[99]. The contribution of EVs in pathological processes is far reaching since tumor-secreted exosomes can mediate angiogenesis, modulate the immune system, and facilitate the generation of pre-metastatic niches[96, 100]. Indeed, EVs have been identified as key mediators of communication networks within and between organ systems, highlighting the clinical importance of exosome function[18,101,102]. Existing web-based online bioinformatic tools including high-throughput techniques (*i.e.* ExoCarta, EVpedia, Vesiclepedia catalog, and Ingenuity Pathway Analysis, IPA) are beneficial to the scientific community in EV research[103, 104]. These resources assist in the characterization of EV molecular and pathophysiology mechanisms through the identification of key functional elements. Based on IPA data, EV cargo delivery depends on the content of bioactive molecules such as mRNA, enzymes, proteins, DNA and lipids that can dictate the role of EVs in disease progression and diagnostic functions (Figure 2).

The clinical assessment of EVs in body fluids provides another measure towards the understanding of exosomes as diagnostic biomarkers and therapeutic targets. Biomolecule-loaded EVs from blood are stable for more than 90 days under normal storage conditions making EV analyses more useful compared to other less-stable measures of cell-free DNAs and circulating tumor cells that are used as liquid biopsies [105,106]. Examples of exosome-related identification in serum samples include prostate cancer-derived exosomes[107]; and exosome cargo containing an androgen receptor variant that is a biomarker of metastatic prostate cancer[108]. Several studies have also reported the sensitivity of EV miRNA composition as biomarkers in disease identification that can be isolated from various body fluids including blood, saliva, and urine[109,110]. A noted example is the oncogenic signature of miR-21 as a biomarker for various cancers including colorectal[111], breast[112], brain[113], and liver[114]. Concerning diseases of the liver, it has been shown that the concentration of EVs in the circulation is enhanced in the setting of AALD, nonalcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma indicating the clinical significance of EV-mediated communication and subsequent effects[66]. Overall, clinical measures as well as bioinformatic programs are valuable in deciphering EV-mediated mechanisms and are useful tools for the characterization of alcohol-associated EVs in development and progression cancers.

Role of exosomes in cancer progression

Mechanisms of tumor development and progression are dynamic, multi-step processes that occur in response to the accumulation of genetic alterations in damaged cells. An integral component of tumor development is thought to be the communication between cancerous and non-cancerous cells that is mediated by nanosized vesicles [115]. Research to date indicates that cancer cell microvesicles actively transfer oncogenic molecules from primary cancer cells to intercellular populations. Indeed, tumor-derived exosomes can regulate cancer progression by stimulating oncogene overexpression, stromal cell remodeling, immune system modulation, and angiogenesis[115]. The transfer of tumorigenic material *via* EVs is implicated in the modulation of morphological changes and the enhancement of anchorage-independent growth capacity of cancer cells. Similarly, tumor-derived exosomes can act as survival factors that bind to and activate anti-apoptotic pathways[116].

The knowledge that exosomes are potential stimulators in cancer progression indicates that EVs can promote angiogenesis and changes in the microenvironment [117]. In this regard, tumor-derived exosomes can influence mesenchymal stem cell differentiation facilitating cancer cell proliferation and disease progression[118]. Moreover, the exosome-mediated transfer of lncRNAs as tumor-promoting material

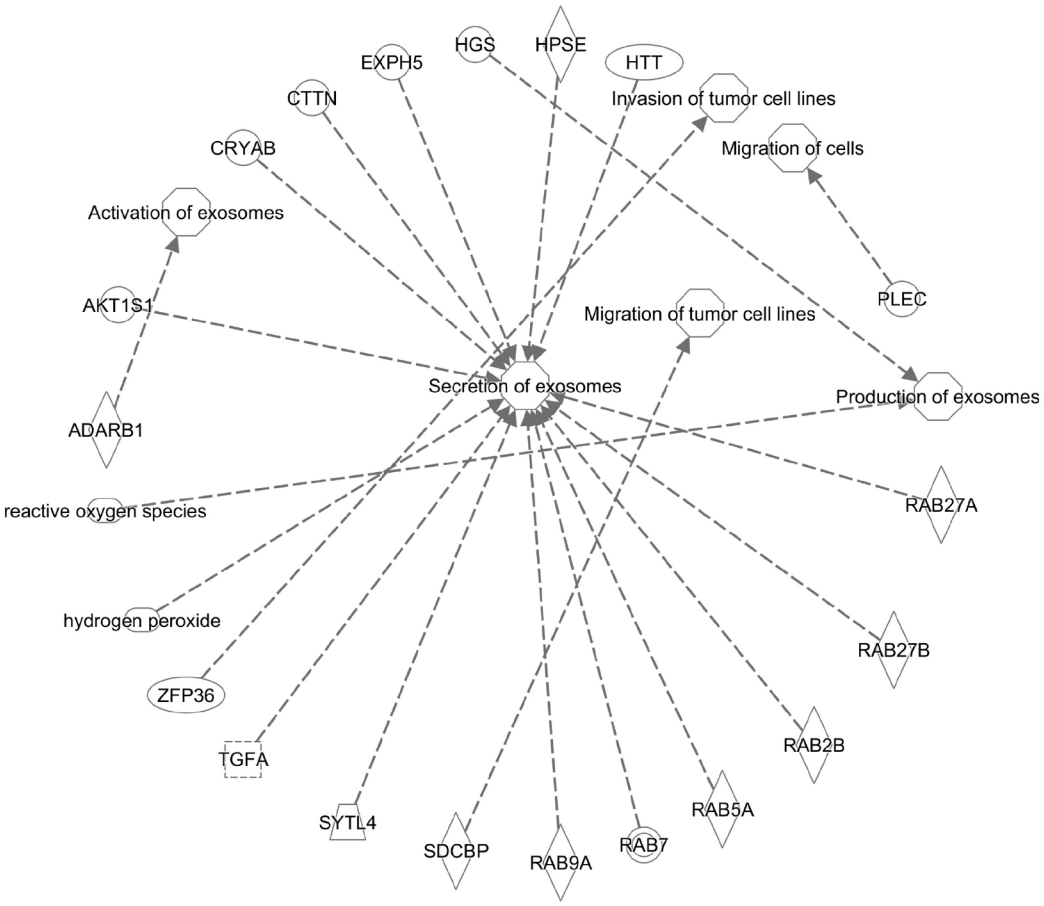

Figure 2 Exosome network analysis. Based on Ingenuity Pathway Analysis, distinct molecules from different tissues and cells can be involved in exosome secretion during cancer progression as well as in diagnostic functions. CRYAB: Chaperone alphaB-crystallin; CTTN: Cortactin; EXPH5: Exophilin 5; HGS: Hepatocyte growth factor; HPSE: Heparinase; HTT: Huntingtin protein; PLEC: Plectin; RAB27A: Ras-related protein Rab-27A; RAB27B: Ras-related protein Rab-27B; RAB2B: Ras-related protein Rab-2B; RAB5A: Ras-related protein Rab-5A; RAB7: Ras-related protein Rab-7; RAB9A: Ras-related protein Rab-9A; SDCBP: Syndecan binding protein; SYTL4: Synaptotagmin like 4; TGFA: Transforming growth factor alpha; ZFP36: Zinc finger protein 36; ADARB1: Adenosine deaminase RNA specific B1; AKT1S1: AKT1 Substrate 1.

has been shown during the transformation of non-malignant cells[119]. The role of EVs enriched with miRNAs has also been shown in cell-cell communications and conversion of cells to populations with enhanced motility[120]. Specific examples include the role of miR-17-92 and miRNA-92a as potent promoters of angiogenesis and oncogenic activity[121]. Likewise, miR-135b-5p[122], miR-30a-5p[40], miR-150-5p [123], miR-183-5p[124], miR-155[125], miR-497[126], miR-181b-5p[127], miR-375[128, 129] and the miR-200 family[110,130,131] have been shown to be effective markers of cancer progression. The clinical evaluation of EV miRNA cargo provides insightful information into processes involved in the various stages of cancer from detection to metastasis as summarized in Table 1.

Another component identified in cancer progression is the release of cancer-associated fibroblasts (CAFs) from exosomes. CAF-derived EVs can play a key role in tumor progression by enabling the transfer of oncogenic molecules such as amino acids, lipids, and TCA-cycle intermediates to confer glycolysis modulation and carboxylation in cancer cells[132]. Tumor-derived exosomes have also been shown to be involved in the stimulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) enhancing the process of neovascularization in endothelial cells in the microenvironment[133]. Moreover, recent studies suggest that EVs are important in mediating cellular communication between cancer cells and other cells of the microenvironment such as immune cells, neutrophils, natural killer (NK) cells, dendritic cells, T cells, and macrophages. For example, cancer-derived exosomes can alter macrophage polarization[134], induce the recruitment of neutrophils to the tumor site[135], decrease the cytotoxic activity of NK cells[136], or inhibit T-cell proliferation mechanisms[137]. Altogether, it is evident that exosomes can mediate cancer progression through a variety of pathways and cellular communications leading to cancer cell proliferation and spread to distant sites.

Table 1 Summary of exosome miRNA signatures as cancer biomarkers

Exosomal miRNA	Expression profile	Mode of action	Type of cancer	Ref.
miR-320d	Upregulated	Predicts metastasis	CRC	Tang *et al*[41]
miR-106b-3p	Upregulated	Promotes metastasis	CRC	Liu *et al*[163]
miR-6803-5p	Upregulated	Prognosis marker	CRC	Yan *et al*[42]
miR-874	Upregulated	Prognosis marker	GC	Zhang *et al*[164]
miR-30a-5p	Downregulated	Diagnostic tool	CRC	Sun *et al*[40]
miR-21	Upregulated	Diagnostic tool	CRC	Bastaminejad *et al*[111]
miR-135b-5p	Upregulated	Metastatic marker	CRC	Li *et al*[122]
miR-150-5p	Downregulated	Prognosis, marker	CRC	Zou *et al*[123]
miR-183-5p	Upregulated	Angiogenesis	CRC	Shang *et al*[124]
miR-155	Upregulated	Diagnostic tool	CRC	Lv *et al*[125]
miR-16-5p	Upregulated	Regulation of ITGA2	CRC	Xu *et al*[165]
miR-497	Downregulated	Prognosis marker	CRC	Zou *et al*[126]
miR-4461	Downregulated	Regulation of COPB2	CRC	Chen *et al*[43]
miR-146a	Upregulated	Invasion and metastasis	BC	Yang *et al*[45]
miR-125a-3p, miR-320c	Upregulated	Stage I marker	CC	Wang *et al*[166]
miR-4772-3p	Upregulated	Stage II & III marker	CC	Liu *et al*[167]
miR-21, miR-10b	Upregulated	Metastatic marker	HCC	Tian *et al*[44]
miR-1290, miR-375	Upregulated	Prognostic marker	CRPC	Huang *et al*[129]
miR-373, miR-200a, miR-200b, miR-200c	Upregulated	Tumor progression	EOC	Meng *et al*[131]
mir-181b-5p	Downregulated	Diagnostic tool	GC	Yun *et al*[127]

CRC: Colorectal cancer; GC: Gastric cancer; ITGA2: Integrin alpha 2; COPB2: COPI coat complex subunit beta 2; BC: Breast cancer; Stage I, II, III: North American Association of Central Cancer Registries Stages I, II, III and IV; CC: Colon cancer; HCC: Hepatocellular carcinoma; CRPC: Castration-resistant prostate cancer; EOC: Epithelial ovarian cancer.

Exosomes role in cancer metastasis

Metastasis is one of the most common causative factors in cancer-related death. Cancer metastasis is a multi-step process for the development of secondary cancers. In 1889, Stephen Paget described the "seed and soil" theory, in which metastasis depends on the interaction between primary cancer cells as the seed and secondary host microenvironments designated as the soil[138]. Involved mechanisms were found to include changes to the extracellular matrix architecture and associated reprograming of normal cells. Clinically significant interactions between cancer cells and the cells of secondary organ sites have been shown to involve hepatocytes, bone marrow progenitor cells, CAFs, macrophages, and neutrophils. However, regulatory mechanisms of secondary organ-specific metastasis are poorly understood. Towards that understanding, studies have indicated that tumor-derived exosomes assist in the priming of premetastatic niches by delivering prometastatic factors. In particular, the integrin expression profile of tumor derived EVs can act as functional "ZIP codes" during metastatic organotropism to direct metastatic cancer cells to target tissue/organs[96]. Proteomic and clinical data support the role of exosome-sorted integrins as vital players in the development of cancer metastasis. For instance, α6β4 and α6β1 integrins are associated with lung metastasis and αvβ5 is involved with liver metastasis[139]. Also, tumor-derived exosomes are involved in the activation of the Src kinase pathway and the upregulation of pro-inflammatory S100 genes during the establishment of premetastatic niches[140]. Thus, cell-cell communication mediated by EVs appears to be a critical element during premetastatic niche formation in cancer development (Figure 3). Review of the literature indicates the variety of cancer types and stromal cell-derived exosome molecules can initiate signals during the reprogramming of the tumor microenvironment (Table 2). Thus, exosome-mediated

Table 2 Extracellular vesicle components in cancer progression and metastasis

EV cargo	Type of molecule	Action on recipient cells/tissue	Type of cancer	Ref.
CEA	Protein	Inflammation	Colorectal	Yokoyama *et al*[162]
KRAS	Protein	Invasiveness in recipient cells	Colorectal	Beckler *et al*[152]
ITG	Protein	Metastatic organotropism	Breast	Hoshino *et al*[96]
TNC	ECM protein	Stem cell niche formation	Breast	Oskarsson *et al*[97]
MIF	Protein	Liver premetastatic niche formation	Pancreatic	Costa-Silva *et al*[168]
ZFAS1	lncRNA	Cancer growth/metastasis	Gastric	Pan *et al*[119]
Amino acids, lipids, TCA-cycle intermediates	Metabolites	Cancer growth	Prostate	Zhao *et al*[132]

CEA: Carcinoembryonic antigen; KRAS: Kirsten rat sarcoma viral oncogene; ITG: Integrins; TNC: Tenascin C; MIF: Macrophage migration inhibitory factor; ZFAS1: ZNFX1 antisense RNA1; LncRNA: Long non-coding RNA; ECM: Extracellular matrix.

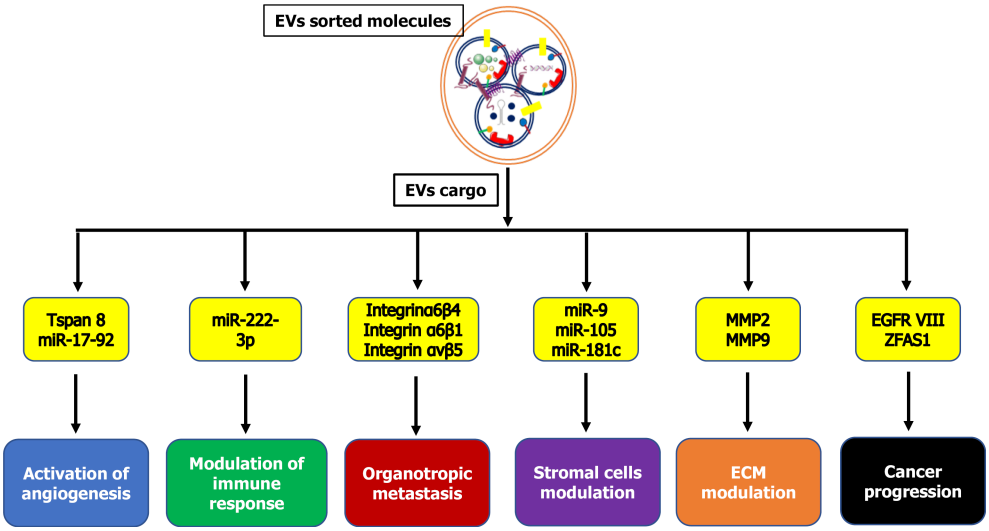

Figure 3 Exosome-mediated functions in the pre-metastatic niche. The role of tumor-derived exosomes along the establishment and progression of metastatic disease. Extracellular vesicle cargo can be involved in the initiation and regulation of cancer by promoting immune responses, angiogenesis, extracellular matrix modulation, stromal cell changes and metastatic organotropism. EVs: Extracellular vesicles; Tspan8: Tetraspanin-8; MMP2: Matrix metallopeptidase 2; MMP9: Matrix metallopeptidase 9; EGFR VIII: Epidermal growth factor receptor variant III; ZFAS1: ZNFX1 antisense RNA1; ECM: Extracellular matrix.

intracellular signaling as well as organ-organ communication can influence cancer progression and changes to host and tumor microenvironments to facilitate metastatic disease.

CLINICAL IMPLICATIONS OF EXOSOME COMMUNICATION NETWORKS

Role of EVs in alcohol and colorectal cancer disease

Recent studies indicate an alarming increased rate of morbidity and mortality from alcohol use disorders in the United States[141]. Of particular clinical significance is the disease burden related to alcohol use in colorectal cancer and associated liver metastasis. CRC is a leading cause of cancer mortality with the majority of deaths due to the development of colorectal liver metastasis (CRLM) as the liver is the foremost site of distant metastatic spread in CRC patients[142,143]. Epidemiological studies suggest that chronic alcohol consumption is one of the major causative factors of colorectal cancer mortality in both men and women[144]. Alcohol use correlates with CRLM at colorectal cancer diagnosis as well as hepatic metastases that occur over time. Further, alcohol-associated CRLM requires intensive follow-up and treatment due to poor liver function and unresectable lesions. Despite advancements in surgical interventions and chemotherapeutics, CRLM morbidity and mortality are leading healthcare concerns

emphasizing the significant need to determine contributing mechanisms. The involvement of EV signaling during CRC progression in the setting of alcohol is not known. Thus, understanding EV communication networks and the role of EVs as biomarkers can significantly contribute to the development of strategies to address the serious public health issues associated with alcohol use and cancers.

The development of CRC is a multi-step process involving the malignant transformation of normal cells of the colon. The contribution of ethanol metabolism and related metabolites in colon carcinogenesis has been investigated for some time. A variety of pathways attributed to the effects of alcohol have been identified in the promotion CRC including genetic abnormities, epigenetic dysregulation, cell signaling, and changes in the tumor microenvironment[13]. However, the role of alcohol during CRC spread to other organs is less understood. In particular, the contribution of alcohol during liver metastasis is emerging as a critical area of study given the substantial mortality associated with CRLM and need to identify targetable mechanisms. Current literature indicates that alcohol creates a hepatic microenvironment susceptible to CRC seeding and growth. Attributable mechanisms include the sensitization of resident macrophages (Kupffer cells, KCs) to endotoxin-induced signaling, the production of inflammatory factors, and the activation of fibroblastic cells that promote disease rather than wound-healing[145]. Moreover, it is likely that targetable mechanisms of CRLM involve communication networks between alcohol-affected macrophages and cancer cell-associated factors. The contribution of EVs in alcohol-associated CRLM is not defined but is clearly considered an important process to characterize.

EVs represent a new form of communication in colorectal cancer progression and liver metastasis. The fact that EVs can deliver cargo (*i.e.* RNAs, lipids, proteins) between cells and organs indicates the potential of playing a key role in metastatic disease[146,147]. CRC proliferation and migration can induce the release of EVs and other tumor-derived factors that can promote prometastatic niche formation, vascular changes, inflammation, and immunosuppression in host microenvironments. Several studies have recently described the contributions of EV cargo as prime mechanisms of CRC metastasis. For example, proteomic data revealed a distinct profile of metastatic factors, signal transduction molecules, and lipid raft-associated components in EVs obtained from metastatic CRC cells[148]. The contribution of mRNA components from CRC-derived EVs in cancer progression has also been shown for miRNAs (i.e. miR-21, miR-192 and miR-221) as well as natural antisense RNAs such as Leucine Rich Repeat Containing 24, MDM2 Proto-Oncogene, and Cyclin Dependent Kinase Inhibitor 1A [149]. Moreover, the role of genetic mutations in CRC patients are of interest. In particular, KRAS mutations are frequently associated with CRC metastasis and the regulation of exosome composition and release in CRC cells[150,151]. In addition, many oncogenic proteins (*e.g.* KRAS, Src family kinases, integrins) are highly enriched in mutant KRAS-derived exosomes indicating a role in CRC progression and metastasis[152]. Together, these observations provide novel insight into the role of EVs and the therapeutic potential of targeting the CRC-generated EVs during metastatic disease.

There is a growing body evidence suggesting that tumor-derived exosomes are crucial factors that influence differentiation in the microenvironment through particular signaling pathways[153]. For example, CRC cell-derived EVs have been shown to promote angiogenesis and tumor growth in the host microenvironment through the hyper-activation of Wnt/β-catenin signaling. As a result, hypoxic metastatic niches provide CRC cells protection from chemotherapy and attack from immune cells[154]. Another signaling pathway implicated in colon tumorigenesis is the activation of proinflammatory cellular kinases. A recent study by Talwar *et al*[155] demonstrated that phosphorylated p38γ is activated in CRC tumorigenesis. Further, it is suggested that the activation of p38γ may be associated with immunoglobulin adhesion molecules such as carcinoembryonic antigen (CEA) and biliary glycoprotein (BGP). In support, the expression of CEA and BGP have been linked to hepatic metastasis in various preclinical models and in CRC patients with ongoing efforts to define the mechanistic role of CEA during CRLM[156-158]. Key studies have shown a direct relationship between CEA and the metastatic potential of CRC cells, and that CEA stimulation results in the production of tumorigenic factors by Kupffer cells[159-161]. Current works are evaluating the role of alcohol on KC function to determine if ethanol-sensitized macrophages are more responsive to CEA leading to advanced metastatic disease. To date, studies have shown that the alcohol-injured liver provides a permissive environment for CRLM and that CEA-mediated inflammatory mechanisms may play a key role[157,162]. However, the role of tumor-derived and alcohol-associated EVs in the process of metastatic mechanisms involving MAPK signaling or

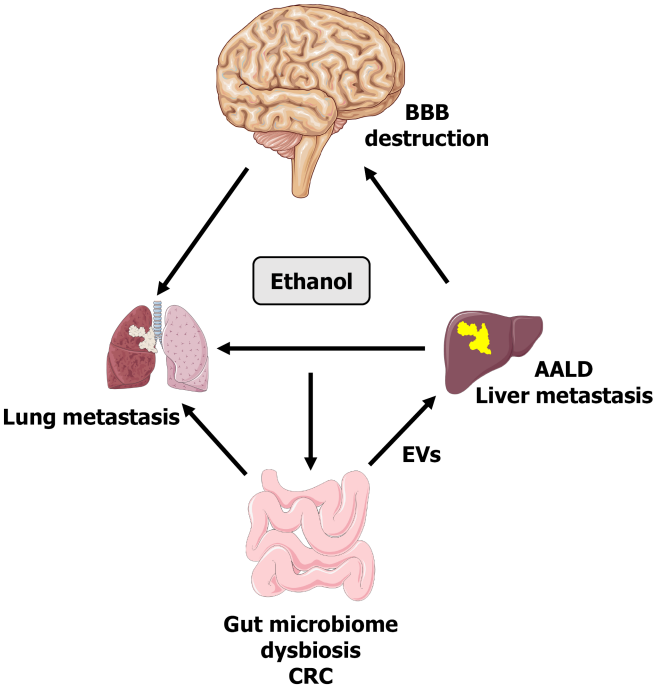

Figure 4 Model of extracellular vesicle interactions along the gut/liver/lung/brain axis during alcohol disorders and cancer progression. Alcohol exposure leads to enhanced gut microbiome dysbiosis, the development of alcohol-associated liver disease, lung inflammation, and neurological manifestations. The potential role of tumor- and alcohol-derived extracellular vesicles (EVs) in advanced malignancies is a potential consequence of EV organ-organ communication during alcohol disorders. BBB: Blood brain barrier; CRC: Colorectal cancer; AALD: Alcohol-associated liver disease.

carcinoembryonic antigen-related cell adhesion molecules is unknown. Further, the effectiveness of blocking EV-mediated communication in the alcohol-injured liver during CRLM also remains to be defined. Overall, the characterization of exosome cargo and communication networks in the transformation of CRC cells and reprogramming of the tumor microenvironment is an important area of translational research, especially in the context of complex comorbidities associated with aberrant alcohol intake.

CONCLUSION

In recent years, investigations into the role of EVs in cancer progression and AALD have increased in a remarkable manner. The elucidation of EV communication networks to date have indicated the powerful role of EVs as metastatic cancer markers and inducers of varied biological effects. Extensive work is ongoing to characterize the biogenesis and effects of distinct EV populations generated from different cell types and diseases. The unique features of EV size and cargo contents can produce hallmark effects on recipient cells. Therefore, the heterogeneity of exosome populations will dictate studies on the role and outcomes of exosome networks during disease states. For example, understanding the diversity of EVs released during gut microbiome dysbiosis, migration, and organ-organ communication aims to reveal the association of AALD and hepatic CRC metastasis. The complexity of interorgan communication and the involvement of mediators such as EVs, cytokines, and chemokines is the ongoing focus of translational research. Related to alcohol-associated diseases, it is proposed that EV-mediated communication affects multi-organ damage as well as cancer metastasis along the liver/gut/lung/brain axis (Figure 4). Future studies will likely focus on the characterization of exosomal components involved in alcohol's effects and cancer cell metastasis to secondary organs. Moreover, further investigation is needed to explore the role of exosome-mediated cell-free networks in the detection of alcohol-related tumors and microenvironment interactions for the development of targeted therapeutics.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7100-7112

DOI: 10.3748/wjg.v27.i41.7100

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

MINIREVIEWS

DNA diagnostics for reliable and universal identification of *Helicobacter pylori*

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Author contributions: Sulo P designed the outline and wrote most of the manuscript; Šipková B summarized the data, prepared the figures and tables, and wrote some of the manuscript; both authors read and approved the final manuscript.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Supported by Slovak Research and Development Agency, No. PP-COVID-20-0051.

Country/Territory of origin: Slovakia

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

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Abstract

Reliable diagnostics are a major challenge for the detection and treatment of *Helicobacter pylori* (*H. pylori*) infection. Currently at the forefront are non-invasive urea breath test (UBT) and stool antigen test (SAT). Polymerase chain reaction (PCR) is not endorsed due to nonspecific primers and the threat of false-positives. The specificity of DNA amplification can be achieved by nested PCR (NPCR), which involves two rounds of PCR. If the primers are properly designed for the variable regions of the 16S rRNA gene, it is not difficult to develop an NPCR assay for the unambiguous identification of *H. pylori*. Elaborate NPCR for a 454 bp amplicon was validated on 81 clinical biopsy, stool, and saliva samples, each from the same individuals, and compared with available *H. pylori* assays, namely histology, rapid urease test, SAT, and ^{13}C-UBT. The assay was much more sensitive than simple PCR, and it was equally sensitive in biopsy samples as the ^{13}C-UBT test, which is considered the gold standard. In addition, it is sufficiently specific because sequencing of the PCR products exclusively confirmed the presence of *H. pylori*-specific DNA. However, due to the threshold and lower abundance, the sensitivity was much lower in amplifications from stool or saliva. Reliable detection in saliva also complicates the ability of *H. pylori* to survive in the oral cavity aside from and independent of the stomach. The reason for the lower sensitivity in stool is DNA degradation; therefore, a new NPCR assay was developed to obtain a shorter 148 bp 16S rRNA amplicon. The assay was validated on stool samples from 208 gastroenterological patients and compared to SAT results. Surprisingly, this NPCR revealed the presence of *H. pylori* in twice the number of samples as SAT, indicating that many patients are misdiagnosed, not treated by antibiotics, and their problems are interpreted as chronic. Thus, it is unclear how to properly diagnose *H. pylori* in practice. In the first approach, SAT or UBT is sufficient. If samples are negative, the 148 bp amplicon NPCR assay should be performed. If problems persist, patients should not be considered negative, but due to threshold *H. pylori* abundance, they should be periodically tested. The advantage of NPCR over UBT is that it can be used universally, including questionable samples taken from patients with achlorhydria, receiving proton pump inhibitors, antibiotics, bismuth compound, intestinal metaplasia, or

Received: March 20, 2021
Peer-review started: March 20, 2021
First decision: July 3, 2021
Revised: July 11, 2021
Accepted: September 19, 2021
Article in press: September 19, 2021
Published online: November 7, 2021

P-Reviewer: Keikha M, Lee SW
S-Editor: Fan JR
L-Editor: A
P-Editor: Yu HG

gastric ulcer bleeding.

Key Words: Chronic diseases; *Helicobacter pylori*; Diagnostics; Nested polymerase chain reaction; DNA sequencing; Detection limit

Core Tip: Polymerase chain reaction (PCR) is not endorsed for *Helicobacter pylori* (*H. pylori*) diagnostics due to nonspecific primers and the threat of false-positives. However, a nested PCR that is as specific and equally sensitive in biopsy samples as the ^{13}C-urea breath test was developed. Due to the threshold of *H. pylori* abundance and the ability to survive in the oral cavity, it is not suitable for saliva samples. Despite DNA degradation in stool samples, nested PCR for a shorter 148 bp amplicon identified twice the number of positive samples as stool antigen test, indicating that many patients are misdiagnosed, not treated by antibiotics, explaining why their problems are interpreted as chronic.

Citation: Sulo P, Šipková B. DNA diagnostics for reliable and universal identification of *Helicobacter pylori*. *World J Gastroenterol* 2021; 27(41): 7100-7112
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7100.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7100

INTRODUCTION

Chronic diseases, such as cardiovascular disease (CVD) and cancer, are the leading causes of death worldwide. In the United States alone, they account for 70% of deaths *per* year, and CVD and cancer account for over 50% of all deaths each year. Additionally, diseases of the joints, such as arthritis, Parkinson's disease, and Alzheimer´s disease, reduce the quality of life for the elderly, and the treatment consumes enormous resources (reviewed in[1,2]). In 2005, 133 million Americans had at least one chronic disease. The economic cost was estimated at $1.3 trillion (sic) *per* year[1]. Of the 12.7 million new cases of cancer in 2008, about 2 million were attributed to infectious agents, such as human papilloma virus, hepatitis B virus, hepatitis C virus, and *Helicobacter pylori* (*H. pylori*)[1].

There is clear evidence that *H. pylori* is a major cause of chronic disease in the gastrointestinal tract (GIT). This helical, gram-negative, microaerophilic bacterium is the most successful human pathogen. The route of infection is not fully understood, but it is transmitted between sexual partners and relatives due to gastroesophageal reflux, often in childhood by an oral–oral or oral–fecal route. The infection persists and remains with the host for life[3]. In most cases, the infection causes mild gastritis, which remains mostly asymptomatic. In approximately 10%–20% of infected people, *H. pylori* causes stomach and duodenal ulcers. The chronic state increases the risk of developing duodenal and gastric cancer. Thus, since 1994, the International Agency for Research on Cancer has classified *H. pylori* as a "group 1 (definite carcinogen)" alongside asbestos and benzopyrene[4-6]. Patients with stomach cancer have a poor prognosis. After lung, breast, colorectal, and prostate cancers, stomach cancer is the fifth most common malignancy in the world, and is the third-leading cause of cancer death in both sexes (723000 deaths in 2012, 8.8%)[7]. The high mortality rate is related to early metastatic expansion through the lymphatic system. Since the discovery of *H. pylori* as the causative agent, its eradication has reduced the incidence of ulcers to almost zero. Additionally, a decrease in the incidence of stomach cancer has been recorded in most European countries over the last decade. Despite this trend, the incidence remains high. In some areas of Eastern Europe, it is more than 20 *per* 100000 inhabitants, while it is approximately 7 *per* 100000 in Central and Western Europe, and approximately 2 *per* 100000 in North America[3,7-10]. Recently, *H. pylori* infection has also been associated with a number of extragastric diseases, such as idiopathic thrombocytopenic purpura, iron deficiency anemia, vitamin B12 deficiency, insulin resistance, and metabolic syndrome[4,10,11].

There are several clinical tests for *H. pylori* identification used differentially, depending on the method of medical examination and considering country-specific preferences. The Maastricht V/Florence Consensus Report recommends only the ^{13}C-urea breath test (UBT), the stool antigen test (SAT), or endoscopy for consistent identification[4,10].

The ^{13}C-UBT detects *H. pylori* indirectly by measuring the activity of bacterial urease in the stomach. The principle is based on the hydrolysis of orally administered ^{13}C- or ^{14}C-labelled urea that is hydrolyzed into ammonia and CO_2, which diffuse into the blood and are exhaled through the lungs. The increase in ^{13}C-labelled CO_2 in breath specimens (analyzed before and 30 min after the consumption of the urea) is the proof of urease activity and can be measured by mass spectrometry or by the less expensive infrared spectroscopy and laser-assisted ratio analysis. False-negative test results can occur if the patient has received a proton-pump inhibitor (PPI) two weeks before the examination or antibiotics four weeks before. Bleeding similarly affects the diagnostic reliability of the UBT, and therefore, the assay should be accomplished only when bleeding is suppressed. Corpus-predominant gastritis can also be the reason for false-negatives[11].

SAT relies on the recognition of *H. pylori* antigens in stool. Two types of SAT, the enzyme immunoassay (EIA) and immunochromatography assay (ICA), have been used for *H. pylori* detection. Either polyclonal or monoclonal antibodies are used, but monoclonal antibody-based and EIA-based tests provide more accurate and reliable data. The accuracy can be affected if the patient has taken PPIs, antibiotics, or *N*-acetylcysteine, or has bleeding ulcers. False-negative results may also occur when the *H. pylori* count is low, also due to the use of antibiotics, bismuth, and PPIs. The SAT is a fast, simple, and inexpensive test that is also useful in epidemiological studies and screening programs[11].

Both tests have good sensitivity and specificity, as well as excellent performance if a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) is used[4,10-12]. Nevertheless, extensive study has shown that 3% of cases examined by UBT and 9% of patients tested by *H. pylori*-specific SAT should be taken as false-negatives [13].

In cases where the patient's medical condition requires endoscopy, *H. pylori* infection is examined in gastric biopsies by a rapid urease test (RUT), histology, or cultivation. RUT depends on the ability of *H. pylori* to secrete the enzyme urease. In the mixture containing urea, phenol red or other pH indicators and stomach tissue samples, urea is decomposed into ammonia and carbon dioxide. The presence of ammonia increases the pH, which changes the color of the indicator. The sensitivity and specificity of this method is considered to be > 90%. Patients with achlorhydria and those treated with PPIs, antibiotics, or bismuth compounds may have false-negative results. The test is also not very reliable in patients with intestinal metaplasia or gastric ulcer bleeding[11].

In histology, the biopsy sample is usually stained with Giemsa to identify pathogens and by hematoxylin and eosin to visualize inflammatory cells. If these stains provide inconclusive images, toluidine blue, acridine orange, and Warthin-Starry silver staining can be beneficial. *H. pylori* is unevenly distributed in the mucus layer; therefore, biopsy samples used to be taken from different parts of the stomach. The sensitivity is 80%–95%, and the specificity is 99%–100%. The diagnostic accuracy of histological examination affects many factors, such as the skill and experience of the gastroenterologist performing the sampling and the pathologist observing the biopsy specimens, the staining technique adopted, the use of PPIs or antibiotics, and the bleeding of peptic ulcers[11,12].

The culture of *H. pylori* from gastric biopsy samples is performed only in specialized laboratories, as it is not a routine technique. However, it is rather useful for the detection of antibiotic susceptibility and for scientific research. Sensitivity and specificity are considered to be about 70%–80% and 100%, respectively, but in our hands, it is possible to cultivate *H. pylori* from only about 8% of positive samples[11,14].

Serology, the main approach to detect bacterial infections in blood, is a controversial topic. The ability to recognize active infections of *H. pylori* relies on age, the clinical conditions of the infection, the antigen used for antibody preparation in the ELISA kit, and the prevalence of infection. Serology has a high negative predictive value; despite its low accuracy, it is cost-effective and due to the availability and simplicity, it is commonly used in epidemiological studies[10,11].

Approaches involving DNA amplification have not been widely accepted in medical practice, due to their higher price in comparison to SAT and UBT and the associated technical demands. The other objections are doubts concerning accuracy, as

alterations in the primer binding site may produce false-negatives; on the other hand, nonspecific primers may generate false-positives[12,13,15,16]. This opinion comes from the article by Sugimoto *et al*[17], who examined 26 various PCR reactions with diverse primers, designed to number of different *H. pylori* genes including 16S rRNA. DNA from biopsy and saliva specimens was amplified and compared to the results from cultivation and histological examination[17]. They concluded that none of the amplification systems were consistent in terms of specificity or sensitivity with classical tests and all provided false-positives.

In addition, multiple cases of positive results in PCR assays were reported without confirmation by other methods[16-19]. Furthermore, there are considerable doubts about interpreting PCR from a single gene, although the reliability can be unambiguously verified by sequencing[15,16]. Despite the ability to detect a few *H. pylori* cells or DNA molecules, the PCR approach was not even accepted for proving the eradication by antibiotics, which is again associated with the danger of the identification of cell debris[16]. These misbeliefs are still held, despite the recent meta-analysis reporting that the sensitivity and specificity of stool PCR tests are similar to those of other diagnostic methods[20].

Several PCR modifications, such as real-time PCR, allow the rapid detection and quantification of target DNA[21-24]. However, the disadvantages of real-time PCR include the high equipment cost, the high levels of technical skill required, the increased chance of false-negative results due to operator error resulting from improper assay development, and improper data analysis[21,25,26].

The alternative of choice is nested PCR (NPCR), which includes two rounds of PCR reactions. The first reaction amplifies a larger DNA region that is used as a template in the second reaction, which amplifies a narrower sub-region (Figure 1)[15,27].

Due to the two sets of primers, NPCR is more specific, and DNA can be amplified from samples with a smaller number of target molecules than simple PCR[27-30]. However, this method is prone to spray contamination and false-positives[27,28]. Nevertheless, NPCR has the potential to become the gold standard in diagnostics when sampling difficulties due to the patchy distribution of *H. pylori* and the recurrent incidence of false-positives are properly addressed[15]. The potential of NPCR is reinforced by the detection of antibiotic-resistant mutations in 23S rRNA in stool samples with no reports of false-positives[31,32].

We have been involved in *H. pylori* diagnostics for a while, and during four master's degree theses and one dissertation, we found that simple PCR was much more sensitive than histology and that many samples that were considered negative were actually positive by NPCR performed in our laboratory. However, before designing this assay, 17 different NPCRs available for *H. pylori* detection were evaluated from the point of view of efficiency and selectivity. In most of them, serious limitations and mistakes were found in the design of primers. The first major drawback was the non-specificity of primers, especially at the 3´ ends, which can be proved by the BlastN comparison if the *Helicobacter* TaxId is excluded. This is typical for oligos designed to amplify ribosomal RNAs and protein genes. A lack of specificity was confirmed in two cases from PCR product sequences by the authors themselves[30,33]. The common cause is a mismatch at the 3´ ends of the primer, resulting from polymorphisms found in the *fliI*, *hpaA*, *hsp60*, *ureA*, *ureC/glmM* genes. Frequently, alterations in the melting temperature (T_m) are greater than the accepted 4 °C. Sporadically, primer oligos are very short and their T_m is consequently low. These differences should have an impact on the efficiency of the amplification and could cause a failure in the amplification of positive samples. This issue is profound for the housekeeping gene *glmM*, which was used to confirm qPCR results[34], where the detection rate was only about half of that in the 16S rRNA-positive samples[35-37]. In another two NPCR assays, specificity was assessed through an experiment[31,38] on the *Helicobacter*-free stool samples and the products targeted to the 16S rRNA gene were sequenced. Their GenBank comparison showed 97% or more identity to the unrelated bacteria *Actinomyces naeslundii*, *Bifidobacterium pseudocatenulatum*, and *Varibaculum cambriense*[38] and to *Bacteroides salanitronis* in the case of 23S rRNA amplification[31]. An identity of 97% or higher is the taxonomic criterion that allows isolates to be assigned to the same bacterial species [39]. Apparently, almost all published NPCR systems are not specific or sensitive enough to spot low-density infection in complex specimens. Only *ureA* gene amplification systems passed the BlastN *in silico* test[40,41], but the *ureA* product is not critical for the *H. pylori* persistence in the stomach[42], although it is indispensable for colonization of the GIT in mice[43]. Furthermore, the *ureA* gene PCR systems were not examined on composite samples, and the PCR products were not sequenced.

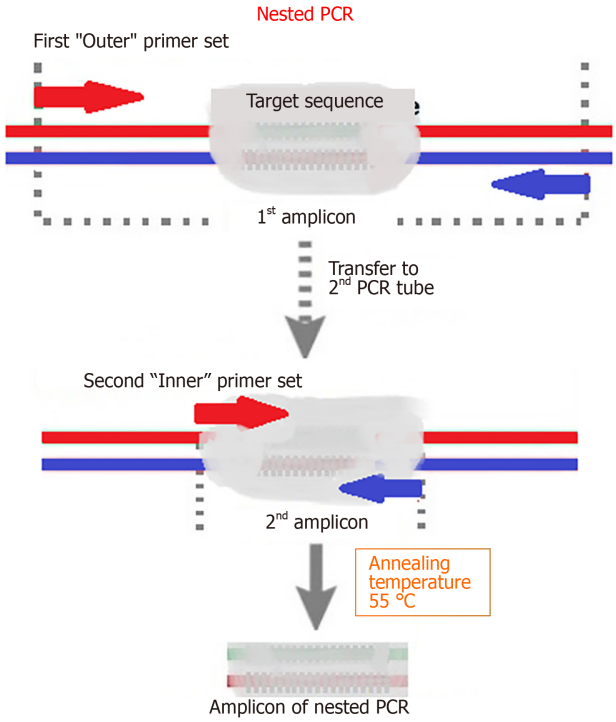

Figure 1 Nested polymerase chain reaction. Nested polymerase chain reaction involves two amplification reactions. The first round targeted a larger DNA region, and the second targeted a narrower sub-region of the products of the first round that were used as a template. PCR: Polymerase chain reaction.

In conclusion, nonspecific primers that amplify the DNA of other biological species are major pitfalls in PCR diagnostics. This current state results from inappropriate primer design, mostly from 30 years ago, and the recurrent use of outdated primers. At that time, only a limited number of sequences were stored in GenBank, so primer design was focused only on unique genes, such as urease genes.

RELIABLE DNA DIAGNOSTICS

Primer design

Many sequences are currently available for various strains, related organisms, and organisms from natural ecological niches, with many for entire genomes. In addition, most of the bioinformatic software offers the option of primer design. However, it is not difficult to design them using common sense.

The first task is gene selection. Various housekeeping genes are considered, but due to habits in taxonomy and clinical microbiology, especially with regard to the identification of new species, our attention should be focus on the 16S rRNA gene for the small ribosomal subunit. This RNA gene present in any bacteria contains conserved regions that are used in metagenomic studies for the universal amplification of bacterial DNA[39,44,45]. In addition, species- or genus-specific hypervariable sections can be found in the 16S rRNA sequence, which favors this gene for primer design[46,47]. Sufficiently selective primers can be designed in the regions discriminating *Helicobacter* from other known bacteria. To select a suitable region, the *H. pylori* 16S rDNA sequence was compared to the corresponding genes from representative stool resident (*Escherichia coli*) and representative of the closely related genera (*Campylobacter jejuni*). If these DNAs are aligned, several unique *H. pylori* regions useful for primer design can be found (Figure 2).

