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REVIEW

Positioning of old and new biologicals and small molecules in the treatment of inflammatory bowel diseases

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Abstract

The past decade has brought substantial advances in the management of inflammatory bowel diseases (IBD). The introduction of tumor necrosis factor (TNF) antagonists, evidence for the value of combination therapy, the recognition of targeting lymphocyte trafficking and activation as a viable treatment, and the need for early treatment of high-risk patients are all fundamental concepts for current modern IBD treatment algorithms. In this article, authors review the existing data on approved biologicals and small molecules as well as provide insight on the current positioning of approved therapies. Patient stratification for the selection of specific therapies, therapeutic targets and patient monitoring will be discussed as well. The therapeutic armamentarium for IBD is expanding as novel and more targeted therapies become available. In the absence of comparative trials, positioning these agents is becoming difficult. Emerging concepts for the future will include an emphasis on the development of algorithms which will facilitate a greater understanding of the positioning of novel biological drugs and small molecules in order to best tailor therapy to the patient. In the interim, anti-TNF therapy remains an important component of IBD therapy with the most real-life evidence and should be considered as first-line therapy in patients with complicated Crohn's disease and in acute-severe ulcerative colitis. The safety and efficacy of these 'older' anti-TNF therapies can be optimized by adhering to therapeutic algorithms which combine clinical and objective markers of disease severity

and response to therapy.

Key words: Inflammatory bowel disease; Small molecule; Positioning; Biologic; Therapeutic

Core tip: Anti-tumor necrosis factor therapy should be considered as first-line therapy in patients with complicated Crohn's disease and in acute-severe ulcerative colitis. Beyond these specific circumstances, the positioning of novel biologics and small molecules depends on the patient's medical history, preference and disease phenotype. The efficacy and safety of using immunomodulatory therapy can be enhanced by adhering to therapeutic algorithms and using a 'treat-to-target' approach. The risks for adverse events due to poor disease control outweigh the risks associated with early aggressive therapy. In the setting of clinical and biochemical remission, following at least 6 mo of combined immunosuppressive therapy, consideration can be made to withdrawing thiopurine therapy in the correct patient with close follow-up.

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INTRODUCTION

Therapeutic trials for inflammatory bowel disease (IBD) began nearly 100 years after the first case report of IBD was published by Sir Samuel Wilks in 1859 who used the term "ulcerative colitis (UC)" to describe a condition similar to what is understood as UC today[1]. Approximately 10 years following the original study by Sir Sidney Truelove which revealed the efficacy of corticosteroid therapy in UC, the first clinical trial evaluating steroids in Crohn's disease (CD) was conducted in 1966 by Jones and Lennard-Jones[2]. Prior to these landmark trials, the treatment of IBD was limited to supportive care and surgical intervention.

Knowledge regarding the adverse effects of chronic steroid therapy in UC ultimately led to the first positive double blind randomized controlled trial (RCT) evaluating the efficacy of sulfasalazine in 1962[3,4]. Unfortunately, many patients were unable to tolerate the side-effects from sulfasalazine which prompted additional studies to uncover the active ingredient, 5ASA[5]. Since, 5ASA has repeatedly demonstrated its efficacy and improved safety profile as compared to sulfasalazine in mild to moderate UC[6-8]. In contrast, 5ASA therapy has been abandoned in CD due to its inability to prevent quiescent disease relapse[9]. As steroid-refractory disease became more prevalent, reports on the use of ciclosporin began appearing and the first successful trials were conducted in 1989 and 1994 for steroid resistant severe CD and UC, respectively[10,11]. Due to ciclosporin's narrow therapeutic window, alternative steroid-sparing agents such as thiopurines were investigated. Although they have demonstrated fair efficacy in IBD, it may take up to 3-6 mo for them to reach their full therapeutic effect thereby limiting their potential as a strong induction agent[12]. Despite their slow onset of action and risks, thiopurines may be used strategically to reduce immunogenicity associated with biologic therapy and augment the rate of remission[13,14] Budesonide, a corticosteroid which undergoes significant first-pass metabolism in the liver resulting in low systemic exposure, has also established its position in the therapeutic armamentarium since Rutgeerts *et al*[15]'s original study demonstrating its non-inferiority to prednisolone therapy for CD patients in 1994. Budesonide has since repeatedly demonstrated its efficacy and safety making it the preferred means of inducing remission in patients with mild Crohn's ileitis[16]. A newer formulation with a delayed release (budesonide-MMX©) can be efficacious in moderate UC as well[17].

Alongside the advent of new biological therapies, the therapeutic approach has evolved over the past decade to include the use of objective markers of disease severity and response to therapy in tandem with the historical clinical scores[18,19]. In this article, authors review the existing data and provide a rationale for the positioning of the 'old' and 'new' biologicals and small molecules. Strategies for the use of available therapies based on recent guidelines will be reviewed.

CD

Anti-tumor necrosis factor

Infliximab: Four years after the FDA approved the use of infliximab in CD, the first large RCT; ACCENT I, was published in 2002 which evaluated infliximab maintenance therapy in 573 patients with a CDAI of at least 220 whom had responded well to an initial infusion of infliximab[20]. At the 30 and 54 wk follow-up, patients receiving infliximab maintenance therapy were more likely to be in remission (CDAI < 150) as compared to those without maintenance therapy (30 wk: OR = 2.7, 95%CI: 1.6-4.6) with a similar incidence of infection across all groups[20]. Besides demonstrating infliximab's efficacy, this study also provided a rationale for dose escalation in patients losing response to therapy[21]. Although effective for luminal disease, it was unclear if infliximab would also be effective for fistulising disease, thus the ACCENT Ⅱ trial was published 2 years later which included 306 patients with one or more draining abdominal or perianal fistulas of at least 3 mo duration[22]. In this trial, the patients who were undergoing infliximab maintenance therapy demonstrated a significant fistula response wherein 36% (*vs* 23%, *P* = 0.009) had

complete resolution of fistula draining at 54 wk[22]. Additionally, ACCENT Ⅱ demonstrated a significant reduction in the requirement for hospitalization and surgery due to fistulising disease (8.6% *vs* 18.9%, $P < 0.05$)[23]. Early initiation of infliximab was further supported in a large study conducted by the GETAID group which evaluated the use of dual therapy *vs* monotherapy over 52 wk in 113 steroid-dependant CD patients[13]. Both GETAID and ACCENT-Ⅰ studies identified incongruence amongst endoscopy and clinical scores, such as the CDAI. In a sub-study of ACCENT-Ⅰ, 18% of moderate to severe CD patients as determined by the CDAI score had no active CD on endoscopy[24]. This prompted a rationale to include more objective end points and markers of disease severity (*e.g.*, CRP and mucosal healing) in future studies, as was included in the Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease (SONIC) conducted in 2010[14]. In this landmark RCT involving 508 biologic- and immunosuppressive-naïve patients, the superiority of infliximab over azathioprine as well as the therapeutic advantage of combining therapies over monotherapy with either infliximab or azathioprine alone at the 30 and 50 wk follow-ups was demonstrated.[14].

Adalimumab: In an attempt to possibly reduce the immunogenic responses induced by chimeric antibodies, such as infliximab which contains 25% mouse sequences, adalimumab was designed as the first fully human monoclonal antibody against tumor necrosis factor (TNF)-alpha[25]. The results of three pivotal trials (CLASSIC-Ⅰ, CHARM and GAIN) established regulatory approval of adalimumab for the induction and maintenance of remission of CD in 2007. CLASSIC-Ⅰ was the first human trial to evaluate induction of remission using adalimumab in 299 moderate to severe CD patients naïve to anti-TNF therapy[26]. A linear dose-response curve was appreciated at the 4 wk follow-up, with the greatest clinical remission rate associated with the highest dose studied (160 mg and 80 mg at weeks 0 and 2, respectively)[25]. As a ceiling effect was not achieved, it is unclear if higher dosing would be more efficacious, studies evaluating this are underway. The use of adalimumab as a second line induction agent following the failure of infliximab due to intolerance or poor response was evaluated in the GAIN trial which included 325 patients who had either lost response or become intolerant to infliximab[27]. At the 4 wk follow-up, 21% (34 of 159) of patients in the adalimumab group *vs* 7% (12 of 166) of those in the placebo group achieved clinical remission.

The efficacy of adalimumab for maintenance therapy was evaluated in the CLASSIC-Ⅱ and CHARM studies. CLASSIC-Ⅱ followed up with 276 patients from the CLASSIC-Ⅰ study at 56 wk after randomizing patients to receive maintenance dosing or placebo. A greater proportion of patients receiving adalimumab 40 mg SC weekly or biweekly were in remission as compared to those receiving placebo (83% and 79% *vs* 44%, respectively)[28]. Additionally, although most patients responded to therapy within the first week, some patients only responded to therapy after week 12[28]. This suggests that an observational period may need to occur prior to modifying therapy in patients who do not respond to induction following 1 wk. In the largest open-label study, CHARM enrolled 854 patients in order to evaluate the efficacy of adalimumab for induction and maintenance in CD patients not responding to alternative immunosuppressive therapy, including those whom had failed infliximab[29]. Although the induction dose of adalimumab was half of that provided in the CLASSIC trials, the response rate was similar. At week 56, biweekly and weekly dosing was equally effective at maintaining remission as compared to placebo (36% and 41% *vs* 12%, respectively). Of note, a greater proportion of patients receiving placebo discontinued treatment due to adverse events as compared to those receiving adalimumab[29]. This suggests the risks of complications associated with poorly controlled disease outweigh the risks associated with therapy. To corroborate the findings from the previous studies demonstrating clinical remission, the EXTEND trial conducted in 2012 which involved 135 patients with moderate to severe ileocolonic CD demonstrated a trend towards mucosal healing with adalimumab at week 12 as compared to placebo (27% *vs* 13%, respectively) as well as a significant difference at week 52 (24% *vs* 0%, respectively ($P < 0.001$))[30]. Again, this suggests 12 wk may not be sufficient in all patients to determine response to therapy.

Certolizumab: Certolizumab pegol is a pegylated humanized monoclonal antibody Fab' fragment linked to polyethylene glycol that has a high affinity to tumor necrosis factor alpha[31]. Certolizumab was proposed as a potential alternative to infliximab due to its ease of delivery (SC as oppose to infusion) and longer half-life which may reduce the need for frequent dosing and risk for immunogenicity, theoretically[32,33]. The risks for side effects were presumed to be lower due to the lack of an Fc region which would be responsible for activating the compliment pathway leading to cellular apoptosis[32,33]. The largest phase Ⅱ trial in 2005 by Schreiber *et al*[33] in 292 patients with moderate to severe CD demonstrated a significant dose-response relationship with clinical benefit demonstrated up until week 10, then lost significance at week 12 which was presumed to be secondary to greater placebo rates in patients with lower CRP values[33]. The potential placebo effect was addressed in the PRECISE-Ⅰ trial which stratified 662 patients with moderate to severe CD based on their CRP prior to randomization to treatment groups[31]. Although response rates at week 6 and 26 were found to be modestly significant, induction of remission rates were not. However, in patients responding to certolizumab, maintenance of remission was successfully demonstrated in the PRECISE-Ⅱ and PRECISE-Ⅲ follow-up trials through 5 years[34,35]. The MUSIC trial conducted in 2013 confirmed certolizumab's efficacy with respect to mucosal

healing following 54 wk of therapy after evaluating 89 patients with active endoscopic disease (ulceration in ⩾ 2 intestinal segments with a Crohn's Disease Endoscopic Index of Severity (CDEIS) score ⩾ 8 points)[36]. As early as week 10, endoscopic remission was achieved in 37% of patients.

Anti-integrin

Natalizumab: Natalizumab blocks the adhesion and subsequent migration of leukocytes from circulation into the gut by binding alpha-4 integrin which is expressed on all circulating leukocytes except neutrophils. Originally designed for multiple sclerosis patients, natalizumab demonstrated good efficacy for induction and maintenance of remission for CD in a large meta-analysis which included 5 trials[37]. The largest trials to be performed were ENACT-Ⅰ, ENACT-Ⅱ and ENCORE. ENACT-Ⅰ included 905 patients with CD randomized to either placebo or natalizumab induction groups[38]. Although there was a subtle but significant difference in the response rate favoring natalizumab (56 percent and 49 percent, respectively), there was no difference in remission rates between groups for induction. ENACT-Ⅱ included 339 responders to natalizumab from ENACT-Ⅰ and randomized them to maintenance therapy every 4 wk or placebo[38]. In contrast to the first trial, significantly higher rates of remission occurred through 36 wk as compared to placebo (44% *vs* 26%). Induction of remission was reassessed in the ENCORE study which included 509 patients with CD evaluated through 3 induction doses over 8 wk. At week 12, a greater proportion of patients on natalizumab were in remission as compared to placebo, 28% *vs* 16% respectively[39].

Although natalizumab demonstrated good efficacy in luminal CD, concerns related to serious infection surfaced. In an open-label extension of the ENACT-Ⅱ trial, one patient died from JC virus-associated progressive multifocal leukoencephalopathy (PML)[40]. The association with PML and natalizumab was described in two other case reports on patients receiving treatment for multiple sclerosis[41,42]. Since the estimated risk for PML is 1 per 1000 patients, JC virus antibody testing should be considered if natalizumab will be used in IBD.

Vedolizumab: Vedolizumab reduces lymphocyte migration into the gut by antagonizing the $\alpha_4\beta_7$ integrin mediated reactions. In contrast to natalizumab it does not act on $\alpha_4\beta_1$ integrin, which is involved in brain lymphocyte trafficking, thus may have lower risk for PML[43]. Efficacy for its use as an induction and maintenance agent in CD was demonstrated in the GEMINI-Ⅱ trial[44]. In the induction component of the trial, 368 patients were randomized to placebo or vedolizumab and 747 patients received open-label vedolizumab. Approximately 50% of all patients had failed at least one anti-TNF prior to enrolling in the study. Although clinical remission was achieved in a significantly greater proportion of patients taking vedolizumab as compared to placebo at week 6 (14.5% *vs* 6.8%, respectively), there was no significant difference in CDAI scores greater than 100 (CDAI-100 score) or CRP levels between groups. However, nearly twice as many patients in the vedolizumab maintenance groups were in clinical remission as compared to the placebo group (39% *vs* 21.6% respectively). Significant differences in favor of maintenance therapy over placebo were demonstrated in the CRP and the CDAI-100 score. Fistulization also improved as compared to placebo in the small group of patients on vedolizumab every 8 wk (n = 17) but not in the small group taking vedolizumab every 4 wk[44]. Acknowledging that subjects recruited for this study had likely more aggressive disease than the aforementioned biologic-naïve anti-TNF studies discussed, vedolizumab is efficacious for luminal and possibly fistulising disease but may not provide as effective and efficient induction as compared to anti-TNF therapy. This has also been supported in network meta-analyses[45]. As such, if rapid induction is required then physicians prescribing vedolizumab should be aware of the potentially slower onset of action and consideration for the concomitant use of faster-acting induction agents (*e.g.*, corticosteroids) to bridge the patient symptomatically.

A common reason for using vedolizumab as first line treatment in IBD is the assumption of the reduced risk for infection given the attenuation of the immune response is localized to the gut. This has been previously supported in a review which included six trials evaluating the use of vedolizumab in UC and CD (2380 patients with 4811 person-years of vedolizumab exposure)[46]. Within this study however, 16 patients with CD in the vedolizumab group developed clostridium difficile infection as compared to none in the placebo group. Additionally, more patients on vedolizumab had gastroenteritis and developed tuberculosis infection (despite negative tuberculosis screening at enrollment). In the aforementioned GEMINI-Ⅱ trial, vedolizumab also had a higher rate of infections (44.1% *vs* 40.2%), and serious infections (5.5% *vs* 3.0%) as compared to placebo[44]. Head to head trials are needed to better describe the risk for infection in patients taking vedolizumab as compared to other biologics.

Ustekinumab: IL-12 p35-p40 and IL-23 p19-p40 are two proinflammatory heterodimeric cytokines that are induced in the inflamed mucosa of CD patients[47,48]. Ustekinumab is a human monoclonal $IgG_{1\kappa}$ antibody which blocks the P40 sub-unit of IL-12 and IL-23 on T cells, natural killer and antigen presenting cells[49]. Originally successful in the treatment for plaque psoriasis and psoriatic arthritis, ustekinumab demonstrated its efficacy for CD in the UNITI trials which included 1300 CD patients with moderate to severe disease[50]. UNITI-Ⅰ included 741 patients whom had failed anti-TNF therapy due to non-response or intolerance. The induction component of the trial revealed a significantly better clinical response in the ustekinumab treatment groups as com-

pared to placebo (34% *vs* 22%, respectively). UNITI-Ⅱ included 628 patients whom were anti-TNF naïve but failed conventional immunosuppressive therapy due to poor response or intolerance. The UNITI-Ⅱ cohort also had a significant improvement in their CDAI scores for induction by approximately 25% as compared to placebo. Patients receiving maintenance therapy every 8 wk and every 12 wk demonstrated a significantly greater remission rate at week 44 as compared to placebo (53% and 49% *vs* 36%, respectively). Of note, the secondary analyses demonstrated a non-significant difference in CDAI scores compared to placebo in the UNITI-Ⅰ group as compared to the UNITI-Ⅱ group, albeit the trend still favored ustekinumab therapy[50]. Lack of significance is most likely due to a lack of power to properly evaluate the difference amongst sub-groups, however this trend is expected; patients in UNITI-Ⅰ have more refractory disease thus less likely to respond to ustekinumab as compared to the biologic-naïve patients in UNITI-Ⅱ. Significant improvements in fecal calprotectin and CRP were also noted and able to be seen as early as 3 wk supporting its usefulness in acute severe flares.

UC

Anti-TNF agents

Infliximab: The first two large-scale studies to assess the therapeutic potential of infliximab were the ACT 1 and ACT 2 trials published in 2005, prior to this, biologic therapy for UC was not established[51]. ACT 1 evaluated 364 patients with moderate to severe UC following their induction and maintenance dosing until 54 wk. ACT 2 evaluated the same number of patients and maintained the same induction, maintenance and follow-up regimen as ACT 1 except maintenance dosing ceased after 22 wk. Nearly 60% of patients in both cohorts were steroid dependent. In both studies, a significant clinical response was demonstrated with remission occurring in approximately 35% and 31% of patients taking infliximab as compared to 15% and 6% of patients on placebo at week 8 in ACT 1 and ACT 2 studies, respectively. Sustained remission was achieved over the study period in approximately 20% of patients on infliximab as compared to 5% of patients in the placebo group. Additionally, a greater proportion of patients were able to be weaned off their steroids following the initiation of infliximab. Mucosal healing, considered to be the greatest risk factor for malignancy, was markedly improved throughout the study period and significantly better than placebo as early as week 8, approximately 60% *vs* 30% respectively. No difference between the two doses prescribed, 10 mg/kg and 5 mg/kg, was identified with respect to efficacy[51].

Given the toxicity associated with cyclosporine and limited therapies available, GETAID compared the efficacy of infliximab against cyclosporine in an open-label RCT involving 115 patients with severe ulcerative colitis whom had failed high dose intravenous steroid therapy. The results were positive for both agents with no significant difference in treatment failure or side effects between the infliximab and the ciclosporin groups (54% *vs* 60%, respectively)[52].

Adalimumab: Five years following the approval for infliximab use in UC, adalimumab became the second biologic approved for use in UC based on the results from the ULTRA trials. ULTRA 1 utilized two different induction regimens (160/80 mg *vs* 80/40 mg SC at weeks 0 and 2 followed by 40 mg every 2 wk) to evaluate if adalimumab was effective in 186 moderate to severe UC patients[53]. At week 8, 19% *vs* 9% were in remission in the 160/80 mg group as compared to placebo, respectively. As noted in the CD trials, a ceiling effect was not achieved thus the optimal dose is still under investigation. ULTRA 2, which included 518 patients with moderate to severe UC, was conducted to evaluate the long-term efficacy of adalimumab as a maintenance agent[53]. Following 1 year, remission was achieved in 17% of patients on regular maintenance dosing as compared to 9% of patients in the placebo group. Similarly, mucosal healing was also higher in the adalimumab group as compared to placebo at both week 8 and 52 follow-up intervals, 41% and 25% *vs* 32% and 15%, respectively. This study also demonstrated that biologic naïve patients were more likely to achieve clinical remission as compared to patients previously on infliximab (Week 8: 21% *vs* 9% and Week 52: 22% *vs* 10%, respectively), which highlights prior biologic use as a potential risk factor for difficult to treat or aggressive disease. Long-term maintenance therapy using adalimumab was further evaluated over 4 years in ULTRA 1 and 2 trials as well as in an open-label study (ULTRA 3)[54]. With respect to patients observed as nonresponder imputation (NRI), 25% and 28% of the 199 patients from ULTRA 1 and 2 whom were still on adalimumab at the 4 year follow-up maintained clinical remission and mucosal healing respectively. In contrast, the ULTRA 3 open-label trial demonstrated clinical remission and mucosal healing rates to be considerably greater (64% and 60%, respectively), albeit difficult to compare in the absence of randomization.

Golimumab: Golimumab is a fully human monoclonal immunoglobulin delivered subcutaneously which targets a unique epitope on the TNF molecule as compared to infliximab and adalimumab. The PURSUIT trials which evaluated 1064 biologic naive patients with moderate to severe UC were responsible for establishing regulatory approval for it in 2014. The induction trial, PURSUIT-SC, revealed a significantly greater proportion of patients in clinical remission following 6 wk using 200/100 mg and 400/200 mg induction doses as compared to placebo, 51% and 55% *vs* 30% respectively[55]. The extension of this trial, PURSUIT-M, which included 464 patients with moderate to severe UC whom had responded favorably to golimumab in the induction trial also demonstrated greater efficacy than placebo at maintaining clinical remission following 54 wk. At study end, 42% of patients

taking golimumab 100 mg every 4 wk were found to be in clinical remission as compared to 27% of patients taking placebo[56]. The rate of mucosal healing was significantly greater for patients taking golimumab in both the induction and maintenance studies, the differences were able to be appreciated as early as 2 wk. Golimumab, although not formally assessed in clinical trials, has been reported to be efficacious as a second and third-line anti-TNF agent in real life settings[57].

Anti-integrin agent

Vedolizumab: GEMINI-1 evaluated the efficacy of vedolizumab in a treatment resistant group of 895 moderate to severe UC patients (approximately 40% of patients failed ⩾ 1 anti-TNF therapy)[58]. In the induction phase of the trial, 17% of patients taking vedolizumab were in clinical remission as compared to 5% of patients taking placebo by week 6. Mucosal healing was also nearly twice as apparent in patients taking vedolizumab as compared to placebo (41% *vs* 25% respectively). At 52 wk, clinical remission was maintained in approximately 44% of patients taking vedolizumab as compared to 16% of patients on placebo. No significant difference was identified between treatment groups receiving every 4 or 8 wk dosing regimens. In contrast to GEMINI-Ⅱ for CD, there was no difference in infection rates in the treatment group as compared to placebo[44,58].

SMALL MOLECULES

JAK inhibitors

Tofacitinib: Tofacitinib is a new oral medication which suppresses cytokine signalling in mucosal immune cells by inhibiting janus kinase's 1 and 3 (JAK 1 and 3). The oral route of administration and ability to target multiple cytokine pathways makes JAK inhibitors an attractive therapeutic option.

Although the efficacy for tofacitinib has not been established in CD yet, it has been established in UC as demonstrated by the OCTAVE trials[59]. Of the 905 patients with moderate to severe UC randomized to treatment in the induction trials, approximately 18% achieved clinical remission as compared to 6% of patients in the placebo group at 8 wk. Onset to effect was rapid, with improvements in their partial mayo score demonstrated as early as 2 wk. Although over 50% of patients within the induction groups had prior exposure to anti-TNF therapy, the treatment effect was similar in comparison to patients whom were biologic naïve despite OCTAVE's more stringent criteria for clinical remission as compared to the aforementioned trials (*i.e.*, partial mayo rectal bleeding subscore of 0). The OCTAVE-Sustain extension trial, which included 593 patients who had a clinical response to induction therapy, also demonstrated good maintenance of remission after 52 wk in both 5 mg and 10 mg twice daily treatment groups as compared to placebo (34% and 41% *vs* 11%, respectively). Mucosal healing and steroid-free remission was achieved and maintained in a similar proportion of patients. With respect to adverse events, serious infections occurred more frequently in the induction but not maintenance trial. However, herpes zoster infection did occur more frequently in the tofacitinib 10mg maintenance group as compared to placebo[59]. Of note, tofacitinib received a recommendation for the treatment of UC by the GIDAC-FDA in March 2018 a final decision is anticipated by June 2018[60].

BIOSIMILARS

According to the FDA, a biosimilar is defined as a biological product that is highly similar to the reference product notwithstanding minor differences in clinically inactive components which result in no clinically meaningful differences in the purity, safety and efficacy of the product[61]. The use of biologic anti-inflammatory medications is increasing and the cost has become a significant economic burden on many national healthcare systems around the world[62]. In Canada, the growth of Canadian sales of biologic anti-inflammatory drugs has nearly doubled since 2010. The top-selling biologic, remicade (infliximab), has cost the Canadian Government $224 million in 2015 and $4.8 billion since it was approved 10 years ago. Based on a Market Intelligence Report published by Health Canada, the use of a biosimilar such as Inflectra could have resulted in a $41.7 million reduction in drug expenditures in 2015[62]. Several biosimilars to remicade (flixabi, inflectra, remsima) and adalimumab (cyltezo and imraldi) have already been approved for use in IBD.

Infliximab-dyyb (or CT-P13), was the first biosimilar for remicade (infliximab) to be approved and has the greatest amount of 'real world' observational data evaluating its efficacy and safety[63]. Infliximab-dyyb was first approved in South Korea and thereafter in Europe in 2013 following the results of two large randomized and double-blind clinical studies evaluating its safety and efficacy in rheumatoid arthritis as compared to remicade, PLANETRA and PLANETAS[64,65]. No significant differences were found with respect to safety, efficacy and immunogenicity thus it was approved for use in all labelled indications remicade was approved for. However, small retrospective studies in IBD have demonstrated mixed results[66-68]. A larger prospective nationwide multicenter study performed in Hungary involving 126 CD and 84 UC patients reported excellent induction rates[69]. At week 14, 81% of patients with Crohn's disease and 78% of patients with ulcerative colitis had a clinical response (CDAI reduction > 70) and 54% and 59% respectively, were in clinical remission (CDAI < 150). Comparable results were also seen in another large observational cohort study including 313 CD and 234 UC patients[70]. Response rates at 8 wk were greater than 90% for all patient groups, including patients whom switched from remicade to infliximab-dyyb. At week 24, response rates were 73.7%, 62.2% and 78.9% for biologic naïve,

Table 1 Currently approved biologic treatments for inflammatory bowel diseases[16,117,118]

Medication	Route of administration (IV, SC, PO)	Approved dose
Infliximab	IV	Induction: 5-10 mg/kg (weeks 0, 2, and 6) Maintenance: 5-10 mg/kg every 4-8 wk
Adalimumab	SC	Induction: 160 mg (week 0), 80 mg (week 2) Maintenance: 40 mg every 7-14 d
Golimumab	SC	Induction: 200 mg (week 0), 100 mg (week 2) Maintenance: 100 mg every 4 wk
Certolizumab	SC	Induction: 400 mg (weeks 0, 2, and 4) Maintenance: 400 mg every 4 wk
Vedolizumab	IV	Induction: 300 mg (weeks 0, 2, and 6) Maintenance: 300 mg every 4-8 wk
Ustekinumab	IV SC	Induction: < 55 kg: 260 mg 55-85 kg: 390 mg > 85 kg: 520 mg Maintenance: 90 mg every 8 wk

pre-exposed and switched respectively. The efficacy, immunogenicity and safety profiles in both studies were considered comparable to that of the originator drug infliximab.

To date, studies which have evaluated switching from originator to biosimilar have been largely positive[71,72]. The longest evaluation period occurred over 52 wk in the NOR-SWITCH study which was a randomised, non-inferiority, double-blind, phase 4 trial involving 482 patients across 40 Norwegian centres with various inflammatory diseases maintained in remission on infliximab for at least 6 mo. Of the 482 patients, 155 (32%) and 93 (19%) were CD and UC respectively[73]. At study end, there was no difference in disease worsening, safety or immunogenicity amongst any of the groups. Although switching therapies in the setting of controlled disease would be a reasonable option and is supported by the evidence as well as the European Crohns and Colitis Organization; switching in the setting of failing the originator drug would be ill-advised[71]. Ben-Horin *et al*[74] studied the cross reactivity of antibodies to remicade and infliximab-dyyb in 125 patients with IBD and healthy individuals as negative controls. They demonstrated that anti-remicade antibodies recognize and inhibit infliximab-dyyb as well. These results suggested that there was similar immunogenicity and shared immunodominant epitopes. Although this supported the safety of biosimilars and the use of the same assay as the originator drug to detect antibodies, this study also supported not using the biosimilar in the setting of originator failure[71,74,75].

Evolution of treatment strategies of IBD and positioning currently approved biologics and small molecules in clinical practice

As the therapeutic armamentarium for IBD continues to expand, so follows the complexity associated with managing IBD patients in clinical practice. The needs for algorithms are required in order to assist health care practitioners determine the relative positioning of each agent and their use in combination with other therapies. Until the results of head to head biologic and small molecule trials become available, we can only speculate the positioning of therapeutic agents based on the current available literature as summarized in this section (Table 1).

Positioning the 'old' biologics: Anti-TNFs first, alone or in combination?

As newer and more targeted therapies in IBD become available, questions related to maintaining anti-TNF agents as first line therapy arise. Based on decades of data, anti-TNFs currently provide the best long-term evidence of efficacy in CD and UC, with a known safety profile. They are effective for both induction and maintenance therapy, decrease corticosteroid exposure and promote sustained mucosal healing[76,77]. The most important safety concern is the risk of serious infection. However, in younger patients without co-existing medical problems, this risk is fairly low[78].

Comparing efficacy of TNF inhibitors is difficult due to the lack of high-quality, head-to-head trials (Table 2). Network meta-analyses indirectly comparing anti-TNF agents have reported mixed results[45,79-81]. Based on 'real world' data, an analysis of retrospective and comparative effectiveness database studies revealed subtle differences regarding hospitalisation and surgery rates as well as the steroid sparing effect between infliximab and adalimumab, favouring infliximab at currently recommended doses. Of note, clinical trials of higher-dose adalimumab for both UC and CD are currently underway[82,83].

Deciding between which anti-TNF agent to use depends on the clinical circumstances, treatment history and patient preference. In the absence of head-to-head comparisons, there exists few specific scenarios in which the evidence supports the use of specific anti-TNF agents. In the setting of a hospitalized patient with severe UC, only infliximab has demonstrated its efficacy as a 'rescue' therapy[84]. Patients with perianal disease can benefit from either infliximab or adalimumab, albeit the evidence is based on a post-hoc analysis for adalimumab and lacking for other anti-TNF agents[23,85]. Golimumab

Table 2 Biologic agents which have demonstrated efficacy in inflammatory bowel diseases and rheumatology

	Mechanism of action	UC	CD	[2]Fistulization	Ankylosing Spondylitis	Psoriasis
Anti-TNF						
[1]Infliximab[20,22,51,119]	Chimeric monoclonal antibody	x	x	x	x	x
Adalimumab[26,28,54,120,121]	Fully human monoclonal antibody	x	x	x	x	x
Certolizumab[31,122,123]	Pegylated humanized monoclonal antibody Fab' fragment		x	+/-	x	x
Golimumab[57,122,124]	Fully human monoclonal antibody	x			x	x
Anti-integrin						
[4]Natalizumab[39]	Chimeric monoclonal antibody against α4 integrin		x			
[3]Vedolizumab[46,96]	Chimeric monoclonal antibody against α4β7 integrin	x	x	+/-		
Ustekinumab[50,125,126]	Fully human monoclonal antibody against P40 sub-unit of IL-12 and IL-23		x	+/-	x	x

[1]Infliximab is the only biologic which has been evaluated to be an effective 'rescue' agent. Evidence is lacking for the remaining biologics; [2]Improvement in fistulizing disease was evaluated as a primary outcome only in infliximab. Efficacy was otherwise determined indirectly from secondary outcomes, subgroup analyses and small scale studies for the remaining biologics; [3]Consider the use of vedolizumab as a first-line biologic agent in patients at high risk for infectious complications. Vedolizumab has a slower onset of action (approximately 6-8 wk) as compared to alternate biologics; [4]Use of natalizumab is contraindicated if the patient is JC virus antibody positive due to the risk of progressive multifocal leukoencephalopathy. UD: Ulcerative colitis; CD: Crohn's disease.

has demonstrated efficacy in UC as a second or third line anti-TNF agent in small cohorts of patients but not for CD. Similarly, certolizumab can be considered in the same context for CD but lacks evidence for UC. Ease of administration may influence one's decision thus patients who would rather less frequent dosing may prefer the Ⅳ infusion infliximab as compared to the other anti-TNF agents which are delivered SC by the patient.

The relatively high costs of anti-TNFs and the expiration of patents have triggered the development of biosimilar monoclonal antibodies. Multiple regulatory agencies have approved the use of biosimilars in IBD based on extrapolation of data on safety and efficacy. Since then, real-word data and randomised controlled trials on switching from originator to biosimilar infliximab has shown similar results in terms of efficacy and safety[72]. Following the introduction of vedoluzimab and ustekinumab, anti-TNF therapy may not be the first-line biologic agent in all IBD patients. However, the lower cost of biosimilars probably makes the use of anti-TNF agents still very attractive.

Optimizing the efficacy of the initial anti-TNF therapy prior to switching to another biologic, either in or out of class, is a critical principle when managing IBD patients. Studies have repeatedly demonstrated that patients failing their first biologic have poorer outcomes following initiation of their second or third biologic[50,58]. The ability to differentiate the cause for a loss of response to anti-TNF therapy has been facilitated with therapeutic drug monitoring (TDM)[86]. Based on TDM results, an educated decision regarding dose optimization and switching in or out of class can now be determined[16,87]. However, the frequency of TDM is still up for debate. Few retrospective studies have demonstrated benefit with proactive TDM[88,89]. The recent multicentre prospective RCT involving 167 patients with active CD, TAILORIX, demonstrated that there was no benefit in patients receiving infliximab dose escalation based on TDM as compared to clinical scoring[89]. Although more patients in the clinical dose escalation group received dose escalation as compared to the TDM group, thus the benefit seen from the clinical group may be over-inflated. Similarly, the TAXIT study, which was a 1 year RCT involving 178 CD and 85 UC patients performed at a single tertiary referral center, did not find benefit in proactive vs reactive (*i.e.*, symptom based) TDM[88]. However, the results from the TAXIT study should be interpreted with caution since dose optimisation occurred in both groups at study start. Prospective, multi-center studies are needed to further investigate the positioning of TDM.

The decision to initiate combination therapy involves balancing the benefits of improved efficacy and lower immunogenicity of therapy against the heightened risks for infection and malignancy. The SONIC trial revealed the steroid-free remission rate in CD patients at week 26 was significantly greater in the combination azathioprine and infliximab group as compared to infliximab or azathioprine alone (57% *vs* 44% *vs* 30%, respectively)[14]. The SUCCESS trial, which was a 16 week RCT involving 239 patients with moderate to severe UC, revealed similar results. Steroid-free remission was achieved in 40% of patients on dual therapy as compared to 22% and 24% on infliximab and azathioprine monotherapy, respectively[90]. Supporting this strategy was the open label prospective DIAMOND study which evaluated 176 Japanese patients with CD over 52 wk. This study demonstrated that the efficacy of using dual therapy was not limited to only infliximab but also to adalimumab. Mucosal healing was significantly better in the combination group as compared to the azathioprine monotherapy group at week 26 (84% *vs* 64%, respectively)[91]. Although the difference in clinical remission was not significant, likely due to a small cohort and lower thiopurine dosing, a trend was maintained in favor of combination therapy. The infection and serious complication risks were not greater on dual therapy as compared to monotherapy in either of the aforementioned studies. In contrast, the SONIC trial

demonstrated the lowest risk for infection to be present in the dual therapy group (3.9%) as compared to the infliximab or azathioprine monotherapy groups (4.9% and 5.6%, respectively). This suggests that poorly controlled disease is a stronger risk factor for infection instead of intensified immunosuppression. Ultimately, the risk for hepatosplenic T-cell lymphoma (especially in young/adolescent males after 2 years of therapy), myelosuppression and opportunistic infections must be weighted individually[92,93]. Consideration can be made to initiating therapy with both combined thiopurine and anti-TNF therapy than stopping thiopurine therapy after 6 mo in the setting of clinical and biochemical remission and a therapeutic drug level, which has been supported in the literature[94,95].

Positioning 'new' agents: First or second-line?

Vedolizumab has emerged as a first-line agent for induction of remission for moderately active UC patients failing conventional therapy[58]. In CD, clinicians should be aware of the potentially slower onset of action of vedolizumab. Concomitant use of corticosteroids may be necessary during the induction period. For these reasons, anti-TNFs or ustekinumab may be more favourable first line choices in CD patients with severe disease activity at present. There is also no considerable data from RCTs on the efficacy of vedolizumab in fistulizing CD and acute severe UC. Ongoing phase Ⅳ trial will determine its effectiveness[96]. Vedolizumab is currently being positioned in some jurisdictions as a second-line biologic agent following anti-TNFs, although the ongoing LOVE studies are evaluating the use of vedolizumab in early vs. late UC and CD[97,98]. Given their effectiveness in the medium to long term and the favourable safety profile, it is expected that gut-selective anti-integrin agents will increasingly be used as maintenance therapy or even as part of a combination biological therapy. A clinical trial evaluating the efficacy of adalimumab, methotrexate and vedolizumab triple combination therapy is ongoing[99].

Ustekinumab is the most recently approved biologic agent for CD[50]. Presently, there is no data available describing its efficacy in UC or fistulising CD. An indirect comparison amongst the anti-TNF and UNITI trials suggests ustekinumab may be safer and have a lower rate of immunogenicity which may make it the preferred biologic for some CD patients[77]. More comprehensive data on efficacy in certain patient subgroups and mucosal healing is needed.

Finally, tofacitinib is a small molecule awaiting final approval for the treatment of UC[59,60]. Their oral route of administration makes them particularly attractive. Their safety profile has been suggested to be similar to that of thiopurines. Due to their mechanism of action, they are not limited by immunogenicity and subsequent loss of response. Their positioning and use as mono- or combination therapy has yet to be elucidated.

The evolution of treatment strategies and objective monitoring: Early aggressive or tailored therapy?

The introduction of highly effective therapies early in the disease course alongside objective patient monitoring can modify the disease trajectory and reduce morbidity. However, it is also important to recognize that approximately 20% of patients with IBD may have an indolent disease course, and available population-based data suggests that approximately half of patients with CD can be symptomatically controlled 10 years after diagnosis[100,101]. Risk stratification can guide early introduction of highly effective therapy in patients with a poor prognosis and prevent overtreatment in low-risk patients. Unfortunately, current patient stratification relies on clinical factors. Most of these are indicators rather than predictors of a complicated disease course (*e.g.*, presence of perianal disease, age < 40 years old at diagnosis and need for steroids during the first flare)[102]. Molecular makers for predicting an aggressive phenotype have yet to be identified but studies are ongoing[103,104].

In the absence of objective predictors for disease severity, studies have attempted to better elucidate the risks and benefits of aggressive therapy. The TOP-DOWN trial was the first to assess and compare different treatment algorithms in IBD[105]. Treatment-naïve early CD patients were randomly assigned to receive early aggressive therapy ('top-down') with an immunosuppressant and anti-TNF agent or less aggressive ('step-up') therapy with steroids and a possible transition to immunosuppressant and biologics if necessary. The authors found that the 'top-down' strategy was more effective than the conventional 'step-up' strategy for achieving corticosteroid-free remission at week 52 (61.5% *vs* 42.2%, $P = 0.027$). Similar conclusions were demonstrated in both the SONIC and UC-SUCCESS trials whereby the efficacy of therapy was improved despite comparable adverse events between groups.

The strengths of objective patient monitoring are becoming more evident as study designs continue to improve and include more objective markers of disease severity and response to therapy. An example is the cluster randomisation trial, REACT[106]. In this trial, 1982 patients with CD were randomized to receive either algorithm-based treatment optimization vs. conventional management (therapeutic decisions based on community physician assessment). The composite endpoint of hospitalization, surgery and serious disease related complications was lower in patients treated with the algorithm-based strategy at 24 mo (27.7% and 35.1%, hazard ratio: 0.73, 95%CI: 0.62 to 0.86, $P < 0.001$), despite no differences in serious drug-related adverse events as compared to the conventional treatment group. In UC, evidence is less straightforward on whether 'top-down' therapy alters the long-term disease outcomes. Although several studies have shown that the severity and extent of UC at diagnosis may have a major impact on the subsequent course of the disease with elevated risks of recurrent hospitalization, colectomy, cancer and mortality[101,107,108]. In a population-based inception cohort from Norway, the extent of disease, need for systemic steroids and high CRP at diagnosis were independently associated with colectomy[109]. Consequently, patients presenting with extensive colitis and signs of severe disease

Table 3 Recommendations for treating to target in Crohn's disease by the International Organization for the Study of Inflammatory Bowel Diseases[19]

Crohn's disease	Ulcerative colitis
The consensus target is a combination of:	
Clinical/[1]PRO remission defined as resolution of abdominal pain and diarrhea or altered bowel habits which should be assessed every 3 mo until resolution then 6-12 mo thereafter.	Clinical/[1]PRO remission defined as resolution of rectal bleeding and diarrhea or altered bowel habits which should be assessed every 3 mo until resolution then 6-12 mo thereafter.
and	and
Endoscopic remission[2] defined as resolution of ulceration at ileocolonoscopy which should be assessed at 6-9 mo intervals during the active phase	Endoscopic remission[2] defined as resolution of friability and ulceration at flexible sigmoidoscopy or colonoscopy[3] which should be assessed at 3 mo intervals during the active phase
Adjunctive measures of disease activity that may be useful in the management of selected patients but are not a treatment target include:	
•Faecal calprotectin	•CRP •Faecal calprotectin •Histology
Measures of disease activity that are not a target:	
•Histology •Cross-sectional imaging	•Cross-sectional imaging

[1]Patient reported outcomes; [2]When endoscopy cannot adequately evaluate inflammation, resolution of inflammation as assessed by cross-sectional imaging can be substituted; [3]While Mayo subscore of 0 may be defined as the target, there is currently insufficient evidence to recommend it in all patients; only Mayo subscore of 0-1 can be systematically recommended in practice.

at diagnosis could benefit from top-down therapy.

'Treat to target', a strategy that uses objective clinical and biochemical outcome measures to assist clinicians in making decisions related to modifying therapy, has been gaining popularity since the REACT study demonstrated that disease activity correlates relatively poorly with objective measures of inflammation, and clinical remission in the absence of mucosal healing may not necessarily decrease the risk of future complications in CD[106]. The CALM study supported this logic as well and demonstrated that early and stringent control of disease using objective markers of inflammation (*e.g.*, CRP and fecal calprotectin) was efficacious and safe in their sample population of 244 patients with CD[18]. Their primary end-point, mucosal healing at 48 wk, was achieved in 46% *vs* 30% of the patients in the 'tight control' group as compared to the 'clinical management' group. Deep, biological and steroid-free remissions were greater in the 'tight control' group as well, whilst the adverse events not significantly different between groups. The recent systematic review and expert opinion of 28 IBD specialists on 'Selecting Therapeutic Targets in Inflammatory Bowel Disease' (STRIDE) also suggested the importance of using objective markers and recommends that therapeutic targets for CD and UC should move away from composite disease activity indices to separate patient-reported outcomes and objective measurements of inflammation (Table 3)[19]. However, the open-label multicentre RCT 'CALM' suggested that biomarkers such as fecal calprotectin be considered as additional targets to therapy in their cohort of 244 patients with active CD. Besides acting as a treatment target, biomarkers can facilitate the monitoring of a patient. For example, elevated c-reactive protein or fecal calprotectin should prompt further endoscopic and/or radiologic evaluation irrespective of clinical scores. Although intensified regimens are efficacious, they are also more likely to encounter difficulties with patient compliance. Additional guidance regarding the use of endoscopic findings as treatment targets will come following the completion of the REACT-Ⅱ prospective trial.