First pick primers were modified for a significant mismatch at the 3′ end, in order to keep the GC content below 50%. Primer length was then trimmed to maintain the T_m at around 55 °C, which can be easily calculated in many programs. These parameters are important to prevent the amplification of false priming sites and to improve efficiency. Primer specificity can be assessed simply *in silico* by BlastN comparison with other GenBank sequences. Good primers match precisely 100 times or more within the *Helicobacter* genus, but exhibit a strong divergence at the 3′ end of other bacteria. This can be done by the exclusion of *Helicobacter* TaxId from the task. According to these

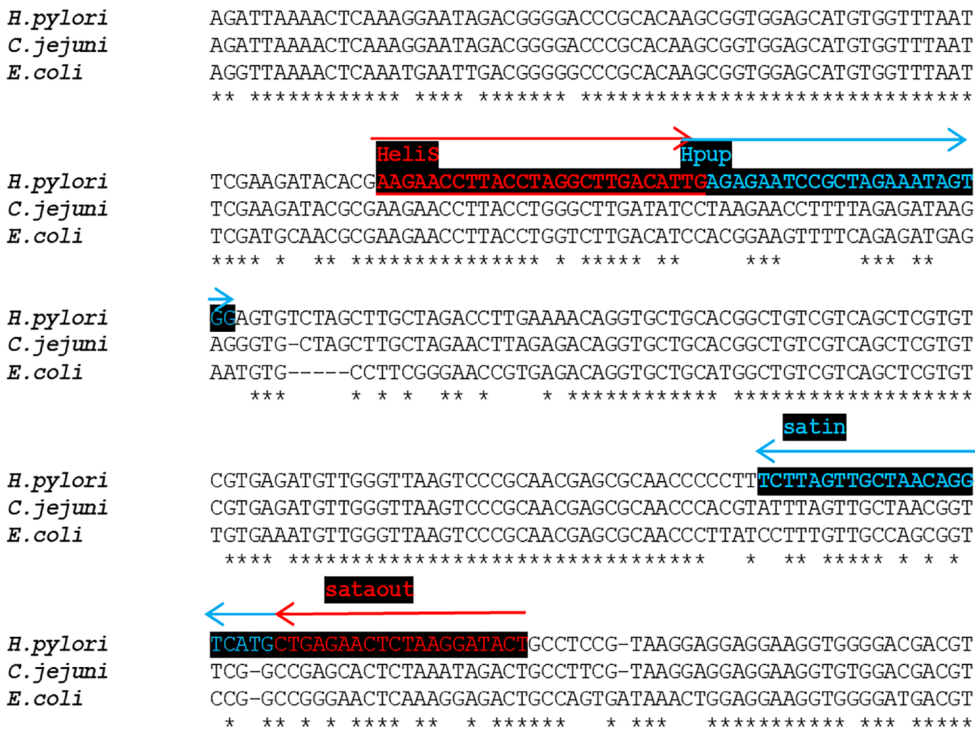

Figure 2 Design of *Helicobacter pylori*-specific primers for shorter 148-bp 16S rRNA amplicon. Alignment of *Helicobacter pylori* (amplified region) to other bacterial species. Selective primers marked in red, and blue were designed in the regions with a high divergence of *Helicobacter* sequence.

principles, two primer sets were selected: External primers for the amplification of a 497 bp region and internal primers for the amplification of a 454 bp fragment. However, the excellent performance of any PCR assay requires the optimization of amplification conditions, such as annealing temperature, the concentration of magnesium and the number of cycles (25–45)[28].

Sensitivity and the limit of detection

The Achilles heel of all identification methods is the absence of a detection limit, which should be understood as the minimal number of cells or DNA copies that can be consistently identified. This can be determined by adding ('spiking') a known number of cells directly into the PCR reaction ('colony PCR') or to the spare samples that previously tested negative. The detection limit for *H. pylori* cells was as low as 0.5 cells in a PCR vial (Figure 3)[28].

This value expresses the smallest DNA amount that can be theoretically amplified, as the *H. pylori* genome contains duplicate 16S rRNA genes[48]. Nevertheless, in reality, when samples are spiked with *H. pylori* culture, the detection threshold is roughly ten times less sensitive; it contained approximately 10 cells in a PCR vial that requires more than 1–5 × 10^3 cells *per* g or mL of biopsy, saliva, or stool specimen[28]. This is apparently the consequence of reduced DNA yield from silica columns. However, due to the unknown elution volumes, these data cannot be compared to those of other studies[17,49]. Nonetheless, the detection limit does not rely on the DNA isolation kit or the enzyme used[28].

Solo PCR is significantly less effective since approximately ten times more cells are needed in the amplification reaction for consistent identification. Another parameter that is extremely important but omitted from almost all diagnostic works is the concentration of target molecules in the analyzed samples. Their actual abundance is possible to determine from dilutions of the sample solutions. The lowest detectable density should be the same for particular NPCRs and specimens as the known threshold limits. The number of target DNA copies in the sample can be estimated by multiplying this value by the dilution factor. The density of *H. pylori* in stomach biopsies was found to be in the range 0.5–2.5 × 10^4 cells/g, while in saliva and feces it corresponded to 5 × 10^3 cells/g or 1 × 10^3 cells/mL, respectively. This was at least 5- to 25-fold lower than that in stomach mucosa[28].

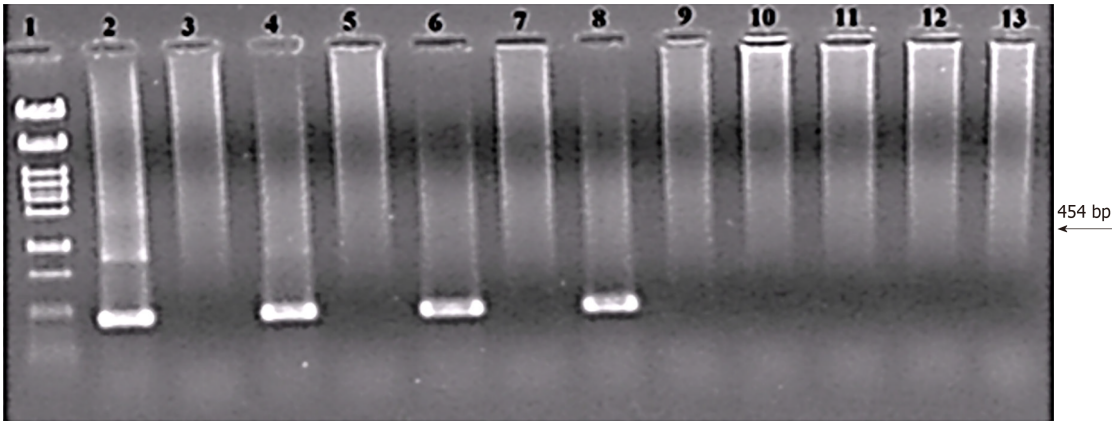

Figure 3 Threshold value of the nested polymerase chain reaction assay for *Helicobacter pylori* detection in cell suspension (colony polymerase chain reaction). Lines: 1: Size marker λ/*Pst1*; 2: 500; 3: Negative control (NC); 4: 50; 5: NC; 6: 5; 7: NC; 8: 0.5; 9: NC; 10: 0.05; 11: NC; 12: 0.005; 13: NC. Numbers express cell counts in the polymerase chain reaction (PCR) reaction. External primers HeliS/HeliN. Internal primers Hpup/Hpdown. Size of PCR product is 454 bp[28,53]. Each sample was tested by PCR separately in two independent experiments, always with the same result. Separated on 2% agarose in TBE.

Reliability of NPCR assays in different specimens

NPCR was validated in biopsy, stool, and saliva samples from the same individuals and compared to other detection methods[28]. Overall, 39.5% of patients were positive for *H. pylori* by UBT considered the gold standard, but only 21% by histopathology, 18.5% by RUT, and 27.2% by immunochromatographic SAT, while 39.5% of the biopsy samples were positive by NPCR (Table 1)[28]. Biopsy specimens were subjected to evaluation by simple PCR (second amplification reaction from NPCR), but only 29.6% were positive (Table 1).

As expected, NPCR was more sensitive than simple PCR. In addition to NPCR, samples that were positive in all other *H. pylori* tests were also positive using UBT. The *H. pylori* origin of PCR products was confirmed by DNA sequencing and their comparison revealed that they belong to at least 32 different strains. The sensitivity of histology, RUT, SAT, and simple PCR tests was lower, so these differences were interpreted as false-negatives (Tables 1 and 2)[28].

Are saliva specimens reliable?

There is an increasing demand for non-invasive diagnostics to circumvent the discomfort of the endoscopic examination required to collect samples[16,17]. The oral cavity is as suitable for a *H. pylori* reservoir as the stomach in adults[18], as are inflamed teeth (pulp) in children[18], but this is still a controversial issue[15,35,50]. It remains unclear whether *H. pylori* colonizes the oral cavity residentially, transiently, or at all[35]. Therefore, saliva and feces from 81 individuals were examined for the presence of *H. pylori*-specific DNA by simple and nested PCR. Simple PCR did not provide specific PCR products from any samples, but NPCR revealed a positive rate of about 12% in stool and 10% in saliva samples. Sequencing confirmed the correct origin, demonstrating high specificity of NPCRs, because several hundred diverse bacteria can be found in saliva[51]. The variability or identity of microbial populations can be distinguished simply by DNA polymorphism. Sequence comparisons of stool and saliva sources confirmed identical strains in the GIT and oral cavity in only three of the eight *H. pylori*-positive samples. Different strains in the stomach and saliva were found in two cases. However, in three individuals, *H. pylori* was identified exclusively in saliva/the oral cavity, but not in stool samples. Apparently, this pathogen can persist in the oral cavity, aside from and independent of the stomach, which was already reported in adolescents[52]. Nevertheless, NPCR of saliva samples appears to be a reproducible, consistent assay because the *H. pylori* 16S rRNA gene can be repeatedly amplified from any positive specimen. To find out how sampling could affect the results, we took advantage of the willingness of one SAT- and saliva-positive volunteer who was keen to provide samples throughout the day. However, besides one sample, which was only positive when the DNA concentration in the reaction was increased to maximum, all other daily saliva assays were negative. This outcome can be explained by variables but especially by the insufficient occurrence of bacteria in the samples. Therefore, saliva cannot be considered as a reliable source to confirm the presence of *H. pylori* in the stomach. This conclusion regarding the consistent detection

Table 1 *Helicobacter pylori* positivity by different diagnostic tests

Patients	^{13}C-UBT	Histology (biopsy)	RUT (biopsy)	SAT (stool)	PCR[1] (biopsy)	NPCR (biopsy)	NPCR (stool)	NPCR (saliva)
5	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	-
7	+	+	+	+	+	+	-	-
2	+	-	-	+	+	+	+	-
5	+	-	-	+	+	+	-	-
2	+	+	-	-	+	+	-	-
6	+	-	-	-	-	+	-	-
2	+	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-
3	-	-	-	-	-	-	-	+
44	-	-	-	-	-	-	-	-
Total 81	**32**	**17**	**15**	**22**	**24**	**32**	**10**	**8**

[1]Simple polymerase chain reaction (PCR).

Hpup/HPdown primers; 37 cycles; PCR products sequenced. GenBank database comparisons confirmed the DNA sequence origin as *Helicobacter pylori*. Plus indicates a positive result, minus indicates a negative result[43]. RUT: Rapid urease test; SAT: Stool antigen; PCR: Polymerase chain reaction; NPCR: Nested PCR.

Table 2 Sensitivity and specificity of diagnostic tests

	Histology (biopsy)	RUT (biopsy)	SAT (stool)	PCR (biopsy)	NPCR (biopsy)
Sensitivity (%)	53.1	46.9	68.8	75	100
Specificity (%)	100	100	100	100	95.6
Positive predictive values (%)	100	100	100	100	93.8
Negative predictive values (%)	74.6	72.1	81.5	84.6	91.3

Sensitivity and specificity related urea breath test[43]. RUT: Rapid urease test; SAT: Stool antigen; PCR: Polymerase chain reaction; NPCR: Nested PCR.

also supports a 50-fold lower (threshold) abundance in the oral cavity in comparison to the stomach[28,53].

The detection of H. pylori DNA in stool

Stool contains several thousand different species of bacteria. Nevertheless, we only amplified *H. pylori* DNA from stool samples using NPCR. These data again demonstrate the specificity of NPCR[54]. According to the SAT test, 22 samples were positive but only 10 were positive by NPCR. When stool samples were spiked with dilutions of *H. pylori* culture, the SAT limit was ≥ 2–5 × 10^5/g, which is 100 times less than that of the NPCR assay[28]. This shows strong inconsistencies between the detection limits and detection capabilities of SAT and NPCR. This paradox could be caused by the breakdown of intact *H. pylori* cells and its DNA in the digestive system. During digestion, DNA from food components is degraded to only about 200 bp fragments[55] which are much smaller than the NPCR product (454 bp). Despite our efforts, we were unable to determine which antigen was used to produce antibody components of the immunochromatographic SAT kits. Hypothetically, the SAT test could be more sensitive if antibodies were prepared against secreted antigens such as urease, *CagA*, *VacA* or surface antigens, which are not extensively degraded in the stool. To explain the SAT/NPCR paradox, we designed a new NPCR that allows the amplification of a shorter 148 bp segment of the 16S rRNA gene. SAT and NPCR for the 148 bp amplicon showed that only about 30% of 106 volunteers and 203 gastroenterological patients were positive by SAT, but 60% by short NPCR[53,56]. The origin of

the PCR product was confirmed by DNA sequencing, indicating a sensitivity for SAT of only 50%.

A comparison of SAT and NPCR indicates that many patients are misdiagnosed. They have health problems and host *H. pylori*, but are diagnosed as negative. They have been using proton pump inhibitors for a long time and see a physician regularly, but their problems become chronic. Gastroenterologists are aware of this phenomenon and often consider alternative pathogens[57]. We and others[51] have not identified any another pathogens, even by metagenomic analysis, and the most plausible explanation is the insufficient sensitivity of *H. pylori* tests due to the threshold of abundance[53,56,58].

Pitfalls of NPCR and H. pylori diagnostics

The major drawback of NPCR is false-positives due to the spray effect, as the tubes are opened after the first PCR to add aliquots to the second amplification reaction[27,59,60]. To avoid contamination, instead of a single negative control, we included two negative controls after each sample and analyzed the samples in triplicate. Only cases with a signal in the sample and without the signal in the negative control were considered positive.

This arrangement is good for the amplification of longer fragments (400–500 bp) but not for the amplification of shorter DNA (100–200 bp). Testing for a short 148 bp amplicon is not routine. The rules of the forensic laboratory and a number of rules, especially for pipetting, must be followed, not all of which can be reported in the protocol. Apparently, this assay cannot be used in practice in medical laboratories for the routine analysis of tens or hundreds of samples. The major source of the spray effect and thus of false-positives is the opening of tubes containing DNA amplified in the first reaction. However, it is possible to simplify NPCR so that it can take place in a single tube according to the rules described in previous studies[61-63]. Moreover, the assay can be modified for real-time PCR using both SYBR Green and TaqMan detection. Preliminary data are promising, and even the SYBR Green variant was shown to be more robust and as sensitive as the 148 bp amplicon NPCR assay. This modification has the potential for use in medical practice in the future. However, there are several other emerging methods, such as CRISPR-based detection, new imaging techniques, and novel fluorescent methods in histology[10,64,65].

CONCLUSION

The diagnosis of *H. pylori* can be divided into two basic categories. The first includes culture, RUT, and UBT and relies more on the good physiological state of metabolically active bacteria than on their abundance. However, this feature cannot be neglected; although in the case of UBT, it involves the extent of stomach colonization. However, these methods have a number of limitations, as errors can occur in patients receiving PPIs, antibiotics, and bismuth compounds or those with intestinal metaplasia or gastric ulcer bleeding. The second category involves PCR, NPCR, SAT, histology, and partial RUT, and relies strictly on cell abundance and the scale of their degradation. RUT and histology likely require bacterial loads of at least 10^4[10-64], an abundance that can be reached only in some biopsy samples[28]. For other methods, cell debris is sufficient for the identification of *H. pylori*, despite the threshold of occurrence in stool.

Generally, the fundamental problems in medical research are methods, their use, and their interpretation. The stumbling block is the common effort to detect various analytes (antigens, antibodies, pathogens) at levels around the detection limit, but the results are interpreted as data from an area of high confidence. The attempt to find the nature of the problem is then replaced by statistics and the comparison of inaccurate results. Its massive and improper use is the reason why half-true data are accumulated, complicating the solution to the problem[66]. Whether or not this is the case, medicine attempts to solve the problem *via* meta-analysis of the already published results (perhaps it is the only science to do so). The problem is significant in the identification of *H. pylori*.

The most common objection about the use of PCR is concerns about false-negatives that could be caused by polymorphism and the risk of false-positive results that could occur if non-specific primers are used[12,14,16].

One source of these misbeliefs is the phenomenon known as the 'gold standard'. This is a reliable method, which is usually histological examination together with urease or breath tests in the case of *H. pylori* detection. The gold standard implies

unanimously positive samples in all tests. Furthermore, the terms sensitivity and specificity are used, given as percentages[14]. The sensitivity expresses the percentage of samples in which the presence of *H. pylori* is detected compared to the gold standard, with 100% indicating that all samples identified as positive by the gold standard are identified as positive in the new test. The second concept, specificity, expresses the ability of the new test to accurately select samples in which *H. pylori* is absent. When a new test identifies samples as positive which were negative by the gold standard, they are considered false-positives. The fact that the new test might have a better detection threshold and is more sensitive, as is the case for NPCR, is disregarded. Samples are simply considered false-positives. However, the origin of the amplified DNA can be confirmed by DNA sequencing and comparison with databases such as GenBank. If the identity is > 97%, the isolates are considered to be the same bacterial species. This criterion is generally used in taxonomy and molecular biology [39,67], but for unknown reasons, it is ignored in medicine.

Apparently, the most promising *H. pylori* DNA detection method is the one-vial modification of short-amplicon NPCR. In addition to sensitivity and specificity, another advantage is that it can be used to verify the presence of *H. pylori* in questionable samples from patients that are SAT-negative but with achlorhydria, those receiving PPIs, antibiotics, or bismuth compounds, or in those with intestinal metaplasia or gastric ulcer bleeding, although all symptoms indicate *H. pylori* infection.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7113-7124

DOI: 10.3748/wjg.v27.i41.7113

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

MINIREVIEWS

Non-alcoholic fatty liver disease in patients with intestinal, pulmonary or skin diseases: Inflammatory cross-talk that needs a multidisciplinary approach

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Author contributions: Perez-Carreras M coordinated the review and wrote the majority of the article; Casis-Herce B and Martinez-Montiel P contributed to writing of the content on inflammatory bowel disease; Fernandez I contributed to writing of the content on non-alcoholic fatty liver disease; Rivera R contributed to writing of the content on psoriasis; Villena V contributed to writing of the content on obstructive apnea syndrome; and all authors have read and approve the final manuscript.

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

Country/Territory of origin: Spain

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common cause of liver disease. Its prevalence is increasing in parallel with the obesity and type 2 diabetes mellitus (DM2) epidemics in developed countries. Several recent studies have suggested that NAFLD may be the hepatic manifestation of a systemic inflammatory metabolic disease that also affects other organs, such as intestine, lungs, skin and vascular endothelium. It appears that local and systemic proinflammatory/anti-inflammatory cytokine imbalance, together with insulin resistance and changes in the intestinal microbiota, are pathogenic mechanisms shared by NAFLD and other comorbidities. NAFLD is more common in patients with extrahepatic diseases such as inflammatory bowel disease (IBD), obstructive syndrome apnea (OSA) and psoriasis than in the general population. Furthermore, there is evidence that this association has a negative impact on the severity of liver lesions. Specific risk characteristics for NAFLD have been identified in populations with IBD (*i.e.* age, obesity, DM2, previous bowel surgery, IBD evolution time, methotrexate treatment), OSA (*i.e.* obesity, DM2, OSA severity, increased transaminases) and psoriasis (*i.e.* age,

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

Received: May 20, 2021
Peer-review started: May 20, 2021
First decision: June 12, 2021
Revised: July 4, 2021
Accepted: September 16, 2021
Article in press: September 16, 2021
Published online: November 7, 2021

P-Reviewer: Ferraioli G, Grefhorst A
S-Editor: Wang JJ
L-Editor: A
P-Editor: Yuan YY

metabolic factors, severe psoriasis, arthropathy, elevated transaminases, methotrexate treatment). These specific phenotypes might be used by gastroenterologists, pneumologists and dermatologists to create screening algorithms for NAFLD. Such algorithms should include non-invasive markers of fibrosis used in NAFLD to select subjects for referral to the hepatologist. Prospective, controlled studies in NAFLD patients with extrahepatic comorbidities are required to demonstrate a causal relationship and also that appropriate multidisciplinary management improves these patients' prognosis and survival.

Key Words: Non-alcoholic fatty liver disease; Liver fibrosis; Psoriasis; Obstructive sleep apnea; Metabolic syndrome; Inflammatory bowel disease

Core Tip: Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common cause of liver disease. Its prevalence is increasing in parallel with the obesity and type 2 diabetes mellitus epidemics in developed countries. Several recent studies have suggested that NAFLD may be the hepatic manifestation of a systemic inflammatory metabolic disease that also affects other organs. This article reviews the currently available literature on issues relating to the co-existence of NAFLD and inflammatory bowel disease, obstructive syndrome apnea or psoriasis, with particular focus on the prevalence, risk factors and impact on clinical multidisciplinary management.

Citation: Perez-Carreras M, Casis-Herce B, Rivera R, Fernandez I, Martinez-Montiel P, Villena V. Non-alcoholic fatty liver disease in patients with intestinal, pulmonary or skin diseases: Inflammatory cross-talk that needs a multidisciplinary approach. *World J Gastroenterol* 2021; 27(41): 7113-7124
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7113.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7113

INTRODUCTION

Non-alcoholic liver disease (NAFLD) has become the leading cause of liver disease in western countries and has attracted researchers' and clinicians' interest in recent years. It affects 20%-30% of the adult population in developed countries and its prevalence rises to 95% among subjects with morbid obesity and 70% among those with type 2 diabetes mellitus (DM2)[1,2]. Close to two-thirds of NAFLD patients have some metabolic factor and one-third have metabolic syndrome (MetS). Although NAFLD has been proposed as the hepatic manifestation of MetS, in actual fact the relationship between these two conditions is complex and reciprocal, mediated by insulin resistance. NAFLD is known to be an independent risk factor for the development of DM2/prediabetes and cardiovascular mortality[2-4].

NAFLD is a dynamic disease that includes an evolving spectrum of anatomoclinical lesions, including hepatic steatosis (fat deposition in hepatocytes, usually in the form of macrovacuoles), steatohepatitis (NASH; which adds cell death and inflammatory infiltrates) and cirrhosis. Although most patients have a mild form of the disease, up to 20%-30% may have NASH lesions and one-third may have progressive fibrosis[1,5].

Excluding alcohol consumption has long been the key criterion to diagnosis of NAFLD[6,7]. However, its close association with metabolic factors has recently led some experts to propose replacing the term NAFLD with "MAFLD" (abbreviating for "metabolic dysfunction-associated fatty liver disease"), which would include patients with metabolic conditions, even if they consume alcohol and/or have other causes of fatty liver disease[8,9]. Likewise, "extra-hepatic manifestations" is probably not a suitable designation for the morbidities that coexist with NAFLD in metabolically unhealthy subjects, including inflammatory bowel disease (IBD), obstructive syndrome apnea (OSA) and psoriasis[7,10,11] (Figure 1).

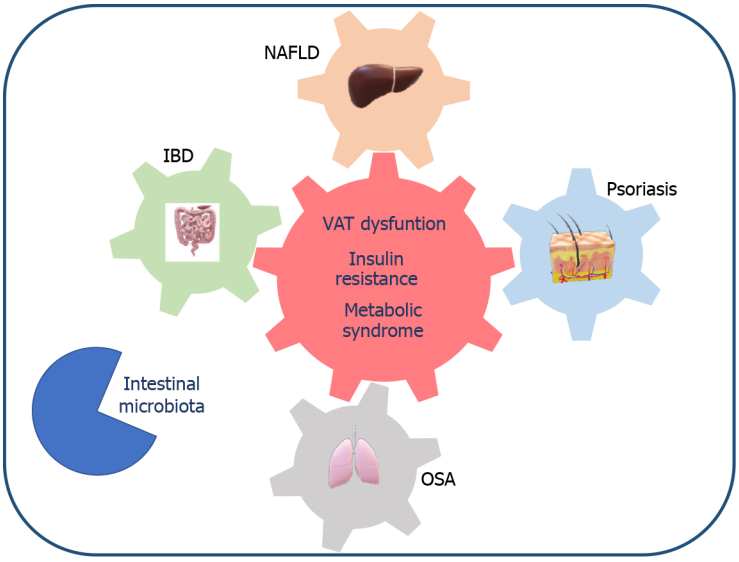

Figure 1 Potential pathogenic factors contributing to the co-existence of non-alcoholic fatty liver disease and extrahepatic comorbidities (*i.e.* inflammatory bowel disease, obstructive syndrome apnea and psoriasis). IBD: Inflammatory bowel disease; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive syndrome apnea; VAT: Visceral adipose tissue.

The mechanisms that link NAFLD with these extrahepatic comorbidities are complex and multifactorial. It appears that the imbalance in favor of proinflammatory *vs* anti-inflammatory cytokines produced by the visceral adipose tissue (VAT) of patients with metabolic dysfunction not only plays a role in the onset of an insulin resistance state, hepatic lipotoxicity and NAFLD but also interacts synergistically with the local production of these same inflammatory mediators in the intestine or skin, as occurs in IBD and psoriasis, or is enhanced by the hypoxemia present in OSA[12-15]. Moreover, there is increasing evidence that changes in the intestinal microbiota and their metabolic interaction with the host also play a role in the multi-hit pathogenesis of NAFLD and related extra-hepatic diseases[12,16].

In this review, our aim was to update the available evidence on the link between NAFLD and three increasingly prevalent diseases: IBD, OSA and psoriasis. Ultimately, we aim to draw attention to the impact that NAFLD may have on the management and prognosis of patients with these extra-hepatic comorbidities in clinical practice.

NAFLD AND IBD

IBD is a complex and multifactorial gastrointestinal disease that usually presents in the form of acute outbreaks on a chronic immune-mediated inflammatory substrate. Although its main symptoms relate to the intestine, in both types of IBD — Crohn's disease and ulcerative colitis — extraintestinal symptoms can appear in organs such as the skin, joints, eyes and liver[15]. Hepatobiliary manifestations have been reported in 3%-50% of patients with IBD and elevated transaminases in up to one-third of them, often being transient and attributable to the immunomodulatory drugs used in IBD. NAFLD is now considered the leading cause of liver disease among IBD patients[17,18].

Since the link between ulcers in the intestine and fatty hepatomegaly was first described in 1873[19], multiple cases, series and observational cohort studies have been published that analyze the prevalence of NAFLD in patients with IBD and the factors that may link the two diseases, both metabolic (*e.g.*, DM2, obesity, arterial hypertension, dyslipemia, MetS) and relating to the IBD itself (*e.g.*, type, duration, inflammatory activity, extension, drugs, intestinal surgery)[20-30].

Zou *et al*[31] conducted a systematic review and meta-analysis covering 19 observational studies published up to 2018, involving a total of 5620 patients with IBD. Although NAFLD prevalence varied significantly (8%-40%), in the most recent studies they found an increase in the frequency of this liver disease among IBD patients, to the point that it now exceeds the level in the general population (33% *vs* 25%, respectively). Furthermore, the authors identify age, metabolic factors, methotrexate use, previous bowel surgery, and chronic kidney disease as risk factors for NAFLD. More

recent publications confirm most of these data and attribute them to the changed metabolic profile of IBD patients in recent years. Thanks to new biologic drugs, these patients have gone from being malnourished to being obesity/overweight in up to a third of cases[27,32,33].

The liver-intestine interaction in IBD and NAFLD patients could be explained by mechanisms such as the synergism between inflammatory mediators produced by hypertrophic adipocytes in the VAT and the increase of proinflammatory cytokines from the intestine[12,15]. It could also be explained by the involvement of intestinal microbiota, including changes in its diversity, interaction of metabolites produced by intestinal microorganisms with the host's lipogenesis and host hydrocarbon metabolism, or changes to intestinal permeability. All these mechanisms could favor the development of insulin resistance, MetS and NAFLD[34]. Animal experiments with drug delivery system (DDS)-induced colitis support this interrelationship between intestinal barrier disruption, endotoxemia, metabolic dysfunction in adipose tissue and NAFLD[35].

It has recently been recognized that patients with IBD, mainly those with Crohn's disease, have a specific type of mesenteric adipose tissue, located in the areas of inflamed bowel, called "creeping fat". This is an immunologically active tissue that behaves similarly to VAT, promoting inflammation of the intestinal mucosa and perhaps playing a role in the metabolic changes involved in the onset of NAFLD[36,37].

There are some practical issues of particular interest to IBD gastroenterologists, such as: (1) Identifying IBD patients with NAFLD risk; (2) The impact of a NAFLD diagnosis on the treatment and prognosis of patients with IBD; and (3) When to refer the patient with IBD to a hepatologist.

NAFLD should generally be suspected in older IBD patients with metabolic conditions, previous bowel surgery, or long-standing bowel disease[31,36]. However, comparing NAFLD patients with and without IBD, it appears that patients with NAFLD and IBD are younger and have less metabolic factors than those NAFLD patients without bowel disease. This is why some authors have proposed defining two phenotypes of patients with IBD and NAFLD: "classic or metabolic" and "IBD-specific". The first group included subjects > 45-years-old with elevated transaminases, obesity, DM2 or arterial hypertension, and a later onset of bowel disease, while the second group included younger individuals with normal transaminases and less metabolic factors[23,25] (Figure 2).

We do not have prospective studies analyzing whether there are differences in natural history and prognosis between these two phenotypes. Sartini *et al*[25] have associated NAFLD severity, measured by the degree of ultrasound steatosis, with fewer metabolic conditions and a "severe" IBD phenotype (*i.e.* more than one annual inflammatory bowel flare, more extensive IBD and previous intestinal surgery). These data suggest that in addition to metabolic conditions, other IBD-specific factors are likely to be involved in the onset and progression of NAFLD.

Based on animals and *in vitro* experimental studies, it has been speculated that tumor necrosis factor-alpha inhibitors may protect against developing NAFLD and also that glucocorticoids and immunomodulators increase the risk of liver disease progression[38,39]. However, few clinical studies have adequately collected the time and dose of treatments and thus conclusive information is not available at present. A synergistic effect of NAFLD and methotrexate treatment has been suggested, favoring liver toxicity and progression of NAFLD to more severe forms, especially in patients with obesity or DM2[40,41]. Lapumnuaypol *et al*[27] found a very high prevalence of NAFLD (54%) in their series of 80 patients with IBD treated only with biologics (*i.e.* infliximab, adalimumab, certolizumab or goligumab), most of whom were male and obese. In the multivariate analysis, they found an association between NAFLD and the clinical activity of IBD but not with drugs.

Considering that liver fibrosis is the main prognostic marker in NAFLD patients, it is important for gastroenterologists to use non-invasive markers of fibrosis in patients with IBD and NAFLD. These markers consist of mathematical algorithms that include clinical and analytical variables whose result enables the identification and stratification of patients with liver fibrosis. Among them, FIB-4 and NFS (non-alcoholic fatty liver disease score) have been validated in patients with NAFLD[4,6,7]. Retrospective and longitudinal studies that include the calculation of both serological markers of fibrosis found that only 2.2% of subjects with IBD and NAFLD have advanced fibrosis, and that it remains stable during a 3-5 year follow-up in most patients. Age, metabolic factors and duration of IBD appear to increase the risk of fibrosis progression[26,42,43].

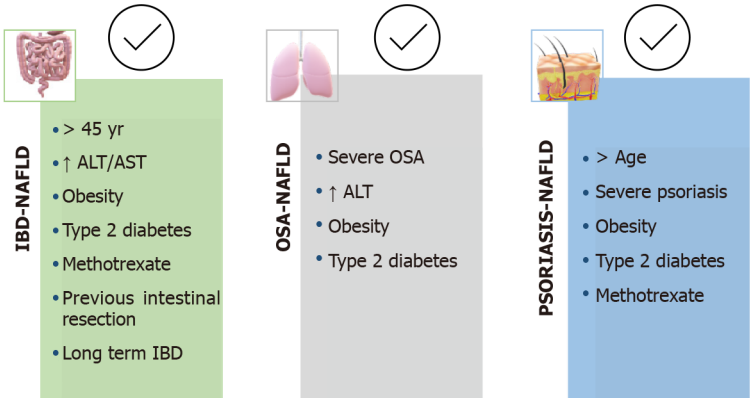

Figure 2 Non-alcoholic fatty liver disease and extrahepatic comorbidities (*i.e.* inflammatory bowel disease, obstructive syndrome apnea and psoriasis) risk phenotypes. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IBD: Inflammatory bowel disease; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive syndrome apnea.

Palumbo *et al*[42] were the authors of the only prospective study designed as part of a screening program for NAFLD and fibrosis involving 384 patients with IBD using control attenuation parameter (CAP) and hepatic elastography available on the FibroScan device probe (Echosens, France). They found any grade NAFLD in 32.8% of patients (CAP ≥ 248 dB/m), severe NAFLD (CAP > 300 dB/m), significant fibrosis in 24.6% (> 7 kPa) and advanced fibrosis in 18% (> 8.7 kPa). These NAFLD prevalence data should be taken with caution as there is evidence that higher CAP cutoffs than those used by these authors improve the diagnostic accuracy of this method[44,45]. The authors compared the presence of significant and advanced fibrosis in patients with NAFLD *vs* non-NAFLD and found a higher prevalence in the NAFLD group (24.6% *vs* 6.2% and 18.3% *vs* 3.1%, respectively; $P < 0.001$). Age, obesity, plasma triglycerides and methotrexate use were factors associated with liver fibrosis. In this way, they stratified the patients and referred a third of them to the hepatologist. In addition, they highlighted the presence of chronic kidney disease and cardiovascular disease among patients with IBD and NAFLD, in many cases related to subclinical atherosclerosis, information which supports the multidisciplinary approach to at-risk patients.

Although we now have scientific evidence to establish the suspicion of NAFLD in patients with IBD, prospective, longitudinal and control group studies are needed to identify those phenotypes at risk of developing fibrosis and advanced liver disease before we can establish an appropriate algorithm for screening, diagnosis and follow-up of patients with IBD and NAFLD.

NAFLD AND OSA

OSA is a clinical condition usually considered a respiratory disease, which has begun to be recognized as a multisystemic disease in the last two decades. It consists of recurrent nocturnal episodes of complete (apnea) and incomplete (hypopnea) obstruction of the upper airway, leading to hypoxemia and reoxygenation phenomena. It affects 1%-4% of the population, mostly males, with obesity and/or MetS, usually as a consequence of increased fat deposition in the upper airways and surrounding soft tissues[46,47]. Although, from a clinical perspective, OSA patients fundamentally consult due to sleep fragmentation and daytime sleepiness, the most important consequence is the chronic intermittent ischemia that occurs in different organs, such as the liver[46-48].

Over the last decade, at least 20 clinical studies have been published linking NAFLD and OSA, including systematic reviews and meta-analyses[49]. Although most include short series of patients and histological information is usually obtained from liver biopsies during morbid obesity surgery, OSA is considered to be an independent risk factor for the development and progression of NAFLD. In a recent meta-analysis and systematic review, Jin *et al*[50] found that patients with severe OSA (number of apnea-hypopnea episodes > 30/h) have higher aspartate aminotransferase values and a greater degree of steatosis, inflammation, ballooning degeneration and fibrosis. Trzepizur *et al*[51] found in their cohort of almost 1300 patients that those with severe

OSA had a 2.5-fold increased risk of liver fibrosis; although, this association was not independent of other factors when logistic regression analysis was performed. When determining the epidemiological and pathogenic relationship between NAFLD and OSA, it is difficult to avoid the impact of obesity and other metabolic conditions that are so frequent in both diseases. Evidence from experimental models shows that chronic intermittent ischemia and increased sympathetic tone triggered by nocturnal hypoxemia phenomena are the main causes of cardiometabolic manifestations linked to OSA (*i.e.* DM2, dyslipemia, arterial hypertension, atherosclerosis) and of the onset and progression of NAFLD lesions[13,46].

The decrease in oxygen tension that occurs during nocturnal apnea-hypopnea episodes primarily affects hepatocytes in zone 3 of the hepatic lobule, where NAFLD lesions predominate, and results in the release of ischemia-induced factors (commonly known as HIF)[52,53]. These HIF favor the expression of genes involved in lipogenesis, with the consequent excess of triglycerides in the hepatocytes (steatosis), free fatty acids and hepatokines. This state of liver lipotoxicity leads to inflammation, mitochondrial dysfunction, oxidative stress, lipoperoxidation, cell damage (steatohepatitis) and fibrosis. In addition to this direct hepatic mechanism, VAT ischemia and sympathetic nervous system stimulation secondary to chronic intermittent ischemia promote a state of insulin resistance, lipolysis and hepatic fatty acid overload, which contribute to the onset of DM2, atherogenic dyslipemia, arterial hypertension and NAFLD[46,51,54]. Finally, different authors have proposed that nocturnal hypoxemia may alter the integrity of the intestinal barrier and favor the hit attributed to dysbiosis and bacterial translocation in the pathogenesis of NAFLD[55,56].

At present, we do not have prospective studies that allow us to determine the prevalence of NAFLD in patients with OSA or *vice versa*. However, considering that chronic intermittent ischemia can promote and aggravate liver damage, some authors propose screening for OSA in patients with NAFLD[47,49]. Taking into account the available information, hepatologists should always ask NAFLD patients about OSA-related symptoms and consider referring to the pneumologist those with elevated transaminases and/or advanced liver lesions, and/or associated metabolic factors, especially DM2 and obesity (Figure 2).

The use of a continuous positive airway pressure (CPAP) device and lifestyle changes to diet and exercise, especially in obesity/overweight patients, comprise the standard treatment of OSA patients. CPAP increases pharyngeal intraluminal pneumatic pressure, prevents hypoxemic events related to airway collapse, improves quality of life and decreases cardiovascular mortality[57,58]. However, its impact on metabolic factors and NAFLD lesions is controversial. The available evidence in this regard is of low quality because it is based on observational uncontrolled studies in patients with OSA-NAFLD and had a follow-up of only 3 mo of CPAP treatment[59]. Prospective, controlled and randomized studies that assess patient adherence to CPAP for more than 12 mo, could establish whether this measure can improve or prevent the progression of NAFLD.

NAFLD AND PSORIASIS

Psoriasis is one of the diseases that dermatologists are most concerned about because of its prevalence (it affects 2%-3% of the adult population in developed countries), the gaps in our knowledge of its pathogenesis, and its relationship with extracutaneous pathologies[14]. Currently, psoriasis is considered a systemic immune-mediated chronic inflammatory disease, since psoriasis patients not only present skin lesions but also frequently have comorbidities that can condition their prognosis and treatment, such as MetS and its hepatic manifestation, NAFLD[60-63].

There is epidemiological evidence and pathogenic hypotheses linking psoriasis to NAFLD[64,65]. In the last 10 years, multiple controlled cross-sectional observational studies have been published, some in large populations, studying the prevalence of NAFLD in subjects with psoriasis and the specific characteristics of the subpopulation of patients with psoriasis and NAFLD[66-69]. Van der Voort *et al*[66] analyzed these data in a Dutch cohort of 2292 individuals > 55 years of age and found that the prevalence of NAFLD among the 118 psoriasis patients was significantly higher than among the 2174 healthy controls (46.2% *vs* 33.3%; $P < 0.005$). Furthermore, after adjusting for confounders, including MetS, they determined that elderly patients with psoriasis are 70% more likely to have NAFLD than those without psoriasis. Gisondi *et al*[67] found that 44% of the 124 psoriasis patients included in their study had NAFLD *vs* 26% of the 79 healthy controls ($P < 0.001$). Comparing patients with psoriasis and

NAFLD *vs* no-NAFLD, they found that those included in the first group were more frequently male and had a higher body mass index, transaminase values (Alanine aminotransferase and aspartate aminotransferase), and psoriasis severity [measured according to Psoriasis Area Severity Index (PASI) score. It combines the assessment of the severity of the lesion and affected area into a single figure between the values of 0-no disease to 72-maximum disease].

In 2015, Candia *et al*[68] published the first meta-analysis and systematic review of seven case-control studies evaluating the psoriasis-NAFLD association in populations from different continents (n = 267761). Their combined analysis indicated that psoriasis patients have twice the risk of developing NAFLD [odds ratio (OR): 2.15] and identified male sex (OR: 2.28), obesity (OR: 12.25), DM2 (OR: 2.63), arterial hypertension (OR: 2.7), MetS (OR: 9.03) and arthritis (OR: 2.25) as predisposing factors. They also found that patients with moderate to severe psoriasis had a 2-fold increased risk of NAFLD. These data have recently been confirmed in an updated systematic review and adjusted meta-analysis (n = 3019308 subjects)[69].

Approximately one-third of psoriasis patients have joint involvement and this has been linked to increased inflammatory burden, severity of skin lesions and risk of NAFLD[14,70]. However, not all authors found an association between arthropathy and liver disease[71]. The systematic review and meta-analysis by Candia *et al*[68] found that patients with psoriatic arthritis had double the risk of NAFLD when compared to those without arthropathy (OR: 2.25, 95%CI: 1.4-3.7; P < 0.05). Although this information would suggest considering patients with psoriasis and joint involvement as a special risk group for NAFLD, a recent meta-analysis raises doubts on the increased risk for NAFLD of such an association[69].

Although most studies found an independent link between psoriasis and NAFLD, those with metabolic factors appear to be particularly at risk[61,62]. The high prevalence of obesity (20%-40%), DM2 (12%) or MetS (20%-50%) among psoriasis patients, and the close relationship between these conditions and insulin resistance and NAFLD, means the same patient having both diseases cannot be seen as totally independent[72-74]. This clinical link has a common pathogenic substrate, the protagonist of which is the imbalance between overproduction of proinflammatory cytokines [*e.g.*, tumor necrosis factor-alpha, interleukin (IL)-1, IL-6, IL-17] and the decrease of anti-inflammatory ones, mainly adiponectin. These mediators act locally by promoting cell growth and differentiation of the epidermis and dermis, the proliferation of keratinocytes and the consequent appearance of psoriatic plaques[63,64]. However, they may also act systemically and contribute to insulin resistance and other inflammatory mediators implicated in the metabolic dysfunction involved in NAFLD [63,65]. Interestingly, it is speculated that modifications in the skin microbiota present in psoriasis and their relationship with gut microbes may play a role in the psoriasis-NAFLD association[75]. These pathogenic hypotheses may explain why patients with psoriasis and NAFLD have more advanced forms of liver disease and severe skin lesions.

Most studies have assessed NAFLD using ultrasound, while few have involved liver biopsy. Roberts *et al*[76] found NAFLD in half of psoriasis patients (n = 103) and NASH histological lesions in 22% (liver biopsy in 52/103), one-third of them with significant advanced fibrosis. Furthermore, they linked NASH lesions with higher PASI scores, obesity and higher transaminase levels. More authors have analyzed the severity of liver lesions in psoriasis using non-invasive markers of fibrosis, serological or technological (FibroScan®)[67,77,78]. Using these scores it was found that 7%-8% of psoriasis patients have advanced liver fibrosis. This risk is multiplied by 4 when NAFLD is associated with this skin disease. Although fibrosis is more frequent in older subjects with metabolic factors, psoriasis appears to be an independent risk factor for fibrosis when adjusting in the multivariate logistic regression analysis, including metabolic conditions and drugs used to treat psoriasis[78].

From a practical perspective, the psoriasis-NAFLD association not only has an impact on the severity of skin and liver lesions, but may also influence dermatologists' decisions when selecting systemic treatment for psoriasis[62,79].