FUTURE PERSPECTIVES

Data obtained from head to head biologic and small molecule trials will eventually be applied to clinical practice in order to better individualize and optimize therapy. The determination of which therapies can be combined best will be further elucidated as well. For instance, combining anti-TNF therapy with vedolizumab is being evaluated in studies for patients with refractory disease because it combines a rapidly acting systemic agent with a slower acting gut-specific therapy. The development of oral medications with specific targets (*e.g.*, filgotinib) will open the door to a large range of potential therapeutic combinations which will enable therapy to be individualized further[110]. Specific therapies such as antifibrotics, SMAD7 inhibitors, sphingosine 1-phosphate receptor modulators and phosphodiesterase inhibitors are quickly making their way through trial phases and can be expected to hold a place in the IBD armamentarium in the near future[111-113]. As in Oncology, omics will enable us to determine which patients are at greatest risk for a complicated disease course thus provide a rationale for initiating intensified immunotherapy at diagnosis and individualize therapy best[114]. Molecular imaging and pretreatment genetic and biomarker analysis may be able to predict response to a proposed therapy in the future and are currently being investigated[103,104,115,116].

CONCLUSION

As the quality of trial designs improved over the decades, so followed our understanding of IBD. This has enabled

us to tailor therapy and develop effective treatment algorithms using clinical symptomps/PROS, biomarkers and endoscopic indices to help guide therapy. Anti-TNF therapy remains an important component of IBD therapy with the most real-life evidence and should be considered as first-line therapy in patients with complicated CD and in acute-severe UC. Novel mono- and combination therapies have only begun to be approved and offer the ability to tailor therapy further. However, clinicians will be faced with important challenges in defining the optimal use of these new therapies and their relative position in treatment algorithms. The next generation of clinical trials will need to ascertain the answers to these questions.

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REVIEW

Prognostic significance of tumor immune microenvironment and immunotherapy: Novel insights and future perspectives in gastric cancer

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Abstract

Despite a decrease in gastric cancer incidence, the development of novel biologic agents and combined therapeutic strategies, the prognosis of gastric cancer remains poor. Recently, the introduction of modern immunotherapy, especially using immune checkpoint inhibitors, led to an improved prognosis in many cancers. The use of immunotherapy was also associated with manageable adverse event profiles and promising results in the treatment of patients with gastric cancer, especially in heavily pretreated patients. These data have led to an accelerated approval of some checkpoint inhibitors in this setting. Understanding the complex relationship between the host immune microenvironment and tumor and the immune escape phenomenon leading to cancer occurrence and progression will subsequently lead to the identification of prognostic immune markers. Furthermore, this understanding will result in the discovery of both new mechanisms for blocking tumor immunosuppressive signals and pathways to stimulate the local immune response by targeting and modulating different subsets of immune cells. Due to the molecular heterogeneity of gastric cancers associated with different

clinico-biologic parameters, immune markers expression and prognosis, novel immunotherapy algorithms should be personalized and addressed to selected subsets of gastric tumors, which have been proven to elicit the best clinical responses. Future perspectives in the treatment of gastric cancer include tailored dual immunotherapies or a combination of immunotherapy with other targeted agents with synergistic antitumor effects.

Key words: Immunotherapy; Prognostic significance; Tumor immune microenvironment; Immune checkpoint inhibitors; Gastric cancer

Core tip: The use of modern immunotherapy, including adoptive cell therapy, vaccines, and especially immune therapy using checkpoint inhibitors, has led to encouraging results in clinical trials including gastric cancer patients. This review analyzes the relationship between immune microenvironment profile of the host and tumor development, identification of the immune prognostic markers and future perspectives of immunotherapeutic strategies. The treatment algorithm should be adapted to the specific molecular profile of the gastric cancer subtype in order to obtain maximum clinical benefits.

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INTRODUCTION

Early estimates situated gastric cancer in the first place as being the most frequent neoplasia (1975). Despite a decrease in gastric cancer incidents during the last decades, it remains a major health problem globally, with almost one million new cases diagnosed in 2012 (6.8% of the total), after tumors of the lung, breast, colorectum and prostate. There is a high geographical variation in gastric cancer incidence. Approximately 70% of cases occur in developing countries; half of the total number occurs in Eastern Asia, especially China; the incidence rates are approximately twice in men *vs* women. Overall, this type of tumor represents the third leading cause of cancer death in both sexes, accounting for 723,000 deaths in 2012 (8.8% of the total number of cases). The highest mortality rates are seen in Eastern Asia, whereas the lowest rates occur in Northern America; also, high mortality rates are encountered in Central and Eastern Europe and in Central and South America, respectively[1].

Most gastric cancers are diagnosed at an advanced stage, whereas another 25%-50% of cases will develop metastases during the outcome of the disease. Although surgical resection remains the main treatment with curative-intent in gastric cancer patients, there is a poor associated 5-year survival rate of approximately 20%-25%. Therefore, additional treatments (neo-adjuvant/adjuvant), such as chemotherapy and radiotherapy where associated with tumor resection, unfortunately lead to only modest survival benefits. In advanced stages, approximately 50% of cases present local/systemic recurrence after adjuvant treatment, and only 10%-15% of cases achieve a 5-year overall survival[2]. In the metastatic stage, the backbone of treatment is represented by palliative chemotherapy, associated with a poor median overall survival, of approximately 8-10 mo[3]. Despite recent advances using novel biologic therapeutic agents, with the exception of trastuzumab [anti-human growth factor receptor 2 (HER2) monoclonal antibody] and ramucirumab [fully humanized monoclonal antibody receptor antagonist to bind vascular endothelial growth factor receptor 2 (VEGFR-2)], showing beneficial results by improving overall survival (OS), and therefore approved in first-line (in association with standard chemotherapeutic regimens) and second-line settings, respectively (as monotherapy, or in association with chemotherapy), in advanced and metastatic gastric cancers, clinical trials assessing other targeted agents showed disappointing results in gastric cancer[4-6].

Recently, the therapeutic algorithm and prognosis of many tumors changed radically by introducing immunotherapy, especially using immune checkpoint inhibitors, and the first drug of this class approved by the United States Food and Drug Administration (FDA) was ipilimumab, an anticytotoxic T lymphocyte antigen-4 (CTLA-4) antibody, used in the treatment of advanced melanoma (2011)[7,8]. Afterwards, immune checkpoint inhibitors, which are antagonists of the programmed death (PD)-1/PD-ligand 1 (PD-L1) pathway, were approved by the FDA for the treatment of different tumors, such as melanoma, non-small cell lung cancer (NSCLC), urothelial/renal cell carcinoma, squamous cell carcinoma of the head and neck, Merkel cell carcinoma and Hodgkin's lymphoma[9].

MOLECULAR CLASSIFICATION OF GASTRIC CANCER

The following main histological classifications of gastric cancer have routinely been used: the World Health Organization (WHO) classification[10] that categorizes four histological subtypes, namely, papillary, tubular, mucinous and poorly cohesive, and Lauren's classification, dividing gastric cancers into intestinal, diffuse and mixed type[11].

Because these two classifications are not able to direct specific therapeutic strategies and, additionally, because the group of gastric cancers includes heterogeneity of tumors, there was a need to elaborate new classifications capable of stratifying patients regarding tumor behavior, prognosis and response to specific treatments. For the first time, the molecular assessment of gastric cancer

patients was proven to add benefits in the context of the TOGA trial in which a combined treatment with classical chemotherapy and trastuzumab showed an improvement of survival in the subgroup of patients overexpressing HER2[4]. Moreover, the behavior of the tumor and the outcome proved to be different in cases of Asian patients *vs* Caucasians included in several clinical trials[12].

In 2013, Singapore researchers identified three different molecular subtypes of gastric cancer: proliferative (high genomic instability, TP53 mutation), metabolic (high response to 5-FU chemotherapy), and mesenchymal (stem cell-like cancers that are sensitive to PIK3CA-mTOR inhibitors)[13].

The aim of "The Cancer Genome Atlas (TCGA)" project (2014) was to develop a new molecular classification of gastric cancer with clinical impact and to identify the main dysregulated pathways of each subtype of gastric tumors. The TGCA research group divided gastric cancer into four genomic subtypes:

Chromosomal instability (CIN): Includes approximately 50% of cases, most of the tumors are located at the gastro-esophageal junction or cardia[14]; leads to a loss or gain of some oncogenes and tumor suppressor genes[15]; has a high frequency of TP53 gene and receptor tyrosine kinase mutations and amplifications of cell cycle genes[16]; and has amplifications in oncogene pathways (HER2, BRAF, EGFR, MET, and RAS)[17].

Microsatellite instability: Tumor testing methodologies include immunohistochemistry for abnormal absence of MMR protein expression or polymerase chain reaction (PCR) for Microsatellite instability analysis (MSI), to evaluate for MSI-H on a tumor specimen. Immunohistochemistry can predict which gene is most likely to be mutated, the gene for the affected protein. Interpretation of immunohistochemical reports can sometimes be confusing, as "positive" should mean the abnormal absence of MMR protein expression, in contrast to normal presence of expression. MSI testing panels may consist of mononucleotide and dincleotid markers. For classifying MSI, a panel of five markers for the analysis of MSI was recommended by the National Cancer Institute, including two mononucleotide and two dinucleotide repeats. In the case that $\geqslant$ 2 of the five markers show instability, the genotypes are grouped into high-frequency (MSI-H); when only one marker shows instability, into low-frequency (MSI-L), and when no marker shows instability, into microsatellite stable (MSS). MSI-H consists of 30%-40% instability markers, while MSI-L of < 30%-40%. Bethesda Guidelines have stated that MSI-H can be defined when instability is present at mononucleotide loci and MSI-L when instability is limited at only dinucleotide loci; mononucleotide repeats were demonstrated to be more sensitive *vs* dinucleotide loci in detecting MSI. Some studies have shown that both immunohistochemistry and MSI are cost-effective and useful for selecting high-risk patients. A review showed that the sensitivities of MSI and immunohistochemical testing are 77% to 89%, and 83%, respectively; specificities are 90% and 89%, respectively. Some patients may have MSI or abnormal immunohistochemistry due to sporadic development of cancer, rather than an underlying genetic (germline) mutation. MSI accounts for 15%-30% of gastric cancers; most often includes tumors of intestinal type, antral location, females and older patients[18,19]; shows mutations in DNA mismatch repair genes, such as MLH1 or MLH2, that lead to the dysfunction of DNA mismatch repair enzymes[20]; is reported in a meta-analysis that demonstrated a 37% reduced mortality risk and prolonged OS in gastric cancer patients with MSI-high (MSI-H) *vs* MSI-low (MSI-L)[21]; is associated in non-colorectal cancer with an increased frequency of somatic mutation and amplification of tumor antigens; therefore, an increased sensitivity to treatment with PD-1 immune checkpoint inhibitors[22,23]; has increased intratumor infiltrate[24]; is reported in studies that show an increased activity of pembrolizumab in gastric cancer[25] and assess the efficacy of nivolumab ± ipilimumab in MSI-H gastrointestinal cancers[26]; and is possibly being considered in the development of a preventive vaccine using neopeptides affecting MSI carcinogenesis[27].

Genomic stability: Accounts for approximately 20% of gastric cancer cases; has an increased frequency of the diffuse type; indicates main somatic genomic alterations: CDH1, ARIDIA, RHOA; and presents a recurrent inter-chromosomal translocation involved in cellular motility[28].

Epstein Barr virus-associated: Epstein Barr virus (EBV) is detected by *in situ* hybridization or PCR in approximately 10% of gastric cancer patients[29]; it seems to increase ten times the gastric cancer risk, especially in Far East Asian patients[30], and is more frequent in younger patients[29]; it has a better response to immunotherapy and better survival[31]; it shows that the PD-L1 gene is frequently amplified; approximately 15% of EBV^+ tumors present amplification of chromosomal region 9p24.1 (locus of PD-L1, PD-L2)[32]; EBV^+ is found in approximately 50% of tumor cells and 94% of immune cells; therefore, PD-1 testing could predict a response to immunotherapy in this subset of patients[12]; and it shows PIK3CA mutations that could be targeted using PI(3)-kinase inhibition, DNA hypermethylation and JAK2 mutations[28].

Taking into account specific characteristics of the four subtypes of gastric cancer, it was highlighted that EBV-associated and MSI categories are associated with the best responses to immune therapeutic strategies.

The Asian Cancer Research Group (ACRG) proposed a molecular classification of four molecular subtypes for gastric cancer (2015): one subtype was related to the epithelial-to-mesenchymal transition (MSS/EMT) phenotype, which was associated with highest recurrence frequency, and another subtype was related to the phenotype of MSI, cytokine signaling, cell proliferation and methylation signals, including hypermutated tumors,

which was associated with the best overall prognosis. The remaining of the non-EMT and non-MSI patients were further divided into MSS/p53$^-$ and MSS/p53$^+$ molecular subtypes and were associated with an intermediate overall prognosis and recurrence[33,34].

Comparing TCGA *vs* ACRG subtypes of gastric cancers, one may notice a resemblance between MSI tumors, GS and MSS/EMT subtypes, EBV and MSS/TP53$^+$ subtypes, and CIN and MSS/TP53$^-$ tumors, respectively[13].

The development of genotyping different subtypes of gastric cancer will provide a guide to molecular targeted drugs that should be investigated in large clinical trials on specific subsets of gastric tumor patients in the future.

HOST IMMUNE RESPONSE IN GASTRIC CANCER PATIENTS

Antitumor immune response of the host

The term "immunosurveillance" refers to the capacity of the host immune system to identify the tumor cells as "non self", and subsequently to kill them[35]. The immune response includes both innate immunity (represented by macrophages, dendritic cells, and natural killer cells), and specific adaptive immunity (T and B lymphocytes).

The host protective response and the capacity of the tumor to surpass the host immune response are defined as "cancer immunoediting", which is a process comprising three progressive steps:

Elimination phase: In this stage, natural killer (NK) cells and T lymphocytes (helper and cytotoxic) secrete interferon IFNγ, leading to a reduction of angiogenesis and proliferation of cancerous cells; moreover, macrophages and dendritic cells (DC) secrete cytokines that activate immune cells to phagocytize dead tumor cells.

Equilibrium phase: Residual cancerous cells remain in a dormancy state because DC and cytotoxic T cells secrete IFNγ and inhibitory cytokines (IL12), suppressing them.

Escape phase: Tumor cells change their features which will be transmitted to the daughter cells, therefore escaping immunosuppression and proliferating, along with the apoptosis of the effector immunocytes[36]. This process is illustrated in Figure 1.

The tumor mechanisms to evade suppression by the immune system may include a reduced expression of the tumor antigens and major histocompatibility class I (MHC) antigen, Fas-L modulation, increased synthesis of inhibitory cytokines such as TGFβ1, IL10, IL6, VEGF, prostaglandin, and an increased number of T regulatory lymphocytes (Treg) and myeloid-derived suppressor cells (MDSC)[37].

Tumor immunosuppressive microenvironment

The most important components of the tumor immunosuppressive microenvironment are represented by the regulatory T cells (Tregs) and mesenchymal- or bone marrow-derived stem cells (BM-MSCs). Tregs represent CD4$^+$CD25$^+$FOXP3$^+$ T lymphocytes that determine reduced activity of cytotoxic and helper T cells, and of NK cells and are involved in the immunological tolerance to self-antigen and the persistence of *Helicobacter pylori (H. pylori)*-related inflammation[38]. BM-MSCs migrate to cancerous tissues and, in some animal models, were shown to create an immunosuppressive environment in chronic *H. pylori* infection and to represent a "seeding point" for gastric carcinogenesis[39-41]. In this regard, immunotherapeutic strategies directed against Tregs and BM-MSCs and against immunosuppressive cytokines seem to be promising.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) play essential roles in the immune checkpoint modulation. Normally, these molecules modulate the response of T lymphocytes to antigens. CTLA-4 represents an inhibitory receptor exhibited by T cells, whereas PD-1 represents a co-inhibitory receptor located on the cell surface, suppressing T cell activity in peripheral tissues in the context of inflammation. PD-1 is widely expressed on T and B lymphocytes, monocytes, and natural killer cells; conversely to CTLA-4, PD-1 is involved in subsequent phases of immune responses[42]. It has been shown that various tumor cells upregulate PD-L1. In gastrointestinal cancers, PD-L1 upregulation has been identified in pancreatic, gastric, and colorectal cancers, correlating in several studies with a poor prognosis[43,44].

Significance and prognostic role of tumor-infiltrating lymphocytes in gastric cancer

Tumor-infiltrating lymphocytes (TILs) comprise the presence of T cells, B cells and NK cells[45]. T cells include cytotoxic lymphocytes (CD8$^+$), helper T cells (CD4$^+$), memory T cells (CD45RO$^+$) and T regulatory cells (FOXP3$^+$). Specific cell membrane antigens of TILs bind to specific cellular types: CD3, CD4, CD8 and FOXP3 bind to T cells, CD20 to B cells and CD57 to NK cells[46].

In the complex relationship between the host immune microenvironment and cancer occurrence and progression, TILs seem to gain bidirectional regulation abilities. In one way, tumor neoantigens captured by the DC are presented on MHC molecules to the T cells, leading to the activation of effector T cells, with subsequent infiltration of the tumor and destruction of the cancerous cells. In addition, these activated cells secrete inhibitory cytokines, with the augmentation of antitumor effects[47,48]. On the other hand, TILs may help cancer to proliferate, either by creating an appropriate environment for tumor growth or by protecting tumor cells to survive[49].

The stromal TILs represent the mononuclear inflammatory cells infiltrating tumor stroma, whereas intratumor TILs define the intraepithelial lymphocytes/mononuclear cells within the tumor. Stromal TILs were shown to predict disease-free survival (DFS) of patients[50].

The assessment of TILs as a prognostic biomarker in gastric cancer patients has led to controversial conclusions. The presence of various subsets of cells seems

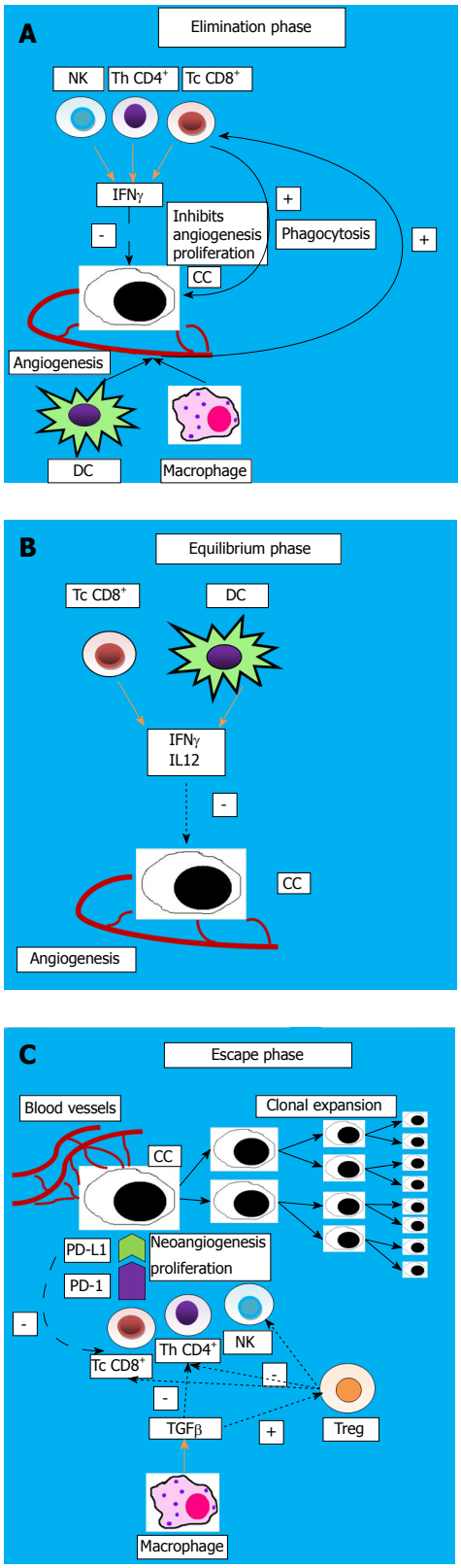

Figure 1 The three phases of cancer immunoediting. A: Phase 1: Elimination; B: Phase 2: Equilibrium; C: Phase 3: Escape. NK: Natural killer cells; Th: T helper lymphocyte; Tc: T cytotoxic lymphocyte; T reg: Regulatory T lymphocyte; DC: Dendritic cell; CC: Cancer cell.

to differently influence the patient's prognosis. Studies have shown that high density of intratumor TILs are associated with better prognosis (HR = 0.55)[51-53]. Additionally, some data in the literature revealed that an increased number of $CD8^+$ T cells, both intra- or extra-tumor located, is associated with an improved DFS and OS[54-57]; in contrast, the results of a recent study showed that an increased number of $CD8^+$ cells correlate with poor overall survival and increased expression of programmed death ligand 1 (PD-L1)[58]. Other studies showed that a high density of intratumor $FOXP3^+$ Treg is correlated with a poor OS, whereas an extratumor high density of this cell type leads to an increased OS[59-64]. The increased intratumor Treg/$CD8^+$ lymphocytes ratio is correlated with a decreased OS[65] and the presence of T helper 17 and T helper 22 with tumor progression[66]. Moreover, an increased T helper 1/T helper 2 of $CD4^+$ T lymphocytes represents a favorable prognostic factor[67]. A better OS was associated with an increased intratumor presence of various immunocytes, such as $CD3^+$ T cells[57,68], CD57 NK [51,57,69,70], $CD45RO^+$ (memory T cells)[71], and T-bet^+ (marker for T helper 1 lymphocytes)[72]; in addition, an increased DFS was observed in the case of high intratumor density of CD20 (surface marker of B cells)[73]. The data show a decrease in $CD3^+$ TILs density along with tumor progression[74]. On the other hand, the subgroup of $CD45RO^+$ T lymphocytes seems to prevent peritoneal spreading of gastric neoplasias[75]. All the data from the literature demonstrate that high densities of $CD8^+$, $CD3^+$, and $CD57^+$ TILs and low densities of $FOXP3^+$ TILs represent favorable prognostic factors in gastric neoplasia.

$CD4^+$ T cells secrete various cytokines, such as IL-17, the role for which the data from the literature reveal controversial results. Some of the studies showed that this cytokine could stimulate tumor angiogenesis, growth, and spreading[76], while other studies show that IL-17 exhibits anticancer effects, either by stimulating the cytotoxic activity of TIL[77] or by stimulating the maturation of DC[78].

The study of Yuan *et al*[79] revealed that gastric cancers present an increased percentage of $CD4^+$ T lymphocytes and lower $CD8^+$ T cells (with an increased $CD4^+/CD8^+$ ratio) compared to blood and, further, to paraneoplastic tissue. In addition, the number of TILs of effector and memory T cell type is significantly higher than in the case of circulating T cells. As we have already mentioned, the involvement of Tregs in antitumor immunity is controversial, and their role may differ according to the type of cancer[80,81]. These authors considered $CD4^+CD25^{high}CD127^{low}$ as being the most specific marker to define the Treg population, with the percentage of these cells being increased among TILs, demonstrating the accumulation of immunosuppressive Tregs at the site of a gastric tumor[79]. Recent studies show that the coexpression of PD-1^+ and Tim-3^+ define the most hypo-functional T lymphocytes[32,82]. The percentage of these cells among TILs was significantly increased in gastric tumors, especially in patients with advanced stages, suggesting that they may be implicated in a tumor immune escape phenomenon and that TILs in gastric cancer show T cell dysfunction. The combined blockade of these molecules seemed to have a

synergistic effect on IFNγ production and, therefore, may provide new promising immune modulating strategies[79].

Finally, the strategies of immunotherapy in gastric cancer are directed on TILs and are based on augmenting anticancer immunity by blocking the interaction of $CD8^+$ T lymphocyte-related receptors such as CTLA-4 and PD-1 and their ligands situated on tumor cells (PD-L2 and PD-L1). Furthermore, it may include the stimulation of the local immune response by targeting and modulating different subsets of $CD8^+$ TILs.

Significance of tumor infiltrating DCs in gastric cancer

Immunotherapy needs the activation of the cellular immune responses following the presentation of the tumor antigen peptides by DCs to T cells[83].

It has been demonstrated that the density of tumor infiltrating DCs correlates with the staging and prognosis in gastric neoplasias, and patients with a high amount of DCs present an improved OS compared with patients with a lower density of DCs[69]. The study of Tsujitani *et al*[84] showed that the use of postoperative adjuvant immunotherapy exhibits beneficial results on the survival of gastric cancer patients with reduced tumor DC infiltration.

Classification of the immune microenvironment of the gastric cancer according to the presence of $CD8^+$ TILs and PD-L1 expression

Based on the existence of tumor-infiltrating lymphocytes (TILs) and PD-L1 expression, it has been proposed to categorize the tumors into four types[85] as follows: type Ⅰ ($TILs^+$ $PD\text{-}L1^+$), presenting adaptive immune resistance; type Ⅱ ($TILs^-$ $PD\text{-}L1^-$), revealing immune ignorance; type Ⅲ ($TILs^-$ $PD\text{-}L1^+$), showing intrinsic induction; and type Ⅳ ($TILs^+$ $PD\text{-}L1^-$) in which other suppressors have a role in initiating immune tolerance. This stratification may have a certain prognostic role and may guide efforts towards a specific immunotherapeutic strategy.

Because tumor response to immunotherapy using PD-1 blockade requires the presence of $CD8^+$ TILs that are downregulated by PD-1/PD-L1 activity[86], this type of therapeutic strategy in type Ⅰ cancer might improve prognosis. As tumors included in type Ⅱ and Ⅲ lack TILs, the combination of immune checkpoint inhibitors with a vaccine that is capable to induce T cell activation, migration and infiltration at the tumor site might improve the clinical outcome in these patients[87].

Correlation between TILs and gastric cancer subtypes

Among the four molecular subtypes of gastric cancer, EBV^+ and MSI tumors often show the activation of immune mechanisms, being associated with a high density of TILs, which has been correlated with an improved cancer-specific survival[88]. An increased number of $CD8^+$ and $FOXP3^+$ TILs were associated with improved OS in MSI-H gastric cancers[55] and EBV-associated cancers[87]. In addition, another study showed that a high number of $CD3^+$ and $CD8^+$ TILs in EBV-associated and MSI gastric cancer subtypes are associated with better survival[56].

Advanced TNM stages of EBV^+ tumors were correlated with a reduced density of $CD4^+$, $CD8^+$ and $Foxp3^+$ TILs and PD-1 expression. Additionally, PD-L1 expression was shown to predict a reduced survival in EBV-associated cancers. Approximately two-thirds of EBV^+ gastric cancers were proved to present a type Ⅰ or Ⅳ microenvironment associated with a better prognosis by inducing adaptive immune responses (type Ⅳ showed the best 5-year OS), whereas more than 70% of negative EBV tumors belong to the type Ⅱ and Ⅲ microenvironment, showing an absence of an immune response and a poor prognosis[87].

All these results are indicating the possibility of different subsets of TILs to be used as prognostic markers in these specific categories of patients. Moreover, EBV-associated and MSI gastric cancer categories might become potential targets of immunotherapy.

Significance of peripheral immune status

Myeloid-derived suppressor cells (MDSCs) represent immune suppressive cells with the ability to inhibit T cell ($CD4^+$ and $CD8^+$) activation and increase T cell apoptosis[89]. The data showed that high intratumor density of MDSCs is related to a poor prognosis in gastric cancer[80,90]. Peripheral blood granulocyte MDSCs are significantly increased in cancerous patients[91]. Shoji *et al*[92] noted that an increased proportion of granulocyte MDSCs prior to chemotherapy represents a negative prognostic factor for PFS in advanced gastric cancer patients receiving cisplatin-based chemotherapy and tends to be associated with poor OS.

Several papers showed that IL-8 is involved in gastrointestinal carcinogenesis by its ability to recruit MDSCs[93]. In addition, an increased number of circulating MDSCs was associated with advanced tumor stages, increased serum IL-8 levels, and dysfunction of T cells[94]. In this light, IL-8 seems to determine an adverse immune status[92].

Currently, numerous papers target defining the host inflammatory response to cancer. Due to a release of cytokines, the systemic inflammatory response seems to be responsible for the promotion of angiogenesis, DNA alteration, and tumor proliferation[95].

Studies have demonstrated that the neutrophil to lymphocyte ratio (NLR) has a prognostic value in patients with solid cancers[96-98]. In oncologic patients, lymphopenia is a marker of deficient cell-mediated immunity, while neutrophilia is a sign of response to systemic inflammation. Furthermore, malnutrition/ hypoalbuminemia show correlation both with immune suppression and systemic inflammation, which are phenomena overexpressed in advanced tumor stages[99,100]. Gonda *et al*[101] consider that NLR might be a useful biomarker for assessing tumor response to chemotherapy, immune suppression, malnutrition and unfavorable prognosis in gastric cancer patients. Moreover, it has been suggested that combining anti-inflammatory agents with chemotherapy provides an enhanced efficiency of the treatment.

HISTORY OF IMMUNOTHERAPY

The concept of immune modulating treatment was used for the first time by Edward Jenner (1798), who demonstrated that inoculating humans with cowpox could prevent smallpox occurrence. Over time, this strategy was implemented in developing serum and vaccinations. The efficacy of immunotherapy in cancer treatment was demonstrated by WB Coley (1891) who, by injecting streptococcal germs into a patient with unresectable cancer, determined the decrease of the tumor size[102]. Furthermore, Ehrlich (1909) has suggested that the host immune system could suppress the tumor growth by recognizing cancerous cells as non-self. Half a century later, Burnett has proposed the theory of tumor immune surveillance[35], recently completed by Schreiber *et al*[49] with the concept of cancer immunoediting.

In recent years, many immunotherapeutic strategies have been developed, including treatments using monoclonal antibodies, cytokines, cytotoxic cells, T cells infusions and gene transferred vaccines[36], having the aim of either increasing the host antitumoral response capacity or increasing the immunogenicity and susceptibility to treatment of the tumor cells[103].

CURRENT IMMUNOTHERAPEUTIC STRATEGIES IN GASTRIC CANCER

The development of an effective immunotherapeutic management for digestive cancers has evolved relatively slowly, with the majority of these immune-modulating approaches still under assessment in early phase clinical trials, mostly because of their well-known lack of antitumor effector T lymphocyte responses and their decreased immunogenicity[104]. Despite these specific aspects, some immunotherapeutic approaches, such as those using anti-PD/PD-L1 immune checkpoint inhibitors, proved to also be effective in cancers defined by a poor immunogenic nature[105].

Immunotherapeutic strategies may be classified into[42]:

Active immunization strategies, including: (1) Adoption of cytokines (*e.g.*, IFNγ, IL-10, IL-2) to date, leading to inconclusive results[103]. (2) Vaccination strategies that include: vaccines using peptides/proteins recognized by $CD8^+$ and $CD4^+$ lymphocytes, such as various tumor-rejection antigens (including melanoma-associated antigen (MAGE-3) or HER-2/neu)[106]; new vaccines using a compound formed by an immunogenic protein fused with peptide (Z12) that determines a persistent antitumor T cell response[107]; DC-based vaccines; RNA-based vaccines, *etc.* (3) Immune checkpoint inhibitors (anti-CTLA4, anti-PD/PD-L1). (4) Combination of different immunotherapeutic strategies. (5) Combination of immunotherapy with standard treatment.

Passive immunization strategies: Adoptive cell therapy (ACT) using TILs- refers to the passive transfer of antitumor T lymphocytes into a tumor-bearing host followed subsequently by the direct destruction of this cancer[108]. Current immunotherapeutic strategies are presented in Figure 2.

Cellular immunotherapies in gastric cancer

Currently, immunotherapy in gastric cancer patients includes cell-based strategies aimed either to activate cytotoxic T lymphocytes directed against cancer cells or to bind molecules expressed by tumor cells.

ACT: This technique refers to injection of different tumor-specific T lymphocytes into a cancer patient, such as cytokine- and anti-CD3 monoclonal antibody- induced killer cells, as well as TILs.

(1) Cytotoxic T cell therapy

In a preclinical study, cytotoxic activity of peripheral blood lymphocytes extracted from gastric tumor patients or from healthy individuals was induced using different HLA-A matched allogeneic gastric cancer cells, exhibiting antitumor efficacy against HLA-A2 and HLA-A24 gastric cancer cell lines[109]. Furthermore, Kawamoto *et al*[110] demonstrated the efficiency of peptide-based immunotherapeutic strategies by proving that cytotoxic T lymphocytes were able to kill HLA-A-0201/2402 colon and gastric cancer cells, which were positive for mitotic centromere-associated kinesin (MCAK) (a new cancer antigen) in an HLA-Ⅰ restricted way. In addition, MHC-1 restricted T cells were obtained from primary tumors, metastatic lymph nodes, and ascites of autologous gastric cancer and were proved to detain different recognition characteristics towards gastric cancer antigens[111]. Moreover, splenic MAGE-specific cytotoxic T lymphocytes against HLA-A2 cancer cell antigen, existing in testis and several neoplasias (including gastric cancer), were successfully obtained and tested[112].

Another preclinical study has revealed that cytokine-induced killer (CIK) cells, mostly by stimulating IFN-γ and tumor necrosis factor-alpha, exhibit antiproliferative effects against the MGC-803 gastric cancer cell line[113]. In addition, Kim *et al*[114] demonstrated the benefits of using ACT with CIK cells in gastric cancer patients. These CIK cells, isolated from the human peripheral blood mononuclear cells and activated by IL-2 and anti-CD3 antibody, were able to suppress the MKN74 human gastric cancer cell line *in vitro* and inhibit tumor proliferation in a nude mouse model.

As gastric cancers usually show paucity of stroma infiltration, *in vivo* studies have suggested administration of immunotherapy using ACT combined with chemotherapy[115]. Furthermore, the chemotherapeutic drug oxaliplatin, by stimulating high-mobility group box 1 protein to induce anticancerous T lymphocytes, is capable of producing an immunogenic cancer cell death[116]. In several *in vitro* and *in vivo* studies on drug-resistant gastric cancer, a high amount of cytokines was

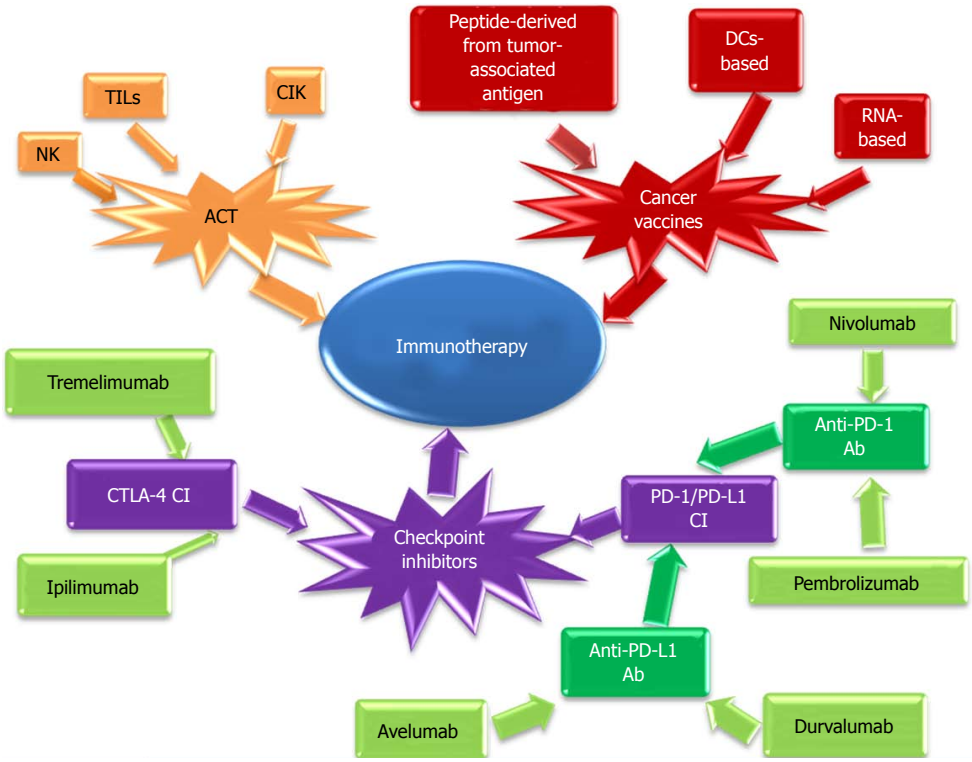

Figure 2 Current immunotherapeutic strategies in gastric cancer. NK: Natural killer cells; TILs: Tumor-infiltrating lymphocytes; CIK: Cytokine-induced killer cells; ACT: Adoptive cell therapy; DCs: Dendritic cells; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; CI: Checkpoint inhibitors.

induced by combining this drug with CIK cells[117]. It was suggested that chemotherapy associated with T lymphocyte reduction would be capable of enhancing the results of ACT therapy by stimulating the persistence of endogenous T cells in circulation, while depleting autoimmune reactions on healthy tissues. However, these results unfortunately occurred at the expense of severe infectious adverse events in these patients[118].

Moreover, the results obtained by combining ACT treatment with an antibody directed against both anti-epidermal growth factor receptor (EGFR) and anti-epithelial cell adhesion molecule (EpCAM), that specifically targets the simian virus 40 (SV40) T antigen-specific T cells (previously transduced with a truncated human EGFR), showed a better tumor reduction and OS than with ACT alone [119].

Du *et al*[120] used a mouse model of gastric cancer and concluded that ACT by peritoneal injection of CIK might be both a beneficial and minimally invasive strategy for treating this type of cancer.

The first clinical trial proving the beneficial results of ACT in humans used lymphokine-activated killer cells plus IL-2 in patients with metastatic melanoma, leading to the approval of the treatment for this group of patients[121-123]. Furthermore, this strategy determined a significant tumor reduction in patients with different tumors[124].

Zhang *et al*[125] showed that administration of expanded activated autologous lymphocytes, which were stimulated by anti-CD3 monoclonal antibody (mAb) and IL-2, to gastric neoplasia patients led to a prolonged OS compared to the group that received only standard chemotherapy.

The efficiency assessment of combined treatment using CIK cells and chemotherapy as adjuvant therapy in stage Ⅱ/Ⅲ of gastric neoplasia after curative gastric resection revealed a significantly prolonged OS *vs* the group using chemotherapy alone[126]. In a similar context, a clinical trial analyzed the possible toxicities of adjuvant ACT associated with chemotherapy (including 5-fluorouracil/capecitabine), showing an improvement in both DFS and OS, without the development of severe adverse reactions[127]. Moreover, a clinical trial assessing this type of combined adjuvant treatment in stage Ⅲ/Ⅳ (M0) gastric cancer patients after R0/D2 resection showed a significantly longer 5-year OS and DFS *vs* adjuvant chemotherapy[128].

In cases of advanced gastric cancer, several clinical studies have also proven an increased response rate, better quality of life and even an increased OS in patients treated with chemotherapy (FOLFOX4) plus CIK cells *vs* chemotherapy alone[129,130].

By assessing the administration of a chemotherapeutic regimen followed by autologous CIK cells, the results highlighted an improved remission rate in gastric cancer patients, associated with tolerable and reversible side effects. Standard chemotherapy using XELOX plus CIK cells administered intraperitoneally in gastric cancer patients showed a marked decrease in ascites volume and OS prolongation[131].

In a pilot study, patients with advanced gastric cancer, who received gamma delta T cells with zoledronate intraperitoneally as a local treatment for carcinomatous ascites, showed a significant reduction both in the number of peritoneal tumor cells and ascetic volume with no serious side effects[132].

Several clinical trials are currently investigating the tumor responses after adjuvant administration of ACT plus chemotherapy after surgical resection in advanced gastric cancer patients[133]. In a phase Ⅱ clinical study involving gastric cancer patients in stages Ⅰ-Ⅲ, the adjuvant combination of autologous tumor lysate-pulsed dendritic and CIK cells (Ag-D-CIK) plus chemotherapy is currently being evaluated following curative resection[134].

A clinical trial is currently investigating the safety and efficiency of infusing chimeric antigen receptor (CAR) T cells specific for EpCAM into relapsed or refractory gastric cancer patients[135].

A phase Ⅰ/Ⅱ clinical trial is currently investigating the benefits of infusing CAR T cells targeting mucin 1 (MUC1) in several solid cancers (including gastric tumors), as its overexpression leads to chemotherapeutic-refractory tumors[136].

A phase Ⅰb clinical trial on advanced gastric cancer expressing CEA assesses the efficacy of injecting anti-CEA CAR T cells into the hepatic artery targeting liver metastasis[137].

Additionally, a phase Ⅰ/Ⅱ clinical study on HER2$^+$ gastric cancer patients (defined as HER2 in immunohistochemical tumor tissue greater than or equal to 2 levels) with liver metastasis is analyzing the cytotoxic potency of engineered pluripotent stem cells and T cells, which specifically bind to HER2[138]. In addition, another phase Ⅰ clinical trial assesses the safety profile of administrating autologous T cells equipped with a bi-specific antibody (HER2 Bi-Armed T cells) in gastric and esophageal neoplasias[139].

Patients with metastatic gastric cancer are also investigated regarding a combination of S-1 plus dendritic cell activated CIK (DC-CIK) (phase Ⅰ/Ⅱ clinical trial)[140].

(2) Adoptive immunotherapy using TILs

The use of ACT with TILs is not associated with an immediate effect because this therapeutic protocol requires approximately six wks, as T cells must undergo the following preparation steps before infusion: first, they are isolated from tumor tissue; next, they are *in vitro* expanded; and finally, tumor-specific T cells are selected[141].

The immunotherapy using TILs has led to encouraging results in preclinical models[142], but not in all clinical studies (except for melanoma)[143,144].

In most gastric cancer patients, TILs exhibit a specific type-1 T cell response to cancer antigens. It is important to note that in order to obtain "*in vivo*" destruction of the tumor cells, the efficacy of tumor-specific T cells usually needs to be enhanced by combining vaccination using specific cancer antigens/peptides or by injecting *in vitro* expanded autologous cancer-specific T cells [103].

Moreover, TILs can sometimes stimulate proliferation of tumor cells. Studies show that HP0175-specific TILs in gastric cancer patients infected with *H. pylori* determine gastric Th17 response, exhibiting a pro-inflammatory low cytotoxic TIL response; Th17 cells promote tumor progression through the promotion of inflammation by secretion of IL-17 and other interleukins, which could induce proliferation and migration of cancer cells; therefore, TILs reveal a correlation between *H. pylori* infection and gastric cancer development[145].

Because studies have demonstrated that a high Tregs/CD8$^+$ ratio in the tumor areas represents an independent factor for poor OS, a combination of the deletion of Tregs plus the stimulation of effector T cells may represent an effective immunotherapy in gastric cancer patients[65].

ACT using TILs isolated from the patient's tumor was also assessed in cases with gastric neoplasia[146]; the results of a clinical trial showed a longer OS using a combined treatment of ACT using TILs and standard chemotherapy *vs* chemotherapy alone[143].

(3) Adoptive immunotherapy using NK cells

The data show that NK cells exhibit cytotoxic activity against allogeneic and autologous cancer cell lines, including gastric cancer cells lines[147], and could prevent tumor metastatic spreading[148,149]; additionally, intra-tumor infiltration of NK cells is associated with a longer survival in neoplastic patients[150]. Patients with advanced gastric adenocarcinoma having a high density of NK cells demonstrated a prolonged survival rate *vs* those with the low density NK[151]. The number of apoptotic NK cells (Fas$^+$ NK cells) is significantly higher in gastric neoplasia patients *vs* normal controls and is correlated to the tumor progression[152].

Clinical data revealed a favorable prognostic role of NK cells in gastric cancer patients, with a high level of CD57 antibody expression in gastric tumors correlated with a reduced size of tumors, N0 tumors, more surgical resections and prolonged 5-year OS[51].