Although some studies associate methotrexate liver toxicity with cumulative dose and treatment time, most find that the risk of liver damage is primarily related to the presence of risk conditions such as obesity, DM2, alcohol consumption, and NAFLD [80,81]. Cyclosporine and acitretin, a retinoid derived from vitamin A, are considered to be diabetogenic and to promote atherogenic dyslipemia and arterial hypertension [79,82].

It has been speculated that new biologic drugs could be beneficial in patients with psoriasis and NAFLD by acting on the proinflammatory cytokines involved in both diseases[63]. Although controlled studies with some tumor necrosis factor-alpha

inhibitors have demonstrated their ability to decrease insulin resistance and the risk of DM2 (etanercept), others appear to favor weight gain and dyslipemia (infliximab, adalimumab)[79]. Recent research studies in animals have shown that IL-17 is involved in the progression of hepatic steatosis to NASH. There is speculation that the use of anti-IL17 monoclonal antibodies (secukinumab) could be beneficial in patients with psoriasis and NAFLD[83,84].

Considering that being overweight or obesity significantly increases psoriasis risk and severity, recent clinical trials have shown that weight loss, both with a hypocaloric diet and physical exercise or with bariatric surgery, improves psoriasis activity and also favors the response to systemic treatment. These interventions could be of particular importance in patients with psoriasis and risk of NAFLD[61,74].

Aware of the impact of NAFLD on psoriasis patients, experts from the European and American academies of dermatology have recently published recommendations for the management of psoriasis comorbidities, including MetS and NAFLD[82,85]. According to the available evidence, they consider NAFLD screening to be indicated in patients with a risk phenotype (*i.e.* moderate-severe psoriasis and metabolic factors) by means of transaminase measures and ultrasound, and propose a monitoring and follow-up algorithm that includes evaluation by the hepatologist in patients with suspected liver disease (Figure 2). In addition, they recommend that a diagnosis of NAFLD be taken into account when selecting psoriasis treatment. However, these recommendations do not appear to be implemented universally in dermatologists' clinical practice, nor has a specific hepatologist referral protocol for patients with psoriasis and NAFLD been established in most hospitals.

CONCLUSION

NAFLD should be considered more than a liver disease and be taken into account not only by hepatologists but also by clinicians caring for patients with other related diseases, such as IBD, OSA and psoriasis. The scientific evidence shows that these comorbidities share an inflammatory background that synergistically impacts the severity and management of these patients. It is essential that screening and referral algorithms for NAFLD subjects are developed from a multidisciplinary perspective in which not only liver, intestinal, respiratory or skin lesions are analyzed but also the risk of morbidity and mortality from metabolic and cardiovascular causes.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7125-7133

DOI: 10.3748/wjg.v27.i41.7125

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

MINIREVIEWS

Current update on molecular cytogenetics, diagnosis and management of gastrointestinal stromal tumors

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Author contributions: Wang MX, Devine C and Segaran N contributed to the manuscript; all authors contributed to the design of the study; Ganeshan D designed the structure of the overall manuscript, made critical revisions related to important intellectual content of the manuscript; and all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests for this article.

Country/Territory of origin: United States

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and are thought to arise from precursors of the interstitial cells of Cajal. GISTs can arise anywhere in the GI tract, but most commonly originate from the stomach and small intestine. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α. Mutational analyses allow for predicting patient prognosis and treatment response. Clinical presentations can vary from no symptoms, typical in the case of small incidentally found tumors, to GI bleeding, abdominal discomfort, and ulcer-related symptoms when the tumor is enlarged. Imaging plays a critical role in the diagnosis and management of these tumors with multiphasic computed tomography serving as the imaging modality of choice. Magnetic resonance imaging and positron emission tomography-computed tomography can serve as imaging adjuncts in lesion characterization, especially with liver metastases, and subsequent staging and assessment for treatment response or recurrence. Surgical resection is the preferred management for small GISTs, while tyrosine kinase inhibitors − imatinib mesylate and sunitinib malate − serve as crucial molecular-targeted therapies for locally advanced and metastatic GISTs. This review article highlights the clinical presentation, pathology and molecular cytogenetics, imaging features, and current management of GISTs.

Key Words: Gastrointestinal stromal tumors; Cytogenetics; Diagnostic imaging; Computed tomography; Magnetic resonance imaging; Imatinib mesylate

Received: June 7, 2021
Peer-review started: June 7, 2021
First decision: June 20, 2021
Revised: July 28, 2021
Accepted: September 15, 2021
Article in press: September 15, 2021
Published online: November 7, 2021

P-Reviewer: Kurokawa R
S-Editor: Wang JJ
L-Editor: A
P-Editor: Xing YX

Core Tip: Gastrointestinal stromal tumors (GISTs) often occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α, allowing for effective molecular targeted therapies for these patients. Mutational analyses help predict patient prognosis and treatment response. Imaging plays a critical role in the diagnosis and management of GISTs. Multiphasic computed tomography serves as the imaging modality of choice in their diagnosis and follow-up. It is crucial to understand and identify the key imaging features of GISTs and their expected appearance with treatment response and disease recurrence.

Citation: Wang MX, Devine C, Segaran N, Ganeshan D. Current update on molecular cytogenetics, diagnosis and management of gastrointestinal stromal tumors. *World J Gastroenterol* 2021; 27(41): 7125-7133
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7125.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7125

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and are thought to arise from the precursors of the interstitial cells of Cajal. GISTs can arise anywhere in the GI tract, most commonly from the stomach and small intestine[1,2]. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α (PDGFRA)[1]. Clinical presentations can vary from no symptoms, typical in the case of small incidentally found tumors, to GI bleeding, abdominal discomfort, and ulcer-related symptoms when the tumor is enlarged. Imaging plays a critical role in the diagnosis and management of these tumors with multiphasic computed tomography (CT) serving as the imaging modality of choice. Magnetic resonance imaging (MRI) and positron emission tomography-computed tomography (PET-CT) can serve as imaging adjuncts in lesion characterization, especially with liver metastases, and subsequent staging and assessment for treatment response or recurrence. Surgical resection is the preferred management for small GISTs, while tyrosine kinase inhibitors − imatinib mesylate and sunitinib malate − serve as crucial targeted therapies for locally advanced and metastatic GISTs[1].

This review article highlights the clinical presentation, pathology and molecular cytogenetics, imaging features, and current management of GISTs.

EPIDEMIOLOGY

The annual incidence of GISTs is estimated to be at least 3000 per year in the United States[1]. GISTs are often diagnosed in older adults ages 50-70 years with a median age at diagnosis ranging from 59 to 66 years[3-5]. GISTs can occur in all geographic and ethnic groups, and men and women are equally affected[6]. There are no known risk factors for developing GIST. A small subset of patients may present with the non-inherited Carney's triad, which is comprised of GIST, often with loss of function of succinate dehydrogenase (SDH), paragangliomas, and pulmonary chondromas[1]. While most GISTs occur sporadically, rare hereditary GISTs have been reported[5]. Familial GISTs are related to inherited germline mutations in either KIT or PDGFRA and also manifest with cutaneous hyperpigmentation, irritable bowel syndrome, dysphagia, and diverticular disease[1]. In Caryney-Stratakis syndrome, patients present with GIST and paragangliomas related to loss of function mutations within SDH genes. Small intestinal GISTs can also be associated with neurofibromatosis type 1, an autosomal dominant disorder in which patients more often present with café au lait spots, gliomas, and neurofibromas[1,5]. A very small group of GISTs (1%-2%) occur in the pediatric population.

CLINICAL FEATURES

Clinical presentations vary depending on the size and location of tumors. GISTs can arise anywhere along the GI tract. They most often arise from the stomach (60%), followed by the jejunum and ileum (30%), duodenum (5%), colorectum (4%), and esophagus or appendix (< 1%)[2]. Rarely, GISTs can develop outside the GI tract in the mesentery, omentum, or retroperitoneum. The majority of GISTs are benign (70%-80%). Patients are often asymptomatic, especially when the tumor size is small (less than 2 cm)[1]. When the tumor is enlarged, symptoms may include abdominal pain, bleeding, abdominal distension, early satiety, fatigue, and palpable mass[7]. Unfortunately, these nonspecific symptoms may result in delayed diagnosis and management of the disease. Infrequently, patients with advanced GISTs may present with severe hypoglycemia and hypothyroidism[8,9]. Laboratory work-up may reveal anemia, which may be related to bleeding or intratumoral hemorrhage. Metastases are uncommon (10%-20% of cases); however, when they do occur, they can occur *via* local or hematogenous spread. The most common metastatic sites include the liver, omentum, and peritoneal cavity[1,2]. Lymph node and extra-abdominal metastases are extremely rare[5]. In severe cases, patients may present with acute abdomen, melena, or hematemesis secondary to frank hemorrhage due to tumor invasion or rupture. Such emergent clinical presentation is more often seen in small intestine GISTs compared to gastric GISTs[5].

Pediatric GISTs occur in approximately 1%-2% of cases and are predominantly seen in girls presenting with multiple nodules in the stomach. These patients typically present with anemia, weakness, and syncope due to GI bleeding[1]. In addition to liver and peritoneal metastases, lymph node metastases uniquely occur in this group of patients.

PATHOLOGY AND MOLECULAR CYTOGENETICS

In gross pathology, GISTs can widely vary in size, ranging from 1-2 cm to more than 20 cm in diameter. The median size at presentation is approximately 5 cm[1]. They are well-circumscribed gray-white to red-brown masses in the bowel wall that can be submucosal, intramural, or subserosal in location[10]. They are generally unencapsulated but may have pseudocapsules. GISTs typically arise from the muscularis propria and exhibit an exophytic growth pattern. Intraluminal or mixed growth patterns may also be seen. There are three main histologic subtypes: (1) Spindle cell (60%-70%); (2) Epithelioid (30%-40%); and (3) Combination of both spindle cell and epithelioid in various proportions[10]. On microscopy, spindle cell subtypes demonstrate highly cellular, fascicular, whorled, storiform, or palisading architecture, while epithelioid tumors appear more fascicular or nested[10]. The mitotic rates can vary widely from virtually absent to high. Other findings may include areas of hemorrhage or necrosis.

The majority of GISTs (80%-90%) occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or PDGFRA. In 1998, Hirota *et al*[11] published the revolutionary finding that the majority of GISTs (94%) expressed activating mutations in KIT (CD117), now a key diagnostic immunohistochemical marker for GIST that distinguishes it from leiomyomas, leiomyosarcomas, or other mesenchymal tumors. KIT belongs to the type II transmembrane receptor tyrosine kinase family that includes PDGFRA and PDGFRB. The c-kit proto-oncogene encodes KIT; in combination with the stem cell factor extracellular ligand, this c-kit product normally plays an essential role in cellular survival, proliferation, and differentiation [10]. Activating mutations in KIT result in altered cell growth. In addition, Hirota *et al* [11] demonstrated that the interstitial cells of Cajal, which are the pacemaker cells involved in regulating the peristalsis located primarily in the muscularis propria, were the only cells that were double-positive for KIT and CD34 in the GI wall. Therefore, GISTs, which share morphological, structural, and immunohistochemical features with interstitial cells of Cajal, are thought to arise from them or their stem cell precursors [10-12]. Germline or sporadic gain of function mutations in c-kit result in both benign and malignant GIST tumorigenesis[10]. Aside from KIT mutation, PDFGFRA mutation can be an alternative cytogenetic change that can also result in similar downstream effects of tumor progression[13]. Hence, imatinib mesylate, a selective adenosine triphosphate-competitive inhibitor of KIT, PDGFRA and PDGFRB, serves as a groundbreaking therapy for GISTs[14]. Furthermore, DOG1, a calcium-dependent, receptor-activated chloride channel protein, has been found to be expressed in GISTs regardless

of mutation type; this marker can aid in the diagnosis of KIT-negative tumors, such as those with PDGFRA mutations that are KIT-negative[15].

Other markers for GISTs include CD34 antigen (70%), smooth muscle actin (30%-40%), desmin (< 5%), and S100 protein (approximately 5%)[16]. The expression of these markers varies depending on the location of the tumor. CD34 is often found in esophageal, gastric, and rectal tumors, while smooth muscle actin is seen in small intestine tumors. Prognostic predictors vary considerably in the literature. It has been suggested that mitotic activity and tumor size are potential prognostic predictors: A mitotic index of at least 5 per 50 high power fields (HPF) and a size greater than 5 cm are suggestive of malignant behavior, while a mitotic index of 5 or less per 50 HPF and a size less than 2 cm are suggestive of benign GIST[1,6,17]. Ki-67 can also be used to predict malignant potential[18]. Tumor location is another prognostic factor; intestinal GISTs demonstrate worse outcomes compared to gastric GISTs with regard to tumor size and mitotic rates[19]. GISTs that carry KIT exon 11 point mutations and insertions have a favorable prognosis, while those with KIT exon 9 mutations or KIT exon 11 deletions have a worse prognosis[19,20]. A small number of patients with GISTs may harbor concomitant *BRAF* gene mutations, which may portend poorer prognosis due to their primary resistance to imatinib mesylate therapy[21]; in such cases, patients may benefit from selective *BRAF* inhibitors. Further genotyping is advised for patients with KIT-negative GIST for management planning.

In the pediatric population, GISTs typically do not have KIT or PDGFRA mutations, and generally demonstrate the epithelioid subtype and express CD117[22]. Compared to adults, these pediatric GISTs uniquely overexpress fibroblast growth factor 4 (FGF4), brain and acute leukemia, cytoplasmic, insulin-like growth factor I receptor, NEL-like 1, cytokine receptor-like factor 1, pleomorphic adenoma gene 1, and FGF3)[23]. With KIT activation, these GISTs are similar to adult GISTs that carry KIT mutations. Although there is limited literature on the clinical benefits of tyrosine kinase inhibitors, sunitinib malate is suspected to be superior to imatinib mesylate in these pediatric cases[23].

IMAGING FEATURES

Fluoroscopic examination

Fluoroscopic examination is not routinely used for identifying GISTs. However, patients who undergo double-contrast barium studies may demonstrate a submucosal or well-circumscribed mass with smooth mucosal surface and obtuse angles at the margins[24]. With necrosis or ulceration, they may demonstrate irregular contours. Evaluation of extraluminal structures is limited with this approach. Further evaluation of the lesion and presence of metastatic disease with cross-sectional imaging is ultimately required.

Ultrasonography

Ultrasonography is not routinely used for imaging GISTs, especially since the tumor origin cannot be well-identified. When small, GIST may be homogeneously hypoechoic. When large, GIST may present as a heterogenous mass, which may reflect internal necrosis or hemorrhage; these findings suggest high malignant potential[25]. Hepatic metastases can be identified, although their sonographic appearance is nonspecific.

CT

CT serves as the imaging modality of choice in the diagnosis and follow-up of GISTs. Multiphasic protocol with noncontrast, arterial, and portal venous phases should be obtained. The noncontrast images help identify hemorrhage and provide a baseline for evaluating tumor enhancement. Adequate gastric distension is essential to help distinguish intramural mass; therefore, negative oral contrast agents can aid in the visualization of the enhancing mucosa[26]. The imaging appearance of GISTs depends on their size and aggressiveness. Classic CT features of GISTs include large, hypervascular, enhancing masses that may demonstrate heterogeneity due to hemorrhage, cystic degeneration, or necrosis[27]. These tumors typically displace adjacent structures and vessels, although they may exhibit direct invasion of adjacent structures resulting in ulceration and fistulization in the GI tract in advanced stages. When small, GISTs appear homogeneous and may be incidentally found on CT or endoscopy. Metastases are present in approximately 50% of patients, and metastases often involve the liver and mesentery; they demonstrate similar imaging features as primary GISTs

[24,27]. Lymph node metastases are extremely rare[27]. Features of high-grade GISTs include liver metastasis, GI wall infiltration, irregular surface, ill-defined margins, inhomogeneous enhancement, and peritoneal spread[28].

Regardless of tumor size, a change from a heterogeneously hyperattenuating mass to a homogeneously hypoattenuating mass with decreased enhancing tumor nodules and intratumoral vessels suggest response to imatinib mesylate[24,27]. The attenuation of treated lesions reaches approximately 20-25 Hounsfield units, which is close to simple density[29]. Although the tumors may enlarge during treatment as a result of intratumoral hemorrhage or myxoid degeneration, this does not suggest disease progression in the setting of decreased tumor enhancement[27]. The Response Evaluation Criteria in Solid Tumors has been found to be insensitive in evaluating treatment response as it does not account for tumor density, intratumoral vessels, or tumor metabolism[30]. Therefore, Choi *et al*[31] proposed a modified CT response evaluation criteria to account for such features on CT as tumor response to tyrosine kinase inhibitor therapy cannot be determined based on size alone (Table 1). Disease recurrence is signified by the development of enhancing tumor nodules within the treated hypoattenuating tumor[27]. A summary of key imaging features is highlighted in Table 2.

MRI

MRI serves as an imaging adjunct, especially for young patients for whom repeated ionizing radiation exposure should be minimized, for evaluating liver metastases, and for evaluating rectal tumors. MRI has been found to be superior in characterizing treated liver metastases compared to CT, especially with identifying foci of hypervascularity[32]. Conversely, MRI is less helpful in identifying mesenteric lesions due to the lack of oral contrast and respiratory gating[32]. Generally, the recommended MRI sequences include T1-weighted in and out of phase axial, T2-weighted coronal turbo spin echo, T2-weighted fat-saturated axial respiratory triggered turbo spin echo, and T1-weighted fat-saturated 3D volumetric acquisition in noncontrast, early arterial, portal venous, and hepatic venous phases[32].

MRI features of GISTs vary depending on the amount of hemorrhage, necrosis and cystic degeneration. Solid tumor components demonstrate low T1 signal, intermediate-to-high T2 signal, and enhancement with contrast[24]. The presence of intratumoral cystic change with low apparent diffusion coefficient (ADC) values are predictors of high malignant potential[33]. A negative correlation between mean ADC values and malignancy risk of GISTs has been demonstrated[33]. Upon treatment response, GIST metastases demonstrate increased T2 signal with increased cystic degeneration of solid tumoral components and increased ADC values[34]. With disease recurrence, new peripheral thickening and enhancement of cystic metastases can be seen[24].

PET-CT

18F-fluorodeoxyglucose (18F-FDG) PET-CT can aid in staging, detecting early response to treatment, and detecting early recurrence of GIST[34]. PET-CT can be helpful in distinguishing tumors from benign tissue given the expected increased glucose metabolism of viable tumor cells. PET-CT is more sensitive than CT in detecting treatment response due to detecting decreased 18F-FDG uptake, which is typically observed before a change in tumor size[35]. Such changes can be detected 24 h to 1 mo after therapy initiation[36]. For patients on imatinib mesylate, increased 18F-FDG uptake may signify treatment resistance or lack of medication compliance.

MANAGEMENT AND SURVEILLANCE

Surgical resection is the mainstay of treatment, especially for small-to-medium sized GISTs without metastasis. Obtaining preoperative biopsy is controversial due to the risk of tumoral hemorrhage and seeding; therefore, postoperative pathology is required for diagnosis[1]. During resection, the tumor should be handled carefully to avoid bleeding, rupture, and peritoneal seeding. Ideally, the tumor resection should include an intact pseudocapsule and negative microscopic margins. Follow-up imaging intervals depend on the GIST's risk group categorization: A very low-risk GIST is likely cured by surgery and does not require follow-up; a low-risk GIST may need annual CT or MRI follow-up for 5 years; an intermediate-risk GIST needs annual CT or MRI follow-up for 5 years with the first scan completed 6-8 mo after surgery; and a high-risk GIST should be followed every 6 mo for the first 5 years, then annually for the next 5 years[35].

Table 1 Modified computed tomography response evaluation criteria for gastrointestinal stromal tumors

Response	Definition
Complete response	Disappearance of all lesions; No new lesions
Partial response	A decrease in size[1] of ≥ 10% or decrease in tumor density (HU) ≥ 15% on CT; No new lesions; No obvious progression of nonmeasurable disease
Stable disease	Dose not meet criteria for complete response, partial response, or progressive disease; No symptomatic deterioration attributed to tumor progression
Progressive disease	An increase in tumor size of ≥ 10% and does not meet criteria of partial response by tumor density (HU) on CT; New lesions; New intratumoral nodules or increase in size of existing intratumoral nodules

[1]The sum of longest diameters of target lesions as defined in RECIST. CT: Computed tomography.

Table 2 Imaging features of gastrointestinal stromal tumors

	CT	MRI
Primary and metastatic GISTs	Small: Homogeneous mass; Large: Hypervascular, enhancing masses with heterogeneity due to hemorrhage, cystic degeneration, or necrosis	Depend on the amount of hemorrhage, necrosis and cystic degeneration; solid tumor components with low T1 signal, intermediate-to-high T2 signal, and enhancement; low mean ADC values may predict high malignancy potential
Treatment response	Homogeneously hypoattenuating mass with decreased enhancing tumor nodules and intratumoral vessels	Increased T2 signal, increased cystic degeneration of solid tumoral components, increased ADC values
Disease recurrence	Development of enhancing tumor nodules within the treated hypoattenuating tumor	New peripheral thickening and enhancement of cystic tumor

CT: Computed tomography; MRI: Magnetic resonance imaging; GISTs: Gastrointestinal stromal tumors; ADC: Apparent diffusion coefficient.

Prior to 2000, cytotoxic chemotherapy had not been found to be clinically effective in the management of GISTs[1]. However, following the Food and Drug Administration approval of imatinib mesylate for treating metastatic and locally advanced KIT-positive GISTs in 2002, the management of GISTs has rapidly expanded. As previously stated, imatinib mesylate is a potent tyrosine kinase inhibitor that acts on enzymes including KIT, leukemia-specific BCR-ABL chimera, and PDGFRA. Imatinib mesylate can be utilized preoperatively to downsize the tumor and/or as adjuvant therapy to prevent recurrence. Preoperative imatinib mesylate can be utilized for large and poorly positioned GISTs that may be marginally resectable; imatinib mesylate has been shown to induce tumor cell apoptosis and decrease tumor glucose metabolism on PET-CT[37]. Postoperatively, 1-year of adjuvant imatinib mesylate has been shown to prolong overall survival, although the optimal duration of postoperative treatment is unclear[38]. For unresectable or metastatic GISTs, a phase II trial of imatinib mesylate therapy demonstrated 68% objective response rate regardless of imatinib dosage, and the median time to at least partial response was 2.7 mo[39]. The median survival of patients with metastatic GISTs improved significantly from 19 mo as reported by DeMatteo *et al*[40] in the pre-imatinib era to 73 mo with imatinib mesylate as reported by Menge *et al*[5]. If there is imaging evidence of disease progression despite using standard-dose imatinib mesylate, dose escalation of imatinib mesylate or utilization of sunitinib malate, a second line tyrosine kinase inhibitor, may be considered[1]. Sunitinib malate acts as a less specific tyrosine kinase inhibitor on KIT, PDGFR, vascular endothelial growth factor receptors, Fms-related tyrosine kinase 3, colony-stimulating factor-1R, and RET; as a result, sunitinib malate demonstrates activity against angiogenesis in addition to tumor activity related to receptor tyrosine kinase inhibition[1]. For imatinib and sunitinib-resistant GISTs, investigational therapeutic options include second generation tyrosine kinase inhibitors, such as sorafenib, dasatinib, and nilotinib[1]. Follow-up CT should be obtained within 3 mo of initiating imatinib mesylate with surveillance scans completed every 3 to 6 mo for unresectable or metastatic GISTs; the follow-up interval can be less frequent for low-risk GISTs[1].

With the advancement of these molecular-targeted therapies, multiple associated adverse effects have been demonstrated, and some of these may be identified on follow-up imaging. Fluid retention is commonly seen with imatinib mesylate and can manifest with pleural effusions, pericardial effusion, ascites, or extensive

subcutaneous edema[27]. Imatinib mesylate can be associated with intratumoral hemorrhage, especially in patients with large bulky tumors[1,27]. Tyrosine kinase inhibitors are associated with pancreatic findings. For instance, imatinib mesylate is associated with asymptomatic pancreatic swelling; a ≥ 22% increase in pancreatic volume has been shown to be a poor prognostic indicator[41]. Conversely, sunitinib malate is associated with pancreatic atrophy, and this finding is associated with poor prognosis[42]. Moreover, there are several case reports of pancreatitis associated with sunitinib malate and sorafenib therapy[43]. It is important to identify these adverse effects on imaging, which would allow for dose reduction, dose interruption, or drug discontinuation in the appropriate setting.

CONCLUSION

GISTs are the most common mesenchymal tumors of the GI tract and often arise from the stomach or small intestine. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases, KIT and/or PDGFRA, leading to tumorigenesis. Mutational analyses allow for predicting patient prognosis and treatment response. Clinical presentations can vary from no symptoms to GI bleeding, abdominal discomfort, and ulcer-related symptoms. While most GISTs are benign, some cases can be aggressive with metastases. Imaging plays a key role in the diagnosis and follow-up of these tumors. It is crucial to understand and identify the key imaging features of GISTs and their expected appearance upon treatment response and disease recurrence. Surgical resection is the preferred management, especially for small tumors, while tyrosine kinase inhibitors, including imatinib mesylate and sunitinib malate, can serve as a neoadjuvant and/or adjuvant therapies.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7134-7143

DOI: 10.3748/wjg.v27.i41.7134

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

ORIGINAL ARTICLE

Basic Study

Circulating tumor DNA dynamics analysis in a xenograft mouse model with esophageal squamous cell carcinoma

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Author contributions: Kinugasa H designed the manuscript; Terasawa H drafted the manuscript; Kinugasa H, Terasawa H, Yamamoto S, Hirai M, Tanaka T and Takaki A were responsible for experiments; Kinugasa H, Nouso K and Okada H supervised the manuscript preparation; all authors approved the final manuscript.

Institutional review board statement: This study was reviewed approved by [the ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital] Institutional Review Board.

Institutional animal care and use committee statement: Xenograft mouse experimental protocols were approved by the Ethical Committee of Okayama University Graduate School of Medicine,

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Abstract

BACKGROUND

It remains unclear which factors, such as tumor volume and tumor invasion, influence circulating tumor DNA (ctDNA), and the origin of ctDNA in liquid biopsy is always problematic. To use liquid biopsies clinically, it will be very important to address these questions.

AIM

To assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels by using a xenograft mouse after treatment, and to determine whether tumor volume and invasion are related to ctDNA levels.

METHODS

Tumor xenotransplants were established by inoculating BALB/c-nu/nu mice with the TE11 cell line. Groups of mice were injected with xenografts at two or four sites and sacrificed at the appropriate time point after xenotransplantation for ctDNA analysis. Analysis of ctDNA was performed by droplet digital PCR, using the human telomerase reverse transcriptase (hTERT) gene.

RESULTS

Mice given two-site xenografts were sacrificed for ctDNA at week 4 and week 8. No hTERT was detected at week 4, but it was detected at week 8. However, in four-site xenograft mice, hTERT was detected both at week 4 and week 6. These experiments revealed that both tumor invasion and tumor volume were asso-

Dentistry and Pharmaceutical Sciences (OKU-2019276).

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Data sharing statement: No additional data are available.

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

Supported by JSPS KAKENHI (19k17433).

Country/Territory of origin: Japan

Specialty type: Gastroenterology and Hepatology

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

Received: May 14, 2021
Peer-review started: May 14, 2021
First decision: July 14, 2021
Revised: July 21, 2021
Accepted: August 30, 2021
Article in press: August 30, 2021
Published online: November 7, 2021

P-Reviewer: Norčič G
S-Editor: Wang LL
L-Editor: A

ciated with the detection of ctDNA. In resection experiments, hTERT was detected at resection, but had decreased by 6 h, and was no longer detected 1 and 3 d after resection.

CONCLUSION
We clarified the origin and dynamics of ctDNA, showing that tumor volume is an important factor. We also found that when the tumor was completely resected, ctDNA was absent after one or more days.

Key Words: Liquid biopsy; Circulating tumor DNA; Xenograft; Esophageal squamous cell carcinoma; Dynamics of circulating tumor DNA

Core Tip: We clarified the origin and dynamics of circulating tumor DNA (ctDNA), showing that not only tumor invasion but also tumor volume was an important factor. The possibility of detecting ctDNA in early-stage cancers with shallow depth was demonstrated. Also, ctDNA could be measured at 1 d after tumor resection to evaluate the residuals, and the half-life of ctDNA was estimated to be 1.8-3.2 h.

Citation: Terasawa H, Kinugasa H, Nouso K, Yamamoto S, Hirai M, Tanaka T, Takaki A, Okada H. Circulating tumor DNA dynamics analysis in a xenograft mouse model with esophageal squamous cell carcinoma. *World J Gastroenterol* 2021; 27(41): 7134-7143
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7134.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7134

INTRODUCTION

Liquid biopsy, a molecular biological diagnostic method for blood and body fluids, has progressed dramatically in recent years. Circulating tumor DNA (ctDNA), one of the targets of liquid biopsy, is expected to be a useful method for screening and detection of cancer, monitoring therapy, prediction of prognosis, and personalized medicine[1-3]. Therefore, in addition to direct biopsy, which is the basis of conventional cancer diagnosis, a hybrid method, which includes non-invasive liquid biopsy, is becoming the mainstream.

Cell-free DNA (cfDNA), which includes ctDNA, is derived from apoptotic or necrotic cells[4,5]. Theoretically, it could be applied regardless of the stage. However, reports of its usefulness for early stages of cancer are controversial. Bettegowda *et al*[6] revealed that the rate of ctDNA detection is generally high in advanced stages of cancer, but ctDNA levels are generally lower in early stages of cancer. On the other hand, some reports indicated that ctDNA was useful for detecting early-stage cancers [6-9]. It remains unclear which factors, such as tumor volume and tumor invasion, influence ctDNA, and the origin of ctDNA in liquid biopsy is always problematic. To use liquid biopsies clinically, it will be very important to address these questions.

In this study, we used a xenograft mouse model to assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels after treatment, and to determine whether tumor volume and invasion are related to ctDNA levels.

MATERIALS AND METHODS

Cell Line

The human esophageal squamous cell carcinoma cell line TE11 was used because we established an experimental system for TE11 previously[10] and used it to show that liquid biopsy is useful in esophageal cancer cells as well as other gastrointestinal cancers. Cells were grown in RPMI 1640 (Thermo Fisher Scientific, Tokyo, Japan) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, Tokyo, Japan) at 37.0 °C in a 5% CO_2 atmosphere. Appropriate passages were made

P-Editor: Yuan YY

such that confluency did not exceed 70% prior to xenotransplantation. A Countess Automated Cell Counter (Thermo Fisher Scientific, Tokyo, Japan) was used to count cells, and 0.2% Trypan blue dye was used to exclude dead cells.

Xenograft mouse model

Xenograft mouse experimental protocols were approved by the Ethical Committee of Okayama University (OKU-2019276). Six-week-old female nude mice (BALB/c-nu/nu) (Charles River Laboratories, Japan) were used. Mice were raised in the animal facility of Okayama University and given food and water. The physical conditions of the mice, including the presence or absence of body movement or the availability of food and drink, were monitored daily. Mice were euthanized with isoflurane if mice stopped moving or eating.

Tumor xenotransplants were established in mice by inoculation in the shoulders or flanks with 1 × 10^6 TE11 cells suspended in 50 μL medium plus 50 μL Matrigel (Corning Product No. 356234). Inoculation was performed at two sites (*i.e.*, both shoulders, two-site xenograft mouse group, 28 mice) or at four sites (*i.e.*, both shoulders and both flanks, four-site xenograft mouse group, 28 mice) in order to determine the effect of tumor volume as well as the degree of invasion (Figure 1).

Tumor formation was confirmed in all xenograft mice; although, the changes in size varied. Differences in tumor volume were evaluated over time. Two-site and four-site xenograft mouse groups were sacrificed for ctDNA analysis at the appropriate time point after xenotransplantation. To minimize the effects of differences in tumor size, four mice were used for each ctDNA time point analysis.

A sample size calculation using power analysis determined 24 mice were needed in xenograft experiments and 32 mice were needed in resection experiments.

Xenograft experiments

Twelve mice received two-site xenografts, and 12 received four-site xenografts. Tumor size was measured every week after xenotransplantation, and ctDNA was evaluated at two time points: 4 wk and 8 wk after xenotransplantation (Figure 1).

Resection experiments

Sixteen mice received two-site xenografts, and 16 mice received four-site xenografts. All tumors were resected at week 7 after xenotransplantation in the two-site xenograft group or at week 5 in the four-site xenograft group. cfDNA and ctDNA were evaluated 6 h, 1 d, and 3 d after resection, or simultaneously with resection in the controls (Figure 1).

Blood and tumor tissue sample collection

For ctDNA analysis, whole blood was collected in BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), and processed within 1 h after collection. The samples were centrifuged at 3000 × g at 4 °C to separate plasma from peripheral blood cells, and stored at -80 °C. DNA was extracted from 1000 μL of blood and the final solution was 25 μL of DNA. Plasma ctDNA was extracted (25 μl) with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. At sacrifice, tumors were collected and divided into two fragments. One tumor fragment was snap-frozen in liquid nitrogen and used for preparation of genomic DNA. The other fragment was formalin-fixed and paraffin-embedded for histopathological diagnosis, morphological evaluation after hematoxylin/eosin staining, and immunohistochemistry. Four slides were made from the largest diameter section, where it was easy to obtain information on invasion.

Telomerase reverse transcriptase assay

The wild-type telomerase reverse transcriptase (TERT) gene was analyzed by a mouse TERT (mTERT) assay (Thermo Fisher Scientific, Tokyo, Japan) or human TERT (hTERT) assay (Bio-Rad Laboratories, Hercules, CA, United States of America) to take advantage of the differences between mTERT and hTERT genes. The verification experiments using a droplet digital PCR (QX200 system; Bio-Rad Laboratories, Hercules, CA, United States of America) was performed.

Droplet digital polymerase chain reaction and data analysis

To evaluate ctDNA, hTERT was detected *via* droplet digital polymerase chain reaction (PCR) according to the following protocol. DNA eluent (5 μL) from plasma was combined with Droplet PCR Supermix (10 μL; Bio-Rad Laboratories, Hercules, CA, United States of America), primer/probe mixture (1 μL), 5M Betaine (2 μL), 80

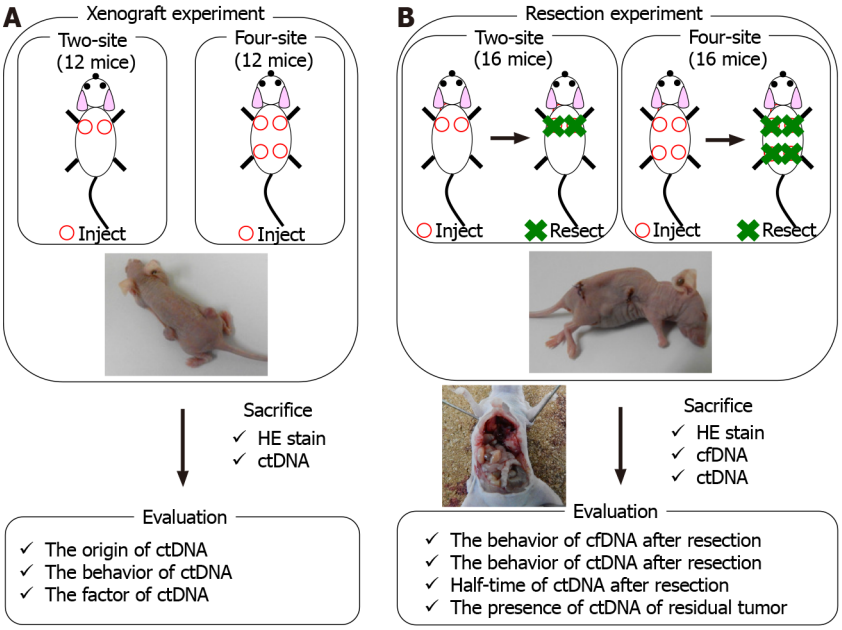

Figure 1 Xenograft mouse model with TE11 cell. A: In the xenograft experiment, groups of 12 mice each were given two-site xenografts or four-site xenografts; B: In the resection experiment, groups of 16 mice each were given two- or four-site xenografts. All tumors were resected at week 7 after xenotransplantation in two-site xenograft mice, or at week 5 in for-site xenograft mice.

mmol/L EDTA (0.25 μL), CviQl enzyme (0.25 μL), and sterile DNase- and RNase-free water (3.5 μL). The mixture (22 μL) was added to Droplet Generation Oil (70 μL; Bio-Rad Laboratories, Hercules, CA, United States of America) to produce droplets. Thermal cycling of the emulsion was as follows: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of 96 °C for 30 s and 62 °C for 1 min. After a final enzyme deactivation step of 98 °C for 10 min, the reaction mixtures were analyzed using a droplet reader (Bio-Rad Laboratories, Hercules, CA, United States of America). For quantification, the fluorescence signal was acquired with QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, United States of America). We set the threshold fluorescence intensity at 7500 (mTERT) or 2000 (hTERT), according to positive and negative controls in this study, i.e., plasma and tissue of healthy human, control mouse, or TE11 cell line.

Statistical analysis

We used JMP version 14.0 (SAS Institute, Cary, NC, United States of America) for statistical analysis and set the threshold of significance at $P < 0.05$. Continuous data were analyzed using the non-parametric Wilcoxon test, and categorical data were analyzed using a Chi-squared test.

RESULTS

Verification experiments

In verification experiments using a droplet digital PCR (QX200 system; Bio-Rad Laboratories, Hercules, CA, United States of America), we confirmed that the mTERT gene was detected in tissue and plasma of control mice, but not in TE11 genomic DNA, whereas the hTERT gene was detected in TE11 genomic DNA, but not in the tissue or plasma of control mice (Figure 2).

Xenograft experiments

Xenograft experiments were designed to reveal the origin of ctDNA and factors contributing to ctDNA increase. Average tumor sizes measured in the two-site xenograft group 1, 2, 3, 4, 5, 6, 7, and 8 wk after xenotransplantation were 1.8, 3.2, 4.6, 6.0, 6.8, 8.0, 8.5, and 12.5 mm, respectively. Two-site xenograft mice were sacrificed 4 or 8 wk after xenotransplantation to evaluate ctDNA. No hTERT was detected at week 4, but hTERT was detected at week 8 (Figure 3). These results indicated that ctDNA was associated with tumor growth.

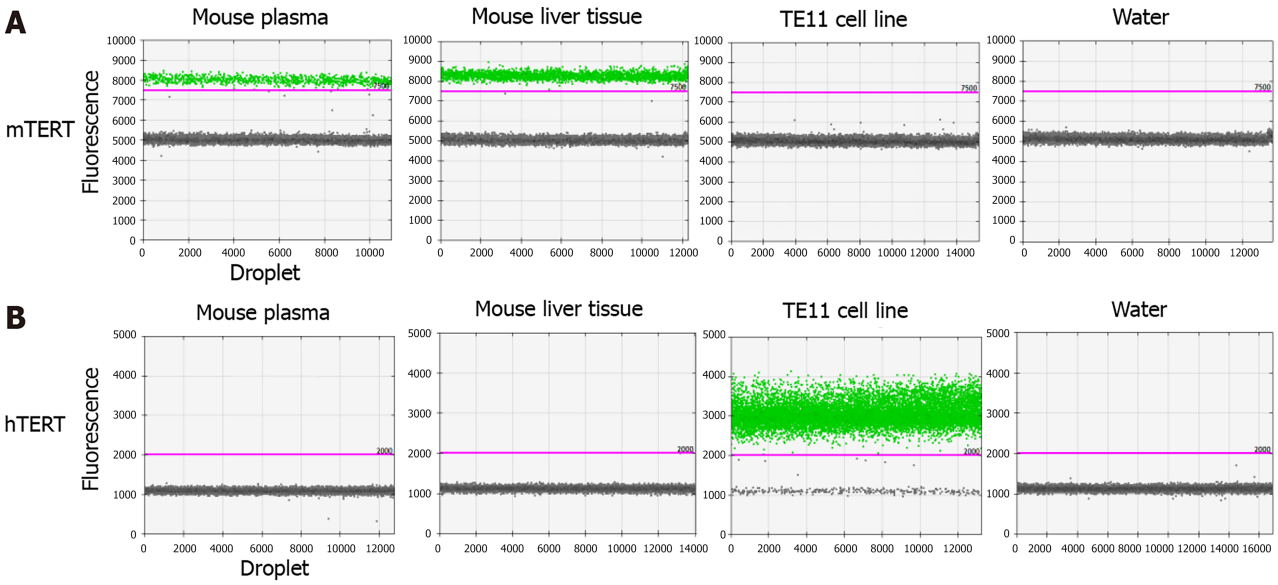

Figure 2 Telomerase reverse transcriptase assay by droplet digital polymerase chain reaction for mouse plasma, liver tissue, TE11 cell and water. The presence of mouse telomerase reverse transcriptase (mTERT) and human TERT (hTERT) forms of the wild type TERT was analyzed by droplet digital polymerase chain reaction. A: The assay correctly detected mTERT in mouse plasma and liver tissue; B: hTERT was detected in the TE11 cell line. Neither mTERT nor hTERT was detected in water.

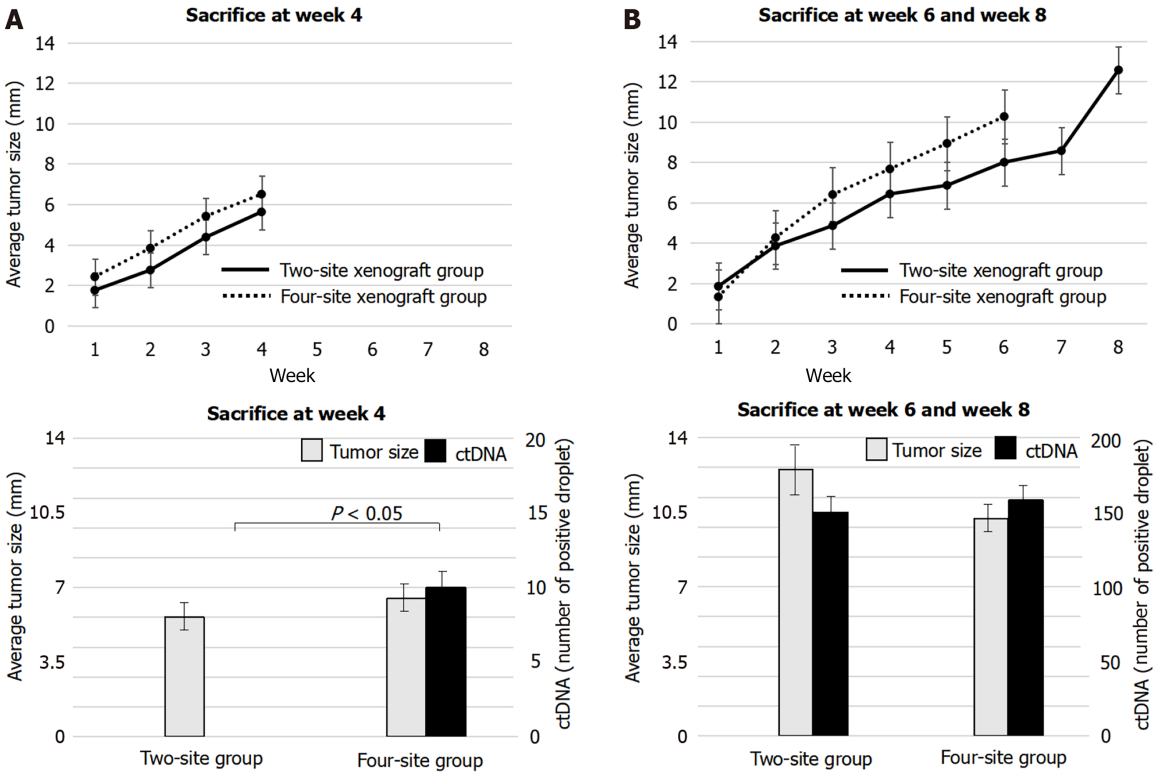

Figure 3 The dynamics of circulating tumor DNA in xenograft experiments. A: Two-site and four-site xenograft mice were sacrificed for circulating tumor DNA (ctDNA) at week 4. Human telomerase reverse transcriptase (hTERT) was detected only in four-site xenograft mice, not in two-site xenograft mice; B: In both two-site xenograft mice sacrificed for ctDNA at week 8 and four-site xenograft mice sacrificed at week 6, hTERT was detected.