By culturing autologous peripheral blood mononuclear cells with K562 cells, researchers were able to obtain cytotoxic NK cells from cancer patients[147], suggesting a possible role of immunotherapy using autologous expanded NK cells in clinical practice. In this regard, a clinical study using a combination of cell-based immunotherapy with autologous NK cells, γδ T cells, and CIK cells plus chemotherapy showed a statistically significant improvement of the 2-year progression-free survival and quality of life, but without demonstrating a significantly prolonged OS in gastric cancer patients[153].

The safety and efficacy of therapy with trastuzumab and NK cells in the treatment of gastric cancer is currently assessed in a clinical trial[154].

It has been demonstrated that lupeol, which exhibits a curative effect on various diseases, has the ability not only to stimulate the proliferation and the cytolytic activity of NK cells against gastric tumor cells, but also to inhibit the proliferation of some gastric cancer cell

lines. Therefore, this agent might be included (either alone or in combination with ACT) using NK cells in the therapeutic strategies of gastric tumors[155].

Cancer vaccines

Cancer vaccines have the aim of activating and increasing the number of effector T lymphocytes, leading to the augmentation of existing immunity, development of novel immunological response, and therefore an improved anticancer immunity[36].

Peptides derived from tumor-associated antigens [HER2/neu-derived peptide[156] and MAGE[157] are captured by antigen-presenting cells (such as DC)] and presented by means of MHC type Ⅰ for presentation to cytotoxic T cells, and by means of MHC type Ⅱ for presentation to T helper cells, determine their activation, followed by the destruction of tumor cells.

There are several types of vaccinations that have shown promising results in gastric cancer patients:

(1) HLA-A*2402-restricted URLC10-A24-177 and vascular epidermal growth factor receptor (VEGFR1-A12-9 1084) epitope peptide cancer vaccines in advanced chemotherapy-resistant gastric cancer patients - these are safe and induce enhanced specific cytotoxic T cell immune responses[158].

(2) Vaccination using survivin epitope peptide - a recent study suggested an excellent efficiency in gastric cancer patients upon inducing survivin-derived peptide-specific cytotoxic T lymphocytes from mononuclear cells isolated from blood of healthy individuals[159].

(3) Autologous gp96 vaccine in addition to chemotherapy as an adjuvant treatment in patients with resected gastric cancer (phase Ⅱ study) - glycoprotein (gp) 96, belonging to the group of autologous tumor-derived heat shock proteins (HSPs), binds tumor-associated antigens, constituting the HSPs-peptide complexes that promote the activation of APCs, as well as the release of various cytokines, enhancing T cell antitumor immune response; the vaccination seemed be well tolerated and is associated with a potential for prevention of gastric cancer recurrence[160].

(4) Trials using DC-based anticancer vaccines - *ex vivo* expanded DCs were able to generate antigen-specific T lymphocytes responses both in animal models[161] and in clinical trials[162]: (1) vaccination using dendritic cells pulsed with HER-2/neu peptide generated tumor shrinkage (phase Ⅰ study)[143]; (2) vaccination using DC pulsed with nanoparticles MAGE-3 peptide-loaded stopped tumors from proliferating in a mouse model of gastric cancer (phase Ⅰ study)[163]; and (3) vaccination with cancer-loaded autologous DCs (stimulated by autologous apoptotic gastric tumor cells) determined the activation of memory T cells[164].

RNA-based DC vaccines: By using stabilized mRNA, DCs transfected with mRNA coding for tumor-associated antigen/whole tumor RNA were able to generate potent immune responses both in mouse models[165,166] and clinical studies involving melanomas and renal cell carcinomas[167,168].

These RNA-based vaccines seem to present some potential benefits over the classical vaccination techniques: they are pharmaceutically safer because of the presence of transient and cytosolic active mRNA (lack of genomic integration); they have the ability to target multiple tumor-associated antigens; they are not associated with severe adverse events; and they are not MHC-restricted[36].

Because the clinical efficiency of DC vaccines is limited because of the short survival of DCs, mostly due to the cytolytic properties of DC-activated $CD8^+$ T cells[169], their efficiency has been increased by using small interfering RNA (siRNA)-targeting phosphatase and tensin homolog (PTEN), which is involved in negative feedback in the signal transduction of the PI3K/AKT pathway[170]. This technique has increased the number of cancer-specific cytotoxic T cells and the antitumor immune response[171].

The results were improved by targeting the immunosuppressive IL-10 receptor in association with the siRNA DC vaccine[172], by using GM-CSF gene-modified DC[173], and by removing Tregs along with DC vaccination[174].

Strategies of combining vaccination with chemotherapy: The combination of an adjuvant Bacille Calmette-Guérin (BCG) vaccine with chemotherapy *vs* chemotherapy alone resulted in a better OS in patients with radically resected locally advanced gastric cancer[175].

Vaccination using gastrin-17 diphtheria toxoid (G17DT)-targeting gastrin peptide combined with chemotherapy (cisplatin plus fluorouracil) increased the OS of patients with advanced gastric cancer (phase Ⅱ clinical trial)[176].

Vaccination using peptides derived from human VEGF receptors 1 and 2 combined with standard chemotherapy (S1+ cisplatin) improved the OS in patients with advanced gastric cancer[177].

Literature data show that vaccination is a safe and tolerable strategy that is associated with better prognosis in gastric cancer patients, especially when it is performed in addition to classical chemotherapy.

Immunotherapy using checkpoint inhibitors

Immune checkpoints represent inhibitory pathways that are critical for maintaining self-tolerance and physiological homeostasis by controlling the intensity of physiological immune responses to prevent tissue injury, particularly when the immune system is fighting an infection. Additionally, they may also allow immune escape of cancer cells[36].

Immune checkpoint molecules, such as cytotoxic T lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1), are involved in the inhibition of T cell activation via different pathways.

CTLA-4 is a co-inhibitory molecule exhibited on activated T lymphocytes and T regulatory cells, whose receptor on T cells interacts with its B7-1/B7-2 ligands

located on antigen-presenting cells (APCs), subsequently suppressing the T cell stimulatory signal mediated by CD28[178]. CTLA-4 expression is stimulated only in the context of T cell activation; afterwards, it competes with CD28 to bind to B7 molecules and decrease the immune response. By inhibiting this interaction using an anti-CTLA-4 antibody, T cell activation and proliferation is promoted, along with a decrease in immunosuppressive Treg cells among TILs[179].

PD-1 represents a co-inhibitory receptor that is found on the surface of several types of cells, such as activated T cells, Treg cells, and monocytes. It has two ligands, PD-L1 and PD-L2. PD-L1 is expressed on both immune and tumor cells, while PD-L2 is mostly expressed on APCs. In tumors, PD-L1 that is expressed on tumor cells binds to PD-1 on activated T cells that reach the tumor and generates a suppression signal for the activation of T cells, which become unable to destroy tumor cells, leading to a decrease in both cellular and humoral immune responses[180,181]. Unlike CTLA-4, which is considered to be necessary for T cell activation, the PD-1/PD-L1/2 pathway seems to protect tumor cells from attack by T lymphocytes. It has been demonstrated that by inducing antibody-mediated blockage of the PD-1/PD-L pathway followed by the inhibition of this checkpoint, treatment is able to enhance the anticancer immune response of the host[22,182].

Several genetic studies have shown a possible correlation between PD-1 or CTLA-4 polymorphisms and the development of gastric cancer[183-185]. Additionally, the role of CTLA-4 gene promoter hypermethylation has been demonstrated as a risk factor for gastric tumor development, with CTLA-4 expression being significantly higher in gastric tumor samples *vs* normal tissue[186].

Prognostic significance of PD-1/PD-L1 and CTLA-4 expression in gastric cancer: Saito *et al*[187] showed that PD-1 expression in $CD8^+$ and $CD4^+$ T cells in gastric tumors was significantly higher *vs* in normal gastric mucosa. Wu *et al*[188] showed that PD-L1 expression was encountered in 42% of gastric cancer tissues but not in normal gastric mucosa.

A meta-analysis by Gu *et al*[189] that was based on 15 studies (most of them conducted in Asia) including 3291 patients, showed a high variability of PD-L1 immunohistochemical expression among studies, ranging between 14.3% and 69.4%, due to the differences in cut-off values (between > 1% and > 50%).

Unlike in other tumors, such as lung cancer or melanoma, there is scattered PD-L1 expression in gastric cancer cells, which mostly occurs in infiltrating myeloid cells at the tumor invasive front[25,58].

There are several papers suggesting that PD-L1 expression in tumor cells may be upregulated by genomic alterations and oncogenic signaling, either through the phosphatidylinositol-3-kinase-protein kinase B (PI3K-AKT) or signal transducers and activators of the transcription (STAT) 3 pathway. Moreover, PD-L1 is upregulated by the microRNA-200/zinc-finger E-box-binding homeobox 1 (ZEB-1) axis, which is closely related to epithelial-mesenchymal transition (EMT) conversion and to IFN-γ produced by TILs[190-192]. Mimura *et al*[193] showed that membranous PD-L1 immunohistochemical expression in gastric tumor cells was significantly correlated with the number of $CD8^+$ TILs and IFN-γ positive cells in the tumor. Additionally, an enhanced cytotoxic effect of anti-PD-L1 monoclonal antibody treatment was observed after prior IFN-γ exposure.

Literature data regarding the expression of PD-1/PD-L1 as a prognostic in gastric cancer showed controversial results. Dai *et al*[53] reported an association between PD-L1 expression and an increased density of TILs; this increased density of TILs and higher levels of PD-L1 mRNA in gastric cancer was significantly correlated with a better prognosis. Some studies showed a significantly improved prognosis in patients with PD-L1 positive tumors[194,195]; conversely, others reported an association between PD-1/PD-L1 expression in cancer cells and TILs and advanced tumors, increased tumor size, the presence of deep invasion, lymph node metastasis, and perineural invasion and a significantly worse prognosis in these patients[196-198]; and the third group of papers found no influence of PD-L1 on the prognosis of gastric cancer[199].

A few recent meta-analyses have shown a correlation between PD-L1 and gastric cancer prognosis, demonstrating that PD-L1 overexpression is a worse prognostic factor in these patients[200-202]. Additionally, Zhang *et al*[203] performed a recent meta-analysis that included ten studies with 1901 gastric cancer patients and showed that PD-L1 expression was associated with a shorter OS and a poor clinicopathological status.

Fang *et al*[204] highlighted that PD-L1 was expressed both in tumor cells and in TILs. PD-L1 positivity in tumor cells was associated with differentiation, while its expression in TILs was correlated with a late stage of the disease, no surgery and the OS. Patients with $PD\text{-}L1^+$ TILs had a significantly poorer 5-year OS than those without PD-L1 expression (14.2 *vs* 18.3; $P = 0.001$). A study by Gao *et al*[205] showed that PD-L1 and PD-L2 positivity in primary tumors and metastatic lymph nodes decreased the number of $CD8^+$ T cells, and the amount of PD-1 positive expression on $CD8^+$ T cells in primary tumors were prognostic factors that were correlated with a poor prognosis in stage Ⅱ/Ⅲ gastric cancer patients.

Again, the results of the meta-analysis by Gu and collaborators[189], showed that gastric cancer patients with deeper tumor infiltration, lymph node metastasis, venous invasion, and EBV^+ and MSI subtypes were more likely to be PD-L1 positive. Moreover, for the subgroups of Asian patients and patients with stage Ⅱ/Ⅲ gastric cancer, cut-off values greater than 50% and cytoplasm/nuclear PD-L1 expression within tumor cells were positively associated with the OS. The results of this meta-analysis demonstrated that PD-L1 overexpression represented a significantly adverse prognostic factor in gastric cancer, fitting the theory of the cancer immunity cycle[47].

Many other studies[32,206] have also demonstrated

that EBV$^+$ and MSI gastric tumors tend to be positive for PD-L1 expression. These subtypes have a rich infiltration of lymphocytes, especially CD8$^+$ T cells, in the tumor stroma; therefore, they may be categorized as medullary carcinomas. Moreover, in this context, PD-L1$^+$ expression is associated with a significant increase in the number of CD8$^+$ T cells at the tumor invasive front and with the ability of immune cells to infiltrate the center of the tumor[32]. Both EBV$^+$ gastric cancers and MSI$^+$ gastric cancers have IFN-γ response genes, therefore, PD-1 pathway signaling seems to be a crucial mechanism for controlling a previous cytotoxic anticancer immune response[189]. The EBV$^+$ subtype is associated with amplification of the 9p24.1 locus, which harbors the PD-L1/PD-L2 genes; PD-L1 positivity is found in 50% of the cancer cells and 94% of immune cells in this subgroup[194,207]. Additionally, approximately 33% and 45% PD-L1$^+$ expression levels are encountered on tumors cells and immune cells, respectively, in MSI gastric tumors[208]. These data suggest that EBV$^+$ and MSI gastric cancers may be preferred candidates for PD-1 blockade immunotherapy. These types of neoplasias that are associated with rich inflammatory infiltrates are considered "hot" or inflamed tumors, while poorly immunogenic cancers are termed "cold." "Hot" tumors are characterized by the expression of immune-inhibitory signals, such as PD-L1, indoleamine-2,3-dioxygenase (IDO), and Treg cells[209,210], which counterbalance the effects of cytotoxic T lymphocytes. Therefore, using combined treatments to convert cold into hot tumors may increase the proportion of patients who benefit from immunotherapy[211].

While some data from non-small cell lung cancer (NSCLC) and other cancers have revealed that PD-L1 immunohistochemical positivity in cancer and/or in immune cells (bioptic specimens) is correlated with beneficial results after checkpoint inhibitor immunotherapy using monoclonal antibodies[212], other studies have shown tumor responses to PD-L1 therapies in tumors with PD-L1- cancer cells[213].

The location of PD-L1 expression in TILs situated at the invasive front or even at the tumor center may negatively influence its use as a biomarker. Furthermore, stromal rather than membranous expression of PD-L1 may be the cause for the slightly poorer responses to single-agent PD-1 checkpoint inhibitors in gastric cancer *vs* other tumor types[208].

Because there are variations in the techniques, the antibody clones used, and the cutoff values for PD-L1 positivity that limit cross-trial comparisons of different tumor types, including gastric cancer, some researchers have proposed harmonization of PD-L1 testing to standardize the results. Recently, Jiang *et al*[214] elaborated an immunohistochemical-based immunoscore on a cohort of 879 Chinese gastric cancer patients and demonstrated that a high immunoscore corresponded to lower recurrence rates and improved OS after adjuvant therapy.

Data suggest that there is racial and geographical variability in the tumor-immune microenvironment that is related to different responses to immunotherapy; for example, non-Asian gastric cancer patients have tumors that are rich in TILs and are associated with high CTLA-4 signaling[215].

The study by Schlößer *et al*[198] found positive CTLA-4 expression in the tumor microenvironment of 86% of gastric cancer patients that was correlated with poor OS.

Clinical trials using checkpoint inhibitors: Clinical trials using CTLA-4 and PD/PD-L1 checkpoint inhibitors are listed in Table 1.

(1) CTLA-4 checkpoint inhibitors

Tremelimumab

Tremelimumab (formerly ticilimumab, CP-675,206) is a fully human monoclonal antibody against CTLA-4 that received FDA approval for the treatment of mesothelioma. It was the first immune checkpoint inhibitor that was investigated in patients with gastroesophageal tumors.

A phase Ⅱ study[216] investigated tremelimumab (at a dose of 15 mg/kg every 90 d, which is presently considered sub-therapeutic) in 18 patients with advanced esophageal, gastroesophageal junction or gastric adenocarcinoma. Although the median time to progression and OS were relatively short, at 2.83 and 4.83 mo, respectively, approximately one-third of patients were alive at one year. Currently, there is an ongoing clinical trial assessing whether tremelimumab is associated with the anti-PD-L1 antibody durvalumab; the dose of tremelimumab used as a monotherapy is 10 mg/kg every 4 wk[9].

Ipilimumab

Ipilimumab (trade name Yervoy, previously known as MDX-010 and MDX-101) was approved by FDA for the treatment of patients with unresectable/metastatic melanoma, after at least one line of systemic treatment has been performed (2011)[217]; the indications for this drug were recently extended to include pediatric patients (12 years and older) (2017) and adjuvant treatment of patients with cutaneous melanoma with pathologic involvement of regional lymph nodes greater than 1 mm in size who have undergone complete resection (including total lymphadenectomy); in addition, it was recently approved as the first-line treatment, in combination with nivolumab, for patients with intermediate or poor risk with advanced renal cell carcinoma (2018).

A randomized phase Ⅱ trial enrolled patients with unresectable, locally advanced/metastatic gastric or gastro-esophageal junction cancer with partial response/stable disease after first-line chemotherapy with a combined fluoropyrimidine plus platinum regimen to receive either the best supportive care (consisting of continuation of fluoropyrimidine) or ipilimumab. The primary end-point of the study was immune-related PFS. Unfortunately, the study was ended earlier due to the lack of clinical efficiency of ipilimumab. The PFS was

Table 1 Clinical trials using CTLA-4 and PD/PD-L1 checkpoint inhibitors

Checkpoint inhibitors	Study number	Phase	Status	Study design	Study population	Primary outcome measures	Secondary outcome measures	No. of patients (estimated enrollment)	Final results
Nivolumab, Ipilimumab	NCT03342417	Ⅱ	Recruiting	NIVO + IPI	-Neoadjuvant breast cancer -Platinum-resistant advanced ovarian/GC	- % AE	- DOR, OS, QOL, recurrence rate	60	Pending
Nivolumab, Ipilimumab	NCT02872116 (CHECKMATE-649)	Ⅲ	Recruiting	- NIVO + IPI/ -NIVO + chemo *vs* chemotherapy (XELOX/ FOLFOX)	-Naive advanced/metastatic GC/GEJ	-OS NIVO + IPI *vs* chemo (PD-L1 + tumors) -OS NIVO + chemo *vs* chemo -ORR, PFS nivo + chemo *vs* chemo	-OS NIVO + IPI *vs* chemo -PFS NIVO + IPI/ NIVO + chemo *vs* chemo (PD-L1+) -ORR NIVO + chemo *vs* chemo	1349	Pending
Nivolumab, Ipilimumab	NCT02935634 (FRACTION-GC)	Ⅱ	Recruiting	- NIVO + IPI -NIVO + RELATLIMAB -NIVO + BMS-986205	-Advanced GC	-ORR, DOR, PFS	-% AE, SAE, discontinuation/death due to treatment	300	Pending
Nivolumab, Ipilimumab	NCT03044613	Ⅰb	Recruiting	- NIVO/NIVO + IPI prior to chemoradiation + NIVO	-Neoadjuvant treatment, resectable stage Ⅱ/Ⅲ EC/ GEJ	-% AE	-Feasibility of induction treatment -Path CR -Quantity of NIVO bound to PD1 receptor -Changes in expression of immune markers -OS, RFS	32	Pending
Nivolumab, Ipilimumab	NCT03443856 (VESTIGE)	Ⅱ	Not yet recruiting	- NIVO + IPI	-Adjuvant treatment GC/ GEJ adenocarcinoma stage Ⅰ b-IVa, ↑risk of recurrence (ypN1-3 + /R1) after neoadjuvant treatment + resection	-DFS	-OS -Relapse rate -Loco-regional/distant failure rates -% AE, QOL, global health status	240	N/A
Nivolumab, Ipilimumab	NCT03409848 (INTEGA)	Ⅱ	Recruiting	-IPI/FOLFOX + NIVO and TRAS	-Advanced/metastatic GC adenocarcinoma, previously untreated	-OS	-% AE -PFS, RR, QOL -Translational research -Central imaging review	97	Pending
Nivolumab, Ipilimumab	NCT02834013	Ⅱ	Recruiting	-NIVO + IPI	-Rare tumors, including gastric NET, SqCC, GIST	-ORR-RECIST	-Toxicities -OS, PFS -Clinical benefit rate -ir-ORR/PFS	707	Pending
Nivolumab, Ipilimumab	NCT03126110	Ⅰ/Ⅱ	Recruiting	-NIVO + INCAGN01876 -IPI + INCAGN01876 -NIVO + IPI + INCAGN01876	-Advanced/metastatic malignancies, including GC	-Toxicities (%AE) -ORR-RECIST	-DR, DDC, PFS, OS- RECIST	450	Pending
Nivolumab, Ipilimumab	NCT03241173	Ⅰ/Ⅱ	recruiting	-NIVO + INCAGN0949 -IPI + INCAGN0949 -NIVO + IPI + INCAGN0949	-Advanced/ metastatic malignancies, including GC	-Toxicities (%AE) -ORR-RECIST	-DR, DDC, PFS, OS- RECIST	651	Pending

Ipilimumab	NCT01585987	Ⅱ	Completed	-IPI *vs* BSC (5-FU)	-Unresectable/ metastatic GC/GEJ adenocarcinoma (following I st line treatment)	-ir-PFS (ir RECIST) -% BOR	-PFS (mWHO) -OS	143	-ir-PFS ↓ (2.92 *vs* 4.89 mo); -mWHO-PFS↓ (2.72 *vs* 4.89) (P = 0.03); -OS at study completion 12.68 *vs* 12.06 mo
Tremelimumab, Durvalumab	NCT02658214	Ⅰ	Recruiting	-TREME + DURVA + chemo (platinum-based SOC)	- I st line locally advanced/ metastatic solid tumor, including GC/GEJ	-Laboratory findings -%AE, safety, tolerability -Tumor assessment (RECIST)	-	42	Pending
Nivolumab	NCT03453164 (CIRCUIT)	Ⅰ / Ⅱ	Recruiting	NIVO + radiotherapy	-Unresectable recurrent GC (3 rd line)	-DCR: -PD: on CT/ MRI/ PET-CT -CR/PR/SD	-Mean survival -% AE -Local control rate -% PL-L1+, MHCI- tumor cells -Cytokines serum concentration -% regT cells -% Ag-specific CTL	40	Pending
Nivolumab	NCT02267343	Ⅲ	Active, not recruiting	NIVO *vs* placebo	-Refractory, unresectable, advanced/ recurrent GC/GEJ	-OS, PFS	-ORR, DOR, %AE, %SAE, safety	480	Pending
Nivolumab	NCT02746796	Ⅱ / Ⅲ	Recruiting	NIVO + chemo	- I st line therapy, unresectable advanced/ recurrent GC/GEJ	-PFS -OS	-ORR, DOR, DCR -TTR, BOR -% AE, SAE, laboratory abnormalities	680	Pending
Nivolumab	NCT03006705	Ⅲ	Recruiting	NIVO + chemo *vs* placebo + chemo	-Adjuvant treatment *p* stage Ⅲ GC/GEJ (after D2 resection)	-RFS	-OS -Safety- % AE, SAE, laboratory abnormalities	700	Pending
Nivolumab	NCT02999295	Ⅰ / Ⅱ	Recruiting	-NIVO + RAMUCIRUMAB	-Advanced/ recurrent unresectable GC/GEJ	-No. of pts with DLT -6 mo PFS	-%AE -ORR -DCR -OS, PFS	44	Pending
Nivolumab	NCT02946671	Ⅰ	Recruiting	-NIVO + MOGAMULIMUMAB (KW-0761 = anti-CCR4)	-Preoperator treatment against solid cancers, including GC	-% AE - FOXp3 + tumors by immunohistochemistry	-ORR-RECIST -% ↓Treg	18	Pending
Nivolumab	NCT02951091 (Biomarker – integrated Umbrella)	Observational	Recruiting	-NIVO/ AFATINIB/ GSK2636771 + PACLITAXEL	-Advanced GC -Different molecular cohorts: PD-L1+, MSI-H, EBV^+ → NIVO	-PFS	-	400	Pending
Nivolumab	NCT02465060 (The MATCH screening trial)	Ⅱ	Recruiting	-NIVO/ other agents (according to genetic testing)	-Advanced/ metastatic solid tumors (including GC), lymphomas, multiple myelomas → mismatch repair deficiency (loss of MLH1/ MLH2)	-ORR	-OS, PFS -TTP	6452	Pending