In four-site xenograft mice, the average tumor sizes at week 1, 2, 3, 4, 5, and 6 after xenotransplantation were 1.8, 4.0, 5.9, 7.1, 8.9, and 10.2 mm. The 8 wk evaluation planned for this group was revised to occur at week 6, because the tumor in one mouse had grown rapidly to cause thoracic invasion, and it was unlikely to survive to week 8. Four-site xenograft mice were sacrificed for ctDNA at week 4 and week 6. hTERT was detected both at week 4 and at week 6 in this group (Figure 3). These results indicated that ctDNA was associated with tumor growth as well as those of

two-site xenograft mice. There were no other unexpected adverse events.

Histopathology of tumors at week 4 showed no invasion in either the two-site or four-site xenograft group, while tumors showed invasion into muscle both at week 8 in the two-site xenograft mice (P = 0.02) and at week 6 in the four-site xenograft mice (Figure 4; P = 0.03). These results indicated that ctDNA was associated with tumor invasion.

The rates of tumor size increase were similar between the two-site xenograft group and the four-site group. Interestingly, the two groups showed similar tumor diameters (P = 0.25) and invasion at week 4 (Figures 3 and 4), but a clear difference in the ctDNA detection rate (Figure 3; P = 0.02). These findings showed that not only invasion but also tumor volume might be related to the rate of ctDNA detection.

Resection experiments

Resection experiments were designed to clarify responses of ctDNA to tumor resection. Tumors in the two-site and four-site xenograft groups were resected when the diameter exceeded 10 mm. cfDNA and ctDNA were examined at sacrifice. In these resection experiments, two mice were excluded from the evaluation: one mouse with rapid tumor growth and a tendency toward paraplegia before resection, and another mouse with high invasion who died after tumor resection and before evaluation.

In two-site xenograft mice, tumor resection was performed at week 7. The average tumor size in the control group was 10.3 mm at the time of resection, and the average tumor sizes measured 6 h, 1 d, or 3 d at the time of resection were 10.1, 10.3, and 10.2 mm, respectively (P = 0.98). We detected hTERT at resection (control), but hTERT had decreased by 6 h, and was undetectable 1 d or 3 d after resection (Figure 5). The control cfDNA concentration was 1.1 μg/mL at the time of resection, and was 1.2, 1.3, and 1.4 μg/mL measured 6 h, 1 d, and 3 d after resection. Pathological autopsy confirmed the absence of macroscopic residual tumor at each evaluation in this experiment. Using data for the number of positive droplets measured 0 and 6 h after tumor resection in the two-site xenograft resection experiment, the half-life of ctDNA may be calculated from y = 155e - 0.368x. In our study, the half-life of ctDNA was estimated to be 1.8–3.2 h (Figure 6).

In four-site xenograft mice, tumor resection was performed at week 5. The average tumor size in the control group was 9.7 mm at the time of resection, while average tumor sizes measured 6 h, 1 d, or 3 d at the time of resection were 11.4, 10.6, and 10.2 mm, respectively (P = 0.34). In this experiment, hTERT was detected in all groups (Figure 5). The control cfDNA concentration was 1.3 μg/mL at resection and 1.2, 1.5, and 1.7 μg/mL measured 6 h, 1 d, and 3 d, respectively, after resection. Here, pathological autopsy revealed the presence of macroscopic residual tumor at each resection evaluation, with tumor invasion and intrathoracic metastasis in all mice. This experiment revealed that residual ctDNA was associated with incomplete resection and metastasis.

DISCUSSION

Because the TERT gene sequence differs between human and mouse, we were able to determine the origin and dynamics of ctDNA in a xenograft mouse model in which human-derived esophageal cancer cells were injected into the epidermis of mice. This model allowed assessment of ctDNA, which has traditionally been difficult to assess in the human body, due to tumor heterogeneity and the influence of other cells. In our experiment, tumor volume was involved in increases or decreases in ctDNA. In addition, if ctDNA was present over 1 d after resection, the presence of residual tumor is suspected.

Although studies of liquid biopsy using xenograft mouse model have been reported mainly in circulating tumor cells [11], we focused on ctDNA in this study. This model seems to be an ideal method because clinical samples contain a variety of cellular information as well as limitations such as ethical issues. Our report is also extremely valuable in providing direct evidence of the origin of plasma ctDNA, which we assessed in the xenograft mouse model by assaying mTERT and hTERT. Based on this ctDNA confirmation, other factors affecting ctDNA dynamics were examined. In our xenograft experiments, the average tumor sizes 4 wk after two-site and four-site xenografts were very similar (5.6 mm and 6.5 mm), and histology showed similar degrees of tumor invasion (Figure 4). However, ctDNA was detected in four-site xenograft mice but not in two-site xenograft mice. These findings revealed that tumor volume may influence ctDNA detection. In both groups, increasing ctDNA with tumor

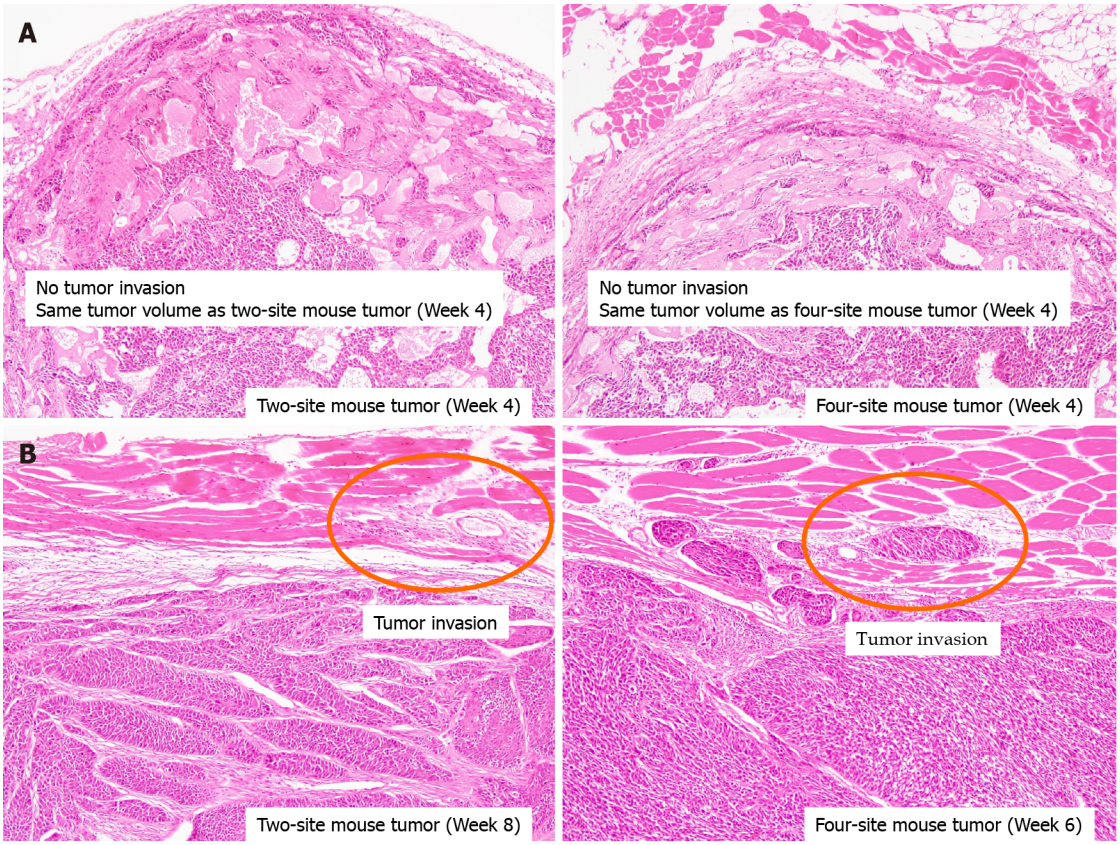

Figure 4 Histopathology of xenograft mouse with TE11. A: Histopathology showed absence of invasion in tumors at week 4 in mice with two-site or four-site xenografts; B: Muscle invasions were observed in tumors at week 8 in two-site xenograft mice, and at week 6 in four-site xenograft mice.

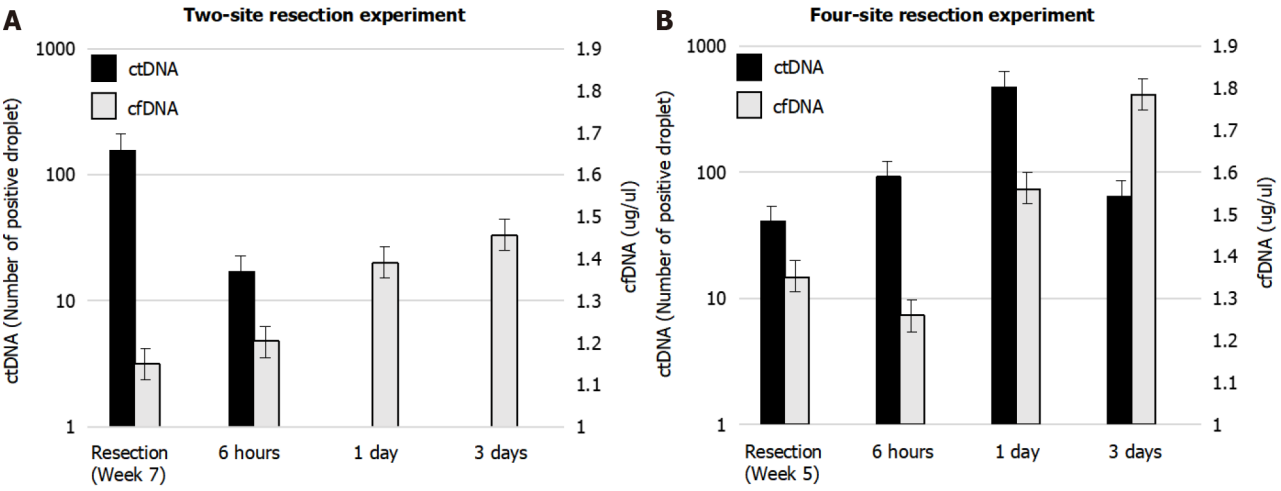

Figure 5 The dynamics of circulating tumor DNA in resection experiments. A: Tumor resection was performed when tumor diameter xenograft mice exceeded 10 mm, at week 7 in two-site xenograft mice, or at week 5 in four-site xenograft mice. Human telomerase reverse transcriptase (hTERT) circulating tumor DNA (ctDNA) was detected at resection (control), had decreased by 6 h, and was undetectable 1 d and 3 d after resection; B: On the other hand, in four-site xenograft mice, hTERT (ctDNA) was detected at resection (control), 6 h, 1 d, and 3 d after resection. cfDNA: Cell-free DNA.

progression was confirmed at week 8 and week 6. The amount and detection rate of ctDNA correlated with tumor progression in a previous clinical study[6], and our results may support that finding. Although detailed studies on the association between tumor volume or invasion and ctDNA have not been conducted, ctDNA is assumed to be detectable in early cancer once the tumor reaches a certain volume.

The presence of ctDNA after surgical resection is observed in clinical samples from cancer patients, and evaluation during the perioperative period is useful for prediction of prognosis[12-14]. Detection of ctDNA after surgery suggests some residual disease [15]. However, these clinical studies may inevitably detect circulating DNA from

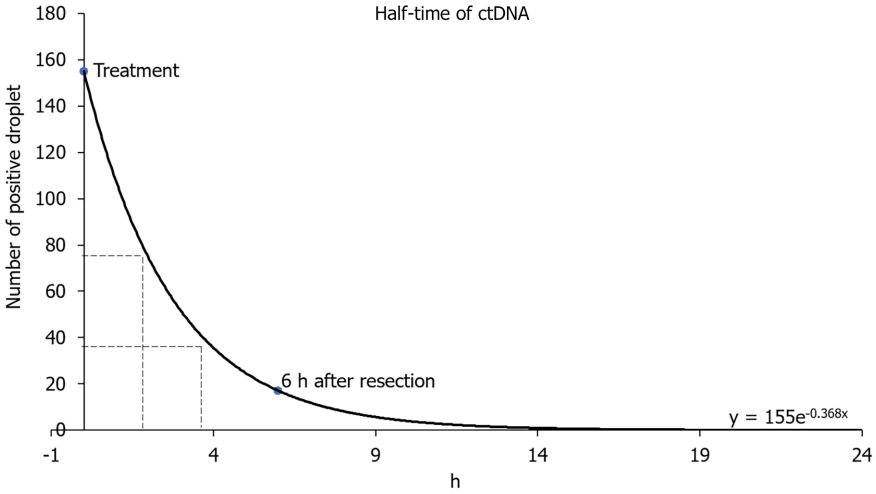

Figure 6 The half-life of circulating tumor DNA in resection experiments. To estimate half-life of circulating tumor DNA in two-site xenograft mice in the resection experiment, the number of positive droplets *vs* time after resection was fit to an exponential curve, y = 155e - 0.368x.

sources other than tumor cells, and there have been no reports to indicate when liquid biopsy should be used. Regarding this point, our resection experiments demonstrated reduced hTERT at 6 h and its absence 1 to 3 d after resection, indicating that ctDNA evaluation 1 d after resection might be useful to detect residual tumor in clinical cases. These experiments also revealed tumor volume was involved in the increase or decrease of ctDNA and that post-tumor resection evaluation requires an interval of one day or more after resection.

The half-life of ctDNA was reported as approximately 2 h in one study[16], but another study found the half-life to be 16 min[17]. The metabolism and excretion of cfDNA is affected by liver and kidney function[18], and ctDNA levels might be regulated by the same mechanism. In our study, we estimated the half-life of ctDNA 1.8–3.2 h, based on ctDNA levels measured 0 and 6 h after resection (Figure 6), which was similar to data from previous reports. Assuming a half-life of 3 h, ctDNA will decline by a factor of 28 after 1 d, and postoperative assessment of ctDNA should be evaluated after 1 d.

cfDNA is derived from apoptotic or necrotic cells[19,20], and its increase is considered to be caused by surgical manipulation, or perhaps cytokines, or cell proliferation in response to invasive therapy. Our results are consistent with these reports, indicating ctDNA decreased after complete resection, while cfDNA increased after resection.

Carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCC-Ag) are biomarkers for esophageal cancer. However, the usefulness of these biomarkers in the early diagnosis of esophageal cancer has not been established. Currently, upper endoscopy is the most useful examination to pick up early-stage esophageal cancer. However, since this examination is invasive, the development of non-invasive methods such as liquid biopsy is eagerly awaited. The combination of this method with conventional methods may lead to the next generation of diagnosis.

Our study had the following limitations. First, the artificial implantation of tumor under the skin in the xenograft model differs from the physiology of actual tumor development. Second, individual mice exhibit differences in tumor growth rates, and therefore, our comparative analyses in the present study used the average values for four animals per group. Third, regarding residual tumor, although pathological autopsies were performed on all mice, complete certainty with respect to residual disease is impossible. Forth, TE11 cell line alone is not necessarily sufficient, other cell lines should be examined as well. Fifth, comparison with conventional biomarkers such as CEA and SCC-Ag needs to be shown.

CONCLUSION

We clarified the origin and dynamics of ctDNA in the xenograft mouse model. We showed that tumor volume was an important factor in ctDNA, and that if the tumor volume was sufficiently large, ctDNA can be detected even in early-stage or superficial

cancers. We also found that, upon complete tumor resection, ctDNA disappeared after at least 1 d, unless residual tumor remained. These findings may indicate future clinical uses of liquid biopsy.

ARTICLE HIGHLIGHTS

Research background

The clinical application of liquid biopsy is becoming more widespread. However, it remains unclear which factors, such as tumor volume and tumor invasion, influence circulating tumor DNA (ctDNA), and the origin of ctDNA in liquid biopsy is always problematic.

Research motivation

It will be very important to address the origin and dynamics of ctDNA for further clinical application of liquid biopsy.

Research objectives

A xenograft mouse model was used to assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels after treatment, and determine whether tumor volume and invasion are related to ctDNA levels.

Research methods

Tumor xenotransplants were established by inoculating BALB/c-nu/nu mice with the TE11 cell line (esophageal squamous cell carcinoma). Analysis of ctDNA was performed by droplet digital polymerase chain reaction, using the human telomerase reverse transcriptase (hTERT) gene.

Research results

Mice given two-site xenografts were sacrificed for ctDNA at week 4 and week 8. No hTERT was detected at week 4, but it was detected at week 8. However, in four-site xenograft mice, hTERT was detected both at week 4 and week 6. These experiments revealed that both tumor invasion and tumor volume were associated with the detection of ctDNA. In resection experiments, hTERT was detected at resection, but had decreased by 6 h, and was no longer detected 1 and 3 d after resection. The half-life of ctDNA was estimated to be 1.8-3.2 h.

Research conclusions

We clarified the origin and dynamics of ctDNA, showing that not only tumor invasion but also tumor volume was an important factor. Also, ctDNA could be measured at 1 d after tumor resection to evaluate the residuals.

Research perspectives

In the clinical application of liquid biopsy, early-stage cancers could be targeted, and post-treatment monitoring should be performed 1 d after treatment.

ACKNOWLEDGEMENTS

We thank all staff in the animal facility of Okayama University, Shinya Ohashi (MD, PhD; Department of Therapeutic Oncology, Kyoto University) and Hiroshi Nakagawa (MD, PhD; Department of Medicine, Columbia University).

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World J Gastroenterol 2021 November 7; 27(41): 7144-7158

DOI: 10.3748/wjg.v27.i41.7144

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

ORIGINAL ARTICLE

Basic Study

Cross-sectional evaluation of circulating hepatitis B virus RNA and DNA: Different quasispecies?

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Abstract

BACKGROUND

interpreted the results; Garcia-Garcia S and Tabernero D drafted the manuscript; Ferrer-Costa R, Rando-Segura A, Trejo-Zahínos J, Casis E, Riveiro-Barciela M, Buti M and Rodríguez-Frías F critically reviewed the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of the Vall d'Hebron Research Institute (PR(AG)146/2020) and all patients provided written informed consent for participation.

Conflict-of-interest statement: Authors have no conflict of interest for this manuscript.

Data sharing statement: Next-generation sequencing data were submitted to the GenBank SRA database (BioProject accession number PRJNA625435).

Supported by Instituto de Salud Carlos III, No. PI18/01436; and European Regional Development Fund (ERDF).

Country/Territory of origin: Spain

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

Received: July 8, 2021

Different forms of pregenomic and other hepatitis B virus (HBV) RNA have been detected in patients' sera. These circulating HBV-RNAs may be useful for monitoring covalently closed circular DNA activity, and predicting hepatitis B e-antigen seroconversion or viral rebound after nucleos(t)ide analog cessation. Data on serum HBV-RNA quasispecies, however, is scarce. It is therefore important to develop methodologies to thoroughly analyze this quasispecies, ensuring the elimination of any residual HBV-DNA. Studying circulating HBV-RNA quasispecies may facilitate achieving functional cure of HBV infection.

AIM

To establish a next-generation sequencing (NGS) methodology for analyzing serum HBV-RNA and comparing it with DNA quasispecies.

METHODS

Thirteen untreated chronic hepatitis B patients, showing different HBV-genotypes and degrees of severity of liver disease were enrolled in the study and a serum sample with HBV-DNA > 5 Log_{10} IU/mL and HBV-RNA > 4 Log_{10} copies/mL was taken from each patient. HBV-RNA was treated with DNAse I to remove any residual DNA, and the region between nucleotides (nt) 1255-1611 was amplified using a 3-nested polymerase chain reaction protocol, and analyzed with NGS. Variability/conservation and complexity was compared between HBV-DNA and RNA quasispecies.

RESULTS

No HBV-DNA contamination was detected in cDNA samples from HBV-RNA quasispecies. HBV quasispecies complexity showed heterogeneous behavior among patients. The Rare Haplotype Load at 1% was greater in DNA than in RNA quasispecies, with no statistically significant differences (P = 0.1641). Regarding conservation, information content was equal in RNA and DNA quasispecies in most nt positions [218/357 (61.06%)]. In 102 of the remaining 139 (73.38%), HBV-RNA showed slightly higher variability. Sliding window analysis identified 4 hyper-conserved sequence fragments in each quasispecies, 3 of them coincided between the 2 quasispecies: nts 1258-1286, 1545-1573 and 1575-1604. The 2 hyper-variable sequence fragments also coincided: nts 1311-1344 and 1461-1485. Sequences between nts 1519-1543 and 1559-1587 were only hyper-conserved in HBV-DNA and RNA, respectively.

CONCLUSION

Our methodology allowed analyzing HBV-RNA quasispecies complexity and conservation without interference from HBV-DNA. Thanks to this, we have been able to compare both quasispecies in the present study.

Key Words: Hepatitis B virus RNA; Hepatitis B X gene; Quasispecies; Next-generation sequencing; Quasispecies conservation; Quasispecies complexity

Core Tip: Hepatitis B virus (HBV) quasispecies composition and its evolution are related to liver disease progression. HBV-RNA in serum is potentially useful for analyzing viral quasispecies, even in patients with low levels of or suppressed serum HBV-DNA. Few studies have analyzed circulating HBV-RNA quasispecies, and similarities and differences with DNA quasispecies should be assessed. We used next-generation sequencing to analyze RNA quasispecies variability/conservation and complexity, without interference of HBV-DNA, in untreated chronic hepatitis B patients. Comparison of both quasispecies showed similar results between them. DNA quasispecies tended toward greater complexity, while RNA quasispecies tended toward higher variability.

Citation: Garcia-Garcia S, Cortese MF, Tabernero D, Gregori J, Vila M, Pacín B, Quer J, Casillas R, Castillo-Ribelles L, Ferrer-Costa R, Rando-Segura A, Trejo-Zahínos J, Pumarola T,

Peer-review started: July 8, 2021
First decision: August 19, 2021
Revised: August 20, 2021
Accepted: October 18, 2021
Article in press: October 18, 2021
Published online: November 7, 2021

P-Reviewer: Wu W
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

Casis E, Esteban R, Riveiro-Barciela M, Buti M, Rodríguez-Frías F. Cross-sectional evaluation of circulating hepatitis B virus RNA and DNA: Different quasispecies? *World J Gastroenterol* 2021; 27(41): 7144-7158
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7144.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7144

INTRODUCTION

The hepatitis B virus (HBV) is the etiological agent of hepatitis B and can cause both acute and chronic liver disease. In 2015, roughly 257 million people worldwide were estimated to suffer from chronic hepatitis B (CHB) infection, the major complications of which are cirrhosis and hepatocellular carcinoma[1].

The HBV genome is a 3.2 kb-long partially double-stranded relaxed circular DNA (rcDNA), with a complete minus-strand and an incomplete plus-strand. After cytoplasmic release of the viral nucleocapsid containing the rcDNA, it is actively transported into the nucleoplasm, where it will be repaired and converted to covalently closed circular DNA (cccDNA). This form of viral genome remains as an episomal minichromosome in the hepatocyte nuclei for their entire life and serves as the template for the transcription of viral messenger RNAs by the cellular RNA polymerase II. One of them, the pregenomic RNA (pgRNA) is encapsidated and then reverse-transcribed to generate new rcDNA (or double stranded linear DNA), which is released as new infective DNA-containing virions[2]. In addition, full and spliced pgRNA, as well as X open reading frame (*HBX)* transcripts have been detected in patients' serum[3]. Serum HBV pgRNA has been reported to be useful for monitoring cccDNA activity in hepatitis B e-antigen (HBeAg)-positive (+ve) entecavir-treated patients[4] and to be predictive of HBeAg seroconversion during polymerase inhibitor treatment[5-7]. In addition, its detection in the absence of detectable serum HBV DNA in patients receiving nuclos(t)ide analogs (NA) therapy may allow inference in cccDNA transcription and has been shown to be a predictive biomarker for viral rebound after treatment cessation[8].

In a single HBV infection, viral genomes are present as a complex mutant spectrum constituted by closely related, but not identical, viral populations (genetic variants), termed viral quasispecies[9]. Those variants can occur through numerous host-virus interactions during the viral replication cycle, encompassing not only cellular factors but also viral factors, essentially the error-prone viral polymerase[10]. Serum circulating HBV-RNA is potentially useful for analyzing viral quasispecies, even in patients with low levels of or suppressed serum HBV-DNA. However, unlike DNA quasispecies, HBV-RNA quasispecies have not been subjected to reverse transcription, which is the main source of variability in the HBV genome[11]. Therefore, both quasispecies may be different in terms of nucleic acid variability/conservation and genetic variant complexity. Few studies to date have analyzed circulating HBV-RNA quasispecies[4,6] and similarities and differences with DNA quasispecies should therefore be assessed in more detail. In addition, serum HBV-RNA has been reported to be genetically homogenous to intrahepatic HBV-RNA[4], the main target for targeted gene therapy, which may favor achieving functional cure of HBV infection, the ideal end point of therapy based on sustained hepatitis B virus surface antigen (HBsAg) loss[12]. In order to interfere with the synthesis of all the viral proteins, these therapies should be directed to hyper-conserved sequence targets shared by all the HBV mRNAs. *HBX* thus provides an ideal target, as its sequence is located at the 3' end of all HBV mRNAs[13,14]. Taking this into account, analysis of serum HBV-RNA quasispecies may be an especially useful tool when looking for hyper-conserved targets for this kind of antiviral therapy. To confirm this, it is necessary to establish a reliable methodology to thoroughly analyze serum HBV-RNA quasispecies.

Taking this in mind, the aim of this study was to establish a methodology, based on next-generation sequencing (NGS), for thoroughly analyzing serum HBV-RNA quasispecies without interference from circulating HBV-DNA. This methodology has been tested in a group of untreated CHB patients to gain a preliminary insight into comparison of genetic variability/conservation and complexity between circulating HBV-RNA and DNA quasispecies.

MATERIALS AND METHODS

Patients and samples

The study was approved by the Ethics Committee of the Vall d'Hebron Research Institute (PR(AG)146/2020) and all patients provided written informed consent for participation. No animals were used.

In this cross-sectional study, we included a serum sample from 13 well-characterized untreated CHB patients attending the outpatient clinic of Vall d'Hebron University Hospital (Barcelona, Spain). All samples were selected taking into account HBV-DNA levels > 5 Log_{10} IU/mL and HBV-RNA levels > 4 Log_{10} copies/mL to ensure sufficient levels of both HBV-DNA and RNA to study their quasispecies. In addition, heterogeneity in terms of HBV genotypes and degrees of severity of liver disease were also taken into account in patient selection, in order to obtain an overall picture of differences between HBV-DNA and RNA quasispecies. Exclusion criteria were positive testing for hepatitis D virus, hepatitis C virus, and human immunodeficiency virus.

Serological and virological determinations

HBV serological markers such as HBsAg, HBeAg, anti-HBe antibodies were tested using commercial chemiluminescent immunoassays on a COBAS 8000 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). HBV-DNA was quantified on a COBAS 6800 analyzer (Roche Diagnostics, Mannheim, Germany) with a lower detection limit of 10 IU/mL. HBV genotypes were determined as previously explained[13]. HBV-RNA was quantified using an in-house method. The standard RNA to create the standard curve for this quantification was obtained as follows: 1.1× HBV genome from pTRiEx1.1-HBV[15] was subcloned downstream of a T7 promoter in a pEF6/V5-His TOPO TA vector (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States) following the manufacturer's instructions, to ensure its *in vitro* transcription (pEF6-HBV). Once cloned, the correct orientation and insertion of HBV genome was analyzed by Sanger Sequencing, and pEF6-HBV plasmid was isolated from bacteria using the NucleoBond Xtra Midi Kit (Macherey-Nagel, Dueren, Germany). The pEF6-HBV plasmid was then linearized by NotI restriction enzyme digestion (New England Biolabs, Beverly, MA, United States), and used as template for *in vitro* transcription reaction using the MEGAscript T7 Transcription Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States), by adding the plasmid pEF6-HBV concentration of 0.5 μg/μL diluted in water. At the same time, the *in vitro* transcription of pTRI-Xef positive control DNA provided with the kit was also performed. The RNA resulting from transcription was then purified using the MEGAclear Transcription Clean-up Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States) following the manufacturer's instructions. A DNase I treatment (Life Technologies, Austin, TX, United States) was then carried out for 15 min at room temperature, followed by heat-inactivation at 65 °C for 10 min and adding 2 μL of 25 mmol/L EDTA solution to the reaction mixture, in order to remove the template DNA. The purity of DNAse I-treated RNA was checked by measuring the absorbance of 1 μL of the sample at 260 nm using the NanoDrop spectrophotometer (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States). The concentration of this RNA was quantified from 3 μL of the sample by Qubit fluorimeter using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States). The absence of DNA in DNAse I-treated RNA template was verified using the DNA Master PLUS HybProbe kit in a LightCycler 480 Instrument II system (Roche, Mannheim, Germany) using primers and probe specified in the Supplementary Table 1.

For absolute quantification analyses, the standard curve was defined using the quantified retrotranscribed RNA, taken to a concentration of 7.51 Log_{10} copies/mL, and serial 1:10 dilutions of this standard covering concentrations until 1.51 Log_{10} copies/mL. The points of the standard curve were defined as the mean Ct of a triplicate measurement of the original 7.51 Log_{10} copies/mL standard and its serial dilutions. The standard curve obtained was saved and imported as an external standard curve in each HBV-RNA quantification experiment. In those experiments, at least one dilution of standard RNA used to define one of the standard curve points must be included, in order to adjust the standard curve. Creation of the RNA standard curve and experiments to quantify HBV-RNA levels quantification were performed using one-step quantitative reverse transcription polymerase chain reaction (RT-qPCR) reaction, using the LightCycler 480 RNA Master Hydrolysis Probes kit (Roche, Mannheim, Germany). RT-qPCR program in the Light Cycler 480 instrument was

programmed as described in the Supplementary Table 1.

Amplification of HBV region of interest using NGS

In this study, we analyzed the region between nucleotides (nt) 1255 to 1611, located at the *HBX* 5′ end. In this region, we previously identified hyper-conserved sequence stretches in the circulating HBV-DNA quasispecies[13,14], which we aimed at analyzing at circulating HBV-RNA level.

For each serum sample, HBV-DNA was extracted from 200 μL of serum using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The region of interest was amplified through a 3-round polymerase chain reaction (PCR) protocol. The first-round PCR covered a 1338-bp region and was performed as previously described by our group[16] (Supplementary Table 1). The second-round and third-round PCRs, were also performed as previously detailed[13] (Supplementary Table 1). The second-round PCR amplified the region of interest between nt 1255 and 1611 of the HBV genome, yielding final products flanked by universal M13 sequences at both ends. In the third-round PCR, a specific pair of primers was used for each sample, consisting of the same M13 universal sequences (forward and reverse) at their 5′ ends and a multiplex identifier (MID) or barcode sequence at their 3′ ends. Each individual patient sample required a different MID. The PCR products obtained in this amplification, also known as amplicons, were visualized as single bands on a 1.5% agarose electrophoresis gel, stained with SybrSafe DNA gel Stain (Life Technologies, Carlsbad, CA, United States) with 1x TAE running buffer (Roche Diagnostics, Mannheim, Germany). PCR products were then purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicon quality was verified using the Agilent 2200 TapeStation System and D1000 ScreenTape kit (Agilent Technologies, Waldbronn, Germany). Purified DNA from each sample was quantified by means of fluorescence, using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, United States) adjusted to the same concentration, and pooled.

In the same serum samples, HBV-RNA was isolated from 140 μL serum using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To remove any residual DNA present, isolated HBV-RNA was subsequently treated with DNAse I (Life Technologies, Austin, TX, United States) for 15 min at room temperature, followed by heat-inactivation at 65 °C for 10 min and adding 25 mmol/L EDTA solution to the reaction mixture, in keeping with manufacturer's instructions. After DNAse I treatment, RNA samples were retrotranscribed into cDNA in 2 steps. The first step involves denaturation of HBV-RNA and Oligo(dT)$_{17}$ primer, which binds to polyA sequence, by incubating at 65 °C for 5 min and 20 °C for 5 min. The second is reverse transcription itself involving Accuscript enzyme (Stratagene, Agilent Technologies, Santa Clara, United States) and RNAse OUT (Thermo Fisher Scientific-Life Technologies, Austin, United States). A 60-min incubation at 42 °C for the first-strand synthesis reaction is required, followed by a short incubation time of 15 min at 70 °C.

At the same time, elimination of any residual HBV-DNA after DNAse I treatment was verified by means of qPCR, as described for the DNAse I-treated RNA obtained from in vitro transcription of pEF6-HBV vector, described above. This qPCR experiment included the DNAse I-treated RNA samples and their respective cDNA obtained after reverse transcription. After obtaining the cDNA, the region of interest between nt 1255 and 1611 was amplified through the same 3-round PCR protocol as HBV-DNA samples, with some modifications according to Agilent's PfuUltra II Fusion HS DNA polymerase insert for cDNA targets.

Finally, amplicon pools obtained from both HBV-DNA and RNA were sequenced using the MiSeq platform (Illumina, San Diego, CA, United States). Those purified pools were processed using the DNA library preparation kit Kapa Hyper Prep kit (Kapa Biosystems-Roche, Cape Town, South Africa), with which amplicon ends were repaired and A-tailed. Each pool was then indexed using the SeqCap Adapter Kit A (Roche Sequencing, Pleasanton, CA, United States). After index ligation pools had been purified with KAPA Pure Beads (Kapa Biosystems-Roche, Cape Town, South Africa) and re-amplified to increase indexed amplicon concentration using the KAPA HiFi HotStart ReadyMix PCR Kit (Roche Sequencing, Pleasanton, CA, United States). PCR products were repurified with another round of KAPA Pure Beads and quantified using LightCycler 480 Instrument II with the Kapa Library Quantification kit (Kapa Biosystems-Roche, Cape Town, South Africa). Each DNA pool was then adjusted to a 4 nmol/L concentration and appropriate volumes of each pool were added to a final pool of all DNA pools, the DNA concentration of which was verified using LightCycler 480 Instrument II (Kapa Library Quantification kit). Finally, this

final pool was sequenced using MiSeq Reagent kit v3 (2 × 300 bp mode with the 600 cycle kit) (Illumina, San Diego, CA, United States).

NGS data analyses: Quasispecies complexity and conservation

Bioinformatics processing and haplotype-centric data analysis pipeline were performed as previously described by our group[16].

The HBV-DNA and RNA quasispecies complexities were evaluated by computation of the Rare Haplotype Load (RHL), a new diversity index that measures enrichment of quasispecies in minority genomes below a given threshold[17]. RHL was calculated using the haplotypes (unique sequences covering the full amplicon observed on the set of sequences) resulting from the intersection of forward and reverse strands without filtering by a minimum abundance. In this study, RHL was computed as the sum of the relative frequencies of all haplotypes obtained from HBV-DNA and RNA quasispecies whose abundance is equal to or less than 1%, as previously described[17].

Sequence conservation at nt level was determined by calculating the information content (IC, bits) of each position in a multiple alignment of all intersected haplotypes obtained in frequencies > 0.25%, as previously explained[13]. Nt IC ranges from 0 bits to 2 bits for a given position, where 2 bits represents the maximum IC value (100% conservation, *i.e.*, no nt changes in a given position in any of all haplotypes analyzed). The mean IC was calculated in windows of 25 nt, moved in steps of 1 nt through nt 1255-1611 (sliding window analysis), the 5% of those windows with the highest mean IC values (the most conserved) and the 5% with the lowest mean IC values (the most variable) were selected. Afterwards, those consecutive windows were connected and their sequences were represented as sequence logos using the R language package motifStack[18]. In each position of the logos, letters representing the nts identified are stacked. The relative sizes of letters indicate the relative frequencies of the nt represented at a given position in the multiple alignments of the haplotypes, and the total height of each stack represents the IC of each position.

Statistical analysis

All statistical analyses were performed in R[19]. Qualitative parameters were expressed as number of cases and percentage. Quantitative parameters were expressed as the median value and interquartile range (IQR) or as mean ± SD, where appropriate. Statistical comparisons between HBV-RNA and DNA quasispecies complexity were performed using the Kruskal-Wallis test, *t* test and two-proportions z-test with Yates continuity correction where appropriate. *P* values < 0.05 were considered significant. The bioinformatics and statistical methods used in this study were reviewed by Dr. Josep Gregori from the Liver Disease Viral Hepatitis Laboratory of Vall d'Hebron Hospital (Barcelona, Spain) and the CIBERehd research group.

RESULTS

Patient clinical and virological characteristics

Clinical, virological, and serological parameters from the 13 patients at the time of obtention of samples included are summarized in Table 1. Most of those patients were HBeAg +ve men, showing clinical characteristics compatible with chronic hepatitis stage of CHB infection[12]. In terms of severity of liver disease, 8 patients were classified as patients with nonsignificant fibrosis [Ishak fibrosis stage (F) < 3 or transient elastography < 7-8.5 kPa], and 5 patients were classified as patients with significant fibrosis (liver biopsy F ≥ 3 and < 5 or transient elastography > 7-8.5 and < 11-14 kPa, *n* = 3) or cirrhosis (liver biopsy F5-6 or transient elastography > 11-14 kPa, *n* = 2), according to World Health Organization Guidelines[20].

Comparison of HBV-DNA and RNA quasispecies using NGS

The qPCR verification of residual HBV-DNA elimination from HBV-RNA isolations confirmed the absence of HBV-DNA from DNAse I-treated RNA samples, while showing the presence of cDNA after their reverse transcription. An illustrative example is shown in Figure 1. No HBV-DNA contamination was therefore detected in cDNA samples derived from HBV-RNA quasispecies.

After NGS using the MiSeq platform, Fastq files obtained were filtered by our bioinformatics procedure to obtain the set of haplotypes common to forward and reverse strands. Before subsequent filtering by abundance, those haplotypes represented a total of 4.35×10^6 sequence reads with a median of 2.45×10^5 reads/sample

Table 1 Clinical and viral characteristics of chronic hepatitis B patients enrolled in the study

	Total, *n* = 13
Age, yr, median (IQR)	41.35 (31.7-47.2)
Male, *n* (%)	11 (84.6)
Genotype, *n* (%)	
A	3 (23.1)
C	3 (23.1)
D	2 (15.4)
E	2 (15.4)
F	3 (23.1)
ALT, IU/L, median (IQR)	124 (66-160)
HBeAg +ve, *n* (%)	11 (84.6)
HBV DNA, Log_{10} IU/mL, median (IQR)	8 (8-8.43)
HBV RNA, Log_{10} copies/mL, median (IQR)	5.18 (4.94-6.02)
Fibrosis, *n* (%)[1]	
Nonsignificant	8 (61.64)
Significant	3 (23.08)
Cirrhosis, *n* (%)	2 (15.39)

[1]Nonsignificant fibrosis indicates Ishak fibrosis stage < 3, significant fibrosis F ≥ 3 and < 5, and cirrhosis F5-6. Nonsignificant fibrosis by noninvasive markers indicates liver stiffness < 7-8.5 kPa, significant fibrosis > 7-8.5 and < 11-14 kPa and cirrhosis > 11-14 kPa by transient elastography according to World Health Organization Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection criteria[20].
F: Fibrosis; IQR: Interquartile range; ALT: Alanine aminotransferase; HBeAg +ve: Hepatitis B e-antigen positive; HBsAg: Hepatitis B virus surface antigen; HBV: Hepatitis B virus.

(IQR, 1.90 × 10^5-3.19 × 10^5) for HBV-RNA and 8.27 × 10^4 reads/sample (IQR, 6.03 × 10^4-1.01 × 10^5) for HBV-DNA and were included in the RHL analysis. By setting the threshold of abundance as equal to or less than 1% HBV quasispecies complexity showed a heterogeneous behavior between patients, and although mean RHL was greater in DNA (45.45 ± 4.80) than in RNA (42.50 ± 5.63), the difference was not significant (P = 0.1641, t test) (Figure 2). Similarly, no statistically significant differences were observed when comparing HBV-DNA and RNA quasispecies in the patients with nonsignificant fibrosis (46.57 ± 4.79 for HBV-DNA and 43.88 ± 6.32 for HBV-RNA, P = 0.4642, Kruskal-Wallis test), and in the patients with significant fibrosis or cirrhosis (43.66 ± 4.72 for HBV-DNA and 40.29 ± 3.92 for HBV-RNA, P = 0.3055, Kruskal-Wallis test).

After RHL calculation, we proceeded to eliminate all haplotypes with abundances below 0.25%, obtaining 2.91 × 10^6 sequence reads with a median of 1.58 × 10^5 reads/sample (IQR, 1.29 × 10^5-2.28 × 10^5) for HBV-RNA and 5.66 × 10^4 reads/sample (IQR, 3.2 × 10^4-5.76 × 10^4) for HBV-DNA. These haplotypes were the basis for conservation analysis through IC calculation position by position in both HBV-RNA and DNA quasispecies (Figure 3). Differences in sequence conservation between the 2 quasispecies were determined by subtracting IC DNA values from IC RNA of 357 nt positions analyzed; between nt 1255-1611. In 218 (61.06%) of these positions, IC values of both HBV-RNA and DNA quasispecies were coincident, while in the remaining 139 (38.93%) nt positions differences between IC of both quasispecies ranged from -0.26 to 0.23, with IC DNA > IC RNA in most of them (102/139, 73.38%) (Figure 4A). These differences were also studied by sliding window analysis of the mean IC RNA-IC DNA values (calculated in windows of 25 nt positions displaced in steps of 1 position between them) confirming that, in general, the HBV-RNA quasispecies was slightly less conserved than the DNA quasispecies (Figure 4B). In keeping with this analysis, HBV-RNA displayed more positions with some variability (IC < 2 bits), 135/357 (38%), than HBV-DNA, 85/357 (24%) (P < 0.01, two-proportions z-test).

The high degree of similarity between IC values in both HBV-DNA and HBV-RNA quasispecies was also observed in the sliding window analysis of IC in the multiple

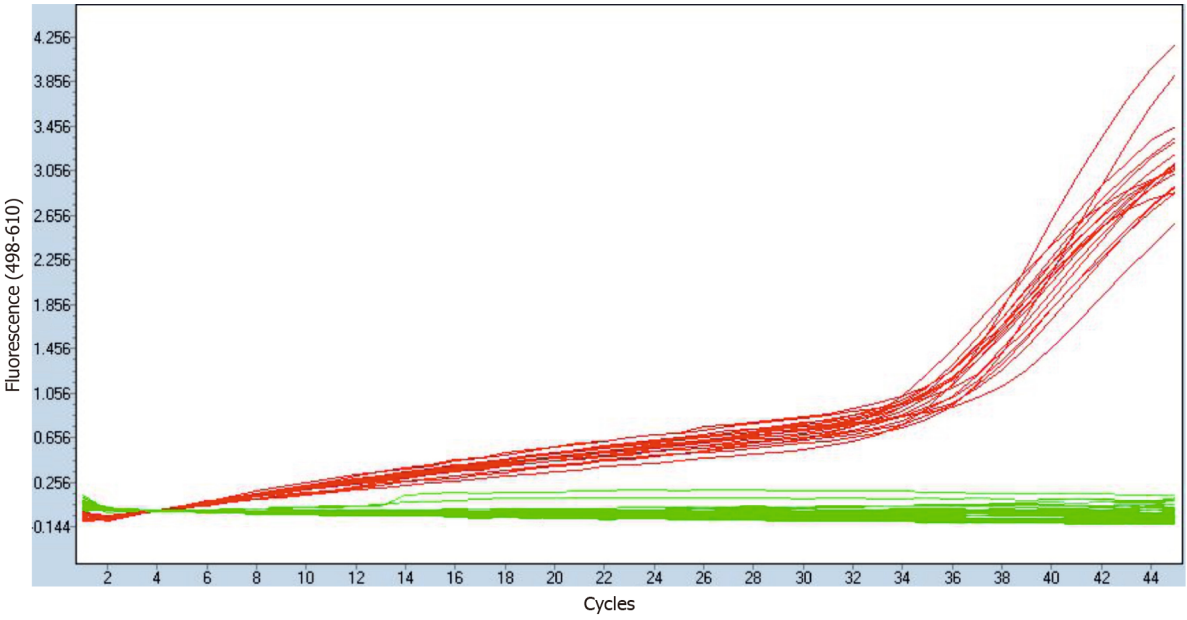

Figure 1 Quantitative polymerase chain reaction verification of residual hepatitis B virus-DNA elimination from hepatitis B virus-RNA isolations after DNAseI treatment. Fluorescence through quantitative polymerase chain reaction amplification samples is shown as green lines for DNAse I-treated RNA samples, and as red lines for their respective cDNA retrotranscribed samples.