Nivolumab	NCT02862535	I b	Active, non-recruiting	-ANDECALIXIMAB (GS-5745) ± NIVO/chemo	-Previously treated, advanced GC/GEJ adenocarcinoma (Japan)	-Safety (% AE)	- Serum concentration of Andecaliximab -% Ab-anti Andecaliximab	36	N/A
Pembrolizumab	NCT03382600 (MK-3475-659/ KEYNOTE 659)	II	Recruiting	-PEMBRO + OXALIPLATIN + TS-1 *vs* -PEMBRO + CISPLATIN + TS-1	-Advanced CG/GEJ adenocarcinoma, HER2(-), PD-L1+	-ORR-RECIST	-ORR (i-RECIST) -DCR, DOR, TTR, PFS (RECIST, iRECIST) -OS, % AE	90	Pending
Pembrolizumab	NCT02901301	I / II	Recruiting	-PEMBRO + TRASTUZUMAB + chemo	- I st line advanced, GC HER2+	-Recommended dose -ORR-RECIST	-DOR -TTR	49	Pending
Pembrolizumab	NCT03342937	II	Recruiting	PEMBRO + XELOX	- I st line metastatic GC adenocarcinoma	-PFS	-OS - % AE	50	Pending
Pembrolizumab	NCT02918161	II	Recruiting	PEMBRO + chemo (SOC)	-Perioperative setting GC/ GEJ	-2 years DFS	- Pathol CR -OS, ORR (RECIST) -DFS	40	Pending
Pembrolizumab	NCT02689284	I / II	Recruiting	PEMBRO + MARGETUXIMAB	-HER2 + advanced, metastatic GC/GEJ	- Expansion phase dose of Margetuximab -Antitumor activity: RD, ORR (RECIST, ir-RECIST)	-OS -PFS	72	Pending
Pembrolizumab	NCT03257163	II	Recruiting	-PEMBRO + CAPECITABINE + radiotherapy (perioperative)	-Mismatch repair deficient, EBV^{+}, operable GC	-RFS	-	40	Pending
Pembrolizumab	NCT03064490 (PROCEED)	II	Recruiting	-PEMBRO + chemoradiotherapy	-Neoadjuvant treatment, locally advanced EG cancers	-Pathol CR	-Toxicity (% AE)	38	Pending
Pembrolizumab	NCT02563548	I b	Recruiting	-PEMBRO + PEGPH2O (Pegylated Recombinant Human Hyaluronidase)	-Hyaluronan-high (HA-H) patients with relapsed/ refractory cancers (adenocarcinoma)	-ORR	-DCR, DOR, PFS (RECIST, ir-RECIST)	81	Pending
Pembrolizumab	NCT02954536	II	Recruiting	-PEMBRO+TRASTUZUMAB+chemo	-Advanced, metastatic HER2+, EG (Ist line)	-PFS (RECIST)	-	37	Pending
Pembrolizumab	NCT03221426 (MK-3475-585) (KEYNOTE-585)	III	Recruiting	-PEMBRO + cemo *vs* placebo + chemo	-Neoadjuvant/adjuvant previously untreated GC/ GEJ adenocarcinoma	-OS - EFS event-free survival) -Pathol CR -% AE + discontinuation of treatment	-DFS	860	Pending
Pembrolizumab	NCT03196232	II	Recruiting	-PEMBRO + EPACADOSTAT	-Metastatic/ unresectable GEJ	-6-mo PFS	-ORR (RECIST) -OS -RR	30	Pending
Pembrolizumab	NCT03019588 (MK-3475-063/ KEYNOTE-063)	III	Recruiting	-PEMBRO *vs* chemo (PACLITAXEL)	-Progression after I st line platinum-fluoropyridine chemo, advanced GC/GEJ adenocarcinoma, PD-L1+ (Asia)	-OS, PFS (RECIST)	-ORR-RECIST -% AE, % discontinuation due to AE	360	Pending
Pembrolizumab	NCT03488667	II	Not yet recruiting	PEMBRO + mFOLFOX	-Neoadjuvant treatment GEJ adenocarcinoma -adjuvant treatment GC	-yp RR (pathologic response) -toxicity (% AE)	-ORR, DFS, OS -PET scan response rate - % PD-L1 + in tumor cells	40	N/A
Pembrolizumab	NCT03413397	II	Recruiting	PEMBRO + LENVATINIB MESYLATE	-Metastatic/ recurrent GC/ GEJ	-ORR-RECIST	-PFS, OS -Characteristic immunologic changes	29	Pending

Pembrolizumab	NCT02730546	Ⅰ / Ⅱ	Recruiting	PEMBRO + chemoradiotherapy	-Locally advanced, operable, GEJ/gastric cardia adenocarcinoma (neoadjuvant setting)	-Path CR -PFS	-R0 resection, DSF -Dose-limiting AE, % surgical complications, OS, PFS, time to relapse	68	Pending
Pembrolizumab	NCT02318901	Ⅰb/ Ⅱ	Active, not recruiting	PEMBRO-TRASTUZUMAB/ ADO-TRASTUZUMAB-ETAMSINE/CETUXIMAB	-Unresectable HER2+, advanced GC/GEJ	-Dose of mAb combined with PEMBRO	-% grade 3-4 AE -RR (RECIST, ir-RECIST) -OS, PFS -Circulating tumor DNA -Imaging changes	90	N/A
Pembrolizumab	NCT03095781	Ⅰ	Recruiting	PEMBRO + XL888 (= Hsp90 inhibitor)	-Advanced gastrointestinal cancer (including GC)	-Recommended dose for combined treatment	-ORR, PFS, RS (RECIST) -OS	50	Pending
Pembrolizumab	NCT02346955	Ⅰ	Terminated	-CM-24[MK-6018 = mAb against CEACAM1] ± PEMBRO	-Advanced/recurrent malignancies (including GC)	-% AE, discontinuation due to AE, DLT	-Maximum drug concentration, half-life elimination, ORR, DOR	27	Pending
Pembrolizumab	NCT02178722 (KEYNOTE-037/ ECHO-202)	Ⅰ / Ⅱ	Recruiting	-PEMBRO + EPACADOSTAT	-Selected carcinomas (including GC)	-% DTL -ORR	-PFS -% AE -OS	508	Pending
Pembrolizumab	NCT02903914	Ⅰ / Ⅱ	Recruiting	-PEMBRO + ARGINASE INHIBITOR INCB001158	- Advanced/metastatic solid tumors (including GC)	-% AE	- Rrecommended dose of arginase Inhibitor ± PEMBRO -Pharmacokinetic profile -Antitumor activity of drugs (RECIST, ir RECIST)	346	Pending
Pembrolizumab	NCT03122548	Ⅱ	Active, not recruiting	PEMBRO + CRS-207	-Recurrent/metastatic GC/EG (1-2 prior lines of systemic treatment)	-% AE	-Tumor response (RECIST) -OS -Characterization of immune response -Analysis of biomarker expression	79	N/A
Pembrolizumab	NCT02393248	Ⅰ / Ⅱ	Recruiting	-INCB054828 + PEMBRO/ chemo/ TRASTUZUMAB	-Advanced malignancies (including GC), progression after prior treatment	-Maximum tolerated dose, pharmacodynamic of INCB054828	-ORR -Maximum/minimum plasma Concentration of NCB054828	280	Pending
Pembrolizumab	NCT02494583	Ⅲ	Active, not recruiting	-PEMBRO *vs* -PEMBRO + chemo *vs* -Placebo + chemo	- Ⅰ st line treatment, advanced GC/GEJ	-PFS (RECIST) -OS	-ORR, DOR (RECIST) -QOL	764	N/A
Pembrolizumab	NCT02370498 (KEYNOTE-061)	Ⅲ	Active, not recruiting	-PEMBRO *vs* chemo (PACLITAXEL)	-Advanced GC/GEJ adenocarcinoma, Progressed after Ⅰ st line (platinum + fluoropyrimidine), PD-L1+	-PFS, OS- in PD-L1+	-PFS, OS -TTP, ORR	592	Pending
Pembrolizumab	NCT02335411 (KEYNOTE-059)	Ⅱ	Active, not recruiting	-PEMBRO or PEMBRO + chemo (CISPLATIN + 5-FU/ CAPECITABINE)	-Recurrent/ metastatic GC/GEJ adenocarcinoma	-% AE, discontinuation of treatment due to AE -ORR	-	316	Pending
Pembrolizumab	NCT03277352	Ⅰ / Ⅱ	Recruiting	-INCAGN01876 + PEMBRO + EPACADOSTAT	-Advanced/ metastatic malignancies	-% AE -ORR, CRR (RECIST)	-ORR, DCR, DOR, PFS, OS (rECIST, mRECIST)	166	Pending

Pembrolizumab	NCT02443324	I	Active, not recruiting	-PEMBRO + RAMUCIRUMAB	-GC/GEJ (NSCLC, transitional urothelial cancer, biliary tract cancer)	-DTL	-% BOR of CR/PR, ORR -% SD -DOR, time to response -PFS, OS -Pharmacokinetics	155	Pending
Avelumab	NCT02625623 (JAVELIN GASTRIC 300)	Ⅲ	Active, not recruiting	-AVE + BSC *vs* -chemo+BSC/BSC	-Unresectable, recurrent, locally advanced/ metastatic GC/GEJ adenocarcinoma (3rd line)	-OS	-PFS, BOR -QOL	37	Pending
Avelumab	NCT03399071 (ICONIC)	Ⅱ	Recruiting	-AVE + chemo (FLOT)	-Perioperative setting, operable EC/GC	-Pathol CR	-% grade 3-4 AE -Radiologic response (RECIST) -median PFS, OS	40	Pending
Avelumab	NCT02625610 (JAVELIN GASTRIC 100)	Ⅲ	Active, not recruiting	-AVE maintenance *vs* I st line continuation of chemo (OXALIPLATIN + FLUOROPYRIMIDINE)	-Unresectable, locally advanced/ metastatic GC/ GEJ adenocarcinoma	-OS, PFS	-BOR (RECIST) -QOL -% AE	499	Pending
Avelumab	NCT01943461 (JAVELIN SOLID TUMOR JPN)	I	Active, not recruiting	-AVE	-Locally advanced/ metastatic solid tumors (Japan) → expansion part GC patients (Asia)	-DLT	-Concentration assessment, elimination half-life -% PD-L1 -BOR+ irBOR, PFS +irPFS -OS % Ab-anti AVE -% AE	57	Pending
Avelumab	NCT03475953 (REGOMUNE)	Ⅰ / Ⅱ	Not yet recruiting	-AVE + REGORAFENIB	-Advanced/metastatic digestive solid tumors (including GC)	-Recommended doses -Assessment of Regorafenib antitumor activity -Pharmacokinetics	-Maximum tolerated dose -DLT -% AE -BOR, ORR, PFS, OS -Blood/tumor growth biomarkers	212	Pending
Avelumab	NCT02554812 (JAVELINE Medley)	Ⅰb/ Ⅱ	Recruiting	-AVE + other immunotherapies → AVE + PD 0360324 (M-CSF mAb) (gastric cancer)	-Locally advanced/ metastatic solid tumors (including GC)	-% DLT -ORR	-Serum concentration of drugs -Ab-anti drugs -TTR, DOR, PFS, OS -Tumor biomarkers (PD-L1, CD8+T cells)	560	Pending
Durvalumab	NCT02734004 (MEDIOLA)	Ⅰ / Ⅱ	Active, not recruiting	-DURVA + OLAPARIB (PARP inhibitor)	-Advanced solid tumors (including GC)	-DCR (at CT/MRI) -% AE -Safety- vital signs, blood samples	-% PD-L1 -DCR (mRECIST) -Time to treatment discontinuation) -OS -% change in tumor size (CT/MRI) -Serum concentration of Ab-anti drug -Pharmacokinetics -ORR, DOR, PFS (mRECIST)	148	Pending

Durvalumab	NCT02572687	Ⅰ	Active, not recruiting	DURVA + RAMUCIRUMAB	-Locally advanced unresectable/ metastatic gastrointestinal (including GC/GEJ adenocarcinoma) and thoracic malignancies	-% DLT	-ORR, DCR -DOR, TTR -PFS -OS -Pharmacokinetics -% Ab-anti drug	114	Pending
Durvalumab	NCT02678182 (PLATFORM)	Ⅱ	Recruiting	-DURVA *vs*. CAPECITABINE *vs*. TRASTUZUMAB *vs*. RUCAPARIB *vs*. surveillance	-Maintenance treatment, locally advanced/ metastatic HER2+/-EG, adenocarcinoma (after Ⅰ st line chemo)	-PFS (RECIST)	-PFR -OS, ORR (RECIST) -% AE -PFS, PFR, OS, ORR according to PD-L1 immunohistochemical status	770	Pending
Durvalumab	NCT02264678	Ⅰ / Ⅱ	Recruiting	-AZD6738 ± Chemo/ OLAPARIB/DURVA	-Advanced malignancies (including GC)	-Safety, tolerability (AE, SAE)	-Pharmacodynamics, biomarker changes -Serum concentration of drug, half-life, etc. -BOR, ORR, % change in tumor size	250	Pending

NIVO: Nivolumab; IPI: Ipilimumab; TREME: Tremelimumab; DURVA: Durvalumab; PEMBRO: Pembrolizumab; AVE: Avelumab, TRAS: Trastuzumab; Chemo: Chemotherapy; AE: Adverse events; SAE: Serious adverse events; DLT: Dose limiting toxicities; DOR: Duration of response; OS: Overall survival; QOL: Quality of life; PFS: Progression free disease; ir-PFS: Immune-related progression free disease; PFR: Progression-free rate; ORR: Objective response rate; RR: Response rate; pathCR: Pathologic complete response, RFS: Relapse-free survival; DR: Disease response; DDC: Duration disease control; BOR: Best objective response; DCR: Disease control rate; PD: Progressive disease; CR: Complete response; PR: Partial response; SD: Stable disease; TTR: Time to response; NET: Neuroendocrine tumors; SqCC: Squamous cell carcinoma; GIST: Gastrointestinal stromal tumors; EBV: Epstein Barr virus; N/A: Not applicable.

shorter, and the toxicities were higher in the ipilimumab group, with similar OS for both groups[218].

(2) PD-1 and PD-L1 checkpoint inhibitors

Anti-PD-1 antibodies - Nivolumab

Nivolumab is a humanized immunoglobulin G4 monoclonal antibody (mAb) that targets PD-1 and has shown efficacy in various cancer types. The FDA approved nivolumab for the treatment of recurrent/metastatic melanoma, metastatic non-small cell lung carcinoma/recurrent or metastatic squamous cell carcinoma of the head and neck (after progression on platinum-based chemotherapy), advanced renal cell carcinoma after antiangiogenic treatment, and Hodgkin lymphoma that has relapsed/progressed after autologous hematopoietic stem cell transplantation.

The phase Ⅰ/Ⅱ CheckMate-032 clinical trial analyzed the safety and efficacy of nivolumab as monotherapy or combined with ipilimumab in heavily pretreated patients with advanced/metastatic solid cancers, including gastric tumors (NCT01928394)[219]. The partial results assessing 160 patients included in three different arms [nivolumab 3 mg/kg (N3), nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1+I3), and nivolumab 3 mg/kg + ipilimumab 1 mg/kg (N3+I1)] showed more frequent toxicities in the N1+I3 group. The most commonly encountered adverse events in all the subsets were fatigue, pruritus, nausea, diarrhea and loss of appetite; thyroid damage was more frequent in the N1+I3 group. The overall objective response rate (ORR) was 16%, which was the highest in the N1+I3 group (26%); the disease control rate (DCR) was 38%. The median OS was longest in the N1+I3 arm (6.9 mo), followed by the N3 (5.0 mo) and the N3+I1 arms (4.8 mo). The response rates for patients with PD-L1$^+$ tumors (> 1% of immunohistochemically-positive cells), both in patients treated with nivolumab monotherapy and combined treatment N1+I3 and N3+I1, were superior compared to patients with negative tumors (19% *vs* 12%, 40% *vs* 22% and 23% *vs* 0%, respectively).

Because of the promising results obtained using N1+I3 combination, the CheckMate-649 phase Ⅲ clinical trial (NCT02872116) was initiated. In this study, the clinicians plan to investigate 1349 patients with naive advanced or metastatic gastric/gastroesophageal junction (GEJ) cancer, with both positive/negative PD-L1 expression to receive

first line nivolumab plus ipilimumab or nivolumab plus standard chemotherapy (XELOX or FOLFOX) *vs* standard chemotherapy (XELOX or FOLFOX), having as a primary endpoint the OS in patients with PD-L1$^+$ tumors[220]. The data from the double-blinded, randomized, multicentric phase Ⅲ trial ONO-12 (ATTRACTION 2) were presented at the ASCO Gastrointestinal Cancers Symposium 2017, demonstrating the benefits of nivolumab as a salvage treatment (third or later line) in patients with advanced unresectable or recurrent gastric or gastroesophageal junction cancer, either PD-1/L1+ or - *vs* placebo (NCT022673430). It was the first time that a prolonged OS was obtained for patients with heavily pretreated tumors using PD-1 inhibition[221]. There were 493 patients randomly assigned (2:1) to receive nivolumab (n = 330) or placebo (n = 163). The median OS was significantly increased with nivolumab *vs* placebo (5.26 mo *vs* 4.14 mo with placebo). The risk of death was lower in the nivolumab group *vs* placebo group (HR = 0.63; P < 0.0001); 68.5% of the patients in the nivolumab group died *vs* 86.5% in the placebo group. Additionally, nivolumab treatment was associated with a significantly better OS rates at 6 and 12 mo compared to placebo (46.1% *vs* 34.7% and 26.2% *vs* 10.9%), Nivolumab treatment led to a longer median PFS (1.61 mo *vs* 1.45 mo) and higher ORR than placebo (11.2% with nivolumab *vs* 0%). Among the approximately 40% (n = 192) of patients with tumor samples, 12.3% in the nivolumab group and 16.1% in the placebo group had PD-L1 positive tumors. The analysis of PD-L1 expression status showed that median OS in patients with PD-L1$^+$ tumors was 5.22 mo in the nivolumab group and 3.83 mo in the placebo group (HR = 0.51). In patients with PD-L1$^-$ tumors, median OS was 6.05 mo in the nivolumab group, and 4.19 in the placebo group (HR = 0.72). Nivolumab resulted in an absolute survival benefit of 1.1 mo in median OS *vs* placebo. Nivolumab led to durable OS benefit that was sustained beyond one year *vs* placebo in heavily pretreated gastric cancer patients, regardless of PD-L1 expression status[222]. Because of the survival benefit demonstrated by the ATTRACTION-2 trial in this subset of difficult-to-treat gastric cancer patients, the approval of nivolumab in Japan was granted as a new treatment option that is beneficial for heavily pretreated advanced gastric cancer.

Anti-PD-1 antibodies - Pembrolizumab

Pembrolizumab (formerly MK-3475, trade name Keytruda) is a humanized IgG4 isotype antibody that targets the PD-1 receptors on lymphocytes. It has FDA approval for the treatment of unresectable or metastatic melanoma, metastatic non-small cell lung cancer (NSCLC) (certain situations), metastatic non-squamous NSCLC (PDL1$^+$/$^-$), as a second-line treatment for head and neck squamous cell carcinoma (HNSCC), after platinum-based chemotherapy, and for the treatment of adult/pediatric patients with refractory classic Hodgkin's lymphoma.

The large multi-cohort, multicenter, nonrandomized, open-label phase Ib KEYNOTE-012 first demonstrated the efficacy of pembrolizumab as monotherapy (administered in a dose of 10 mg/kg every 2 wk or a 200 mg fixed dose every 3 wk) in PD-L1$^+$ recurrent or metastatic gastric or gastroesophageal cancer (NCT01848834)[25]. Of all the patients assessed, 39 patients (40%) had PD-L1$^+$ tumors, most of them being heavily pretreated patients. Treatment with single-agent pembrolizumab determined partial response (PR) in 22% and sustained disease (SD) in 13% of patients. The median PFS was 1.9 mo, with a 6 mo PFS of 26% and a median OS of 11.4 mo. The most common adverse events included loss of appetite, fatigue, pruritus, arthralgia and hypothyroidism. Pembrolizumab was well-tolerated, with 13% of patients developing grade 3-4 toxicity. It is important to stress that approximately 60% of patients enrolled in this trial have previously received more than three lines of chemotherapy, representing a group of patients without any therapeutic response demonstrated by other studies. Almost two-thirds of the tumors revealed genomic profiling of a microsatellite-instability high (MSI-H) status. This genomic status correlates with high tumor mutational burden, and patients with MSI-H cancers, colorectal and non-colorectal, have developed encouraging responses to anti-PD1 therapy in various solid tumors[23]. This aspect remains to be established if present in gastroesophageal cancers. Additionally, in this study, tumor response correlated with an increased interferon-γ gene expression[223].