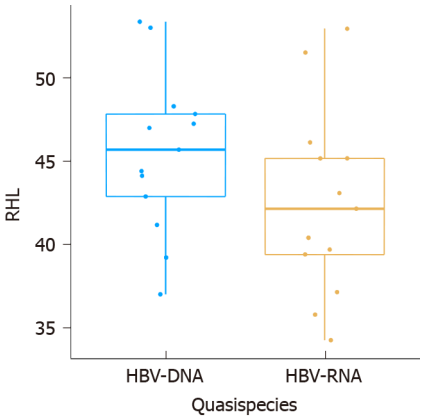

Figure 2 Comparison of mean hepatitis B virus quasispecies complexity. Hepatitis B virus (HBV) quasispecies complexity analyzed by Rare Haplotype Load of all 13 patients in HBV-DNA quasispecies (blue-framed boxes) and HBV-RNA quasispecies (yellow-framed boxes). Differences were statistically non-significant (P = 0.1641, t test). RHL: Rare Haplotype Load; HBV: Hepatitis B virus.

alignments of haplotypes obtained in both quasispecies. Concatenation of the 5% of windows with higher mean IC values (the most conserved), yielded 4 sequence logos in both quasispecies 3 (75%) of which coincided between both quasispecies: nts 1258-1286 (1283 in RNA quasispecies), 1545-1573 and 1575-1604 (Figure 5). In addition, sliding window analysis also showed the sequence stretch 1519-1543 to be hyper-conserved only in HBV-DNA quasispecies and 1559-1587 only in HBV-RNA quasispecies. Regarding the 5% of windows with lower IC values (the most variable), 2 sequence logos, which coincided in both quasispecies, were obtained: nts 1311-1344 and 1461-1485 (Figure 6). Thus, conservation was highly coincident between HBV-RNA and DNA quasispecies although, interestingly, HBV-RNA quasispecies showed slightly higher variability than HBV-DNA quasispecies.

DISCUSSION

Serum HBV RNA results from a mixture of different viral RNA species: Intact and spliced pgRNA, and truncated and polyA-free RNAs, with varying levels depending on the phase of HBV infection and antiviral treatment[21]. In addition, *HBX* transcripts

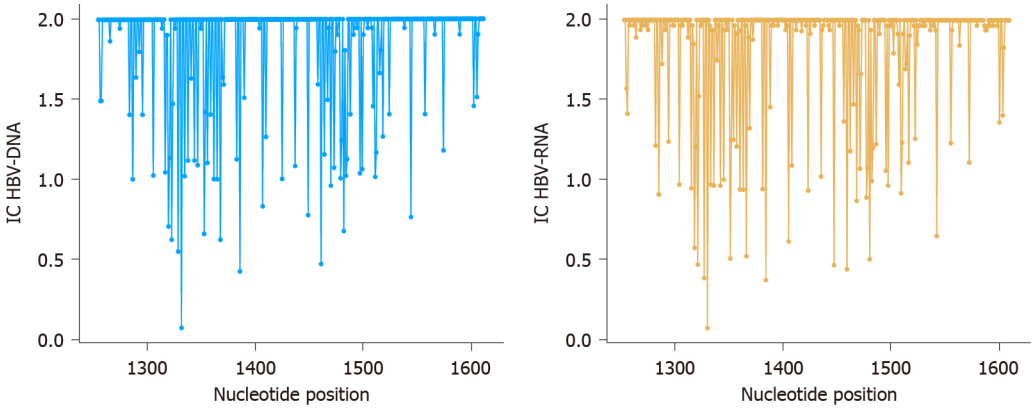

Figure 3 Conservation and variability of 357 nucleotide positions analyzed. Information content of nucleotide positions from 1255 to 1611 for hepatitis B virus (HBV)-DNA (blue lines) and HBV-RNA (orange lines) quasispecies. IC: Information content; HBV: Hepatitis B virus.

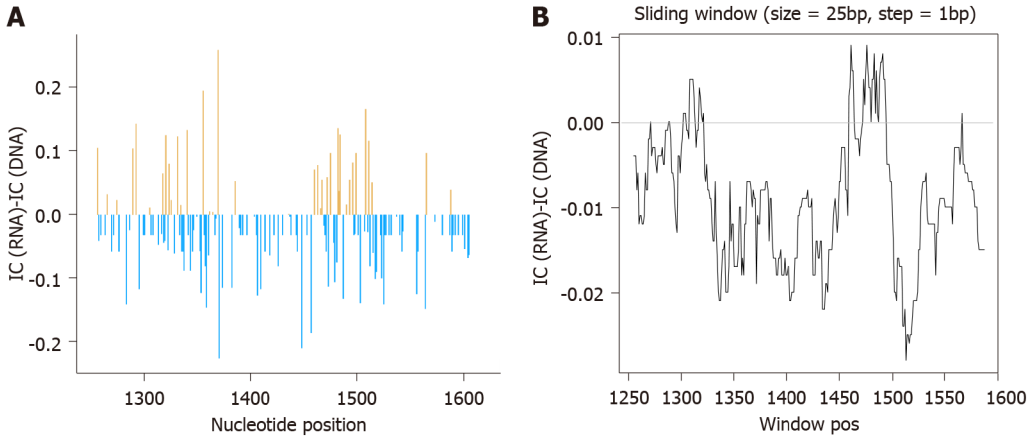

Figure 4 Differences between information content of hepatitis B virus-DNA and RNA quasispecies. A: Nucleotide positions in which information content (IC) hepatitis B virus (HBV)-RNA > HBV-DNA are depicted in orange while positions where IC HBV-DNA > HBV-RNA in blue; B: Sliding window analysis of the subtraction of mean IC RNA-IC DNA values, in windows of 25 nucleotide positions, displaced in steps of 1 position between them. IC: Information content.

have also been detected[3]. This serum HBV RNA has been suggested as a potential surrogate marker both to reflect intrahepatic cccDNA levels during the natural course of CHB infection[22,23] and under NA treatment[4], and to predict treatment outcome [4,21] such as HBeAg seroconversion[5-7]. During antiviral treatment, the kinetics of serum HBV-DNA and HBV-RNA seem to be dissociated. While serum HBV-DNA levels rapidly decline after starting treatment, HBV-RNA levels fall more slowly and the HBV-DNA/HBV-RNA ratio increases significantly. This allows inference in cccDNA transcription in the absence of detectable serum HBV DNA and may predict viral rebound after NA cessation[8]. In addition, the slower decline of serum HBV RNA levels may also make it possible to study circulating viral quasispecies, even when serum HBV-DNA levels are too low to do so. However, few studies to date have analyzed circulating HBV-RNA quasispecies[4,6] and little data is therefore available on their similarities and differences. Indeed, it is reasonable to think that both quasispecies may display significant differences between them, as they are subjected to different sources of genetic variability, due to the error-prone viral polymerase, contributing to an estimated error rate of approximately 1 per 10^4-10^5 bp for minus-strand DNA synthesis and approximately 1 per 3.6-15 × 10^4 bp for second-strand DNA synthesis[24]. Hence, reverse transcription becomes an outstanding source of variability in new HBV-DNA virion formation. However, HBV-RNA quasispecies are not affected by this source of variability.

The first aim of this study was to establish a reliable methodology to thoroughly analyze serum HBV-RNA quasispecies, without interference from HBV-DNA quasispecies. Previous studies have found RNA levels of between 0.8 and 2.8 Logs lower than DNA levels[5,25,26]. Although we measured HBV-DNA and RNA levels with different units (IU/mL for DNA and copies/mL for RNA) in this study, the results appear to be in line with those previous studies. Even if HBV-DNA is present at low

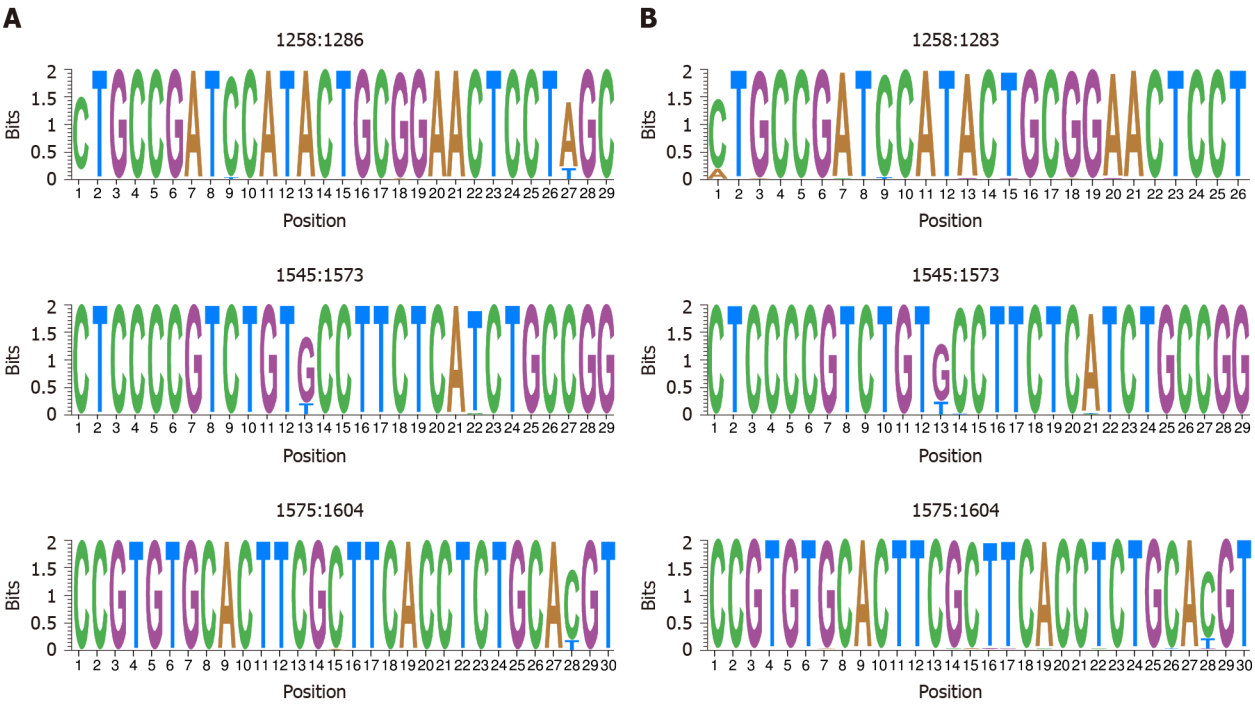

Figure 5 Representation by sequence logos of the information content of the most conserved regions in hepatitis B virus-DNA and hepatitis B virus-RNA quasispecies. The relative sizes of the letters in each stack, each of them representing a nucleotide (nt) position, indicate their relative frequencies at each position within the multiple alignments of nt haplotypes. The total height of each stack of letters depicts the IC of each nt position, measured in bits (Y-axis), therefore 0 bits is the minimum and 2 the maximum conservation. A: Hepatitis B virus-DNA; B: Hepatitis B virus-RNA.

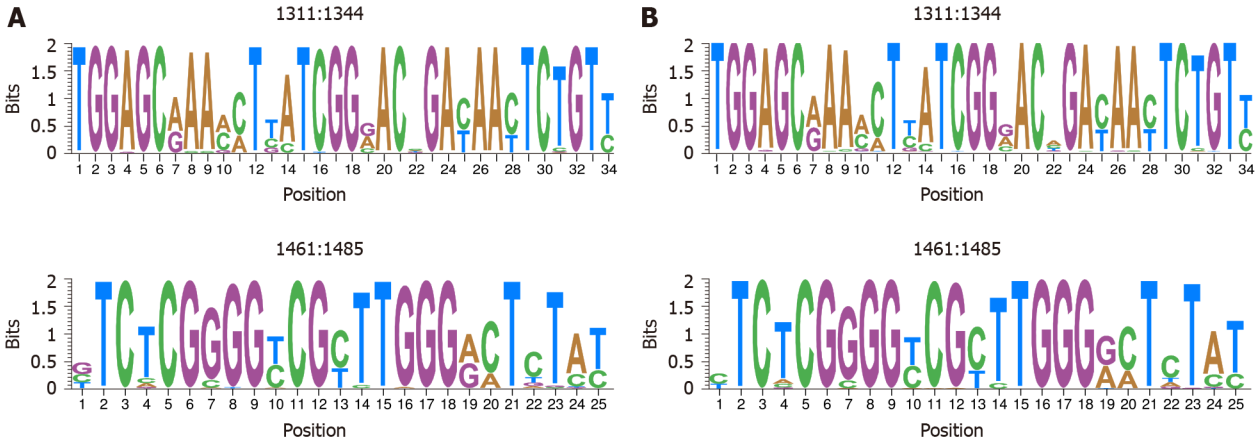

Figure 6 Representation by sequence logos of the information content of the most variable regions in hepatitis B virus-DNA and hepatitis B virus-RNA quasispecies. The relative sizes of the letters in each stack, each of them representing a nucleotide (nt) position, indicate their relative frequencies at each position within the multiple alignments of nt haplotypes. The total height of each stack of letters depicts the IC of each nt position, measured in bits (Y-axis), therefore 0 bits is the minimum and 2 the maximum conservation. A: Hepatitis B virus-DNA; B: Hepatitis B virus-RNA.

levels in patient serum, amplicons derived from it may bias NGS-obtained results to some extent, taking into account the high sensitivity of this technology. Thus, to ensure that results obtained from HBV-RNA quasispecies amplification procedure are free from HBV-DNA contamination, we pre-treated RNA isolates with DNase I, and verified the HBV-DNA elimination from these isolates by means of a qPCR assay. This verification step allowed us to confirm that no residual HBV-DNA was present in the HBV-RNA isolates, making it unlikely that reads obtained from HBV-RNA libraries were actually derived from serum HBV-DNA amplification.

Since viral polymerase errors are considered an important source of genetic variability for the HBV genome, we considered RHL to be a diversity index especially suitable for comparing complexities between HBV RNA and DNA quasispecies and assessing the effect of viral polymerase activity on the latter. This is because this

diversity index refers to the fraction of the quasispecies with the lowest fitness, indicative of the intensity with which replication errors accumulate. High viremia and long infection times would tend to result in high RHL values. High values may also be given to mutagen treatments[17]. In our study, HBV-DNA quasispecies showed a higher mean RHL than RNA quasispecies, which suggests an increased presence of low frequency HBV genomes in DNA quasispecies, probably due to the error-prone reverse-transcription origin of HBV-DNA. However, these differences were not statistically significant, either when including all patients or when separating them according to their degree of fibrosis (nonsignificant fibrosis and significant fibrosis or cirrhosis). The RHL shows high correlation with Shannon entropy, an abundance diversity index[17], which was used in a recent study by Yu *et al*[6] to compare HBV-DNA and RNA quasispecies in sequential serum samples in a group of HBeAg+ve patients receiving entecavir or pegylated interferon. Similarly to our results, no significant difference was found between HBV RNA and HBV DNA quasispecies complexity at baseline quasispecies. While the results were similar, we analyzed a different region of the HBV genome (the region of the *HBX* between nt 1255 to 1611) than the study by Yu *et al*[6] (a sequence stretch within the region encoding for the terminal protein domain of the viral polymerase), and our group of patients is more heterogeneous, especially in terms of viral genotype composition. Thus, results from the 2 studies are not directly comparable, and further studies in larger groups of patients are required to confirm the tendency of HBV DNA quasispecies toward higher quasispecies complexity, as shown by mean RHL values.

Comparison of both HBV-DNA and RNA quasispecies conservation yielded similar results, with HBV-DNA slightly more conserved than HBV-RNA despite the effect of reverse-transcription over DNA quasispecies. This observation may indicate that only a fraction of packaged pgRNA is reverse-transcribed and released. In addition, in this study, we identified 3 hyper-conserved regions which coincided in both HBV-RNA and HBV-DNA quasispecies. The first hyper-conserved region identified was between nt 1258-1286, while the second hyper-conserved region consisted of 2 nt fragments (1545-1573 and 1575-1604) spanning a region between nt 1545-1604. Interestingly, these 3 conserved sequence fragments almost overlapped with hyper-conserved sequence fragments described in a previous study carried out by our group at circulating DNA quasispecies level (1255-1286, 1545-1573 and 1575-1603)[13]. Likewise, a recently published study by our group[14] also performed in HBV-DNA quasispecies, reported some of these hyper-conserved regions (1258-1286 and 1575-1605). In addition, these 2 previous studies[13,14] also reported as hyper-conserved the sequence stretch between positions 1519-1543, which we found among the 5% most conserved windows at HBV-DNA quasispecies level but not at HBV-RNA quasispecies level. Conversely, we identified the sequence stretch 1559-1587 as hyper-conserved only in HBV-RNA quasispecies. This fragment was not identified in our previous studies, although it is included in the region between nt 1519 and 1603 defined by 3 conserved nt fragments (1519-1543, 1545-1573, and 1575-1603) described in one of them[13]. Thus, despite the high degree of similarity between sequence conservation in HBV-DNA and RNA quasispecies, this may follow different trends in some sequence stretches. Finally, assessment of the most variable windows (the 5% with the lowest mean IC values) identified the fragments between nt 1311-1344 and 1461-1485 as the most variable in both DNA and RNA quasispecies, similar to the most conserved stretches identified, which confirmed the similarities between both quasispecies.

Given the important and wide role of hepatitis B X protein (HBx), a multifunctional transactivator protein encoded by the *HBX* gene, in the replication of HBV, particularly in intrahepatic cccDNA stability and transcription, it is an interesting target for new therapies to treat CHB-infected patients[8]. Moreover, HBx is located next to the 3′ end of all the HBV mRNAs, which means that targeting its sequence may interfere in the synthesis of all the viral proteins[13]. This concept is in line with other strategies aiming for functional cure. Thus, identifying highly conserved regions may suggest valuable targets for pan-genotypic approaches with minimum likelihood of antiviral drug resistance. With this in mind, we analyzed a region of the *HBX* gene where we had previously identified hyper-conserved regions in the circulating HBV-DNA quasispecies[13,14], which we intended to confirm in circulating HBV-RNA. In fact, serum HBV-RNA was found to be genetically homogenous with intrahepatic HBV-RNA[4] the main target for this kind of antiviral therapies. Therefore, given that some sequence fragments may follow different conservation trends in both HBV-DNA and RNA quasispecies, when looking for targets for directed gene therapy, it may be worth verifying their conservation not only at serum HBV-DNA level but also at RNA level. This would make it possible to ensure their effect on their main target. In this study, we confirmed that most of the hyper-conserved regions identified by our group in

previous studies at DNA level[13,14] coincided with those identified in serum HBV-RNA, and may thus also be conserved at intrahepatic HBV-RNA level.

The broad range of HBV genotypes and degrees of severity of liver disease from all patients included in the present study enabled to get an overview of complexity and conservation of HBV-DNA and RNA quasispecies. Nevertheless, the main limitation of the present study was that the comparison with this small and heterogeneous group of patients just enabled to take a preliminary picture of HBV-DNA and RNA quasispecies, which needs to be complemented with further studies exploring both of them in larger and more homogeneous groups of patients in terms of severity of liver disease and CHB clinical stage, in order to verify whether the present results could be extrapolated to any stage of the disease. In addition, the implications of HBV quasispecies parameters analyzed in the context of both treatment and prognosis of CHB are still uncertain. Thus, longitudinal follow-up studies of circulating HBV-RNA quasispecies evolution are necessary to assess its adaptation to different evolutive pressures and to compare with that of HBV-DNA. In this way, much more robust conclusions regarding the comparison between both quasispecies could be drawn. Anyway, the results obtained demonstrate that the methodology established in the present study allows detailed analysis of serum HBV-RNA quasispecies, being a useful tool to perform those studies. Finally, *in vitro* functional studies should be performed to test the potential usefulness of the hyper-conserved regions described here as potential candidates for targeted gene therapy. Gene silencing could be a valuable strategy and *HBX* gene, thanks to its co-terminal localization, an optimal target. These hyper-conserved sequences were highly coincident with the findings of the present study[13,14]. Based on data obtained in those previous studies we have already tested several proposals about gene therapy based on antisense locked nucleic acid Gapmer and small interference RNA (siRNA)[27,28].

CONCLUSION

In summary, we developed a methodology for analyzing serum HBV-RNA quasispecies without HBV-DNA interference. By including patients with different clinical and virological characteristics we have been able to make a preliminary comparison of complexity and conservation of HBV-DNA and RNA quasispecies. Complexity analysis by the RHL index indicated an increased presence of low frequency HBV genomes in DNA quasispecies, which should be verified in larger groups of patients.

Interestingly, although HBV-DNA and RNA quasispecies showed a similar degree of conservation in the *HBX* 5′region, HBV-RNA quasispecies tended toward higher variability than HBV-DNA. Most hyper-conserved and hyper-variable regions identified were almost overlapped between HBV-DNA and RNA, and were highly coincident with our previous findings. However, we observed different conservation trends between both quasispecies in some sequence fragments, suggesting that serum HBV-RNA quasispecies should also be considered when looking for targets for directed gene therapy.

ARTICLE HIGHLIGHTS

Research background

Recent studies show that hepatitis B virus (HBV)-RNA detected in serum is mainly encapsidated pregenomic RNA (pgRNA), which could be a useful biomarker for monitoring covalently closed circular DNA activity. During nuclos(t)ide analogs (NA) therapy it is predictive of hepatitis B e-antigen seroconversion and for following viral rebound after treatment cessation. However, few studies have analyzed the serum HBV-RNA quasispecies, a complex mutant spectrum constituted by closely related, but not identical, viral populations. Analysis of serum circulating HBV-RNA quasispecies in those previous studies showed no significant difference between HBV-RNA and HBV-DNA quasispecies complexity before antiviral treatment and indicated that serum HBV-RNA is genetically homogenous with intrahepatic HBV-RNA, which is the main target for targeted gene therapy. Such therapies should be directed to hyper-conserved sequence targets, ideally located at hepatitis B X gene (*HBX*), located next to the 3′ end of all the HBV mRNAs.

Research motivation

Composition and evolution over time of HBV quasispecies is closely linked to liver disease progression, and serum circulating HBV-RNA is potentially useful for its analysis, even in situations with low or undetectable serum level of HBV-DNA. However, HBV-RNA has not been subjected to reverse transcription, which is the main source of variability in the HBV genome and it may therefore present significant differences with respect to the serum HBV-DNA quasiespecies. Nonetheless, little data is available on the comparison of genetic variability/conservation and complexity of both quasispecies. Moreover, analysis of serum HBV-RNA quasispecies may be a very useful tool when looking for hyper-conserved targets for targeted gene therapy, due to its similarities with intrahepatic HBV-RNA, the main target of this kind of antiviral therapy. Thus, in previous studies, we identified hyper-conserved sequence stretches in the circulating HBV-DNA quasispecies, which we aimed to analyze at the circulating HBV-RNA level. Studying circulating HBV-RNA quasispecies may thus favor achieving functional cure of HBV infection. However, it is necessary to establish a reliable methodology to thoroughly analyze this quasispecies, without interference from serum HBV-DNA.

Research objectives

This study aimed to establish a methodology to achieve an in-depth analysis serum HBV-RNA quasispecies without interference from circulating HBV-DNA, using next-generation sequencing (NGS). With this methodology, we aimed to compare both serum HBV-RNA and DNA quasispecies in a group of untreated chronic hepatitis B (CHB) patients. Considering the potential of HBV-RNA for analyzing HBV quasispecies, even in situations of low or undetectable serum HBV-DNA, this thorough analysis may serve as prognostic factor for clinical follow-up of CHB patients. Furthermore, it may be a useful tool for looking for hyper-conserved targets for targeted gene therapy.

Research methods

Serum samples were taken from 13 untreated CHB patients attending the outpatient clinic of Vall d'Hebron University Hospital (Barcelona, Spain). HBV-DNA levels > 5 $\log_{10}$ IU/mL, HBV-RNA levels > 4 Log_{10} copies/mL and heterogeneity in terms of HBV genotypes and degrees of severity of liver disease were considered as inclusion criteria. HBV-RNA and DNA were extracted differently using specific manual isolation protocols. In addition, HBV-RNA, which was quantified by an in-house method, was treated with DNAse I (Life Technologies, Austin, United Ststes) to remove any residual DNA present. The elimination of residual DNA after DNAse I digestion was verified by means of quantitative PCR (qPCR). In parallel, these samples were retrotranscribed into cDNA, and along with DNA isolates the 5' end region of *HBX*, between nucleotides 1255-1611 (the amplicon analysed), was amplified by a 3-nested PCR protocol and later sequenced using NGS (MiSeq, Illumina, United States). HBV-RNA and DNA quasispecies complexity was evaluated using Rare Haplotype Load (RHL) index, which measures enrichment of quasispecies in minority genomes. Sequence conservation and variability was determined by calculating the information content (IC) of each position by aligning all unique sequences covering the full amplicon (*i.e.*, haplotypes) in a multiple alignment. After this analysis, the most conserved and variable sequence stretches were represented as sequence logos, which were compared between both quasispecies.

Research results

After treatment of RNA isolates with DNase I, we confirmed that no residual HBV-DNA was present in the HBV-RNA isolates and that contamination by HBV-DNA in sequences obtained from HBV-RNA was therefore unlikely. HBV quasispecies complexity showed heterogeneous behavior among patients. While RHL was greater in DNA than in RNA quasispecies, differences were not statistically significant. This tendency of HBV DNA quasispecies toward higher quasispecies complexity than HBV-RNA needs to be studied further in larger groups of patients. In general, conservation was highly coincident between HBV-RNA and DNA quasispecies; the majority of nt positions showed the same IC value in both of them. Interestingly, HBV-RNA quasispecies was slightly less conserved than DNA and displayed more positions with some variability (IC < 2 bits). Sliding window analysis in HBV-DNA and RNA quasispecies showed 4 sequence fragments as the most conserved in each of them. Of those fragments 3 coincided between both quasispecies, but 1 was found to be among the most conserved only in HBV-DNA and 1 only in HBV-RNA. The most variable

sequence fragments coincided in both quasispecies.

Research conclusions

In this study, we describe a methodology for analyzing serum HBV-RNA quasispecies without HBV-DNA interference. This methodology allowed us to compare HBV-DNA and RNA quasispecies. For quasispecies complexity, we analyzed the RHL index, a new reliable diversity index to diagnose mutant spectrum expansions, such as may have occurred with the error-prone reverse transcription of HBV-RNA into DNA. However, although we detected a tendency to greater quasispecies complexity in HBV-DNA than in RNA, the differences were statistically non-significant, which may indicate an increased presence of low-frequency HBV genomes in DNA quasispecies. For this reason, we suggest that further studies in larger groups of patients be performed to confirm this observation. We also found a highly coincident conservation between HBV-RNA and DNA quasispecies in the *HBX* 5′ region. Interestingly, HBV-RNA quasispecies showed slightly higher variability than HBV-DNA quasispecies, which may indicate that only a fraction of packaged pgRNA is reverse-transcribed and released. The hyper-conserved sequences identified were highly coincident to what was observed in previous studies by our group in HBV-DNA quasispecies. Nevertheless, we observed that some sequence fragments were differently conserved between HBV-DNA and RNA quasispecies, which suggest that serum HBV-RNA quasispecies should also be considered when looking for targets for directed gene therapy, specially taking into account the similarities between serum and intrahepatic HBV-RNA.

Research perspectives

In this study, data were obtained from patients with a broad range of HBV genotypes and degrees of severity of liver disease. This allowed us to make a preliminary comparison of complexity and conservation of HBV-DNA and RNA quasispecies. However, further studies with a larger sample size are warranted to confirm our results. The main goal of the methodology for HBV-RNA quasispecies described here is to use it in groups of patients where HBV-DNA levels are usually too low for PCR amplification (*e.g.*, patients under NA treatment, since the generation of HBV-RNA is not inhibited by NA directly), in order to be able to monitor HBV quasispecies in those patients.

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World J Gastroenterol 2021 November 7; 27(41): 7159-7172

DOI: 10.3748/wjg.v27.i41.7159

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Short-term and long-term outcomes of laparoscopic *vs* open ileocolic resection in patients with Crohn's disease: Propensity-score matching analysis

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Author contributions: Pak SJ and Kim YI contributed equally to this article; Pak SJ contributed to data acquisition, analysis, and interpretation, and wrote the paper; Yoon YS, Lee JL, Kim YI, and Yu CS generated the data; Lee JB analyzed the data; Yoon YS had the original idea for the paper and provided critical comments, Yoon YS and Kim YI revised the manuscript; and all authors approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Asan Medical Center (IRB No. 2019-0972).

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: This study has no conflict of interest.

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Abstract

BACKGROUND

Laparoscopic ileocolic resection (LICR) is the preferred surgical approach for primary ileocolic Crohn's disease (CD) because it has greater recovery benefits than open ICR (OICR).

AIM

To compare short- and long-term outcomes in patients who underwent LICR and OICR.

METHODS

Patients who underwent ICR for primary CD from 2006 to 2017 at a single tertiary center specializing in CD were included. Patients who underwent LICR and OICR were subjected to propensity-score matching analysis. Patients were propensity-score matched 1:1 by factors potentially associated with 30-d perioperative morbidity. These included demographic characteristics and disease- and treatment-related variables. Factors were compared using univariate and multivariate analyses. Long-term surgical recurrence-free survival (SRFS) in the two groups was determined by the Kaplan-Meier method and compared by the log-rank test.

RESULTS

During the study period, 348 patients underwent ICR, 211 by the open approach and 137 laparoscopically. Propensity-score matching yielded 102 pairs of patients. The rate of postoperative complication was significantly lower (14% *versus* 32%, P = 0.003), postoperative hospital stay significantly shorter (8 d *versus* 13 d, P =

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: South Korea

Specialty type: Gastroenterology and hepatology

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

Received: March 16, 2021
Peer-review started: March 16, 2021
First decision: May 1, 2021
Revised: May 8, 2021
Accepted: October 18, 2021
Article in press: October 18, 2021
Published online: November 7, 2021

P-Reviewer: Brisinda G, Shi Y
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

0.003), and postoperative pain on day 7 significantly lower (1.4 *versus* 2.3, $P < 0.001$) in propensity-score matched patients who underwent LICR than in those who underwent OICR. Multivariate analysis showed that postoperative complications were significantly associated with preoperative treatment with biologics [odds ratio (OR): 3.14, $P = 0.01$] and an open approach to surgery (OR: 2.86, $P = 0.005$). The 5- and 10-year SRFS rates in the matched pairs were 92.9% and 83.3%, respectively, with SRFS rates not differing significantly between the OICR and LICR groups. The performance of additional procedures was an independent risk factor for surgical recurrence [hazard ratio (HR): 3.28, $P = 0.02$].

CONCLUSION
LICR yielded better short-term outcomes and postoperative recovery than OICR, with no differences in long-term outcomes. LICR may provide greater benefits in selected patients with primary CD.

Key Words: Crohn's disease; Laparoscopic; Surgery; Postoperative complications; Recurrence; Propensity score; Retrospective study

Core Tip: The laparoscopic approach to ileocolic resection can be safely performed in patients with primary Crohn's disease (CD), resulting in fewer postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate when compared with open surgery. Postoperative complications were significantly associated with preoperative use of biologics and open ileocolic resection. Additional procedures were found to be independent risk factors for surgical recurrence in patients with CD.

Citation: Pak SJ, Kim YI, Yoon YS, Lee JL, Lee JB, Yu CS. Short-term and long-term outcomes of laparoscopic *vs* open ileocolic resection in patients with Crohn's disease: Propensity-score matching analysis. *World J Gastroenterol* 2021; 27(41): 7159-7172
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7159.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7159

INTRODUCTION

Ileocolic resection (ICR) is the most frequently performed operation for patients with abdominal Crohn's disease (CD) with involvement of the terminal ileum. Since the introduction of laparoscopic colectomy in 1991, experience with laparoscopic ICR (LICR) for CD has increased[1,2]. LICR has become the preferred surgical approach for primary ileocolic CD because it shows greater recovery benefits than open ICR (OICR). These benefits include reduced pain, lower rates of overall morbidity, shorter hospital stay, earlier return to full activity, lower costs, and improved quality of life and cosmesis compared with OICR[1-7].

Conventionally, patients with penetrating type or complex CD have not been candidates for laparoscopic surgery, with open surgery remaining the generally accepted approach for these patients. The laparoscopic approach is regarded as more technically challenging than open surgery in CD patients with complex features, including huge phlegmons, multiple enteric fistulas, and dense adhesions, as well as those requiring repeated surgery[8]. In addition, patients with complex CD have higher rates of host related risks, such as homeostasis disturbance, infection, and severe malnutrition prior to surgery caused by external or internal fistulas[9]. Therefore, utilization of LICR in patients with complex CD remains problematic.

In South Korea, the number of laparoscopic operations in CD patients has increased dramatically, from 11.6% in 2009 to more than 31% in 2015[10]. Although our institution is the highest volume center for CD in South Korea, laparoscopic surgery for CD was conservative, with performance increasing since 2014.

The present study compared the short- and long-term outcomes of LICR and OICR in patients with primary CD over a 12-year period. To overcome possible selection bias, patients in the two groups were analyzed after propensity-score matching.

MATERIALS AND METHODS

Patients and clinical variables

Patients who underwent LICR or OICR for primary CD at Asan Medical Center in Seoul, Korea, from January 2006 to December 2017, were retrospectively identified. Ileocolic resection included patients undergoing resection of the ileum and colon with ileocolic anastomosis within the right/transverse colon. Patients who underwent previous bowel resection for CD, those without anastomosis, those with ileocolic anastomosis distal to the transverse colon, and patients with missing data or loss to follow-up were excluded (Figure 1). Patients who initially underwent laparoscopic surgery but required conversion to open surgery were included in the intention-to-treat analysis. Factors recorded from patients' electronic medical records and charts included demographic characteristics, preoperative disease characteristics, operative details, and perioperative outcomes. Demographic characteristics included patient sex, age at time of surgery, body mass index (BMI), duration of disease from the time of diagnosis, smoking history, and comorbidities. Preoperative disease characteristics included Montreal classification at the time of surgery[11], extra-intestinal manifestations of disease, family history of CD, history of perianal CD, previous history of abdominal surgery, and specific history of intestinal resection for CD. Operative details included intraoperative findings from operation reports (adhesions, strictures, intestinal fistulas, abscesses, phlegmons), indications for surgical intervention, conversion to open surgery, diverting stoma, estimated blood loss, intraoperative red blood cell (RBC) transfusion, operation time, urgent surgery, and American Society of Anesthesiologists (ASA) score. Perioperative outcomes included intraoperative and postoperative morbidity and mortality within 30 d after surgery, readmission, reoperation, length of hospital stay, pain scale, and time to recovery of bowel function [12]. Additional variables included preoperative hemoglobin and albumin levels, preoperative CD medications, anastomosis configuration (side-to-side, end-to-side, side-to-end, or end-to-end), type of anastomosis (stapled or hand-sewn), and synchronous additional surgical procedures. Postoperative morbidity was graded according to the Clavien-Dindo classification. The study protocol was approved by the Institutional Review Board of Asan Medical Center (approval number: 2019-0972).

Definitions

Comorbidities included hypertension, diabetes mellitus, and others. Major intraoperative bleeding was defined as intraoperative hemorrhage reported in operation notes and requirement for transfusion of packed RBCs. However, postoperative transfusion without a need for surgical intervention was not regarded as a postoperative complication. Anemia was defined as hemoglobin concentrations < 11.5 g/dL in women and < 13 g/dL in men, and hypoalbuminemia was defined as albumin concentrations < 3.5 g/dL according to institutional guidelines. Details of additional, concomitant procedures were limited to intra-abdominal surgery, whereas perianal procedures were excluded. Recovery of bowel movement was defined as the day of first flatus.

Laparoscopic or open surgery was selected for each patient according to surgeon preference. A history of previous abdominal surgery with or without bowel resection was an important consideration when choosing the surgical method. Because patients with previous bowel resection were excluded, multiple factors such as age, general condition, and disease extent were taken into consideration. Anastomosis configurations were classified as side-to-side, end-to-side, side-to-end, and end-to-end. Anastomosis materials were categorized as stapled and hand-sewn. ICR involves the removal of the ileocecal valve and was categorized as right colectomy or ileocecal resection depending on the involvement of the hepatic flexure of the colon.

Preoperative CD medications were divided into four categories: Systemic steroids, biologics [infliximab (Remicade®, Janssen Biotech, Inc., Horsham, PA, United States) or adalimumab (Humira®, AbbVie Inc. Chicago, IL, United States)], immunomodulators (azathioprine, 6-mercaptopurine, or methotrexate), and anti-inflammatory agents (5-aminosalicylate acid or budesonide). Preoperative treatment with steroids and anti-inflammatory agents was defined as the administration of each medication within 1 mo before surgery. Treatment with biologics was defined as the administration of at least one infusion of anti-TNF agents within 3 mo before surgery, whereas treatment with immunomodulators was defined as administration within 2 mo before surgery [5]. Immunosuppressive medications included steroids, biologics, and immunomodulators, but not anti-inflammatory agents.

Complications within the first 30 postoperative days included inadvertent intraoperative injury, anastomosis leak, fistula formation, prolonged postoperative ileus,

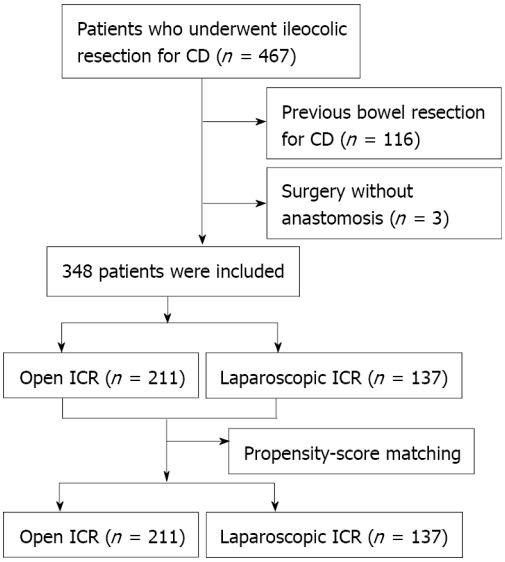

Figure 1 Flow chart of patient selection and propensity-score matching. CD: Crohn's disease; ICR: Ileocolic resection.

wound or intra-abdominal infection requiring antibiotics or drainage, readmission, or return to the operating room. Septic complications included anastomosis leakage, abdominal abscess, and resulting sepsis or septic shock. Ileus was defined as the absence of bowel function by postoperative day 5 and/or the need for nasogastric tube insertion due to abdominal distension, nausea, or vomiting, without evidence of mechanical bowel obstruction.

Surgical recurrence was defined as a repeat operation on any part of the bowel for pathologically confirmed CD or for pathologically confirmed anastomotic disease, including manifestations of the small bowel at the anastomosis or stoma site. Operations not performed to treat CD exacerbations (*e.g.*, adhesiolysis or stoma closure only) were not considered reoperations for surgical recurrence.

Propensity score matching analysis

To minimize the impact of selection bias for the surgical approach and potential confounding in this observational study, patients who underwent LICR and OICR were subjected to propensity-score matching, with rigorous adjustment for significant differences in patient characteristics. Propensity scores were estimated by multiple logistic regression analysis. All pre-specified covariates were included in the full non-parsimonious models. The covariates included demographic characteristics (age, gender, BMI, smoking history, previous history of abdominal surgery, previous history of comorbidity, and ASA score), disease-related variables (Montreal classification, disease duration, perianal CD, family history of CD and extra-intestinal CD manifestations), and treatment-related variables (preoperative hemoglobin and albumin concentrations, preoperative RBC transfusions, preoperative medications, and indications for surgery). These variables were selected because they can affect the choice of surgical approach and perioperative outcomes. The operative approach was entered into the regression model as a dependent variable. A 1:1 "nearest neighbor", case-control match without replacement was used. The discrimination and calibration abilities of the propensity-score model were 0.7332 by C-statistics and $P = 0.1219$ by Hosmer-Lemeshow statistics. Following propensity-score matching, short- and long-term results were compared in the two groups.

Statistical analysis

Continuous variables were reported as mean ± standard deviation (SD) or as median (min, max) and compared by *t*-tests or Wilcoxon rank sum tests, whereas categorical variables were reported as frequency (%) and compared by Pearson's χ^2 test or Fisher's exact test.