With the goal of improving immune treatment efficacy and having the knowledge that chemotherapy is capable of promoting immunogenic cell death[224], PD-1 inhibitors were associated with standard chemotherapy, which is a combined regimen proven to increase response rates in lung cancer[225]. The KEYNOTE-059 phase Ⅱ clinical trial included 259 patients with advanced gastric or gastroesophageal cancer. Cohort 1 assessed the efficacy and safety of pembrolizumab monotherapy in patients with previously treated advanced gastric cancer. Approximately half the patients who enrolled in cohort 1 had received two or more prior treatments for metastatic cancer, whereas others received three or more prior therapies. Among 259 patients, 148 (57.1%) were PD-L1$^+$ (by immunohistochemistry). Pembrolizumab elicited sustained ORR in 30 of 259 patients (11.6%) and complete response in 2.3%. These responses were observed irrespective of PD-L1 expression. The ORR was higher in patients with PD-L1$^+$ *vs* PD-L1$^-$ tumors, 23 of 148 (15.5%) *vs* 7 of 109 (6.4%), respectively. A T cell-inflamed gene expression profiling score was developed in this study, demonstrated to be significantly associated with pembrolizumab response; also, a significant non-linear association was found between this score and PD-L1 expression. Of 174 patients (67.2%) assessed for MSI, seven patients (4.0%) had samples that were MSI-high. It was observed a higher ORR in patients with MSI-high tumors than in patients with non-MSI-high tumors (57.1% *vs* 9.0%). However, prevalence of MSI high tumors was very low in this population (4%), and most responses were observed in non-MSI-high

patients. Thus, in cohort 1, pembrolizumab monotherapy showed good responses and manageable toxicities after ⩾ 2 prior lines of treatment[226,227]; these results led to an accelerated FDA approval of pembrolizumab for PD-L1$^+$ advanced gastric cancer patients as third-line treatment. In cohort 2, the enrollees were HER2$^-$ naïve patients with advanced gastric/gastroesophageal junction adenocarcinoma, who receive pembrolizumab associated with combined chemotherapy (5-fluorouracil/capecitabine plus cisplatin) for six cycles, and subsequent maintenance treatment with pembrolizumab plus 5-FU/capecitabine for a maximum period of two years/until disease progression[228]. The preliminary data showed that 94% of patients have experienced adverse events (*e.g.*, anorexia, nausea, neutropenia); of these patients, two thirds developed grade 3-4 toxicities. The results of the study proved manageable adverse event profiles for patients receiving combined therapeutic schemes, with none of the patients discontinuing the treatment. The data showed an ORR was 60% and 68.8% in PD-L1$^+$ patients[229].

The KEYNOTE-028 phase Ⅰb study evaluated the role of pembrolizumab, administered in up to two years or until progression, in PD-L1$^+$ advanced solid tumors, including esophageal and gastroesophageal junction cancers (adenocarcinoma and squamous cell cancer), with most of them having at least two prior lines of chemotherapy[230]. The interim data on 23 patients showed an ORR of 30%, with 6- and 12-mo PFS rates of 30.4% and 21.7%, respectively. The response rates were better for adenocarcinomas.

An ongoing phase Ⅱ study is assessing the efficiency of pembrolizumab monotherapy, along with the analysis of immune-related gene profiles and PD-L1 expression as biomarkers for treatment response in patients with advanced cancer of the esophagus or gastroesophageal junction (adenocarcinoma or squamous cell carcinoma) (KEYNOTE-180, NCT02559687)[231], progressing on standard chemotherapy.

KEYNOTE-061 is an ongoing phase Ⅲ open-label clinical trial comparing pembrolizumab versus paclitaxel in the second-line setting for patients with advanced gastric or gastroesophageal cancer (progression after first-line therapy with a platinum plus fluoropyrimidine combination) (NCT02370498)[232]. The treatment administration continues until the disease progression or the occurrence of severe toxicities; the primary endpoints are PFS and OS in the PD-L1$^+$ population. The study randomized 592 patients to receive pembrolizumab or standard-dose paclitaxel. PD-L1 positivity was encountered in 395/592 patients enrolled. Median OS was 9.1 mo with pembrolizumab *vs* 8.3 mo with paclitaxel (HR = 0.82, P = 0.042); 12-mo OS rates in pembrolizumab group compared with paclitaxel group were 39.8% *vs* 27.1%, and 18-mo rates were 25.7% *vs* 14.8%. There was no difference in PFS or ORR, but pembrolizumab responses proved more durable, and the treatment effect was more prominent in patients with ECOG PS 0 (HR = 0.69), gastroesophageal junction tumors (HR 0.61) and with increasing PD-L1 expression. The safety profile observed in KEYNOTE-061 was consistent with that observed in previously reported studies of pembrolizumab, this drug demonstrating a better safety profile than paclitaxel. The agent reduced the risk of death by 18% *vs* paclitaxel in patients with previously treated gastric cancer and PD-L1$^+$, although this difference did not achieve statistical significance[233].

Furthermore, the ongoing phase Ⅲ KEYNOTE-062 study is randomizing PD-L1$^+$/HER2$^-$ advanced, metastatic gastric/GEJ adenocarcinoma patients to receive as a first-line regimen either pembrolizumab or pembrolizumab, associated with fluorouracil and cisplatin (NCT02494583)[234]. The primary endpoints are represented by OS and PFS.

A phase Ⅲ study (KEYNOTE-181) aims to include approximately 600 patients with previously treated advanced adenocarcinoma or squamous cell carcinoma of the esophagus or gastroesophageal junction to receive either pembrolizumab or monochemotherapy (paclitaxel, docetaxel, or irinotecan) (NCT02564263)[235].

Anti-PD-L1 antibodies - Avelumab

Avelumab (MSB0010718C, trade name Bavencio) represents a fully human anti-PD-L1 IgG1 antibody which is approved by the FDA for the treatment of metastatic Merkel cell carcinoma (patients older than 12 years) and locally advanced/metastatic urothelial carcinoma under progression, after platinum-containing treatment (2017).

Avelumab is currently assessed in JAVELIN program (NCT01772004)[236]. Patients received 10 mg/kg of avelumab every two wks as second-line treatment. For Japanese gastric cancer patients, the obtained ORR was 15%, with 43.3% of patients presenting PFS at 12 wk[237]. In this context, phase Ⅰb trials are currently investigating avelumab in patients with advanced gastric/gastroesophageal junction cancers. The results, presented at the American Society of Clinical Oncology (ASCO) 2016 meeting, showed that the most encountered adverse events were infusion-related reactions and fatigue. In the first context (patients who had at least one prior therapy), the ORR, DCR, and median PFS were 9.7%, 29.0%, and 6.0 wk, respectively, whereas in the second setting (patients who received avelumab as first-line switch maintenance after chemotherapy), they were 9%, 57.3%, and 12.0 wk, respectively. ORR in the PD-L1$^+$ tumors was higher *vs* PD-L1$^-$ tumors (18.2% *vs* 9.1% for the third-line treatment group and 10% *vs* 3.1% for the maintenance group). Starting from these data, there are two phase Ⅲ ongoing studies assessing avelumab in the treatment of gastric cancer patients. One study is comparing avelumab as a third-line of treatment to best supportive care (phase Ⅲ JAVELIN Gastric 300 trial) (NCT02625623), while another is assessing patients receiving avelumab as a switch maintenance after chemotherapy with 5-FU or capecitabine plus oxaliplatin (phase Ⅲ JAVELIN Gastric 100 study) (NCT02625610)[238]. The Javelin Gastric 300 trial enrolled 371 patients from 147 sites in Asia,

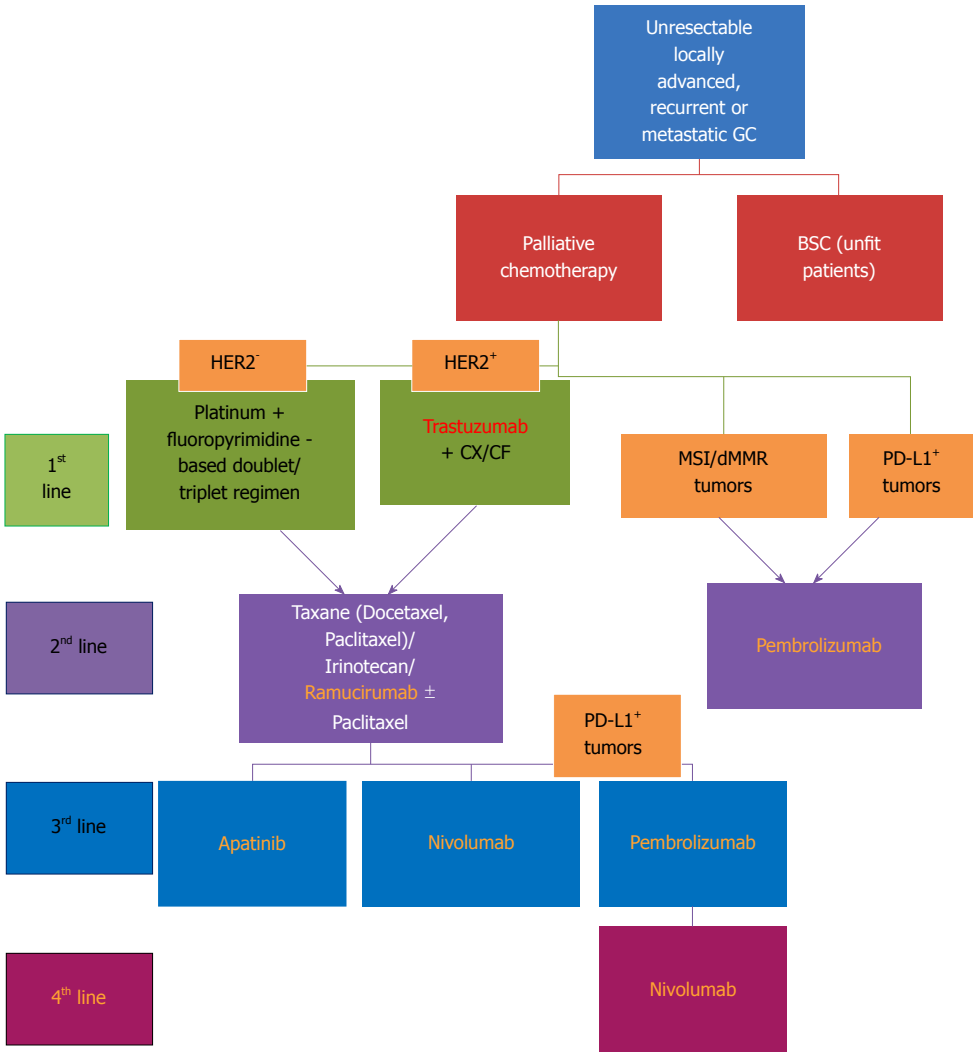

Figure 3 Therapeutic algorithm in unresectable locally advanced, recurrent or metastatic gastric cancer. HER2: Human epidermal growth factor receptor 2; CX: Cisplatin and capecitabine; CF: Cisplatin and fluorouracil; MSI: Microsatellite instability; dMMR: Deficient mismatch repair gene; PD-L1: Programmed death ligand-1.

Australia, Europe, North America and South America. Recently, the companies stated that the pivotal phase Ⅲ Javelin trial investigating avelumab as third-line treatment for patients with unresectable, recurrent or metastatic gastric cancer did not meet its pre-specified primary endpoint of superior OS *vs* chemotherapy. It represents the first trial of a checkpoint *inhibitor vs* an active chemotherapy comparator rather than placebo in this hard-to-treat patient population. The safety profile was consistent with that observed in previously reported studies of avelumab. The JAVELIN Gastric 300 data will be further evaluated in an effort to better understand the results.

Anti-PD-L1 antibodies - Durvalumab (MEDI4736)

Durvalumab represents an engineered human anti-PD-L1 IgG1 antibody that prevents PD-L1 binding to PD-1 and CD80. It has received FDA approval for previously treated advanced bladder carcinoma and unresectable stage Ⅲ NSCLC.

A phase Ⅰ study assessed the efficiency of administering durvalumab in doses up to 10 mg/kg intravenously every two weeks for up to one year in patients with solid tumors, including gastric cancer (NCT01693562)[239]. Fatigue, nausea, vomiting, rash and pyrexia were the most frequent adverse events. An ORR of 25% was obtained in patients with gastric tumors. Additionally, two patients with heavily pretreated tumors surpassed the current median PFS that was obtained with standard treatments.

An ongoing phase Ⅰ B/Ⅱ study is investigating patients with recurrent/metastatic gastric/gastroesophageal junction adenocarcinomas for monotherapy with durvalumab, tremelimumab, or a combination of durvalumab and tremelimumab (anti-CTLA-4) in second- or third-line setting[240].

An algorithm of current treatment in unresectable locally advanced, recurrent or metastatic gastric cancer is presented in Figure 3.

Checkpoint inhibitors in the adjuvant /neoadjuvant setting (stage Ⅱ or Ⅲ tumors): Because multiple

lines of standard chemotherapy may harm the immune system, and immune biomarkers may be stimulated by previous chemoradiation, it was suggested that addition of checkpoint inhibition in the adjuvant or even neoadjuvant setting in earlier stages of the disease may induce a significantly higher tumor response rate[241]. It has been demonstrated that after chemoradiation neoadjuvant therapy, there is a higher number of TILs that are agglomerated in perivascular areas and arranged in lymphoid-like structures; these are features that favor the tumor response to immunotherapy[58].

A randomized phase Ⅲ study (CheckMate-577) NCT02743494 is assessing the role of adjuvant nivolumab for patients with resected esophageal/gastroesophageal cancer. Investigators aim to enroll approximately 760 patients to receive either nivolumab or placebo. The primary endpoints are OS and DFS[242]. Moreover, a phase Ⅰ study is currently investigating nivolumab combined with an anti-CCR4 (mogamulizumab) in the preoperative setting (NCT02946671)[243].

The combination of nivolumab and ipilimumab is going to be assessed in the adjuvant treatment of gastric/gastroesophageal junction adenocarcinomas with a high risk of recurrence - a phase Ⅱ study (NCT03443856) (VESTIGE)[244].

Several ongoing phase Ⅰ/Ⅱ clinical trials are assessing the safety and efficacy of neoadjuvant treatment using different PD-1/PD-L1 checkpoint inhibitors, administered either concomitantly or sequentially with neoadjuvant chemoradiation. For example, a phase Ⅰ trial is assessing neoadjuvant administration of nivolumab and ipilimumab in stage Ⅱ/Ⅲ patients (NCT03044613)[245]; another Ⅰb phase clinical trial is assessing pembrolizumab and chemoradiation in the neoadjuvant setting of locally advanced esogastric tumors (NCT03064490/PROCEED)[246]. Several Ⅱ and Ⅲ phase ongoing studies are investigating the efficacy of pembrolizumab in association with chemotherapy as neoadjuvant/adjuvant treatment in gastric cancer patients[247-249]. Additionally, a phase Ⅱ study is evaluating the results of administering avelumab plus chemotherapy (FLOT) in the perioperative setting[250].

FUTURE PERSPECTIVES

The synergistic action of the PD-1/PD-L1 checkpoint inhibitors and the vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) blockade was demonstrated by preclinical data[251]; the combined treatment induced an improved tumor response and better OS. This combination treatment assessed in phase Ⅰ/Ⅱ studies was associated with an acceptable safety profile [252,253].

A phase Ⅰa/Ⅰb study (NCT02443324)[254] is investigating the safety and efficacy of combining anti-PD-L1 (durvalumab) and anti-VEGFR2 antibodies (ramucirumab) in patients with refractory gastric/gastroesophageal junction tumors. The interim data of 40 patients showed a 45% DCR and a median PFS of 2.60 mo. The most frequently encountered toxicities included fatigue, infusion-related reaction, loss of appetite, pruritus, rash, and hypertension; 25% of patients had severe toxicities.

Additionally, the efficacy of associating nivolumab with ramucirumab in patients with advanced, unresectable gastric/gastroesophageal junction cancers is currently under investigation in a phase Ⅰ/Ⅱ study (NCT02999295)[255]. The clinical trial of nivolumab with paclitaxel and ramucirumab in patients with advanced, unresectable gastric cancer is ongoing under investigation in a phase Ⅰ/Ⅱ study (UMIN000025947)[256].

A phase Ⅱ study is currently recruiting patients with metastatic/recurrent gastric/gastroesophageal cancer to receive pembrolizumab plus lenvatinib mesylate (anti-VEGFR2 tyrosine kinase inhibitor) (NCT03413397)[257], whereas another phase Ⅰ/Ⅱ study is planning to combine avelumab with regorafenib (another anti-VEGFR2 tyrosine kinase inhibitor) in advanced gastric tumors (NCT03475953/REGOMUNE)[258].

Because $HER2^+$ tumors develop resistance to trastuzumab, studies have been evaluating a combination of PD-1 blockade plus anti-HER2 agents. Preclinical studies indicate that the HER2 blockade stimulates T cell activation and enhances interferon-γ secretion by NK cells and antibody-dependent cellular toxicity; therefore, it boosts the PD-1/L1 inhibitor efficacy[259].

The combination of pembrolizumab plus the anti-HER2 monoclonal antibody margetuximab in patients with advanced $HER2^+$ gastric cancers that are resistant to classical trastuzumab-based chemotherapy is currently under evaluation in a phase Ⅰb/Ⅱ dose-escalation trial (NCT02689284)[260].

Other promising options include dual immunotherapies, such as the administration of PD-1/PD-L1 checkpoint inhibitors combined with agents suppressing other immune checkpoints (*e.g.*, TIM3, LAG3) or T cell costimulatory antibodies (*e.g.*, GITR, OX40, and 4-1BB). Additionally, other directions investigate the combination of the PD-1/PD-L1 blockade with radiation and other cytotoxic and targeted treatments (PARP inhibitors, ATR serine/threonine protein kinase inhibitors, pegylated recombinant human hyaluronidase, anti-CEACAM1, arginase inhibitor INCB001158, FGFR inhibitors, immunotherapy using *Listeria* bacteria to activate an immune response against specific tumor-associated antigens, claudiximab, *etc.*), and enzymatic inhibitors such as IDO-1[211].

Therapeutic intervention upon IDO1-mediated immune suppression involves inhibition of the catalytic activity of IDO1. Preliminary data from the ongoing studies investigating dual inhibition of both PD-1 and IDO1 in solid tumors are promising, showing an increased efficiency of the checkpoint blockade[261,262]. Currently, there are two studies of phase Ⅰ/Ⅱ (NCT02178722/KEYNOTE-037/ECHO-202)[263] and Ⅱ (NCT03196232)[264], respectively investigating the IDO1 inhibitor epacadostat in combination with pembrolizumab in gastric tumors.

Additionally, CDC20 encoding cell division cycle protein 20 homologue[265], as well as PLK1 and TTK,

which represent checkpoints in mitosis, may be used as therapeutic targets[266]. PLK1 is upregulated in tumors, and data reported from both preclinical and clinical studies suggest encouraging benefits from PLK-1 inhibition[267]. Maternal embryonic leucine zipper kinase (MELK) decreases the apoptosis of tumor cells; therefore, additional MELK inhibition may also be offered in PD-L1$^+$ cancers[268].

The negative checkpoint regulator VISTA represents a V-domain Ig suppressor of T cell activation (VISTA), known as PD1 homolog, which belongs to the B7 family. VISTA is mostly exhibited on hematopoietic cells[269]. In murine tumor models, anti-VISTA monoclonal antibodies activated intratumoral T cells, enhancing antitumor immunity. Moreover, the combined VISTA/PD-1 blockade was proven efficient[270,271]. A phase Ⅰ study, combining VISTA and PD-L1/PD-L2 inhibition in solid tumors (CA-170 molecule; NCT02812875), has been initiated[272]. Böger *et al*[273], investigated VISTA expression in 464 gastric cancer patients, with an increased expression associated with the intestinal type (VISTA is a regulator of differentiation), proximal gastric tumors, and KRAS- and PIK3CA mutant cancers. Additionally, a significantly higher VISTA expression in immune cells was noticed in association with PD-L1 expression and in EBV$^+$ tumors. These observations might stress that a subset of tumors may use multiple checkpoint pathways to escape immunity and that the combined VISTA/PD-1 inhibition may be a promising novel cancer approach.

Another study is investigating nivolumab plus a matrix metalloproteinase 9 inhibitor (GS-5745 = andecaliximab) in patients with unresectable/recurrent gastric or gastroesophageal junction adenocarcinoma (NCT02864381)[274].

Due to the permanent development of novel immunotherapeutic agents, classical trials do not have the ability to appropriately assess all possible drug combinations. In this regard, FRACTION (Fast Real-time Assessment of Combination Therapies in Immuno-Oncology) is a new clinical trial program with a dynamic design that provides the possibility both for the inclusion of new immunotherapeutic combinations, as well as exclusion of ineffective ones[275].

Cancer radiotherapy may seldom exhibit the clinical phenomenon of the abscopal response, meaning that no irradiated metastases diminish after radiation to the primary tumor. Data from preclinical trials highlighted that radiotherapy plus PD-1/PD-L1 blockade exhibited synergistic anticancer effects. Currently, trials on gastric cancer patients are investigating the efficiency of pembrolizumab plus palliative radiotherapy (metastatic tumors), and additional neoadjuvant chemoradiotherapy (resectable tumors) (NCT02730546)[276], respectively. A phase Ⅰ/Ⅱ trial is also investigating nivolumab$^+$ radiotherapy in unresectable, recurrent gastric cancer (as third-line treatment) (NCT03453164) (CIRCUIT)[277].

PREDICTIVE BIOMARKERS AND GENETIC PROFILES

The combination of immune checkpoint inhibitors with other types of immunotherapies, targeted agents, radio- or chemotherapies, seems to generate promising results against gastrointestinal cancers but at the expense of the occurrence of immune-related side effects, some of them potentially fatal[278,279]. Therefore, identification of prognostic biomarkers and genetic profiles to define subgroups of gastric cancer patients who are most likely to respond to specific immunotherapeutic regimens is an urgent need[280].