Univariate analyses were performed to assess the risk factors associated with postoperative complications and surgical recurrence. Multivariable models were created to identify factors independently associated with postoperative complications. Surgical recurrence-free survival (SRFS) was calculated using the Kaplan-Meier method and compared using log-rank tests. Multivariate analyses to assess the risk

Table 1 Patient demographic and clinical characteristics

	All patients			Propensity-score matched patients		
	Open (*n* = 211)	Laparoscopy (*n* = 137)	*P*	Open (*n* = 102)	Laparoscopy (*n* = 102)	SMD
Age (yr)	29.2 ± 9.7	29.2 ± 9.0	0.976	28.8 ± 9.0	28.6 ± 8.7	-0.026
Gender, male	153 (72.5)	95 (69.3)	0.546	78 (76.5)	72 (70.6)	-0.134
BMI (kg/m^2)	18.8 ± 3.2	18.9 ± 2.8	0.822	19.0 ± 3.3	18.9 ± 2.8	-0.041
Any smoking history	41 (19.4)	33 (24.1)	0.348	21 (20.6)	20 (19.6)	-0.025
Previous abdominal surgery	23 (10.9)	10 (7.3)	0.349	10 (9.8)	8 (7.8)	-0.069
Montreal classification						
Behavior			< 0.001			-0.044
Inflammatory (B1)	2 (0.1)	2 (1.5)		2 (2.0)	2 (2.0)	
Stricturing (B2)	38 (17.5)	52 (38.0)		30 (29.4)	26 (25.5)	
Penetrating (B3)	172 (82.0)	83 (60.6)		70 (68.6)	74 (72.5)	
Location			0.218			-0.060
Terminal ileal (L1)	68 (32.2)	50 (36.5)		36 (35.3)	37 (36.3)	
Colonic (L2)	10 (4.7)	2 (1.5)		6 (5.9)	2 (2.0)	
Ileocolic (L3)	133 (63.0)	85 (62.0)		60 (58.8)	63 (61.8)	
Disease duration (mo)	49.8 ± 50.9	68.1 ± 59.3	0.003	61.5 ± 57.4	67.0 ± 57.8	0.095
Perianal CD	103 (48.8)	57 (41.6)	0.226	45 (44.1)	45 (44.1)	0.000
Family history of CD	5 (2.4)	5 (3.6)	0.523	4 (3.9)	4 (3.9)	0.000
Extra-intestinal CD manifestation	31 (14.7)	18 (13.1)	0.754	13 (12.7)	10 (9.8)	-0.093
Comorbidity	21 (10.0)	5 (3.6)	0.036	2 (2.0)	4 (3.9)	0.116
Hypertension	4 (1.9)	0 (0.0)	0.157	1 (1.0)	0 (0.0)	
Diabetes mellitus	2 (0.9)	1 (0.7)	1.000	0 (0.0)	1 (1.0)	
Others	15 (7.1)	5 (3.6)	0.239	1 (1.0)	4 (3.9)	
ASA score, 3-4	7 (3.3)	2 (1.5)	0.397	3 (2.9)	2 (2.0)	[2]
Emergency	17 (8.1)	9 (6.6)	0.680	6 (5.9)	7 (6.9)	0.040
Preoperative data						
Hemoglobin (g/dL)	11.5 ± 1.5	11.5 ± 1.9	0.780	11.4 ± 1.4	11.4 ± 1.8	0.037
Albumin (g/dL)	3.1 ± 0.5	3.2 ± 0.5	0.140	3.1 ± 0.5	3.2 ± 0.5	0.075
Transfusion	40 (19.0)	26 (19.0)	1.000	22 (21.6)	20 (19.6)	[2]
Preoperative medications						
Steroids	44 (20.9)	25 (18.2)	0.584	22 (21.6)	15 (14.7)	-0.179
Immuno-modulators	96 (45.5)	67 (48.9)	0.583	48 (47.1)	50 (49.0)	0.039
Biologics	29 (13.7)	26 (19.0)	0.229	17 (16.7)	16 (15.7)	-0.027
Indication for surgery			< 0.001			
[1]Fistula *versus* others	83 (39.3)	34 (24.8)	0.022	33 (32.4)	32 (31.4)	0.021
[2]Obstruction *versus* others	39 (18.5)	54 (39.4)	< 0.001	31 (30.4)	26 (25.5)	-0.042

[1]Fistula and obstruction were both selected in some patients.
[2]These variables were excluded from the propensity-score matched set because of the small numbers.
Results are reported as mean ± SD or as number (%). SMD: Standardized mean difference; BMI: Body mass index; CD: Crohn's disease; ASA: American Society of Anesthesiologists.

factors associated with SRFS were performed using Cox proportional hazards model with 95% confidence interval (CI). All statistical analyses were performed using the Statistical Package for the Social Sciences, version 24.0 for Windows (SPSS, IBM Corp., Armonk, NY, United States), with *P* values < 0.05 considered statistically significant.

RESULTS

Patient characteristics, matching, and operative details

A total of 467 patients underwent ICR for CD during the study period. Of these, 119 were excluded, including three patients without anastomosis or with ileocolic anastomosis distal to the transverse colon and 116 patients who had previously undergone bowel resection for CD. Of the 348 eligible patients, 211 underwent OICR and 137 underwent LICR. Patient characteristics before and after propensity-score matching are summarized in Table 1. Compared with patients who underwent OICR for CD, those who underwent LICR had significantly lower rates of penetrating behavior ($P < 0.001$) and comorbidities ($P = 0.036$), longer disease duration ($P = 0.003$), a higher rate of surgical indication of obstruction ($P < 0.001$), and a lower rate of fistula as a surgical indication ($P = 0.022$). Crohn's Disease Activity Index (CDAI) scores were compared between the two groups. LICR and OICR groups had moderate CDAI scores (230.8 ± 9.5 and 269.1 ± 10.8, respectively). Because the demographic data differed between the OICR and LICR groups, these patients were subjected to 1:1 propensity-score matching to reduce selection bias. A total of 102 pairs was therefore included in the propensity-score matched population.

A comparison of the propensity-score matched groups showed that the rate of intraoperative transfusion was significantly lower ($P = 0.017$), and the length of small bowel resection significantly shorter ($P < 0.001$), in the LICR than in the OICR group. Three patients (2.9%) in the OICR group, but none in the LICR group, underwent a diverting ileostomy. Although most patients in both groups underwent side-to-side anastomosis, end-to-side anastomosis was more frequently performed in the LICR than in the OICR group ($P = 0.014$). The open conversion rate in the LICR group was 4.9%. The reasons for open conversion were adhesions in two patients, huge phlegmons in two, and an abscess in one (Table 2).

Short-term outcomes

Of the 348 CD patients who underwent ICR, 75 (21.6%) experienced complications within 30 d, but none died. Of the 204 matched patients, 47 (23%) experienced postoperative complications, with 15 cases being classified as class III Clavien-Dindo complications. All six patients who required reoperation had undergone OICR, including three for anastomotic leakage and one each for an intra-abdominal abscess, luminal bleeding, and a surgical wound complication.

The overall complication rate was significantly lower in the LICR than in the OICR group (13.7% *versus* 32.4%, $P = 0.003$). The rates of septic complications, including intra-abdominal abscess ($P = 0.035$) and/or anastomosis leakage ($P = 0.014$), were also lower in the LICR group. In addition, the rates of reoperation ($P = 0.029$) and blood transfusion ($P = 0.021$) were significantly lower, hospital stay ($P = 0.003$) significantly shorter, and postoperative pain on postoperative day 7 significantly lower ($P < 0.001$) in the LICR than in the OICR group (Table 3).

Univariate analysis showed that an open approach ($P = 0.003$), previous abdominal surgery ($P = 0.037$), preoperative use of biologics ($P = 0.023$), additional procedures ($P = 0.050$), and longer operation time (> 135 min) ($P = 0.016$) were associated with postoperative complications. In multivariate analysis, an open approach [odds ratio (OR): 2.86; 95%CI: 0.17-0.73; $P = 0.005$], previous abdominal surgery (OR: 3.61; 95%CI: 1.23-10.60, $P = 0.020$), preoperative use of biologics (OR: 3.14; 95%CI: 1.33-7.40; $P = 0.009$), and longer operation time (> 135 min) (OR: 2.38; 95%CI: 1.17-4.84; $P = 0.017$) were found to be independent risk factors for postoperative complications (Table 4).

Sixteen patients experienced septic complications, 15 in the OICR and one in the LICR group. Preoperative use of steroids (OR: 4.19; 95%CI: 1.19-14.71; $P = 0.025$) and fistula as a surgical indication (OR: 4.03; 95%CI: 1.23-13.22; $P = 0.021$) were significantly associated with septic complications, whereas preoperative use of biologics was not. The laparoscopic approach was also associated with a lower risk of septic complications (OR: 0.06; 95%CI: 0.01-0.45; $P = 0.007$).

Table 2 Operative details of propensity-score matched patients

	Open (*n* = 102)	Laparoscopy (*n* = 102)	*P*
Operation time (min)	136.6 ± 4.5	130.4 ± 2.7	0.241
Estimated blood loss (mL)	201.8 ± 19.2	145.7 ± 22.1	0.057
Intraoperative transfusion	13 (12.7)	3 (2.9)	0.017
Diverting ileostomy	3 (2.9)	0 (0.0)	0.246
Anastomosis configuration			0.014
Side-to-side	89 (87.3)	74 (72.5)	
End-to-side	9 (8.8)	26 (25.5)	
Side-to-end	3 (2.9)	1 (1.0)	
End-to-end	1 (1.0)	1 (1.0)	
Stapled anastomosis	100 (98.0)	101 (99.0)	1.000
Operation type			0.322
Right colectomy	48 (47.1)	40 (39.2)	
Ileocecal resection	54 (52.9)	62 (60.8)	
Additional procedure	40 (39.2)	30 (29.4)	0.184
Strictureplasty	16 (15.7)	11 (10.8)	
Small bowel resection	20 (19.6)	19 (18.6)	
Colon resection	4 (3.9)	0 (0.0)	
Length of small bowel resected (cm)	60.6 ± 4.2	43.9 ± 2.8	0.001

Results are reported as mean ± SD or as number (%).

Long-term outcomes

The median follow-up duration was 74.8 mo for all patients, 88.13 mo in the OICR group, and 61.45 mo in the LICR group. By the end of the study period, 21 patients (10.3%) in the propensity-score matched cohort had experienced surgical recurrence of CD, including 14 (13.7%) who underwent OICR and seven (6.9%) who underwent LICR; these included 12 patients with fistula/abscess, four with perforation, and five with stricture. The median time to surgical recurrence was 70.3 mo for all patients, 81.7 mo in the OICR group, and 58.9 mo in the LICR group.

The overall 5- and 10-year SRFS rates were 92.9% and 83.3%, respectively, in the 204 propensity-score matched patients, 92.6% and 82.4%, respectively, in the OICR group, and 92.8% and 84.3%, respectively, in the LICR group. Kaplan-Meier analysis showed that the difference between the two groups was not statistically significant (P = 0.407, Figure 2). Univariate analysis showed that preoperative treatment with biologics (P = 0.024), side-to-side anastomosis (P = 0.042), and additional procedures (P = 0.049) were associated with surgical recurrence. Multivariate analysis revealed that the performance of simultaneous additional procedures was a risk factor for surgical recurrence [hazard ratio (HR): 3.28; 95%CI: 1.20-8.91; P = 0.020; Table 5].

DISCUSSION

This propensity-score matched case-control study compared short and long-term outcomes of LICR and OICR for primary CD at a single tertiary center specializing in CD. The results confirmed that LICR can be safely performed in these patients, resulting in a lower rate of postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate compared with open surgery.

The laparoscopic approach is the preferred surgical approach for simple CD, as it is associated with a lower postoperative morbidity rate, a shorter hospital stay, earlier return to full activity, and improved quality of life[1-4]. Laparoscopic surgery is being performed more frequently in patients with complex or recurrent CD, with ran-

Table 3 Short-term outcomes of the propensity-score matched patients

	Open (*n* = 102)	Laparoscop (*n* = 102)	*P*	OR	95%CI	*P*
Total complications	33 (32.4)	14 (13.7)	0.003	0.379	0.189-0.759	0.006
Intra-abdominal abscess	8 (7.8)	1 (1.0)	0.035			
Anastomotic leakage	7 (6.9)	0 (0.0)	0.014			
Wound complication	12 (11.8)	4 (3.9)	0.065			
Ileus	2 (2.0)	7 (6.9)	0.170			
Bleeding	7 (6.9)	2 (2.0)	0.170			
Other	0 (0.0)	2 (2.0)	0.498			
Septic complications[1]	15 (14.7)	1 (1.0)	< 0.001	0.091	0.012-0.704	0.006[a]
Reoperation	6 (5.9)	0 (0.0)	0.029	0.107	0.006-2.039	0.121[a]
Readmission	4 (3.9)	2 (2.0)	0.689	0.250	0.053-1.177	0.109[a]
Recovery of bowel movement (d)	2.97 ± 1.0	2.82 ± 1.0	0.295	0.971	0.694-1.359	0.864
Duration of NPO (d)	4.2 ± 1.9	3.5 ± 2.2	0.026	0.573	0.400-0.820	0.002
Total hospital stay (d)	20.7 ± 17.3	16.1 ± 8.3	0.016	0.594	0.404-0.875	0.008
Postoperative hospital stay (d)	13.1 ± 16.3	8.2 ± 3.3	0.003	0.443	0.298-0.660	< 0.001
Pain scale (NRS)						
POD#1	5.1 ± 2.0	4.9 ± 1.9	0.458	0.386	-0.183-0.956	0.181
POD#2	3.9 ± 1.8	3.9 ± 2.1	0.756	0.159	-0.392-0.710	0.567
POD#3	3.8 ± 2.1	3.3 ± 1.8	0.057	0.602	0.00-1.198	0.048
POD#7	2.3 ± 1.9	1.4 ± 1.4	< 0.001	0.628	0.156-1.100	0.010
Postoperative transfusion	23 (22.5)	10 (9.8)	0.021	0.278	0.103-0.748	0.011

[1]Septic complications, including intra-abdominal abscess and anastomosis leakage.
[a]Exact test. Results are reported as mean ± SD or as number (%).
NRS: Numeric Pain Rating Scale; NPO: Nothing Per Oral; POD: Postoperative day; OR: Odds ratio; CI: Confidence interval.

domized-controlled studies and meta-analyses providing evidence that LICR is safe and effective, even in patients with severe CD[5-7]. The present study showed that LICR was associated with a lower rate of postoperative complications, a shorter postoperative hospital stay, and reduced postoperative pain, with no difference in the behavior or severity of CD. These results support recent trends showing that LICR may be feasible in selected patients with CD.

Operation time was comparable in the LICR and OICR groups. Although several studies have reported that operation time is longer for laparoscopic than for open surgery[1,13,14], other studies have found that operation time is shorter for LICR than for OICR[15,16]. Our finding, of no difference in operation time between laparoscopic and open surgery, may have been due to lack of calibration of selection, even after propensity-score matching. For example, the resected length of the small bowel differed between the LICR and OICR groups, and additional procedures were more frequent in patients who underwent open surgery.

The present study found that preoperative use of corticosteroids was associated with higher rates of intra-abdominal abscess and anastomosis leakage. Moreover, preoperative treatment with biologics was associated with a higher risk of short-term complications. These results are similar to those of the large observational TREAT registry (n = 6273), in which use of infliximab and corticosteroid increased the risks of serious infection[17-19]. In addition, our study found that a history of previous abdominal surgery was associated with postoperative complications. The specific impact of previous intestinal resection on postoperative complications in patients with CD has not been determined. One study reported that 47.6% of postoperative complications occurred in patients with a history of previous abdominal surgery (n = 10, P = 0.310), although the difference was not statistically significant[20]. Other studies have found that previous abdominal surgery is a significant risk factor for postoperative

Table 4 Univariate and multivariate regression analyses of risk factors associated with postoperative complications in propensity-score matched patients

	Univariate analysis			Multivariate analysis		
	No complication (*n* = 157, %)	Complication (*n* = 47, %)	*P*	OR	95%CI	*P*
Demographics						
Male	115 (73.2)	35 (74.5)	1.000			
Family history	6 (3.8)	2 (4.3)	1.000			
Smoking history	32 (20.4)	9 (19.1)	1.000			
Comorbidity	6 (3.8)	0 (0.0)	0.340			
Fistula-in-ano	73 (46.5)	17 (36.2)	0.243			
Previous abdominal surgery	10 (6.4)	8 (17.0)	0.037	3.61	1.23-10.60	0.020
Penetrating type	112 (71.3)	34 (72.3)	1.000			
Open approach	69 (43.9)	14 (70.2)	0.003	2.86	0.17-0.73	0.005
Preoperative medications						
Biologics	20 (12.7)	13 (27.7)	0.023	3.14	1.33-7.40	0.009
Steroid	24 (15.3)	13 (27.7)	0.082			
Immunomodulators	77 (49.0)	21 (44.7)	0.622			
Operation details						
Indications						
Fistula	53 (33.8)	20 (42.6)	0.300			
Obstruction	52 (33.1)	16 (34.0)	1.000			
Anastomosis configuration						
Side-to-side	126 (80.3)	37 (78.7)	0.837			
End-to-side	29 (18.5)	6 (12.8)	0.508			
Stapled anastomosis	155 (99.7)	46 (2.1)	0.546			
Additional procedures	45 (28.7)	21 (44.7)	0.050	1.38	0.61-3.14	0.444
Operation time[1] > 135 min	52 (33.1)	25 (53.2)	0.016	2.38	1.17-4.84	0.017

[1]Average operation time for CD was 135.6 min.

Results are reported as number (%). OR: Odds ratio; CI: Confidence interval.

complications[21,22]. Moreover, patients who had previous surgery were more inclined to develop postoperative complications (P = 0.047), particularly anastomotic leak (P = 0.021) and severe (Clavien-Dindo grade III/IV) complications (P = 0.038)[23]. A previous abdominal surgery history may result in prolonged dissection during surgery because of the atypical planes of disrupted normal anatomy, increasing the risks of accidental enterotomy and additional bowel devascularization[23].

In our study, the rate of conversion from laparoscopic to open surgery was about 5%. Pooled conversion rates have been found to range from 0% to 21.5%[13,24]. Recurrent disease with dense adhesions, pelvic sepsis with fistulizing disease, large inflammatory mass, and thickened mesentery are all conditions predisposing to conversion open surgery[24]. The low conversion rate in the present study may have been due to the relatively mild complexities and complications in patients who underwent laparoscopic surgery. Laparoscopy can be attempted in patients with CD, even if they have risk factors for open conversion. For safety reasons, however, patients should be converted to open surgery without delay.

Prevention of long-term postoperative recurrence in CD patients is a major challenge, especially as 10-15 year post-surgical recurrence rates are approximately 45% to 50%[25]. The long-term effects of laparoscopy have not been determined, as few studies have compared long-term outcomes, especially surgical recurrence, in CD patients who have undergone laparoscopic and open surgery. A meta-analysis

Table 5 Univariate and multivariate regression analyses of risk factors associated with surgical recurrence in propensity-score matched patients

	Univariate analysis			Multivariate analysis		
	No recurrence (*n* = 183)	Recurrence (*n* = 21)	*P*	HR	95%CI	*P*
Demographics						
Male	134 (73.2)	16 (76.2)	1.000			
Family history	8 (4.4)	0 (0.0)	1.000			
Smoking history	38 (20.8)	3 (14.3)	0.579			
Comorbidity	6 (3.3)	0 (0.0)	1.000			
Fistula-in-ano	81 (44.3)	9 (42.9)	1.000			
Previous abdominal surgery	15 (8.2)	3 (14.3)	0.407			
Penetrating type	129 (70.5)	17 (81.0)	0.445			
Open approach	95 (48.1)	14 (66.7)	0.166			
Preoperative medications						
Biologics	26 (14.2)	7 (33.3)	0.024	2.64	0.89-7.86	0.081
Steroid	31 (16.9)	6 (28.6)	0.229			
Immunomodulators	88 (48.1)	10 (47.6)	1.000			
Operation details						
Indication						
Fistula	69 (37.7)	4 (19.0)	0.099			
Obstruction	62 (33.9)	6 (28.6)	0.808			
Anastomosis configuration						
Side-to-side	150 (82.0)	13 (61.9)	0.042	3.82	1.24-11.78	0.078
End-to-side	30 (16.4)	5 (23.8)	0.370			
Stapled anastomosis	180 (98.4)	21 (100.0)	1.000			
Additional procedures	55 (30.1)	11 (52.4)	0.049	3.28	1.20-8.91	0.020
Operation time > 135 min	66 (36.1)	11 (52.4)	0.159			

Results are reported as number (%). HR: Hazard ratio; CI: Confidence interval.

reported that laparoscopic surgery is associated with a lower rate of late reoperations for CD recurrence (OR: 0.46; 95%CI: 0.27-0.80)[14], and laparoscopy was found to protect against surgical recurrence (HR: 0.24; 95%CI: 0.10-0.53; $P = 0.04$)[26]. Another study, however, reported no difference in surgical recurrence rates between surgical techniques (OR: 0.78; 95%CI: 0.54-1.11; $P = 0.17$)[27]. The present study also showed no difference in long-term outcomes between the LICR and OICR groups. Because this study was a retrospective analysis of a small number of patients, future large randomized-controlled trials are needed to assess the impact of laparoscopy on surgical recurrence.

Simultaneous surgery at the time of ICR was also associated with a higher risk of surgical recurrence. The extent of disease at diagnosis had an impact on recurrence, with higher recurrence rates in patients with small bowel and continuous ileocolonic CD than in patients with ileocecal and colorectal disease[28]. Disease extent > 50 cm is considered a risk factor for postoperative recurrence of CD[29]. An additional surgical procedure may be related to surgical recurrence because these patients may have more severe disease. Prospective clinical studies in larger numbers of patients are needed to further evaluate the results of the present study.

Since the introduction of the first anti-TNF agent in the late 1990s, biologic therapy has revolutionized the medical treatment of patients with CD[30]. Although administering biologics prior to surgery has been reported to reduce clinical/endoscopic recurrence rates[31-33], most of these trials had small sample sizes and limited follow-

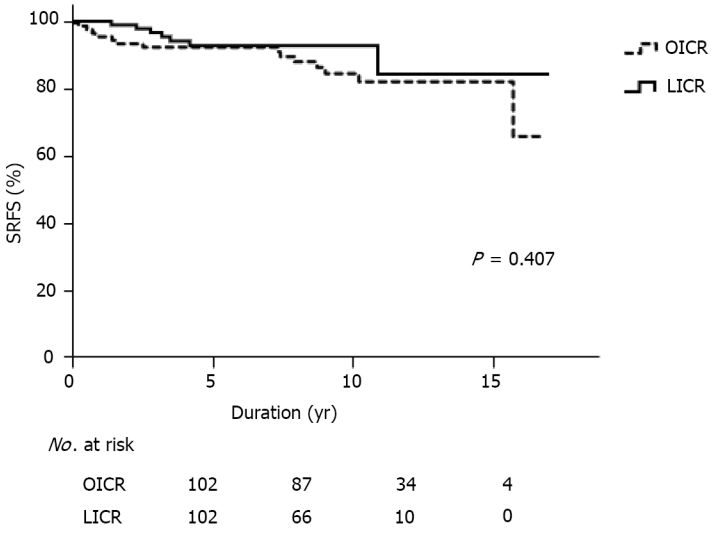

Figure 2 Kaplan-Meier analysis of surgical recurrence-free survival according to type of surgery in patients with Crohn's disease. SRFS: Surgical recurrence-free survival; LICR: Laparoscopic ileocolic resection; OICR: Open ileocolic resection.

up, and focused on endoscopic findings and clinical scores rather than repeat operations[30,34]. In our study, preoperative treatment with biologics was related to higher risk of surgical recurrence. South Korea strictly regulates the use of biologics due to health insurance policies, and prophylactic treatment with biologics is not available[35]. Therefore, the correlation between administration of biologics and higher risk of recurrence may be due to the greater severity of disease in patients selected for treatment with these agents. Large randomized-controlled trials are needed to determine the ability of routine prophylactic biologics after surgery to prevent surgical recurrence.

Stapled side-to-side anastomosis has been associated with lower rates of leakage and surgical recurrence than other types of anastomosis[36-39]. Side-to-side anastomoses maintain better lateral blood flow and a wide lumen, which prevents luminal stenosis and fecal pooling, thereby preventing early disease recurrence[40]. By contrast, another study reported that side-to-side anastomoses (both hand-sewn and stapled) did not reduce short-term complications and postoperative recurrence[41]. Moreover, anastomotic configuration or the material used was not significantly related to reoperations or complications[40]. The present study found that the type of anastomosis did not significantly affect short-term complications. Although the surgical recurrence rate tended to be higher in patients who underwent side-to-side than other types of anastomosis, the difference was not statistically significant (HR: 3.82; $P = 0.078$). However, most patients in the present study underwent side-to-side anastomosis, with only a few receiving end-to-side or end-to-end anastomosis. Surgeons in our center prefer side-to-side anastomosis in CD patients with extensive inflammation. Thus, to evaluate the role of anastomosis configuration, a more controlled study, adjusting for time period and surgeon, is needed.

The present study had several limitations. First, this study was a retrospective evaluation of patients at a single center. Randomized-controlled trials are required to specifically evaluate the ability of a laparoscopic approach to minimize postoperative complications. Although propensity-score matching can reduce selection bias, resulting in a situation similar to a randomized-controlled trial, our propensity-score matching models could not eliminate all selection biases. For example, the most frequent reasons for conversion to open surgery, such as adhesions and huge phlegmons, could not be calibrated by propensity-score matching analysis. Also, although the CDAI scores for both groups were moderate, the laparoscopic group had a significantly lower CDAI score (230.8) than the open group (269.1) ($P = 0.008$)[42]. Inevitably, a randomized controlled trial will be required to evaluate the role of the laparoscopic approach with more reliable evidence. Second, the study included only East Asian patients from a single country, thus not representing a global population. Korean and western CD patients differ in gender distribution, disease location, and perianal fistula occurrence[43]. Third, the use of biologics in this study was less frequent than in western studies because the health insurance reimbursement policy of the Korean government was strict during the study period. This study period was dominated by 'step-up treatment'. Although 'top-down treatment' has been more

frequent in recent years, its use in Korea is limited.

CONCLUSION

LICR yielded better short-term outcomes, including more rapid postoperative recovery, than open surgery. Long-term outcomes, however, did not differ between the two groups. Laparoscopic surgery might be a better surgical option in selected patients with CD.

ARTICLE HIGHLIGHTS

Research background

Ileocolic resection (ICR) is the most frequently performed operation for patients with abdominal Crohn's disease (CD) with involvement of the terminal ileum. Laparoscopic ICR (LICR) has become the preferred surgical approach for primary ileocolic CD because it has greater recovery benefits than open ICR (OICR).

Research motivation

The laparoscopic approach is regarded as more technically challenging than open surgery in CD patients with complex features, including huge phlegmons, multiple enteric fistulas, and dense adhesions, as well as those requiring repeated surgery. Utilization of LICR in patients with complex CD remains problematic.

Research objectives

This study aimed to compare the short- and long-term outcomes of LICR and OICR in patients with primary CD.

Research methods

A total of 348 eligible patients who underwent LICR or OICR for primary CD at Asan Medical Center in Seoul, Korea, from January 2006 to December 2017, were retrospectively analyzed. Data on demographic characteristics, preoperative disease characteristics, operative details, perioperative outcomes, and long-term surgical recurrence were collected. Patients were propensity-score matched 1:1 by factors potentially associated with 30 d perioperative morbidity.

Research results

During the study period, 348 patients underwent ICR, 211 by the open approach and 137 by the laparoscopic approach. Propensity-score matching yielded 102 pairs of patients. The rate of postoperative complications was significantly lower, postoperative hospital stay significantly shorter, and postoperative pain on day 7 significantly lower in patients who underwent laparoscopic than OICR. Surgical recurrence free survival (SRFS) rates in the OICR and LICR groups were not significantly different.

Research conclusions

LICR yielded better short-term outcomes and postoperative recovery than OICR, with no differences in long-term outcomes. LICR may provide greater benefits in selected patients with primary CD.

Research perspectives

The laparoscopic approach to ileocolic resection can be safely performed in patients with primary CD, resulting in fewer postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate when compared with open surgery.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7173-7189

DOI: 10.3748/wjg.v27.i41.7173

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

ORIGINAL ARTICLE

Retrospective Study

Comprehensive radiomics nomogram for predicting survival of patients with combined hepatocellular carcinoma and cholangiocarcinoma

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Author contributions: Tang YY and Zhao YN provided the study concept and designed this study; Tang YY acquired the data; Zhang T and Zhao YN carried out data analysis and interpretation; Tang YY and Zhang T were responsible for drafting and preliminarily revising the manuscript; Ma XL and Chen ZY performed study supervision and final approval.

Institutional review board statement: This retrospective study was approved by the West China Hospital Ethics Committee (Approval No. 2019903).

Conflict-of-interest statement: The authors declare that they have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Data sharing statement: The clinical data and radiomics data were available from the corresponding author at

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Abstract

BACKGROUND

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) is defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma and has a poor prognosis.

AIM

To develop a radiomics nomogram for predicting post-resection survival of patients with cHCC-CCA.

METHODS

Patients with pathologically diagnosed cHCC-CCA were randomly divided into training and validation sets. Radiomics features were extracted from portal venous phase computed tomography (CT) images using the least absolute shrinkage and selection operator Cox regression and random forest analysis. A nomogram integrating the radiomics score and clinical factors was developed using univariate analysis and multivariate Cox regression. Nomogram performance was assessed in terms of the C-index as well as calibration, decision, and survival curves.

RESULTS

Chenzheyu@scu.edu.cn. And no additional data are available.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

Received: April 2, 2021
Peer-review started: April 2, 2021
First decision: June 24, 2021
Revised: June 26, 2021
Accepted: September 3, 2021
Article in press: September 3, 2021
Published online: November 7, 2021

P-Reviewer: Antwi SO, Farid K
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Yuan YY

CT and clinical data of 118 patients were included in the study. The radiomics score, vascular invasion, anatomical resection, total bilirubin level, and satellite lesions were found to be independent predictors of overall survival (OS) and were therefore included in an integrative nomogram. The nomogram was more strongly associated with OS (hazard ratio: 8.155, 95% confidence interval: 4.498-14.785, $P < 0.001$) than a model based on the radiomics score or only clinical factors. The area under the curve values for 1-year and 3-year OS in the training set were 0.878 and 0.875, respectively. Patients stratified as being at high risk of poor prognosis showed a significantly shorter median OS than those stratified as being at low risk (6.1 *vs* 81.6 mo, $P < 0.001$).

CONCLUSION

This nomogram may predict survival of cHCC-CCA patients after hepatectomy and therefore help identify those more likely to benefit from surgery.

Key Words: Radiomics; Nomogram; Combined hepatocellular carcinoma and cholangiocarcinoma; Risk strata; Prognosis

Core Tip: Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) is defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma. Studies vary regarding the prognosis of cHCC-CCA patients after potentially curative hepatectomy, with 5-year postoperative overall survival rates ranging from 8% to 63%. A reliable method to predict prognosis after resection may help select cHCC-CCA patients more likely to benefit from surgery. We established an integrative nomogram based on radiomics features and clinical variables to predict the survival of cHCC-CCA patients after potentially curative resection. The nomogram showed good predictive potential and may help guide treatment decisions.

Citation: Tang YY, Zhao YN, Zhang T, Chen ZY, Ma XL. Comprehensive radiomics nomogram for predicting survival of patients with combined hepatocellular carcinoma and cholangiocarcinoma. *World J Gastroenterol* 2021; 27(41): 7173-7189
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7173.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7173

INTRODUCTION

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA), which arises in hepatic progenitor cells, accounts for 0.8%-6.5% of primary liver carcinoma cases[1-5]. The World Health Organization defines the condition as the presence of a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma (ICC)[6,7]. There is disagreement in the literature on whether the prognosis of cHCC-CCA patients is worse or similar to that of patients with only HCC. Several studies concur that the prognosis of cHCC-CCA patients is comparable to that of patients with only ICC[8-11]. Studies vary regarding the prognosis of cHCC-CCA patients after potentially curative hepatectomy, with 5-year postoperative overall survival (OS) rates ranging from 8% to 63%[12-15]. A reliable method to predict prognosis after resection may help select cHCC-CCA patients more likely to benefit from surgery.

Radiomics is a promising comprehensive analysis to predict the prognosis of liver cancer patients after hepatectomy, which is a post-processing method to quantitatively evaluate imaging features in order to assess cancer heterogeneity non-invasively and objectively[16,17]. Radiomics features have proven effective in predicting the survival of patients with HCC or ICC alone[18-21]. Radiomics can also differentiate cHCC-CCA from common HCC or ICC[18,22], although no radiomics models have been established for predicting long-term survival of cHCC-CCA patients after resection.

The predictive performance of radiomics features may improve when combined with clinical factors, as demonstrated for patients with ICC[23-25]. Therefore, the current study aimed to construct and validate a nomogram based on radiomics and clinical features for predicting postoperative survival of cHCC-CCA patients. This prognostic model may help guide treatment decisions for these patients.

MATERIALS AND METHODS

Study design and patient selection

This retrospective study was approved by the West China Hospital Ethics Committee, and the requirement for informed consent was waived. All patients agreed to undergo medical examination and were informed that their anonymized medical data would be analyzed and published for the purposes of medical research. We retrospectively reviewed the data of all patients: (1) Who were diagnosed with cHCC-CCA based on the 2019 guidelines of the World Health Organization which defined cHCC-CCA as a single nodule showing differentiation into HCC and ICC; (2) Who underwent hepatectomy with curative intent at West China Hospital between February 2012 and May 2017; and (3) For whom complete medical records were available during hospitalization and during follow-up, as well as computed tomography (CT) data within 2 wk before surgery.

Patients were excluded if they were diagnosed with morphologically typical HCC or ICC based on the expression of markers for cholangiocytes, hepatocytes, or progenitor cells (*e.g.*, keratins 7 and 19 based on immunostaining). Patients were considered to have common HCC if they showed trabecular growth (often accompanied by bile production), hyaline bodies, prominent nucleoli, immunoreactivity against HepPar1 or alpha-fetoprotein, and expression of keratin 19[26,27]. Patients presenting typical adenocarcinoma together with abundant stroma and mucin production were considered to have ICC only. Patients diagnosed with cholangiolocellular carcinoma were excluded from this study as the latest guidelines[7] no longer consider this condition a subtype of cHCC-CCA.

Patients were also excluded if they had received transcatheter arterial chemoembolization or any other type of chemotherapy before CT, or if they had other malignancies simultaneously with cHCC-CCA. The primary endpoint of this study was OS, defined as the time from the date of surgery until the date of all-cause death or last follow-up. Patients were routinely followed at 1 mo after surgery and then every 3-6 mo thereafter, until April 30, 2020.

Computed tomography examination

Enhanced CT of the abdomen was performed with a single 64-detector row scanner (Brilliance 64, Philips Medical Systems, Eindhoven, The Netherlands) in all the patients. The scan parameters were as follows: Beam pitch, 0.891; tube voltage, 120 kV; tube current, 200 mA; detector collimation, 0.75 mm; slice thickness, 1.0 mm; reconstruction increment, 5.0 mm; and rotation time, 0.42 s. Arterial phase scanning began at 25 s and portal venous phase scanning began at 60 s[22].

Extraction of radiomics features

All patients were randomly divided into a training set and validation set at a ratio of 7:3. All CT images from portal venous phase scanning were loaded into LIFEx software (version 3.74; CEA-SHFJ, Orsay, France)[28]. Working independently, two radiologists manually drew regions of interest for each patient within the hepatic neoplasm in all portal venous phase CT images. Radiomics features in the CT images were screened using the Least Absolute Shrinkage and Selection Operator (LASSO) and Cox regression, followed by random forest analysis[29]. The selected radiomics features were linearly combined with their own weighting coefficients, generating a radiomics score for each patient.

Selection of clinical factors

All clinical variables in the training set were subjected to univariate analysis followed by multivariate Cox analysis with step-wise selection in order to identify independent predictors of OS. In these analyses, total bilirubin level was converted into a categorical variable.

Development and validation of an integrative nomogram

To develop the nomogram, radiomics scores were categorized as "high" or "low" based on whether they were greater or smaller than the median score. Then the nomogram was constructed based on the radiomics score and the clinical risk factors identified in multivariate Cox regression. Within the nomogram, each variable was scored ranging from 0 to 100, and the variable associated with the greatest hazard ratio (HR) was assigned 100 points[30]. Using the nomogram, we classified patients as being at high or low risk based on the maximum Youden index[31].

The performance of the nomogram was assessed in terms of a calibration curve related to the predicted and observed OS, the C-index used to assess model discrimination, and receiver operating characteristic (ROC) curve[32]. The clinical usefulness of the nomogram was assessed using decision curve analysis[33].

Statistical analysis

Differences in continuous variables were assessed for significance using the Wilcoxon rank-sum test if the data were skewed, or Student's *t* test if the data showed a normal distribution. Differences in categorical variables were assessed using the χ^2 or Fisher's exact test. OS was plotted using the Kaplan-Meier method, and groups were compared using the log-rank test. All statistical analyses were performed with EmpowerStats (version 2.20; 2011 X&Y Solutions) and R software (version 4.0.0; The R Foundation). The following packages in R were used: glmnet, cmprsk, rms, survival, rmda, and devtools. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Patients

A total of 118 eligible patients (86.4% men) were enrolled (Table 1). Their mean age was 51.6 years, and 90 patients had been diagnosed when they were younger than 60 years. Follow-up data were complete for 110 patients, who were followed for a median of 25.1 mo (95% confidence interval [CI]: 17.3-59.7 mo). Median OS was 21.6 mo, and OS rates were 61.0% at 1 year, 48.3% at 3 years, and 37.4% at 5 years.

Patients were randomly assigned to either the training or validation set, and the two sets did not differ significantly in terms of clinical features, except for tumor size, American Joint Committee on Cancer stage and T stage. OS rates at 1 and 3 years were 58.3% and 46.4% in the training set, compared to 67.7% and 52.9% in the validation set.

Feature selection and construction of radiomics score

The integrative nomogram flow chart is depicted in Figures 1 and 2. For each patient, data on 49 radiomics features were extracted from portal venous phase CT images. Among these 49 features, LASSO regression selected nine with non-zero coefficients, of which random forest analysis selected three (MeanValue, NGLDM Busyness and GLZLM HGZE) (Supplementary Table 1) that showed the highest prediction values (variable importance > 0.01, Figure 3A). Radiomics scores were calculated based on these three features, and scores were subsequently categorized into "high" or "low" based on whether they were lower or higher than the median score (Figure 3).

Selection of prognostic clinical factors

In total, 31 clinical variables were initially considered in the univariate analysis; and seven variables with $P < 0.1$ were then entered into the multivariate Cox analysis (Table 2). The multivariate analysis identified four predictors of OS: Vascular invasion, anatomical resection, total bilirubin level, and satellite lesions. Total bilirubin level (> 17.1 μmol/L) resulted in a larger HR (13.94) than the other three risk factors. Nevertheless, all four factors were subsequently included in the nomogram.

Construction and validation of a radiomics nomogram model

Based on the above-mentioned four clinical factors and the radiomics score, we developed a comprehensive integrative nomogram to predict 1-year and 3-year OS of cHCC-CCA patients after surgical resection with curative intent (Figure 4A). The area under the ROC curve (AUC) for 1-year OS was 0.878 in the training set and 0.937 in the validation set (Figure 4B). The calibration curve of 1-year OS showed good agreement between predicted and observed values in both the training and validation sets (Figure 4C). The AUC for 3-year OS was 0.875 in the training set and 0.866 in the validation set. The C-index was 0.807 (95%CI: 0.756-0.858) in the training set and 0.820

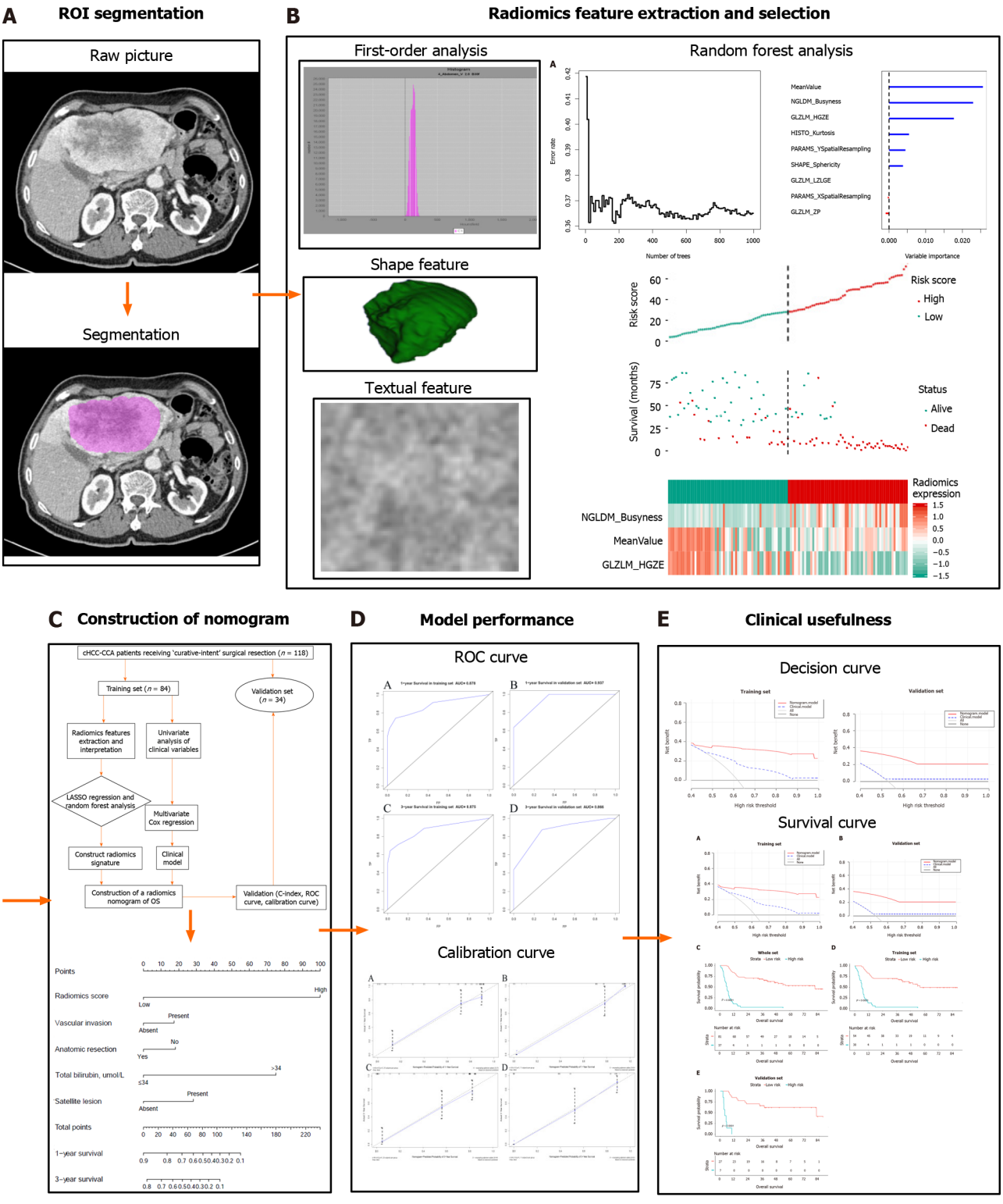

Figure 1 Study workflow. A: Segmentation of the region of interest; B: Extraction and selection of radiomics features; C: Construction of nomogram; D: Comparison of model performance; E: Decision curve analysis and overall survival comparisons between the training and validation sets. ROI: Region of interest; cHCC-CCA: Combined hepatocellular carcinoma and cholangiocarcinoma; LASSO: Least absolute shrinkage and selection operator; OS: Overall survival; ROC: Receiver operating characteristic.

(95%CI: 0.723-0.917) in the validation set. An example of predicting 1- and 3-year OS using the nomogram is shown in Figure 5.

In decision curve analysis, the nomogram showed higher "net benefit" than a model based only on the four clinical factors or models based on "treat-all-patients" or "treat-no-patients" approaches. These results were observed at nearly all threshold probabilities in the training set (Figure 6A) and validation set (Figure 6B).

Table 1 Baseline characteristics of patients with combined hepatocellular carcinoma and cholangiocarcinoma in the training and validation sets

Variable	Entire cohort (*n* = 118)	Training set (*n* = 84)	Validation set (*n* = 34)	*P* value
Male sex	102 (86.4)	73 (86.9)	29 (85.3)	0.817
Age, yr	51.6 ± 10.5	51.2 ± 10.5	52.7 ± 10.6	0.484
Hypertension	11 (9.3)	7 (8.3)	4 (11.8)	0.561
Diabetes mellitus	7 (5.9)	6 (7.1)	1 (2.9)	0.382
Hepatitis B/C	61 (51.7)	40 (47.6)	21 (61.8)	0.164
Child-Pugh, A/B	116/2	83/1	33/1	0.495
Liver cirrhosis	47 (39.8)	35 (41.7)	12 (35.3)	0.522
Hypersplenia	15 (12.7)	11 (13.1)	4 (11.8)	0.844
ALT (U/L)	55.2 ± 100.4	46.1 ± 29.3	77.6 ± 181.1	0.807
AST (U/L)	59.8 ± 136.6	48.1 ± 28.8	88.6 ± 250.7	0.513
ALB (g/L)	42.1 ± 4.6	42.3 ± 4.0	41.5 ± 5.7	0.643
TB (mmol/L)	15.9 ± 10.1	15.7 ± 10.1	16.5 ± 10.0	0.597
AFP (ng/mL)	285.2 ± 475.1	256.3 ± 454.6	356.5 ± 522.4	0.156
CA19-9 (U/mL)	106.8 ± 251.2	109.7 ± 258.3	99.6 ± 236.2	0.184
CA125 (U/mL)	117.0 ± 624.6	152.9 ± 727.5	18.3 ± 11.9	0.541
CEA (ng/mL)	6.4 ± 30.3	7.5 ± 35.5	3.4 ± 3.2	0.444
Liver fibrosis				0.871
No significant fibrosis	15 (13.8)	11 (13.8)	4 (13.8)	
Significant fibrosis	37 (33.9)	26 (32.5)	11 (37.9)	
Advanced fibrosis	57 (52.3)	43 (53.8)	14 (48.3)	
Not mentioned	8 (6.8)	3 (3.6)	5 (14.7)	
Tumor size, ≤ 5 cm	38 (32.2)	20 (23.8)	18 (52.9)	0.002
Tumor number, ≥ 2	67 (56.8)	52 (61.9)	15 (44.1)	0.077
Satellite lesions	42 (35.6)	29 (34.5)	13 (38.2)	0.703
Vascular invasion	46 (39.0)	35 (41.7)	11 (32.4)	0.347
Lymph node infiltration	15 (12.7)	10 (11.9)	5 (14.7)	0.679
Differentiation				0.578
Well	44 (37.3)	30 (35.7)	14 (41.2)	
Moderate	22 (18.6)	18 (21.4)	4 (11.8)	
Poor	1 (0.8)	1 (1.2)	0 (0.0)	
Undifferentiated	51 (43.2)	35 (41.7)	16 (47.1)	
8th AJCC stage				0.027
I	9 (7.6)	7 (8.3)	2 (5.9)	
II	28 (23.7)	14 (16.7)	14 (41.2)	
III	66 (55.9)	53 (63.1)	13 (38.2)	
IV	15 (12.7)	10 (11.9)	5 (14.7)	
T stage				0.042
T1	13 (11.0)	9 (10.7)	4 (11.8)	
T2	29 (24.6)	15 (17.9)	14 (41.2)	
T3	45 (38.1)	37 (44.0)	8 (23.5)	

T4	31 (26.3)	23 (27.4)	8 (23.5)	
N stage				0.762
N0	103 (87.3)	74 (88.1)	29 (85.3)	
N1	15 (12.7)	10 (11.9)	5 (14.7)	
Transfusion	17 (14.4)	14 (16.7)	3 (8.8)	0.388
Blood loss ≤ 400 mL	71 (60.2)	49 (58.3)	22 (64.7)	0.522
Margin, R1	13 (11.0)	9 (10.7)	4 (11.8)	0.869
Surgical method				0.285
Major resection	57 (48.3)	44 (52.4)	13 (38.2)	
Minor resection	50 (42.4)	32 (38.1)	18 (52.9)	
Resection + ablation	11 (9.3)	8 (9.5)	3 (8.8)	
Anatomical resection	50 (43.9)	39 (48.1)	11 (33.3)	0.148
Postoperative TACE	35 (29.7)	28 (33.3)	7 (20.6)	0.17
Hospital stay (d)	12.2 ± 4.5	12.3 ± 4.4	11.9 ± 5.0	0.608
Overall survival (mo)	30.8 ± 26.3	29.6 ± 26.2	33.6 ± 26.9	0.462

[1]Values are *n*, *n* (%), or mean ± SD, unless otherwise noted.

AFP: Alpha fetoprotein; AJCC: American Joint Committee on Cancer; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CEA: Carcinoembryonic antigen; TACE: Transhepatic arterial chemotherapy and embolization; TB: Total bilirubin.

Risk stratification using the nomogram

A total risk score was calculated for each patient by summing the scores for each variable in the nomogram. The maximum Youden index of 105 points in the nomogram led us to determine a cut-off value of 39.66, and patients were categorized as being at "high" or "low" risk based on whether their risk score was above or below this cut-off. Kaplan-Meier curves showed that OS was significantly longer for low-risk patients than for high-risk patients, regardless of whether the analysis included all patients (Figure 6C) or only the training set (Figure 6D) or validation set (Figure 6E). Across all patients, OS rates at 1 year were 10.8% for the high-risk group and 84.0% for the low-risk group ($P < 0.001$), while the corresponding OS rates at 3 years were 2.7% and 69.1%, respectively ($P < 0.001$).

Table 3 compares HRs obtained with the integrated nomogram, the radiomics score alone, or a model based only on clinical factors. The model based only on the four clinical risk factors resulted in an HR of 2.65 (95%CI: 1.53-4.60), even though total bilirubin level resulted in an HR of 13.94 (95%CI: 3.56-54.60) in multivariate analysis. The nomogram HR was higher than that provided by models based on the radiomics score or on clinical factors alone.

DISCUSSION

In the present study, we developed a comprehensive integrative nomogram that takes into account CT radiomics scores and four clinical risk factors that independently predict OS (vascular invasion, anatomical resection, total bilirubin, and satellite lesions), and we showed that this nomogram can predict OS in cHCC-CCA patients following potentially curative hepatectomy. The AUC for 1-year OS was 0.878 in the training set and 0.937 in the validation set. To our knowledge, this is the first CT-based radiomics model to predict postoperative survival of cHCC-CCA patients.

Our results extend the number of situations in which radiomics has shown potential in predicting the survival of patients with liver tumors[34,35]. The patients in our study who were assigned a high radiomics score had a 5.91-fold higher risk of death than those with a low score, consistent with a previously reported association between high radiomics score and risk of recurrence in patients with HCC or ICC[24,36]. These findings imply that radiomics scores may be able to identify patients preoperatively who are more likely to benefit from surgical resection.

Table 2 Univariate analysis and multivariate Cox regression to identify clinical factors associated with overall survival after curative hepatectomy

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Male sex	0.470 (0.203-1.088)	0.078	1.767 (0.244-1.316)	0.186
Age, yr				
≤ 60	Ref.			
> 60	1.173 (0.644-2.139)	0.602		
Liver cirrhosis				
Absent	Ref.			
Present	1.370 (0.852-2.203)	0.194		
AFP (ng/mL)	0.990 (0.597-1.643)	0.970		
CA 19-9 (U/mL)	0.987 (0.586-1.662)	0.960		
Albumin (g/L)	2.496 (0.997-6.244)	0.051	1.025 (0.968-1.085)	0.403
TB (μmol/L)				
≤ 34	Ref.		Ref.	
> 34	17.994 (4.726-68.509)	< 0.001	13.943 (3.561-54.602)	< 0.001
Tumor number, multiple	0.766 (0.473-1.240)	0.277		
Satellite lesions				
Absent	Ref.		Ref.	
Present	2.037 (1.267-3.268)	0.003	1.762 (1.079-2.877)	0.024
Vascular invasion				
Absent	Ref.		Ref.	
Present	2.009 (1.247-3.239)	0.004	1.725 (1.049-2.834)	0.032
T stage				
T1	Ref.			
T2	1.171 (0.705-1.942)	0.542		
T3	2.424 (0.704-8.348)	0.161		
T4	3.823 (1.158-12.615)	0.028		
Anatomy resection				
Yes	Ref.		Ref.	
No	2.011 (1.344-3.006)	0.006	1.731 (1.083-2.767)	0.028
Margin				
R0	Ref.			
R1	1.032 (0.446-2.387)	0.941		
Postoperative TACE				
Yes	Ref.			
No	1.597 (0.924-2.759)	0.093	1.6051 (0.3546-1.0947)	0.100

AFP: Alpha-fetoprotein; CI: Confidence interval; HR: Hazard ratio; Ref.: Reference; TB: Total bilirubin; TACE: Transhepatic arterial chemotherapy and embolization.

Our results further support previous work indicating that combining clinical variables with radiomics features may predict prognosis better than either the variables or the features separately[37,38]. Combining the radiomics score with clinical variables allowed us to classify patients into a high-risk group that had an 8.16-fold

Table 3 Comparison of hazard ratios describing risk for different predictive models

Model	HR (95%CI)	*P* value
Radiomics score		< 0.001
Low risk	Ref.	
High risk	5.908 (3.285-10.626)	
Clinical model		< 0.001
Low risk	Ref.	
High risk	2.653 (1.532-4.595)	
Radiomics nomogram		< 0.001
Low risk	Ref.	
High risk	8.155 (4.498-14.785)	

CI: Confidence interval; HR: Hazard ratio; Ref.: Reference.

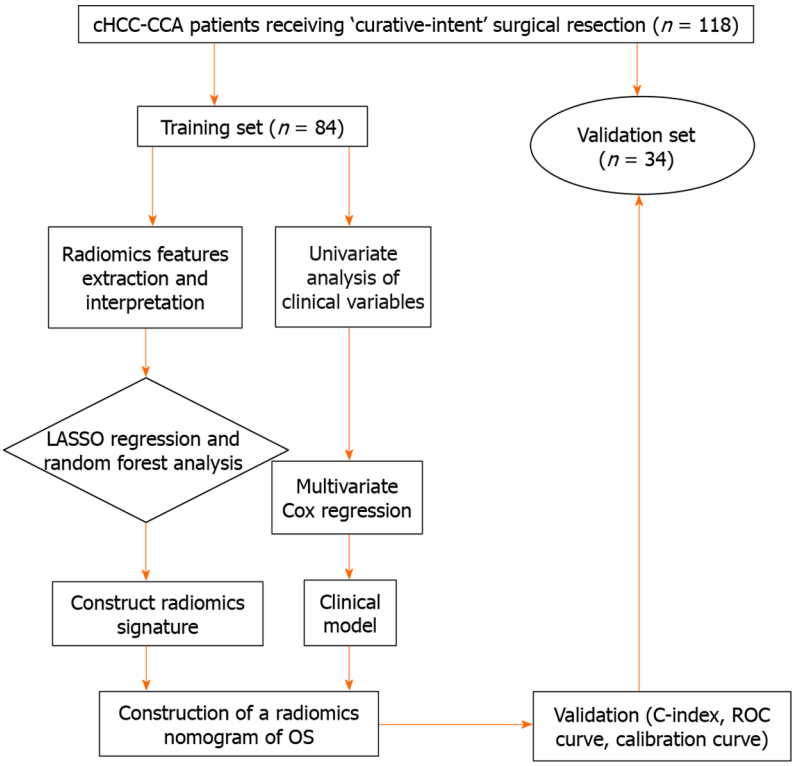

Figure 2 Flow diagram of patient selection. cHCC-CCA: Combined hepatocellular carcinoma and cholangiocarcinoma; LASSO: Least absolute shrinkage and selection operator; OS: Overall survival; ROC: Receiver operating characteristic.

higher risk of death than the low-risk group, with the two groups showing a median OS of 6.1 and 81.6 mo, respectively ($P < 0.001$). This integrative nomogram may help identify cHCC-CCA patients who are more likely to benefit from resection.

The rate of vascular invasion in our patients was 39.0%, similar to previous studies and within the prevalence of 9%-89.5% reported for cHCC-CCA[3,39,40]. As shown in Supplementary Figure 1, the OS rate at 3 years was 56.8% among our patients without vascular invasion, compared to only 36.8% among those with invasion, consistent with the association between vascular invasion and worse postoperative prognosis[2,13,41]. Indeed, vascular invasion has been shown to be an independent predictor of postoperative survival in patients with combined hepatocellular-cholangiocarcinoma and it increases the risk of death in these patients by 1.6- fold to 5.2-fold[42,43].

In addition, elevated total bilirubin level (> 34 μmol/L) and no anatomic surgical resection were considered to be independent risk factors related to the poor prognosis of cHCC-CCA patients. Total bilirubin level is one element of the Child-Pugh classi-

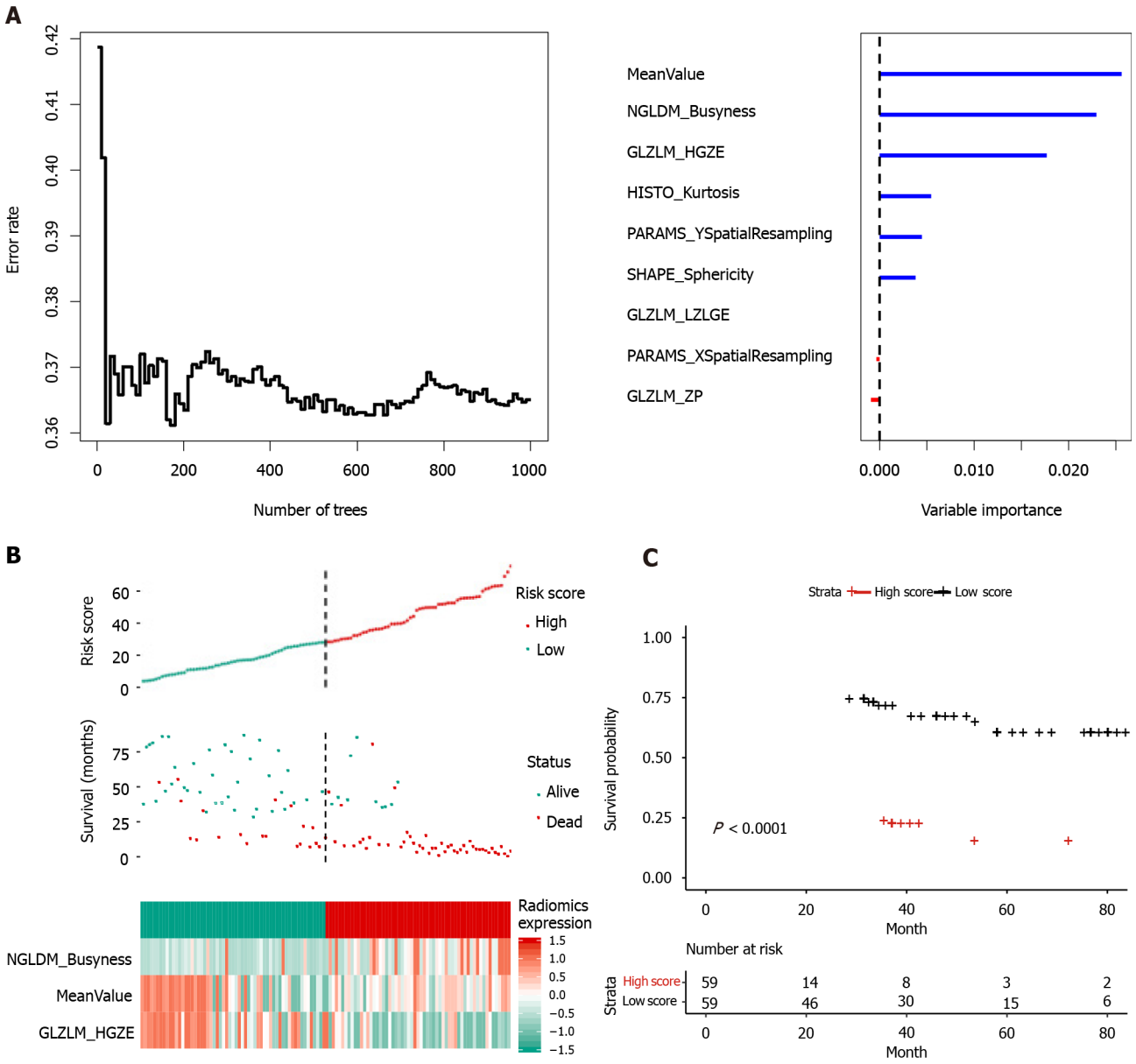

Figure 3 Radiomics feature selection. A: Random forest analysis. Least absolute shrinkage and selection operator regression selected nine radiomics features, of which three were chosen by random forest analysis; B: Weights of MeanValue, NGLDM Busyness, and GLZLM HGZE in each patient; C: Overall survival curves for the entire cohort of patients, stratified by low or high radiomics score.

fication which plays a remarkable role in survival prediction of liver malignancy. In a previous study, Chen *et al*[44] revealed that elevated total bilirubin level (> 17.1 μmol/L) was an independent risk factor resulting in poor prognosis in advanced HCC patients. Peak postoperative bilirubin > 7.0 mg/dL was significantly related to liver-related death and worse outcomes after major hepatectomy. The group of patients with a total bilirubin level higher than the cut-off value (22.7 μmol/L) was also associated with a poorer OS in another study[45]. Moreover, Chantajitr *et al*[46] found that dilation of the intrahepatic bile duct was related to a poor prognosis in cHCC-CCA patients, and Lee *et al*[47] suggested that an increased Child-Pugh score (mean score: 5.8) was related to early death in cHCC-CCA patients. The role of anatomical hepatectomy in the prognosis of cHCC-CCA patients has rarely been evaluated, and some studies have reported that anatomical hepatectomy can prolong the survival time of HCC, but had no benefit in ICC patients[48,49]. These findings imply that the impact of anatomical hepatectomy on OS in cHCC-CCA is unclear and further large scale studies with a prospective design should be conducted to verify the results of this study.

Studies have suggested that anatomical hepatectomy can prolong survival in HCC but not ICC patients[48,49]; however, we are unaware of studies that have examined this issue in cHCC-CCA patients. The impact of anatomical hepatectomy on OS of

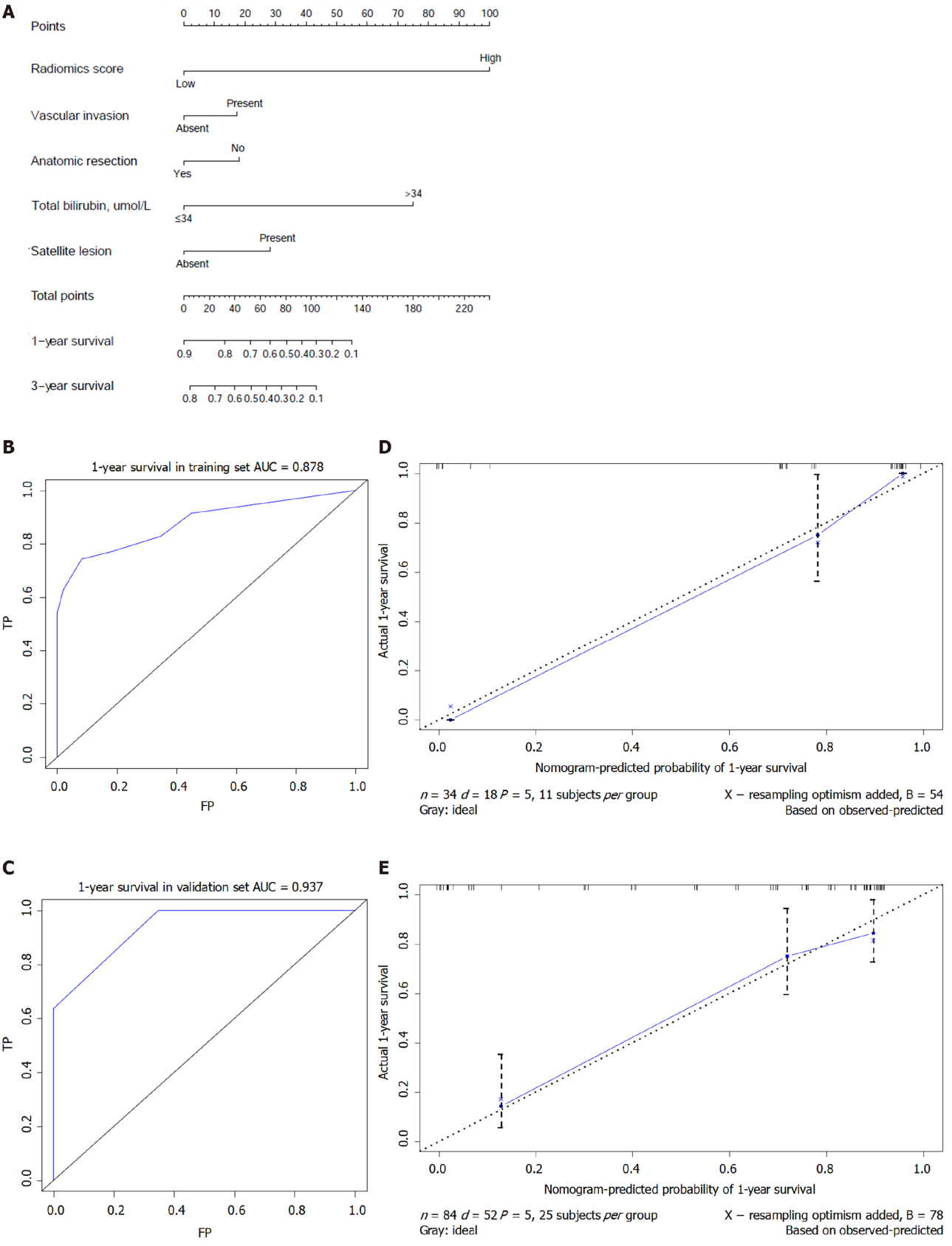

Figure 4 Construction and validation of a radiomics nomogram to predict overall survival of combined hepatocellular carcinoma and cholangiocarcinoma patients after surgical resection. A: Radiomics nomogram to predict overall survival (OS) at 1 and 3 years; B and C: Receiver operating characteristic curves for predicting 1-year OS in the training or validation set. The area under the curve in both cases was > 0.85; D and E: Calibration curves for 1-year OS in the training and validation sets. The horizontal axis is the survival rate predicted by the nomogram, and the vertical axis is the actual survival rate. The black dashed line indicates the case of perfect agreement between the two rates.

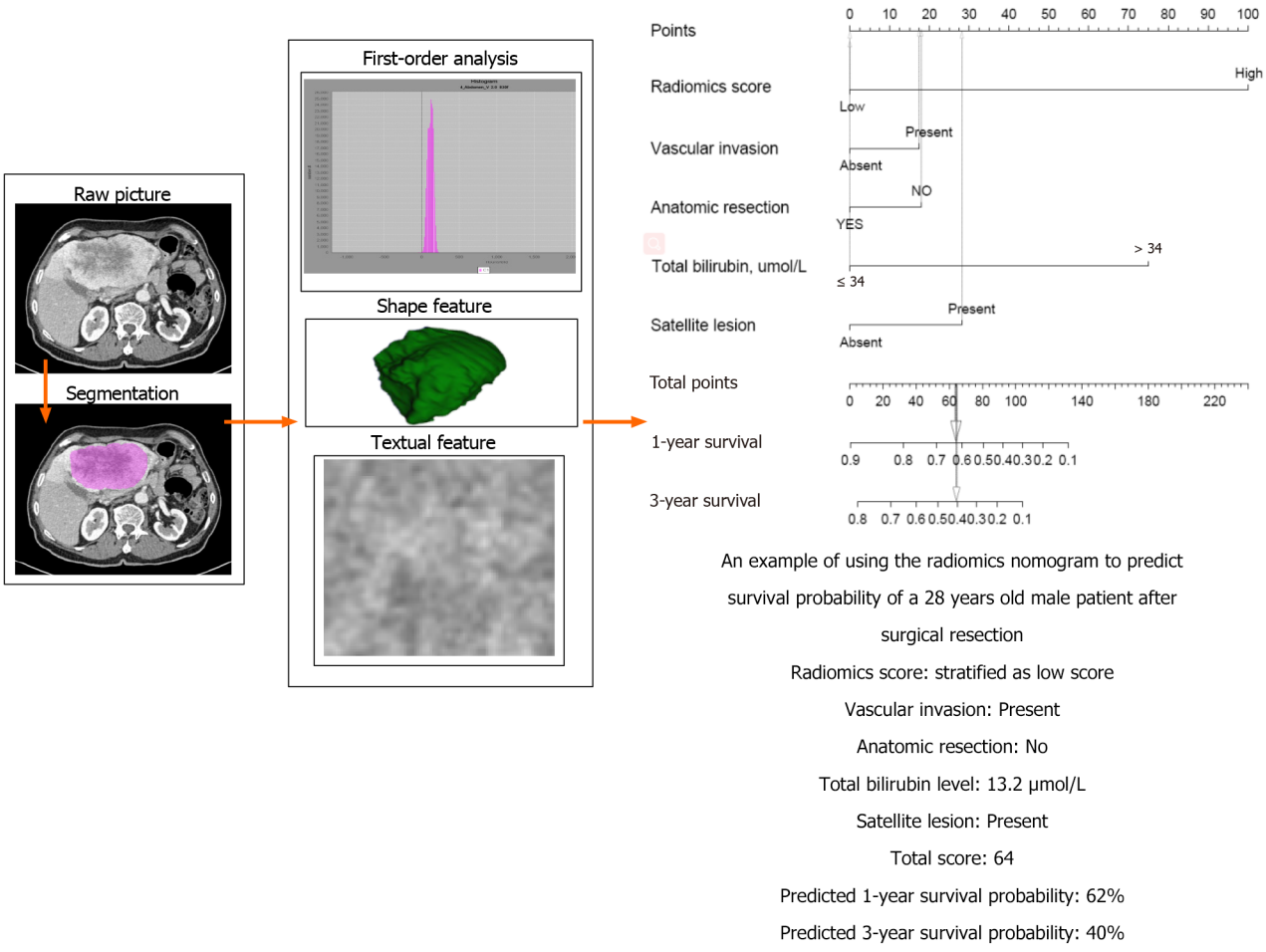

Figure 5 Example of using the radiomics nomogram to predict the overall survival of a 28-year-old man with combined hepatocellular carcinoma and cholangiocarcinoma.

cHCC-CCA patients after resection should be explored in large, prospective studies.

The present study has some limitations. First, its retrospective nature may be associated with a greater risk of selection bias and loss to follow-up, although only eight (6.8%) patients were lost to follow-up. Second, we validated the nomogram internally, not externally; nevertheless, AUCs were > 0.85 for both training and validation sets. Third, the study involved a small sample; thus, the nomogram described here should be validated and optimized using larger samples.

CONCLUSION

This study established a nomogram which combined the CT radiomics score with clinical risk factors to predict OS in patients with cHCC-CCA after resection with curative intent. The radiomics score was strongly associated with postoperative prognosis, and the integrative nomogram predicted OS well: High-risk patients showed a significantly shorter OS than low-risk patients. This integrative nomogram may aid in predicting the prognosis of cHCC-CCA patients after resection, and may support clinical decision-making.

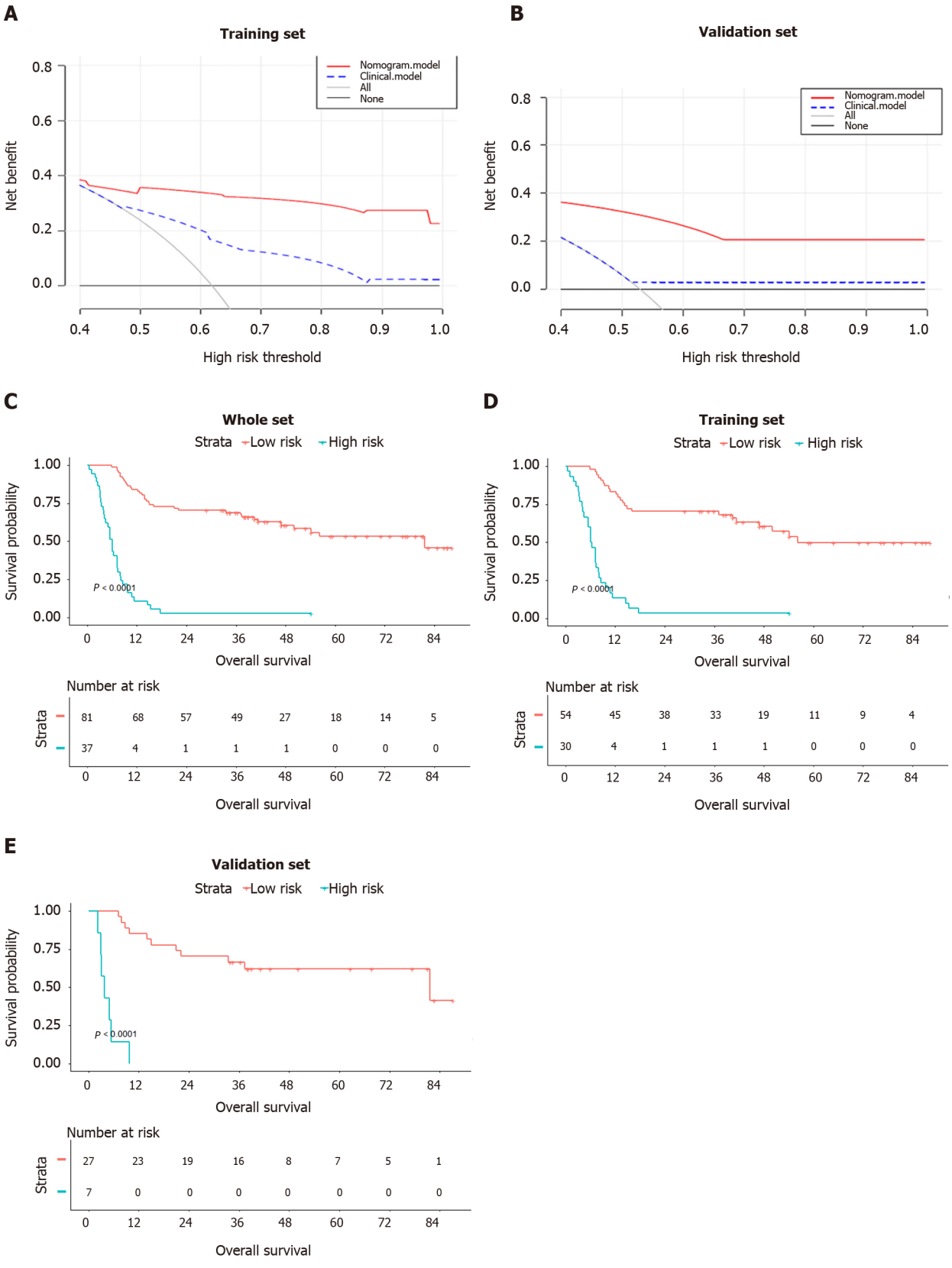

Figure 6 Clinical usefulness of the radiomics nomogram. A and B: Decision curve analysis assessing the ability of the radiomics nomogram or a model based on four clinical factors to predict overall survival (OS) in the training and validation sets. The y-axis indicates "net benefit"; the red line, the radiomics nomogram; the blue dotted line, the model based on clinical factors; the gray dotted line, the result in the event that all patients died; and the black dotted line, the result in the event that no patient died; C-E: OS comparison between patients classified by the radiomics nomogram as at "low risk" or "high risk" of poor OS; C: All patients; D: The training set; and E: The validation set.

ARTICLE HIGHLIGHTS

Research background

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) arises in hepatic progenitor cells and are defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma (ICC) with 5-year postoperative overall survival (OS) rates ranging from 8% to 63%. There are different opinions in the literature on whether the prognosis of patients with cHCC-CCA is worse than that of patients with simple HCC or similar ICC.

Research motivation

Due to the poor prognosis of cHCC-CCA and absence of a promising way to predict prognosis of cHCC-CCA, the authors aimed to construct a radiomics nomogram for predicting postoperative survival of cHCC-CCA patients. This prognostic model may help guide treatment decisions for these patients.

Research objectives

The purpose of this study was to construct and validate a nomogram based on radiomics and clinical characteristics to predict the postoperative survival rate of patients with cHCC-CCA.

Research methods

We collected the clinical data and computed tomography (CT) imaging data of patients with cHCC-CCA. Radiomics features were extracted from portal venous phase CT images using the least absolute shrinkage and selection operator Cox regression and random forest analysis. A nomogram integrating radiomics score and clinical factors was developed using multivariate Cox regression and each patient got a risk score. And patients were categorized as being at "high" or "low" risk based on their risk scores.

Research results

A total of five factors, which were Radiomics score, vascular invasion, anatomical resection, total bilirubin level, and satellite lesions, were independent predictors of prognosis and the nomogram was associated with OS more strongly than a model based on radiomics score or only clinical factors. Patients stratified as being at high risk showed a significantly shorter median OS than those stratified as being at low risk (6.1 *vs* 81.6 mo, $P < 0.001$).

Research conclusions

This nomogram have potential usefulness in predicting postoperative survival of cHCC-CCA patients and may therefore help identify those more likely to benefit from it, which may facilitate clinical decision-making.

Research perspectives

Considering the high AUC of this radiomics nomogram in predicting prognosis of cHCC-CCA, this prognostic model may help guide treatment decisions for these patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7190-7206

DOI: 10.3748/wjg.v27.i41.7190

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

ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of gastrointestinal immune-related adverse events of immune checkpoint inhibitors and their association with survival

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Abstract

BACKGROUND

Despite the popularity of immune checkpoint inhibitors (ICIs) in the treatment of advanced cancer, patients often develop gastrointestinal (GI) and non-GI immune-related adverse events (irAEs). The clinical characteristics and survival outcomes of GI-irAEs have not been fully elucidated in previous reports. This necessitates the evaluation of the impact of GI-irAEs on patients receiving ICI treatment.

interpretation of the data; Yamada K drafted the article; Ishikawa E, Maeda K, Kakushima N, Furukawa K, Honda T, Iida T, Mizutani Y, Ishikawa T, Ohno E, Kawashima H, Ishigami M, Furune S, Hase T, Yokota K, Maeda O, Hashimoto N, Akiyama M, and Ando Y critically revised the article for important intellectual content; Yamamura T performed the statistical analysis; Fujishiro M approved the final version of the article to be published; All authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Nagoya University Hospital (No. 2018-0438, 15006).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: Hase T received personal fees from AstraZeneca, Chugai Pharmaceutical Co. Ltd., Ono Pharmaceutical Co. Ltd., and Bristol-Myers Squibb Co. and grants from AstraZeneca and Chugai Pharmaceutical Co. Ltd., outside the submitted work. Hashimoto N received a grant from Boehringer Ingelheim, outside the submitted work. Ando Y received grants from Mochida Pharmaceutical Co. Ltd.; grants and personal fees from Chugai Pharmaceutical Co. Ltd., Kyowa Kirin Co. Ltd., Nippon Kayaku Co. Ltd., Yakult Honsha Co. Ltd., Ono Pharmaceutical Co. Ltd., Taiho Pharmaceutical Co. Ltd., Daiichi Sankyo Company Ltd., Eisai Co. Ltd.; and personal fees from Eli Lilly Japan K.K., Novartis Pharma K.K., Bayer Holding Ltd., Bristol-Myers Squibb, Sawai Pharmaceutical Co. Ltd., Tsumura & Co., Otsuka Holdings Co. Ltd., Roche Diagnostics K.K., AstraZeneca K.K., and MSD K.K, outside the submitted work. We

AIM

To evaluate the clinical characteristics of GI-irAEs and their impact on survival in patients treated with ICIs.

METHODS

In this single-center, retrospective, observational study, we reviewed the records of 661 patients who received ICIs for various cancers at Nagoya University Hospital from September 2014 to August 2020. We analyzed the clinical characteristics of patients who received ICI treatment. We also evaluated the correlation between GI-irAE development and prognosis in non-small cell lung cancer (LC) and malignant melanoma (MM). Kaplan-Meier analysis was used to compare the median overall survival (OS). Multivariate Cox proportional hazards models were used to identify prognostic factors. A P value < 0.05 was considered statistically significant.

RESULTS

GI-irAEs occurred in 34 of 605 patients (5.6%) treated with an anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1) antibody alone and in nine of 56 patients (16.1%) treated with an anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies. The cumulative incidence and median daily diarrhea frequency were significantly higher in patients receiving anti-CTLA-4 antibodies (P < 0.05). In 130 patients with MM, OS was significantly prolonged in the group that continued ICI treatment despite the development of GI-irAEs compared to the group that did not experience GI-irAEs (P = 0.035). In contrast, in 209 patients with non-small cell LC, there was no significant difference in OS between the groups. The multivariate analyses showed that a performance status of 2-3 (hazard ratio: 2.406; 95% confidence interval: 1.125–5.147; P = 0.024) was an independent predictive factor for OS in patients with MM.

CONCLUSION

Patients receiving anti-CTLA-4 antibodies develop GI-irAEs more frequently and with higher severity than those receiving anti-PD-1/PD-L1 antibodies. Continuing ICI treatment in patients with MM with GI-irAEs have better OS.

Key Words: Colitis; Cytotoxic T-lymphocyte antigen 4; Diarrhea; Drug-related side effects and adverse reactions; Immune checkpoint inhibitors; Prognosis

Core Tip: We compared the clinical characteristics of gastrointestinal immune-related adverse events (GI-irAEs) in patients receiving anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1), anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4), or a combination of anti-PD-1 and anti-CTLA-4 antibodies. We also examined the correlation between GI-irAE development and the prognosis of patients with non-small cell lung cancer (LC) and malignant melanoma (MM). GI-irAEs occurred more frequently and with higher severity in patients receiving anti-CTLA-4 antibodies than in those receiving anti-PD-1/PD-L1 antibodies. Patients with MM, but not non-small cell LC, who continued immune checkpoint inhibitor treatment after developing GI-irAEs had better overall survival.

Citation: Yamada K, Sawada T, Nakamura M, Yamamura T, Maeda K, Ishikawa E, Iida T, Mizutani Y, Kakushima N, Ishikawa T, Furukawa K, Ohno E, Honda T, Kawashima H, Ishigami M, Furune S, Hase T, Yokota K, Maeda O, Hashimoto N, Akiyama M, Ando Y, Fujishiro M. Clinical characteristics of gastrointestinal immune-related adverse events of immune checkpoint inhibitors and their association with survival. *World J Gastroenterol* 2021; 27(41): 7190-7206

URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7190.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7190

declare that there are no conflict of interest.

Data sharing statement: No additional data are available.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

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

Received: July 4, 2021
Peer-review started: July 4, 2021
First decision: July 14, 2021
Revised: July 27, 2021
Accepted: August 31, 2021
Article in press: August 31, 2021
Published online: November 7, 2021

P-Reviewer: Soldera J, Yu F
S-Editor: Yan JP
L-Editor: Filipodia
P-Editor: Yuan YY

INTRODUCTION

In recent years, the prognosis of various malignancies has been improved with the introduction of immune checkpoint inhibitors (ICIs)[1-4] Monoclonal antibodies targeting programmed cell death-1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) exhibit antitumor activity by specifically activating T-cells and suppressing the inhibition of the immune system.

Adverse events (AEs) specific to ICIs, which are different from those of conventional chemotherapy, have been observed in patients receiving ICIs. These AEs are called immune-related AEs (irAEs) and include skin disorders, gastrointestinal (GI) disorders, liver disorders, interstitial lung disorders, and endocrine disorders. Among these, GI-irAEs are the most frequent and substantial. GI-irAEs occur between 0.6 and 120 wk after the initiation of therapy and are characterized by symptoms such as diarrhea, abdominal pain, and bloody stools. Inflammatory sites are found throughout the GI tract[5], and endoscopic findings have been reported to range from normal to ulcerative colitis-like findings[6].

These clinical features reportedly differ between anti-CTLA-4 antibodies, anti-PD-1/anti-PD-L1 antibodies, and their combinations[7-10]. However, there are only a few coherent reports, and the clinical features of GI-irAEs for each ICI have not yet been fully clarified.

The development of irAEs is associated with an improved prognosis in different malignancies[11-14]. In contrast, a previous study[15] showed a poor prognosis with the development of irAEs; hence, a consensus has not yet been reached. In addition, few studies observed prolongation of overall survival (OS) with GI-irAE development [16-18].

Therefore, this study aimed to investigate differences in the clinical characteristics of GI-irAEs associated with anti-PD-1/PD-L1 antibodies, anti-CTLA-4 antibodies, and combination therapy with anti-PD-1 and anti-CTLA-4 antibodies. Further, we examined the correlation between the development of GI-irAEs and patient prognosis.

MATERIALS AND METHODS

Subjects and study design

This single-center, retrospective, observational study included consecutive patients who received ICIs (*i.e.* nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab, or ipilimumab) for various cancers [lung cancer (LC), malignant melanoma (MM), gastric cancer (GC), renal cell carcinoma (RCC), head and neck cancer, urothelial cancer, gynecological cancer, breast cancer, or colorectal cancer] at the Nagoya University Hospital from September 2014 to August 2020. Patients who received ICIs as maintenance therapy after curative chemoradiation for non-small cell lung (NSCLC) or postoperative adjuvant therapy for MM were excluded from the prognostic analysis. The background of the patients, presence or absence of irAEs, time of onset, clinical characteristics at the time of onset, and laboratory findings were collected and reviewed using medical records.

Eligible patients were those treated with ICIs at standard doses. Nivolumab was administered at 3 mg/kg or 240 mg/body every 2 wk. However, some MM patients were administered 2 mg/kg nivolumab every 3 wk. Pembrolizumab was administered at 2 mg/kg or 200 mg/body every 3 wk. Atezolizumab was administered at 1200 mg/body every 3 wk. Avelumab was administered at 10 mg/kg every 2 wk. Durvalumab was administered at 10 mg/kg every 2 wk. Ipilimumab was administered at 3 mg/kg every 3 wk for four cycles. Combination therapy consisted of nivolumab (3 mg/kg or 240 mg/body) and ipilimumab (3 mg/kg) every 3 wk for four cycles.

GI-irAEs were defined as diarrhea or bloody stools after ICI administration in patients in whom infectious enteritis could be excluded. Infectious enteritis (*Clostridium difficile*, other bacterial infections, or viral pathogens, such as cytomegalovirus) was ruled out using blood tests and stool samples. The National Cancer Institute Common Terminology Criteria for AEs (version 5.0) was used to assess the severity of enterocolitis and diarrhea.

Treatment of GI-irAEs was based on the strategy of the attending physician, but in most cases was based on the American Society of Clinical Oncology clinical practice guideline[19]. In the case of Grade 1 GI-irAEs, ICIs were continued or were stopped temporarily and resumed if toxicity did not exceed Grade 1. In the case of Grade 2 or higher GI-irAEs, ICIs were discontinued until the patient's symptoms recovered to Grade 1 or lower, at which point restarting ICIs was considered depending on the

patient's condition.

Outcome measures

We divided the patients into those who received an anti-PD-1/PD-L1 antibody alone (PD-1/PD-L1 group) and those who received an anti-CTLA-4 antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies (CTLA-4 group). The clinical characteristics of GI-irAEs [incidence, cumulative incidence, severity, computed tomography (CT) findings, endoscopic findings, and treatments] were compared between the PD-1/PD-L1 and CTLA-4 groups.

We examined the correlation between the incidence of GI-irAEs and the prognosis of patients with NSCLC and MM, which included more cases of ICI administration than other cancers. Patients who developed GI-irAEs were categorized into two groups: The ICI continuation group in which ICI administration was continued or resumed after improvement of GI-irAEs and the ICI discontinuation group in which ICI administration was permanently discontinued after the development of GI-irAEs. Prognosis was assessed in three groups: Non-GI-irAE, ICI continuation, and ICI discontinuation. Prognosis was also examined by cancer stage (stage III or IV).

Statistical analysis

To compare each group, the Mann–Whitney *U* test was used for continuous variables and Fisher's exact test was used for categorical variables. The Kaplan–Meier method and log-rank tests were used to compare the cumulative incidence and median OS among the groups. Univariate and multivariate Cox proportional hazards models were used to identify prognostic factors associated with GI-irAEs. SPSS Statistics software (version 27.0; IBM Corp., Armonk, NY, United States) was used for analysis. For all analyses, a P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

Overall, there were 605 patients in the PD-1/PD-L1 group and 56 patients in the CTLA-4 group. The clinical characteristics of the PD-1/PD-L1 and CTLA-4 groups are described in Table 1. The median ages were 69 (range: 22–87) and 65 (range: 21–85) years in the PD-1/PD-L1 and CTLA-4 groups, respectively. Patients in the PD-1/PD-L1 group were significantly older than those in the CTLA-4 group (P = 0.039). There were no significant differences in sex, body mass index (BMI), or Eastern Cooperative Oncology Group performance status (ECOG PS) between the groups. The PD-1/PD-L1 group included patients with LC, MM, RCC, GC, and other cancers (head and neck cancer, urothelial cancer, gynecological cancer, breast cancer, and colorectal cancer); the CTLA-4 group included patients with MM and RCC.

Incidence of GI-irAEs

Among 47 patients who developed diarrhea or bloody stools after ICI administration, 39 had diarrhea, one had bloody stools, and seven had diarrhea and bloody stools. Forty-three patients (excluding four patients diagnosed with infectious enteritis) were included in the analysis. GI-irAEs occurred in 34 of 605 patients (5.6%) in the PD-1/PD-L1 group and 9 of 56 patients (16.1%) in the CTLA-4 group. The incidence of GI-irAEs in the CTLA-4 group was significantly higher than that in the PD-1/PD-L1 group (P = 0.008). We compared the cumulative incidence of GI-irAEs in the PD-1/PD-L1 group with that in the CTLA-4 group for all cancers (Figure 1). The cumulative incidence was significantly higher in the CTLA-4 group than in the PD-1/PD-L1 group (P = 0.003). The median observation periods until the development of GI-irAEs were 1683.1 ± 28.3 d [95% confidence interval (CI): 1627.6-1738.5] in the PD-1/PD-L1 group and 1299.7 ± 77.7 d (95%CI: 1147.5-1452.0) in the CTLA-4 group.

Severity of GI-irAEs

The clinical characteristics of patients with GI-irAEs in the PD-1/PD-L1 and CTLA-4 groups are shown in Table 2. There were no differences in age, sex, or median ICI duration before the development of GI-irAEs between the groups. In the PD-1/PD-L1 group, nine patients (26%) had Grade 1 GI-irAEs, 18 (53%) had Grade 2 GI-irAEs, and seven (21%) had Grade 3 GI-irAEs. In the CTLA-4 group, no patients had Grade 1 GI-irAEs, five (55.6%) developed Grade 2 GI-irAEs, and four (44.4%) had Grade 3 GI-irAEs.

Table 1 Clinical characteristics of all patients in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Characteristic	PD-1/PD-L1, *n* = 605	CTLA-4, *n* = 56	*P* value
Age, yr, median (range)	69 (22-87)	65 (21-85)	0.039
Sex, *n* (%)			0.228
Male	419 (69.3)	34 (60.7)	
Female	186 (30.7)	22 (39.3)	
BMI, kg/m^2	21.3 (12.0-37.0)	21.6 (13.9-43.0)	0.532
ECOG PS, *n* (%)			0.073
0-1	534 (88.3)	54 (96.4)	
2-3	71 (11.7)	2 (3.6)	
Cancer type, *n* (%)			
NSCLC	241 (39.8)	0 (0.0)	
MM	110 (18.2)	39 (69.6)	
RCC	52 (8.6)	17 (30.4)	
GC	49 (8.1)	0 (0.0)	
Others	153(25.3)	0 (0.0)	
Drugs, *n* (%)			
Nivolumab	317 (52.4)	0 (0.0)	
Pembrolizumab	180 (29.8)	0 (0.0)	
Atezolizumab	74 (12.2)	0 (0.0)	
Durvalumab	32 (5.3)	0 (0.0)	
Avelumab	2 (0.3)	0 (0.0)	
Ipilimumab	0 (0.0)	28 (50.0)	
Nivolumab + ipilimumab	0 (0.0)	28 (50.0)	

BMI: Body mass index; CTLA-4: Cytotoxic T-lymphocyte antigen 4; ECOG PS: Eastern Cooperative Oncology Group performance status; GC: Gastric cancer; MM: Malignant melanoma; NSCLC: Non-small cell lung cancer; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1; RCC: Renal cell carcinoma.

Grade 2 or 3 GI-irAEs were more frequent in the CTLA-4 group than in the PD-1/PD-L1 group, but there was no statistically significant difference (P = 0.166). The median daily diarrhea frequencies were 5.0 (range: 0-10) times and 6.5 (range: 4-15) times in the PD-1/PD-L1 and CTLA-4 groups, respectively. The median daily diarrhea frequency was significantly higher in the CTLA-4 group than in the PD-1/PD-L1 group (P = 0.03).

Treatment

Thirteen patients (38.2%) in the PD-1/PD-L1 group and one (11.1%) in the CTLA-4 group improved without medication (Table 2). More patients in the CTLA-4 group than in the PD-1/PD-L1 group improved without medication (P = 0.017). Corticosteroids were required in 11 patients (32.4%) in the PD-1/PD-L1 group and eight (88.9%) in the CTLA-4 group. More patients in the CTLA-4 group than in the PD-1/PD-L1 group required steroid therapy (P = 0.006). All patients in this study improved with observation or treatment with steroids, and no patient was treated with biological agents or surgery.

CT and endoscopic findings

Twenty-three patients in the PD-1/PD-L1 group underwent abdominal CT, which showed many inflammation sites, especially between the descending colon and rectum. Eight patients in the CTLA-4 group had diffused CT findings between the jejunum and rectum (Table 3). There was no statistically significant difference between

Table 2 Clinical characteristics of patients who developed gastrointestinal-immune-related adverse events in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Characteristic	PD-1/PD-L1, *n* = 34	CTLA-4, *n* = 9	*P* value
Age, yr, median (range)	69 (37-86)	56 (46-80)	0.187
Sex, *n* (%)			0.427
Male	29 (85.3)	7 (77.8)	
Female	5 (14.7)	2 (22.2)	
Drugs, *n* (%)			
Nivolumab	12 (35.3)	0 (0.0)	
Pembrolizumab	16 (47.0)	0 (0.0)	
Atezolizumab	2 (5.9)	0 (0.0)	
Durvalumab	4 (11.8)	0 (0.0)	
Ipilimumab	0 (0.0)	6 (66.7)	
Nivolumab + ipilimumab	0 (0.0)	3 (33.3)	
Median ICI duration before GI-irAE onset (d), median (range)	77 (4-733)	42 (11-92)	0.127
Diarrhea frequency per day, times (range)	5.0 (0-10)	6.5 (4-15)	0.031
CTCAE Grade, *n* (%)			0.288
1	9 (26.5)	0 (0.0)	
2-3	25 (73.5)	9 (100)	
GI-irAE treatment, *n* (%)			
Improvement without medication	13 (38.2)	1 (11.1)	0.017
Corticosteroids	11 (32.4)	8 (88.9)	0.006
Loperamide	8 (23.5)	1 (11.1)	0.657

CTCAE: Common Terminology Criteria for Adverse Events; CTLA-4: Cytotoxic T-lymphocyte antigen 4; GI-irAE: Gastrointestinal-immune-related adverse event; ICI: Immune checkpoint inhibitor; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1.

the PD-1/PD-L1 group and the CTLA-4 group in each segment from the jejunum to the rectum.

Lower GI endoscopy was performed in 16 patients (12 in the PD-1/PD-L1 group and four in the CTLA-4 group). Endoscopic findings were classified as follows, according to a previous report[6]: (1) Large deep ulcer [PD-1/PD-L1 group (*n* = 1)]; (2) Diffuse erythema with exudate [PD-1/PD-L1 group (*n* = 2); CTLA-4 group (*n* = 1)]; (3) Patchy erythema [CTLA-4 group (*n* = 1)]; (4) Aphtha [PD-1/PD-L1 group (*n* = 2)]; (5) Edema [PD-1/PD-L1 group (*n* = 4); CTLA-4 group (*n* = 2)]; and (6) Normal [PD-1/PD-L1 group (*n* = 2)]. There was no specific trend in endoscopic findings based on treatment group. There was an ischemic change [PD-1/PD-L1 group (*n* = 1)] that could not be classified using the classifications above (Figure 2).

Development of GI-irAEs and prognosis

The study included 209 patients with NSCLC and 130 patients with MM, excluding 30 patients who received durvalumab as maintenance therapy after curative chemoradiation for unresectable advanced NSCLC and 19 patients with MM who received ICIs as postoperative adjuvant therapy.

Twelve patients with NSCLC and 13 with MM developed GI-irAEs. The clinical characteristics of all patients and those who developed GI-irAEs are shown in Tables 4 and 5, respectively.

There were eight and four patients with NSCLC in the ICI continuation and ICI discontinuation groups, respectively, and 10 and three patients with MM in the ICI continuation and ICI discontinuation groups, respectively. Among patients with NSCLC, the median OS was 488.0 ± 20.6 d (95%CI: 447.6-528.4) in the ICI continuation group, 829.0 ± 558.3 d (95%CI: 0-1923.3) in the ICI discontinuation group, and 521.0 ±

Table 3 Site of inflammation on abdominal computed tomography in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Site of inflammation	PD-1/PD-L1, *n* = 23	CTLA-4, *n* = 8
Jejunum, *n* (%)	1 (4.3)	2 (25.0)
Ileum, *n* (%)	2 (8.7)	2 (25.0)
Cecum, *n* (%)	2 (8.7)	2 (25.0)
Ascending colon, *n* (%)	3 (13.0)	4 (50.0)
Transverse colon, *n* (%)	3 (13.0)	3 (37.5)
Descending colon, *n* (%)	6 (26.1)	3 (37.5)
Sigmoid colon, *n* (%)	7 (30.4)	2 (25.0)
Rectum, *n* (%)	8 (34.8)	2 (25.0)
No findings, *n* (%)	9 (39.1)	2 (25.0)

CT: Computed tomography; CTLA-4: Cytotoxic T-lymphocyte antigen 4; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1.

102.4 d (95%CI: 320.2-721.8) in the non-GI-irAE group. There was no significant difference in OS among the three groups. The results were similar when patients were stratified by disease stage (stage III or IV). Among patients with MM, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group [not reached *vs* 481.0 d (95%CI: 329.1-632.9); P = 0.035]. There was no significant difference in OS based on the development of GI-irAEs among patients with stage III disease. Among patients with stage IV disease, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group [not reached *vs* 427.0 d (95%CI: 248.5-605.5); P = 0.017] (Figure 3).

Prognostic factors for OS in NSCLC and MM were examined using Cox proportional hazards models (Tables 6 and 7). The following factors were considered: Age (< 75 *vs* ≥ 75 years), sex, BMI [underweight (< 18.5 kg/m^2) *vs* normal weight (18.5−24.9 kg/m^2) *vs* overweight (≥ 25.0 kg/m^2)], stage (III *vs* IV), ECOG PS (0-1 *vs* 2-3), ICIs (PD-1/PD-L1 *vs* CTLA-4), and GI-irAEs (ICI continuation *vs* ICI discontinuation *vs* non-GI-irAE groups). In NSCLC, the univariate analysis showed significant associations of OS with age, BMI, stage, and ECOG PS. In the multivariate analysis of these factors, stage IV disease [hazard ratio (HR): 2.182; 95%CI: 1.085–4.387; P = 0.029] and a ECOG PS of 2-3 (HR: 12.772; 95%CI: 7.067-23.085; P < 0.001) were identified as independent predictors of OS. Similarly, in MM, the univariate analysis showed significant associations of OS with age, sex, ECOG PS, and GI-irAEs. In the multivariate analysis of these factors, only ECOG PS (HR: 2.406; 95%CI: 1.125-5.147; P = 0.024) was identified as an independent predictor of OS.

DISCUSSION

In this study, patients in the CTLA-4 group had a higher incidence of GI-irAEs and a higher frequency of diarrhea than those in the PD-1/PD-L1 group. In addition, treatment of GI-irAEs required more steroids in the CTLA-4 group than in the PD-1/PD-L1 group. In a previous report[7], the onset of GI-irAEs has been reported to occur 6-7 wk after the initiation of ipilimumab, an anti-CTLA-4 antibody. Patients who received anti-PD-1 monotherapy developed GI-irAEs at a median of 25.4 wk (range: 0.6-119.9), whereas those who received combination therapy with anti-PD-1 and anti-CTLA-4 antibodies developed GI-irAEs earlier, at a median of 7.2 wk (range: 0.7-51) [8]. Regarding incidence, a meta-analysis by Wang *et al*[20] reported overall incidences of colitis of 9.1% among patients treated with anti-CTLA-4 antibody monotherapy, 1.3% among those treated with anti-PD-1/PD-L1 antibody monotherapy, and 13.6% among those treated with a combination of anti-PD-1 and anti-CTLA-4 antibodies. Similarly, our study showed that the incidence of GI-irAEs in the CTLA-4 group was indeed higher than that in the PD-1/PD-L1 group.

There are several possible explanations for the higher incidence, severity, and use of steroids among patients treated with anti-CTLA-4 antibodies compared to those treated with anti-PD-1/PD-L1 antibodies. CTLA-4 is required for the accumulation of

Table 4 Clinical characteristics and frequency of each type of immune-related adverse event in patients with non-small cell lung carcinoma and malignant melanoma

Characteristic	NSCLC, *n* = 209	MM, *n* = 130
Age, yr	66 ± 11	66 ± 13
Sex, *n* (%)		
Male	143 (68.4)	75 (57.7)
Female	66 (31.6)	55 (42.3)
BMI, kg/m^2	21.7 ± 3.4	22.4 ± 4.3
ECOG PS, *n*		
0-1	184	119
2-3	25	11
Drugs, *n* (%)		
Nivolumab	61 (29.2)	58 (44.6)
Pembrolizumab	87 (41.6)	35 (26.9)
Atezolizumab	61 (29.2)	0 (0.0)
Ipilimumab	0 (0.0)	27 (20.8)
Nivolumab + ipilimumab	0 (0.0)	10 (7.7)
History of ICI use, *n* (%)	11 (5.3)	34 (26.2)
Follow-up, d	365 ± 335	466 ± 419
Total irAEs, *n* (%)		
GI-irAEs	9 (4.3)	13 (10.0)
Liver-irAEs	7 (3.3)	13 (10.0)
Lung-irAEs	10 (4.8)	11 (8.5)
Skin-irAEs	9 (4.3)	9 (6.9)
Thyroid-irAEs	12 (5.7)	9 (6.9)

BMI: Body mass index; ECOG PS: Eastern Cooperative Oncology Group performance status; GI: Gastrointestinal; irAE: Immune-related adverse event; ICI: Immune checkpoint inhibitor; MM: Malignant melanoma; NSCLC: Non-small cell lung carcinoma.

regulatory T cells in the bowel[21], and is thought to play an essential role in the maintenance of bowel homeostasis, even in the absence of inflammation. In contrast, PD-1 depends on PD-L1/PD-L2 expression to regulate T-cell activation, and PD-L1 and PD-L2 are upregulated by inflammation, inflammatory cytokines, and chemokines [22-25]. When this mechanism is blocked by anti-PD-1/PD-L1 antibodies, it may be difficult to control inflammation, given that blocking with ICIs leads to a partial block of the mechanisms that trigger inflammation. Some reports suggest that these mechanistic differences may account for the difference in the frequency of enteritis caused by anti-CTLA-4 and anti-PD-1/PD-L1 antibodies[9].

There were no reported differences in the CT or endoscopic findings of enteritis caused by anti-CTLA-4 and anti-PD-1/PD-L1 antibodies. There was no specific trend, and a variety of findings were found in this study. In one case in the PD-1/PD-L1 group, an ischemic colitis-like endoscopic finding, which is rarely reported as a GI-irAE, was observed. Since necrotizing gastritis has been previously reported as an AE in the upper GI tract[9], it was included as a GI-irAE in this study. When GI-irAEs are suspected, CT and endoscopy should be considered, as they are useful for confirming the extent of the lesion and excluding other diseases[19].

In this study, OS was prolonged in the ICI continuation group compared to the non-GI-irAE group among patients with MM, but not among those with NSCLC. According to the multivariate Cox regression analysis, continued administration of ICIs after the development of GI-irAEs was not an independent factor that affected the survival of patients with MM (HR: 3.081; P = 0.058). However, a statistically significant difference may be expected with the accumulation of future cases. Many studies have

Table 5 Clinical characteristics of patients with non-small cell lung carcinoma and malignant melanoma who developed gastrointestinal-immune-related adverse event

Characteristic	NSCLC, *n* = 12	MM, *n* = 13
Age, yr	67 ± 11	67 ± 12
Sex, *n*		
Male	10	9
Female	2	4
BMI, kg/m^2	22.2 ± 3.7	22.1 ± 4.4
ECOG PS, *n*		
0-1	12	13
2-3	0	0
Stage, *n*		
III	1	2
IV	11	11
Latest ICI, *n*		
Nivolumab	2	5
Pembrolizumab	8	3
Atezolizumab	2	0
Ipilimumab	0	5
Nivolumab + ipilimumab	0	0
Diarrhea frequency	4.3 ± 1.8	5.5 ± 2.5
CTCAE Grade, *n*		
1	4	3
2	7	8
3	1	2
Median ICI duration before GI-irAE onset (d), median (range)	60 (7-567)	75 (24-733)
Treatment with ICIs after the onset of GI-irAEs		
Continued or resumed	8	10
Discontinued	4	3

BMI: Body mass index; CTCAE: Common Terminology Criteria for Adverse Events; ECOG PS: Eastern Cooperative Oncology Group performance status; GI-irAE: Gastrointestinal-immune-related adverse event; ICI: Immune checkpoint inhibitor; MM: Malignant melanoma; NSCLC: Non-small cell lung carcinoma.

reported a correlation between the development of irAEs and a favorable prognosis [26-28]. A shared antigen is one promising hypothesis for the mechanism, which was reported in a case study of fatal myocarditis after ipilimumab and nivolumab treatment for MM[29]. Autopsy of the patient revealed infiltration of myocardial T cells and macrophages. Receptor sequencing of the infiltrating T cells revealed that the selective clonal T-cell population infiltrating the myocardium was identical to that in tumors and skeletal muscle. According to another report[30], in patients with NSCLC and MM under PD-1 blockade, lung irAE lesions were infiltrated by T cells with similar specificity to tumor-infiltrating T cells. These reports suggest that specific autoimmune T cell clones recognize shared antigens between normal and tumor tissues, triggering an immune response that results in irAEs. IrAEs may represent a strong immune response to both tumor and healthy tissues, leading to stronger antitumor effects and improved prognosis. However, GI-irAEs may develop due to excessive activation of intestinal immunity by T cells as described above. There may be a common underlying factor between the susceptibility to ICIs due to enhanced activation of tumor immunity and the susceptibility to GI-irAEs due to activation of

Table 6 Univariate and multivariate analyses of clinical factors related to overall survival in non-small cell lung carcinoma

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr						
< 75	1			1		
≥ 75	0.520	0.277-0.976	0.042	0.658	0.345-1.253	0.203
Sex						
Male	1					
Female	1.301	0.845-2.003	0.233			
BMI, kg/m^2						
Underweight (< 18.5)	1			1		
Normal (18.5-24.9)	0.527	0.316-0.878	0.014	0.635	0.377-1.067	0.086
Overweight (> 25.0)	0.394	0.195-0.795	0.009	0.506	0.250-1.040	0.064
Stage						
III	1			1		
IV	2.447	1.227-4.881	0.011	2.182	1.085-4.387	0.029
ECOG PS						
0-1	1			1		
2-3	15.197	8.486-27.214	< 0.001	12.772	7.067-23.085	< 0.001
GI-irAE						
Continued administration of ICIs	1					
Discontinued administration of ICIs	0.904	0.165-4.945	0.907			
Non-GI-irAEs	1.334	0.489-3.642	0.574			

BMI: Body mass index; CI: Confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; GI-irAE: Gastrointestinal-immune-related adverse event; HR: Hazard ratio; ICI: Immune checkpoint inhibitor.

intestinal immunity.

Conversely, patients who discontinued ICIs after developing GI-irAEs did not have prolonged OS compared to those who did not develop GI-irAEs in this study. We consider this result to be caused by the difficulty in continuing ICIs in patients with no antitumor effects at the time of developing GI-irAEs. We also consider the possibility that OS may have been shortened by not administering ICIs for a sufficient period.

ICI administration often cannot be continued depending on the extent of irAEs. According to consensus guidelines[19], careful follow-up after the onset of irAEs or the administration of steroids and continuation of ICIs after improvement of irAEs to Grade 1 can be considered. However, severe irAEs (Grade 3 or higher) may result in permanent discontinuation of ICIs, necessitating hospitalization or surgery, and may be fatal in some cases.

As irAEs are life-threatening, depending on severity, it is difficult to determine whether to continue ICIs in patients with Grade 1 irAEs or to resume ICIs after recovery from Grade 2 or higher irAEs. However, it is expected that the number of cases in which resuming ICIs is considered will increase in the future when irAEs occur and no other alternative treatments are available. IrAEs that occur during ICI reintroduction are less severe than initial irAEs and can be tolerated if monitored appropriately[31,32]. GI-irAE recurrence in cases of ICI re-administration after GI-irAE onset has been observed in about one-third of patients[33], and GI-irAEs occurring at re-administration were less frequent and less severe than the initial GI-irAEs[34].

This study showed that the continuation of ICI administration after GI-irAEs in MM may contribute to enhanced patient prognosis. However, which patients with severe GI-irAEs (Grade ≥ 3) should continue ICIs is a topic for future studies. In addition, we believe that the decision to reinitiate ICI treatment after irAEs should be based on the clinical judgment of the treating physician, considering the physical health of the

Table 7 Univariate and multivariate analyses of clinical factors related to overall survival in malignant melanoma

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr						
< 75	1			1		
≥ 75	1.717	1.067-2.761	0.026	1.474	0.816-2.663	0.199
Sex						
Male	1			1		
Female	0.593	0.354-0.993	0.047	0.793	0.418-1.506	0.479
BMI, kg/m^2						
Underweight (< 18.5)	1					
Normal (18.5-24.9)	1.252	0.671-2.336	0.48			
Overweight (> 25.0)	1.044	0.510-2.137	0.906			
Stage						
III	1					
IV	1.758	0.838-3.686	0.135			
ECOG PS						
0-1	1			1		
2-3	3.014	1.427-6.366	0.004	2.406	1.125-5.147	0.024
GI-irAE						
Continued administration of ICIs	1			1		
Discontinued administration of ICIs	3.818	0.767-18.996	0.102	4.079	0.779-21.368	0.096
Non-GI-irAEs	3.25	1.020-10.360	0.046	3.081	0.963-9.861	0.058
ICI						
Anti PD-1/PD-L1 antibody	1					
Anti CTLA-4 antibody	1.366	0.837-2.228	0.212			

BMI: Body mass index; CI: Confidence interval; CTLA-4: Cytotoxic T-lymphocyte antigen 4; GI-irAE: Gastrointestinal-immune-related adverse event; HR: Hazard ratio; ICI: Immune checkpoint inhibitor; PD-1: Programmed cell death-1.

patient, and appropriate informed consent.

Once the mechanism of irAE development and predictive markers for irAE development are identified, it will be possible to continue ICI treatment for a longer period while monitoring patients prone to irAE development and preventing irAEs. Since this may improve the prognosis of patients, further research to clarify the pathogenesis is desirable.

There are several limitations to this study. First, it is a single-center, retrospective study. Second, abdominal CT scans and lower GI endoscopy were not performed in all patients in this study, and the number of patients who had abdominal CT scans and lower GI endoscopy was not enough. More cases are expected in the future. Third, the OS analysis for each ICI regimen was not statistically significant, probably due to the small sample size. Therefore, patients who received ICI monotherapy and those who received multiple ICIs sequentially were included in the analysis.

CONCLUSION

GI-irAEs tended to occur more frequently and with higher severity in patients treated with anti-CTLA-4 antibodies or combination therapy than in those treated with anti-PD-1/PD-L1 antibodies. Abdominal CT and lower GI endoscopy did not show any characteristic findings or trends. We found evidence that the clinical characteristics

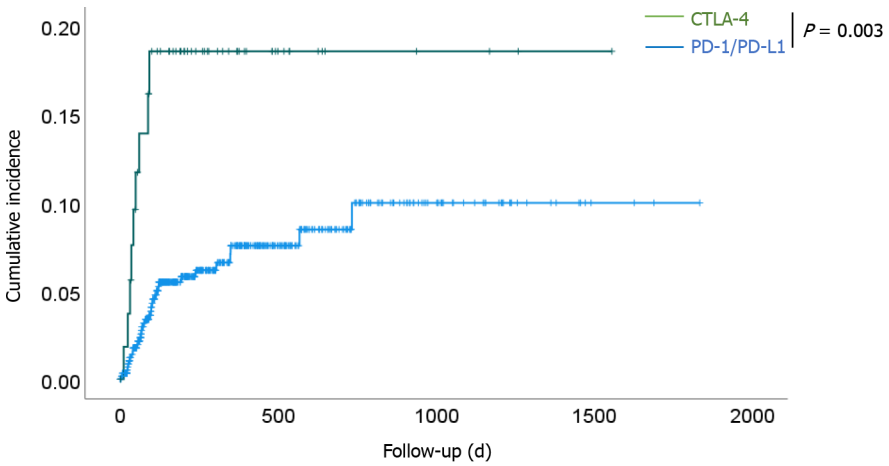


Figure 1 Kaplan–Meier curves of the cumulative incidence of gastrointestinal- immune-related adverse events for all patients in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups. The cumulative incidence was significantly higher in the cytotoxic T-lymphocyte antigen 4 group than in the programmed cell death-1/programmed death-ligand 1 group (P = 0.003). CTLA-4: Cytotoxic T-lymphocyte antigen 4; PD-1: Programmed cell death-1; PD-L1: Programmed death-ligand 1.

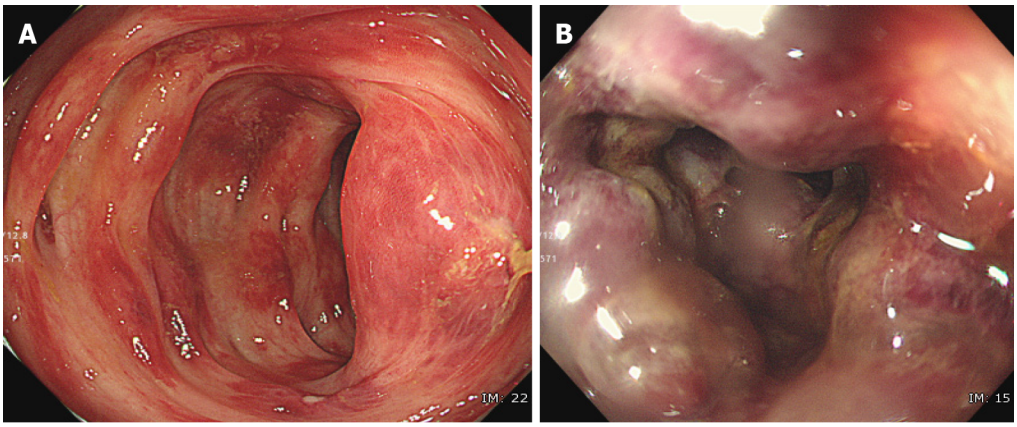
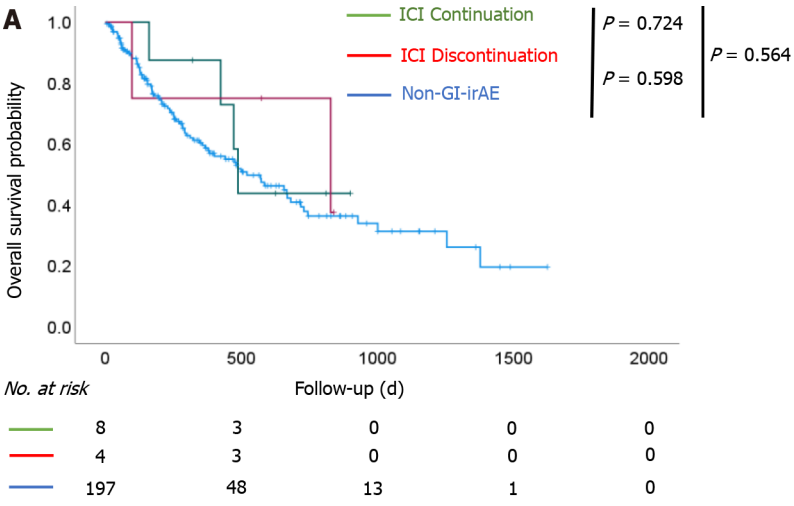
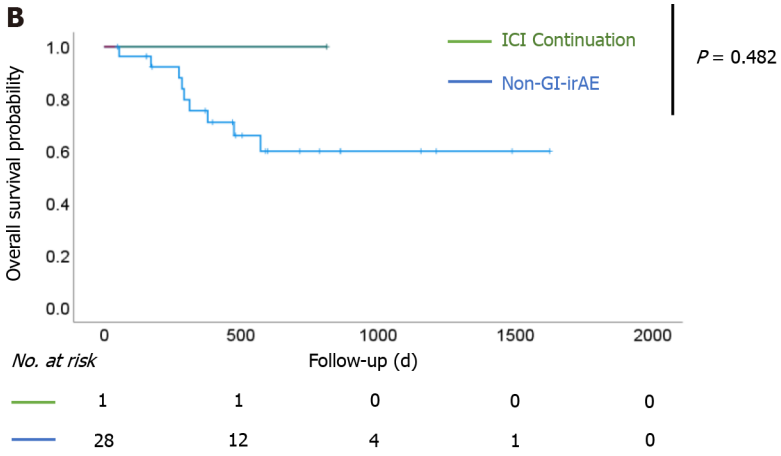
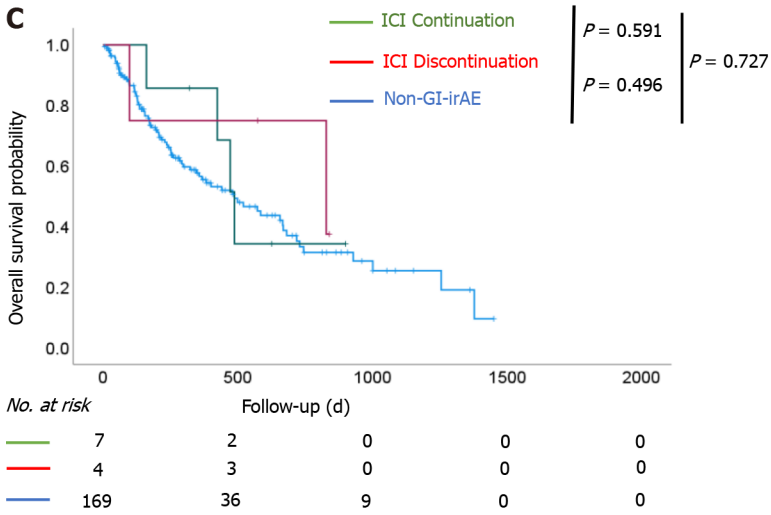
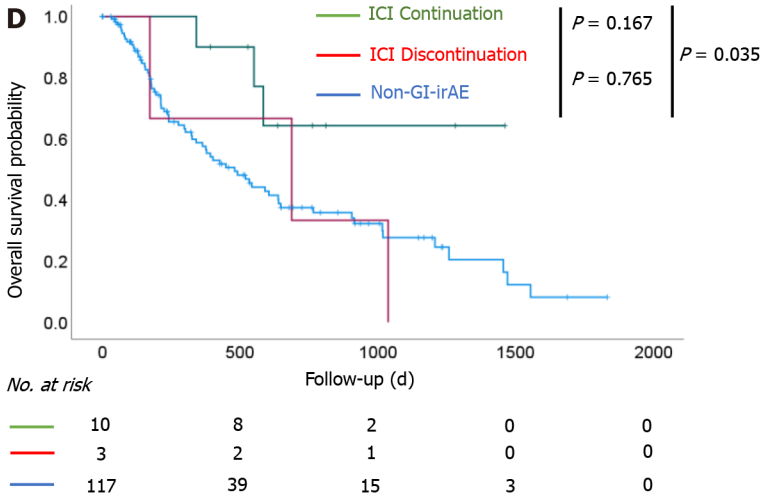


Figure 2 Ischemic change caused by an immune checkpoint inhibitor. Endoscopic images of gastrointestinal-immune-related adverse events in the sigmoid colon of a patient. A: The mucosa of the sigmoid colon shows redness, erosion, hemorrhage, and edema; B: Some of the mucosa is pale, with submucosal edema and bleeding.




B
ICI Continuation
Non-GI-irAE
P = 0.482
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
1 1 0 0 0
28 12 4 1 0


C
ICI Continuation
ICI Discontinuation
Non-GI-irAE
P = 0.591
P = 0.496
P = 0.727
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
7 2 0 0 0
4 3 0 0 0
169 36 9 0 0


D
ICI Continuation
ICI Discontinuation
Non-GI-irAE
P = 0.167
P = 0.765
P = 0.035
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
10 8 2 0 0
3 2 1 0 0
117 39 15 3 0

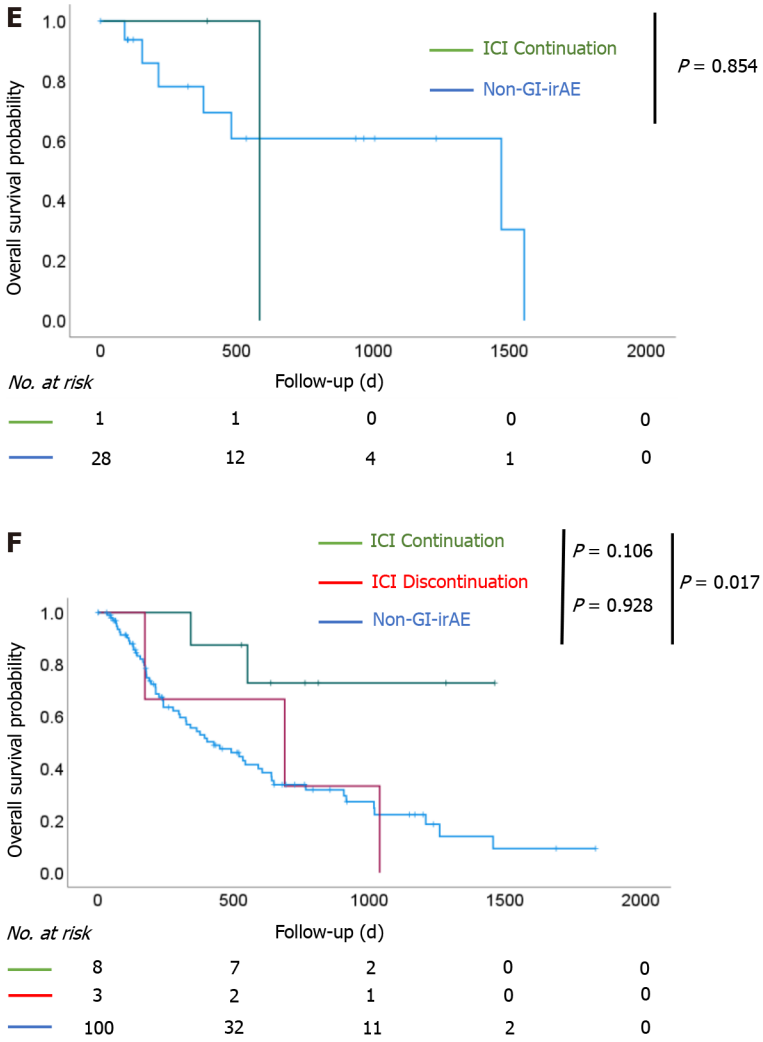


Figure 3 Overall survival after initiation of immune checkpoint inhibitor treatment. Overall survival (OS) of patients with non-small cell lung cancer (NSCLC) and OS of patients with malignant melanoma (MM). A: In patients with NSCLC, there was no significant difference in OS among the three groups; B: The results were similar when stratified in stage III; C: The results were similar when stratified in stage IV; D: In patients with MM, there was a significant prolongation of OS in the immune checkpoint inhibitor (ICI) continuation group compared to the non-gastrointestinal immune-related adverse event (non-GI-irAE) group (P = 0.035); E: There was no significant difference in OS between the ICI continuation group and the non-GI-irAE group among patients with stage III disease; F: Among patients with stage IV disease, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group (P = 0.017). ICI continuation group: Patients who continued ICI treatment after developing GI-irAEs; ICI discontinuation group: Patients who discontinued ICI treatment after developing GI-irAEs; non-GI-irAE group: Patients with no GI-irAEs.

and pathologies may be different between the groups, but a further investigation with a larger sample size is necessary.

In terms of prognosis, OS was not prolonged in NSCLC patients with or without GI-irAEs; however, in MM patients, OS was significantly prolonged in patients who developed GI-irAEs and continued ICI treatment compared with that in other patients.

ARTICLE HIGHLIGHTS

Research background

Immune checkpoint inhibitors (ICIs) are gaining popularity as a treatment for advanced cancer. However, among immune-related adverse events (irAEs), gastrointestinal-related immune AEs (GI-irAEs) have limited their use.

Research motivation

There are only a few coherent reports on the clinical characteristics of GI-irAEs for each ICI. In addition, the correlation between the development of GI-irAEs and patient prognosis has not been fully elucidated.

Research objectives
We aimed to evaluate the clinical characteristics of GI-irAEs and its influence on survival in patients treated with ICI.

Research methods
We retrospectively reviewed the records of 661 patients who received ICIs at Nagoya University Hospital from September 2014 to August 2020. We analyzed the clinical characteristics of patients who received an anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1) antibody, anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody, or combination therapy with anti-PD-1 and anti-CTLA-4 antibodies. We also evaluated the correlation between GI-irAEs development and the prognoses.

Research results
GI-irAEs occurred in 34 of 605 patients (5.6%) treated with an anti-PD-1/PD-L1 antibody alone and in nine of 56 patients (16.1%) treated with an CTLA-4 antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies. The cumulative incidence and median daily diarrhea frequency were significantly higher in patients receiving anti-CTLA-4 antibodies ($P < 0.05$). In 130 patients with malignant melanoma (MM), overall survival was significantly prolonged in the group that continued ICI treatment despite the development of GI-irAEs compared to the group that did not experience GI-irAEs ($P = 0.035$).

Research conclusions
GI-irAEs occurred more frequently and with a higher severity in patients using anti-CTLA-4 antibodies than in those using anti-PD-1/PD-L1 antibodies. In patients with MM who developed GI-irAEs and continued treatment with ICIs, overall survival was significantly prolonged.

Research perspectives
Multicenter studies with large samples are expected to evaluate clinical characteristics of GI-irAEs and their association to long-term survival outcomes.

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World J Gastroenterol 2021 November 7; 27(41): 7207-7209

DOI: 10.3748/wjg.v27.i41.7207

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

LETTER TO THE EDITOR

Pancreatic cyst dilemma: Between physical and biochemical markers

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Author contributions: Khamaysi I designed and wrote the letter; and Eyal Zussman revised the letter; the manuscript has been read and approved by all the authors.

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

Country/Territory of origin: Israel

Specialty type: Gastroenterology and Hepatology

Peer-review report's scientific quality classification

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

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Abstract

Physical analysis of the pancreatic cystic lesions (PCLs) fluid as expressed by the rheological behavior ("string sign") can improve the diagnostic yield and should be integrated in every multimodal PCLs workup.

Key Words: Pancreatic cyst; Fluid analysis; String sign; Rheology

Core Tip: No single optimal test reliably determines the pancreatic cyst subtype including all imaging modalities and biochemical fluid analysis. Physical analysis of the fluid as expressed by the string sign can improve the diagnostic yield and should be integrated in every multimodal pancreatic cystic lesions workup. The string sign as it is currently performed, suffers from significant shortcoming due to its subjective nature. Rheological (physical) properties, instead, can overcome the disadvantages of the standard string sign and replace it in clinical practice.

Citation: Khamaysi I, Zussman E. Pancreatic cyst dilemma: Between physical and biochemical markers. *World J Gastroenterol* 2021; 27(41): 7207-7209
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7207.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7207

Received: June 6, 2021
Peer-review started: June 6, 2021
First decision: June 30, 2021
Revised: July 1, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Kikuyama M, Masuda A
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

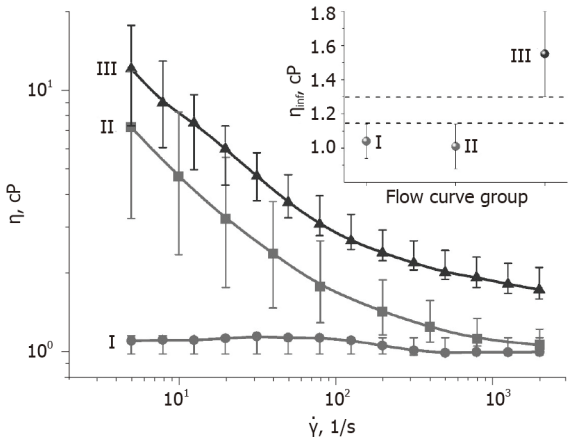

Figure 1 Representative types I, II and III flow curves. The graph inset shows the values of infinite viscosity, ηc, depicting the difference between the minimal value of type III and the maximum value of types I and II.

TO THE EDITOR

We read with great interest the frontier by Okasha *et al*[1] regarding the diagnosis of pancreatic cystic lesions (PCLs) and the benefits of various diagnostic models.

The authors described a vast array of available diagnostic test and concluded that the combination of both endoscopic ultrasound-fine needle aspiration (EUS-FNA) findings with cystic fluid tumor markers analysis, along with clinical, radiologic, histologic, genetic, and molecular characteristics, enhances the diagnostic accuracy and helps to construct a novel model in the era of PCLs[1].

Unfortunately, the authors did not mention the viscosity of the cystic fluid as an important marker for differentiation between PCLs subtypes (mucinous and non-mucinous cysts).

The string sign, as a surrogate marker of fluid viscosity, is a useful and reliable test that can be used to improve the diagnostic accuracy of other pancreatic cyst fluid studies when used in combination[2], however, the sting sign suffers from, relatively, high interobserver variability regarding its positivity and should be interpreted with caution and not used as a single test but in combination with other tests to differentiate mucinous from non-mucinous cysts[3].

String sign is inherently a subjective test and lacks a theoretical framework for predicting the viscoelastic nature of the fluid, which can be objectively characterized by the viscous and elastic response of a fluid under deformation (rheological behavior).

In order to overcome the subjective nature of the string sign, we developed a new rheological assay in which (using a rheometer) a wide array of viscoelastic properties (rheological curves) can be generated and recorded.

Use of a rotational viscometer supports simulation of true rheological conditions (the stepping change of either the shear stress or the shear rate is programmed but the parameter remains constant during each step). The viscosity of the samples was measured with a DHR-2 Rheometer (TA Instruments, USA) at 25 °C. The preferred geometry was cone-and-plate, with a cone diameter of 40 mm and a surface-plate angle of 1°. The rheometer was operated in shear rate control mode. Several time sweep tests at different constant shear rates (5-2000 1/s) were performed. The measured steady-state shear viscosity values (when the viscosity was constant in time) were used to construct flow curves of the fluids

In our study[4], we found that the cutoff value of pancreatic cyst fluid viscosity, ηc, can serve as an independent marker to distinguish between mucinous and non-mucinous cysts. It was found that ηc > 1.3 cP characterizes mucinous cysts, whereas ηc > 1.3 cP is typical for non-mucinous cysts. Moreover, we could differentiate between three distinct flow curves of the rheological behavior of pancreatic cyst fluids according to dynamic viscoelastic properties. Types I and II hypothesized to correlate with non-mucinous cysts, and type III with mucinous cysts (Figure 1). This simple and rapid diagnostic tool can be immediately implemented after EUS-FNA sampling, and provides for a low variability rate compared to the commonly used, subjective string sign technique. Although the findings are promising, they must be further confirmed in a large-scale study.

In conclusion, no single optimal test reliably determines the pancreatic cyst subtype including all imaging modalities and biochemical fluid analysis. Physical analysis of the fluid as expressed by the string sign can improve the diagnostic yield and should be integrated in every multimodal PCLs workup.

The string sign as it is currently performed, suffers from significant shortcoming due to its subjective nature. Rheological (physical) properties, instead, can overcome the disadvantages of the standard string sign and replace it in clinical practice.

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