In this regard, an ongoing observational study NCT02951091 (Biomarker - integrated Umbrella)[281] is investigating different molecular cohorts in oncologic patients, the cases of PD-L1$^+$, MSI-H, and EBV$^+$ advanced gastric cancers being assigned to receive either nivolumab or other agents, such as AFATINIB (EGFR tyrosine kinase inhibitor or GSK2636771 (PI3K beta inhibitor) plus PACLITAXEL). A phase Ⅱ study compares nivolumab with other novel agents, according to genetic testing, in gastric cancer patients with mismatch repair deficiency (loss of MLH1/ MLH2) (NCT02465060 - The MATCH screening trial)[282].

CONCLUSION

Because of the well-known heterogeneity of tumors, it is essential to assess the particular molecular biology of different subtypes of gastric cancers that are associated with different clinico-biologic parameters and prognosis to identify innovative treatment approaches that will improve current results in gastric cancer. Modern treatments, such as the promising immunotherapy, should be applied in this way to selected patients who have been proven to have the best response to a specific therapy (individualized treatment). In this context, it is also mandatory to discover prognostic tumor markers and predictive biomarkers for treatment response.

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REVIEW

Helicobacter pylori infection and liver diseases: Epidemiology and insights into pathogenesis

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Abstract

Both *Helicobacter pylori* (*H. pylori*) infection and liver diseases, including nonalcoholic fatty liver disease (NAFLD), viral hepatitis, and hepatocellular carcinoma (HCC), have high prevalences worldwide, and the relationship between *H. pylori* infection and liver disease has been discussed for many years. Although positive correlations between *H. pylori* and NAFLD have been identified in some clinical and experimental studies, negative correlations have also been obtained in high-quality clinical studies. Associations between *H. pylori* and the pathogenesis of chronic viral hepatitis, mainly disease progression with fibrosis, have also been suggested in some clinical studies. Concerning HCC, a possible role for *H. pylori* in hepatocarcinogenesis has been identified since *H. pylori* genes have frequently been detected in resected HCC specimens. However, no study has

revealed the direct involvement of *H. pylori* in promoting the development of HCC. Although findings regarding the correlations between *H. pylori* and liver disease pathogenesis have been accumulating, the existing data do not completely lead to an unequivocal conclusion. Further high-quality clinical and experimental analyses are necessary to evaluate the efficacy of *H. pylori* eradication in ameliorating the histopathological changes observed in each liver disease.

Key words: *Helicobacter pylori*; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Hepatitis C virus; Hepatitis B virus; Viral hepatitis; Hepatocellular carcinoma

Core tip: Both *Helicobacter pylori* (*H. pylori*) infection and liver diseases have high prevalences worldwide, and their relationship has been discussed for a long time. In this review, we comprehensively summarize positive and negative correlations suggested in clinical and experimental studies, and conclude that existing data cannot fully lead us to make a decision. We also point out the necessity of further analyses evaluating the efficacy of *H. pylori* eradication on histopathological changes in each liver disease. We believe this paper would help readers to gain a better understanding of the relationship between *H. pylori* and liver diseases.

Okushin K, Tsutsumi T, Ikeuchi K, Kado A, Enooku K, Fujinaga H, Moriya K, Yotsuyanagi H, Koike K. *Helicobacter pylori* infection and liver diseases: Epidemiology and insights into pathogenesis. *World J Gastroenterol* 2018; 24(32): 3617-3625 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i32/3617.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i32.3617

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most well-known microbes in the world. Warren and Marshall reported the possible virulence of *H. pylori* in patients with gastritis, gastric ulcer, and duodenal ulcer in 1984[1]. Approximately 50% of the global population is estimated to be infected with *H. pylori*[2], and chronic infection with *H. pylori* is one cause of chronic atrophic gastritis, peptic ulcer diseases, and gastric cancer[3,4]. Recently, findings concerning the influence of *H. pylori* on various extra-alimentary organs have accumulated[5-12]. Among these putative extra-alimentary disorders caused by *H. pylori*, the relationship with metabolic disorders remains controversial[13-23].

Liver diseases, including nonalcoholic fatty liver disease (NAFLD), chronic viral hepatitis, and hepatocellular carcinoma (HCC), also have high prevalences worldwide. Consequently, the relationship between *H. pylori* and liver diseases has been discussed and still remains controversial[10]. Although the presence of *H. pylori* or *Helicobacter* species has been observed in liver samples from patients with various liver diseases[24-30] and findings regarding possible roles for *H. pylori* in the pathogenesis of liver diseases have been accumulating, few studies have reported a direct contribution of *H. pylori* to the pathogenesis of liver diseases. Additionally, negative correlations have been identified in high-quality clinical studies[31-34].

Currently, *H. pylori* is efficiently eradicated by various short-term treatments with combinations of antibiotics[35]. On the other hand, despite the remarkable progress in research and therapy, curative treatments have not yet been established for almost all liver diseases. Therefore, a discussion of whether *H. pylori* has a possible role in the pathogenesis of liver diseases and a clarification of the efficacy of *H. pylori* eradication in treating liver diseases are important. In this review, we present current insights into the relationship between *H. pylori* and liver diseases, such as NAFLD, chronic viral hepatitis, and HCC.

H. PYLORI AND NAFLD

NAFLD is an emerging liver disease worldwide, including in Asian countries[31,36,37]. NAFLD is a spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). The latter is progressive and considered a causative factor of cirrhosis, HCC, and systemic metabolic disorders[38-40].

The initial description of NASH pathogenesis, which was previously defined as the "two-hit" theory, was presented by Day *et al*[41] in 1998 and has been discussed by other researchers. In addition to a "first-hit" of hepatic steatosis, a "second-hit," such as gut-derived endotoxins, proinflammatory cytokines, dysregulation of adipokines, oxidative stress, endoplasmic reticulum stress, and lipotoxicity, is necessary for NASH development. Considering the complicated mechanisms of NAFLD, however, the "two-hit" theory had been thought to be insufficient, and instead, the "multiple-parallel hits" hypothesis was proposed by Tilg *et al*[42]. According to this hypothesis, inflammatory mediators derived from various tissues, including the gut and adipose tissue, play a central role in the inflammatory cascade. However, the detailed pathogenesis largely remains unclear.

The relationship between *H. pylori* and NAFLD in the context of gastrointestinal tract inflammation has been long discussed but remains controversial (Table 1)[10,31-34,43-51]. Some cross-sectional or retrospective studies have not identified correlations between NAFLD and *H. pylori*[31-34]. Previously, we examined the associations of causative background factors with NAFLD by analyzing 13737 subjects in a cross-sectional study in Japan, but no correlations were observed between NAFLD and *H. pylori,* regardless of gender[31]. On the other hand, opposite results have also been reported[45-48]. In a meta-analysis, Wijarnpreecha *et al*[49] found a significantly increased risk of NAFLD among patients with *H. pylori* infection, with pooled odds ratios

Table 1 Summary of relevant studies between *Helicobacter pylori* and nonalcoholic fatty liver disease

Ref.	Year	Country	Study design	Number of subjects	Conclusion
Okushin *et al*[31]	2015	Japan	Cross-sectional study	13737	Negative
Baeg *et al*[32]	2016	South Korea	Cross-sectional study	3663	Negative
Fan *et al*[33]	2018	China	Cross-sectional study	21456	Negative
Cai *et al*[34]	2018	China	Cross-sectional study	2051	Negative
Polyzos *et al*[45]	2013	Greece	Cross-sectional study	53	Positive
Doğan *et al*[46]	2013	Turkey	Cross-sectional study	174	Positive
Kim *et al*[47]	2017	South Korea	Retrospective study	17028	Positive
Chen *et al*[48]	2017	China	Cross-sectional study	2263	Positive
Wijarnpreecha *et al*[49]	2016	Various countries	Meta-analysis	38622	Positive
Jamali *et al*[50]	2013	Iran	Prospective study (RCT)	49	Negative
Polyzos *et al*[51]	2014	Greece	Prospective study	12	Negative

RCT: Randomized controlled trial.

of 1.21 (95%CI: 1.07-1.37).

To the best of our knowledge, only two randomized prospective studies have attempted to reveal the direct correlation between *H. pylori* eradication and NAFLD. As shown in the study by Jamali *et al*[50], eradication does not exert significant effects on the liver fat content, liver function tests, lipid profiles, and homeostasis model assessment of insulin resistance (HOMA-IR) index in patients with NAFLD, although one limitation of this study was that it was conducted on dyspeptic patients with NAFLD. Polyzos *et al*[51] performed a small-scale prospective study of *H. pylori* eradication in patients with biopsy-proven NASH. In this study, eradication had no long-term effect on hepatic steatosis but showed a trend toward improving the noninvasive NAFLD fibrosis score[52]. Namely, in the *H. pylori*-eradicated group, the fibrosis scores decreased from -0.34 at baseline to -0.24 at month 12 (P = 0.116), whereas the scores increased in the control group from -0.38 at baseline to -0.56 at month 12 (P = 0.249). Larger-scale randomized prospective studies focusing on *H. pylori* eradication are needed.

NAFLD is closely related to metabolic syndrome. The relationship between *H. pylori* and metabolic syndrome has also been discussed for many years. Recently, Refaeli *et al*[53] analyzed 147936 individuals aged 25-95 years who performed the urea breath test during 2002-2012 using a large computerized database of a health maintenance organization in Israel. In this study, the prevalences of *H. pylori* infection and metabolic syndrome were 52.0% and 11.4%, respectively. Compared to noninfected patients, *H. pylori*-infected patients exhibited an increased likelihood of developing metabolic syndrome (adjusted OR: 1.15, 95%CI: 1.10-1.19). Similar results have been obtained in a meta-analysis[54], middle-sized community-based studies[55,56], and hospital-based studies[17,57,58]. On the other hand, Takeoka *et al*[59] reported unique controversial results focusing on the quantification of *H. pylori*-specific IgG concentrations. Namely, the subjects were stratified into 4 groups according to the concentration of *H. pylori*-specific IgG as follows: *H. pylori* seronegative (< 10 U/mL), low *H. pylori*-specific IgG levels (10-30 U/mL), moderate *H. pylori*-specific IgG levels (30-50 U/mL), or high *H. pylori* -specific IgG levels (> 50 U/mL). After stratification, patients with low IgG levels had the lowest risk of metabolic syndrome, after adjusting for age, sex, smoking, drinking, and physical activity status. Using patients with the low IgG levels as the reference, patients with negative, moderate, and high IgG levels had ORs (95%CIs) of 2.15 (1.06-4.16), 3.69 (1.12-16.7), and 4.05 (1.05-26.8), respectively. Indeed, *H. pylori*-specific IgG levels do not always reflect disease severity; further discussion is needed to determine why the group with low IgG levels, but not negative for IgG, exhibited the lowest risk of metabolic syndrome. Another cross-sectional study in Japan, which analyzed 7394 cases, evaluated the correlations between *H. pylori* infection with the development of metabolic syndrome and each parameter[17]. In this study, *H. pylori* seropositivity was a significant and independent predictor of metabolic syndrome (OR: 1.39, 95%CI: 1.18-1.62, P < 0.001), as determined by a multivariate logistic regression analysis. Furthermore, according to the multivariate linear regression analysis, *H. pylori* seropositivity was significantly correlated with metabolic syndrome-related variables, such as higher systolic blood pressure (β coefficient = 1.03, P = 0.014), a lower high-density lipoprotein (HDL) cholesterol level (β coefficient = -2.00, P < 0.001), and a higher LDL cholesterol level (β coefficient = 2.21, P = 0.005). In addition, successful eradication of *H. pylori* significantly improves disturbances in these metabolic parameters[60-63]. However, some reports contradict an association between *H. pylori* and these metabolic risk factors[64-68]. Therefore, we are not able to reach a definitive conclusion, and the effect of *H. pylori* on metabolic factors may depend on the subjects examined, due to differences in factors such as country of residence, dietary habits, culture, and fitness habits.

Since obesity is closely linked to NAFLD, a relationship between *H. pylori* and obesity has also been hypothesized. A meta-analysis by Lender *et al*[69] concluded that the rates of obesity and overweight were inversely and significantly correlated with the prevalence of *H. pylori* infection (r = 0.29, P < 0.001). However, this meta-analysis only selected studies conducted in developed countries [GDP > 25000 USD/(person·year)].

Contradictory results were obtained in rather large-scale studies performed in other countries, such as China[70,71]. The reason for this discrepancy remains to be elucidated, but the difference in dietary habits and culture is probably responsible. In addition, the subjects' appetites and actual food intake levels will presumably be changed after successful eradication of *H. pylori* and may affect body weight. To determine whether the presence of *H. pylori* itself triggers body weight gain, detailed studies without exogenous factors are necessary to determine whether an *H. pylori* infection itself triggers body weight gain. Nwokolo *et al*[72] presented interesting data in a study examining this point. In a small-scale pilot trial, plasma ghrelin, leptin, and gastrin levels were measured before and after the cure of *H. pylori* in 10 subjects. After *H. pylori* cure, plasma ghrelin levels increased significantly by 75% (P = 0.002). On the other hand, leptin and gastrin levels have decreased by 11% and 30%, respectively, although the differences were not significant. Ghrelin is known to stimulate appetite and induce a positive energy balance, leading to body weight gain[73]; therefore, an increase in plasma ghrelin levels might be associated with the development of obesity following the eradication of *H. pylori.*

One of the important manifestations of NAFLD is insulin resistance (IR)[74]. Higher HOMA-IR scores were recorded for *H. pylori*-infected patients[55,75-78], while opposite results have also been obtained in other studies[19,79]. Cytokine production was suggested as a mechanism by which *H. pylori* induced IR. *H. pylori* infection stimulates the release of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8[80,81]. TNF-α induces IR by suppressing insulin-induced tyrosine phosphorylation of insulin receptor and its substrate, insulin receptor substrate (IRS)-1, in a hepatoma cell line[82]. In fact, neutralization of increased TNF-α levels in obese fa/fa rats significantly increases the peripheral uptake of glucose in response to insulin[83]. Adiponectin and fetuin-A are also regarded as key factors contributing to IR. Adiponectin, an adipocyte-derived hormone, antagonizes excess lipid storage in the liver and protects against inflammation and fibrosis[84]. According to Ando *et al*[85], successful eradication of *H. pylori* significantly increases total adiponectin levels from 5.61 μg/mL to 6.16 μg /mL (P < 0.0001) as well as the levels of each multimer form (high-, middle-, and low-molecular-weight) of adiponectin. Fetuin-A, a glycoprotein produced by the liver, is correlated with impaired insulin sensitivity, glucose metabolism, and the onset of diabetes mellitus[86,87]. *H. pylori*-positive subjects have higher fetuin-A levels and HOMA-IR scores than *H. pylori*-negative subjects. In a cross-sectional study, the mean fetuin-A values were 0.77 g/L and 0.58 g/L in *H. pylori*-positive and *H. pylori*-negative subjects, respectively. Mean HOMA-IR scores were 3.1 and 2.2 in *H. pylori*-positive and *H. pylori*-negative subjects, respectively. In addition, a significant positive correlation between fetuin-A and HOMA-IR was observed after adjusting for other factors (adjusted coefficient β = 0.23, P < 0.01)[88]. Based on these results, levels of inflammatory cytokines, adiponectin and fetuin-A may be associated with *H. pylori*-related IR, although that relationship has not been completely acknowledged.

Recently, the gut microbiota has been the focus of studies on the pathogenesis of various diseases and has also been suggested to play key roles in NAFLD pathogenesis[89,90]. Cytotoxin-associated gene A antigen (CagA), the known virulence factor of *H. pylori*, has been reported to alter the gut microbiota, resulting in the exacerbation of cell proliferation and immune phenotypes[91]. Furthermore, increased mucosal permeability of the intestine induced by *H. pylori* infection was reported[92]. These alterations in the gut environment, such as the microbiota and mucosal barrier, by *H. pylori* may influence the pathogenesis of NAFLD.

In summary, positive correlations between *H. pylori* and NAFLD have been reported in some clinical and experimental studies, but other studies have presented contradictory data. Further analyses focusing on the effect of *H. pylori* eradication on histopathological changes in patients with biopsy-proven NAFLD are necessary.

H. PYLORI AND CHRONIC VIRAL HEPATITIS OR CIRRHOSIS

The involvement of *H. pylori* in the pathogenesis of chronic viral hepatitis has been speculated (Table 2). Esmat *et al*[30] evaluated the presence of the *H. pylori* CagA gene in liver samples from patients with hepatitis C virus (HCV)-related chronic hepatitis or cirrhosis by the polymerase chain reaction (PCR). In this study, the *H. pylori* gene was detected in 28.2% cases of late fibrosis (F3 + F4) and 5.9% cases of early fibrosis (F1 + F2) (P = 0.0001) by PCR. The influence of *H. pylori* on the progression of HCV-related liver diseases has also been examined. Anti-*H. pylori* antibody positivity was significantly and independently associated with cirrhosis in patients with HCV-related chronic hepatitis or cirrhosis in multivariate analyses (OR: 2.42, 95%CI: 1.06-5.53, P = 0.037)[93]. Rocha *et al*[94]. examined liver tissues from *H. pylori*-infected patients and revealed that the *Helicobacter* 16S rDNA was only detected in 4.2% of liver samples from control patients and in 3.5% of samples from patients with noncirrhotic chronic hepatitis C. The *Helicobacter* 16S rDNA was detected in 68.0% of liver samples from patients with HCV-positive cirrhosis without HCC as well as in 61.3% of patients with HCC. In a meta-analysis, Wang *et al*[95] analyzed the prevalence of *H. pylori* infection in a total of 1449 patients with chronic hepatitis C and 2377 control cases. The prevalence of *H. pylori* was significantly higher in patients with chronic hepatitis C than in those without chronic hepatitis C (pooled odds ratio 2.93). In a subgroup analysis, the odds ratios were 4.48 for HCV-related cirrhosis and 5.45 for HCC. These results suggest an association between *Helicobacter* species and HCV-related disease progression, but these findings only show the presence of *H. pylori,* not its pathogenicity

Table 2 Summary of relevant studies between *Helicobacter pylori* and chronic viral hepatitis, cirrhosis, and hepatocellular carcinoma

Ref.	Year	Country	Study design	Number of subjects	Conclusion
HCV					
Esmat *et al*[30]	2012	Egypt	Cross-sectional study	85	Positive
Queiroz *et al*[93]	2006	Argentina	Cross-sectional study	106	Positive
Rocha *et al*[94]	2005	France	Cross-sectional study	109	Positive
Wang *et al*[95]	2016	Various countries	Meta-analysis	3826	Positive
HBV					
Ponzetto *et al*[96]	2000	Italy	Case-control study	355	Positive
Huang *et al*[97]	2017	China	Cross-sectional study	608	Positive
Mohamed *et al*[98]	2018	Egypt	Cross-sectional study	170	Positive
Wang *et al*[99]	2011	China	Cross-sectional study	1872	Negative
Wang *et al*[100]	2016	China	Meta-analysis	4645	Positive
HCC					
Nilsson *et al*[24]	2001	Sweden	Cross-sectional study	36	Positive
Pellicano *et al*[25]	2004	Italy	Cross-sectional study	26	Positive
Huang *et al*[26]	2004	China	Cross-sectional study	36	Positive
Xuan *et al*[27]	2006	China	Cross-sectional study	50	Positive
Xuan *et al*[101]	2008	Various countries	Meta-analysis	522	Positive

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

in the liver. *H. pylori* may putatively flow into the liver *via* the portal vein and be caught and eliminated by intrahepatic immune cells such as Kupffer cells in the normal liver. Since the number of these cells decreases with the progression of fibrosis, *H. pylori* is speculated to be present in the liver as a result of immune escape. Accordingly, the prevalence of *H. pylori* might simply be high in patients with cirrhosis compared with that in control or noncirrhotic patients. Additional in-depth studies are required to confirm the actual involvement of *H. pylori* in the progression of liver fibrosis.

Clinical findings suggesting relationships between *H. pylori* and hepatitis B virus (HBV)-related liver diseases have also been reported[96-100]. A higher prevalence of *H. pylori* infection in HBV-infected patients has been reported in several studies[96-98], but these findings may just reflect the hygienic environments in childhood. Actually, Wang *et al*[99] reported that the prevalence of *H. pylori* infection in asymptomatic HBV carriers was 38.67% in Shandong Province, China, which was not different than that in the normal adult population recruited from the same region (35.94%, $P = 0.352$). Possible associations between the progression of HBV-related liver disease and liver-related complications, such as variceal bleeding, ascites, and encephalopathy, have also been reported[97]. In a meta-analysis of a Chinese population, the prevalence of *H. pylori* infection among patients with HBV-related liver diseases increased as the disease severity increased[100]. Namely, the *H. pylori*-positive rate in patients with chronic hepatitis B patients but not cirrhosis or HCC was 2.44-fold higher than that in healthy controls (pooled OR: 2.44, 95%CI: 1.85-3.24; $P < 0.01$). Furthermore, the *H. pylori*-positive rate in patients with HBV-induced cirrhosis was 4.28-fold higher (pooled OR: 4.28, 95%CI: 2.99-6.13, $P < 0.01$) than that in healthy controls, while it was 6.02-fold higher (pooled OR: 6.02, 95%CI: 4.33-8.37, $P = 0.821$) in patients with HBV-related HCC. Therefore, the presence of *H. pylori* may accelerate the progression of HBV-related liver pathogenesis, but the precise pathogenicity in the liver remains to be elucidated.

In summary, although *H. pylori* infection and chronic viral hepatitis seem to be associated in limited situations, further studies are necessary to obtain a final conclusion since researchers have not yet clearly determined whether *H. pylori* itself directly contributes to the progression of viral hepatitis.

H. PYLORI AND HEPATOCARCINOGENESIS

Several clinical studies have reported an association between *H. pylori* and HCC (Table 2). *H. pylori* and similar species were detected in liver samples from patients with HCC[24-27]. Additionally, a positive association between *H. pylori* and the risk of HCC was reported in a meta-analysis[101]. The overall prevalence of *H. pylori* in the liver was 53.3% (129 of 242) in patients with HCC and 10.4% (29 of 280) in controls, and the odds ratio for the association between *H. pylori* infection and the risk of HCC was 13.63 (95%CI: 7.90-23.49). These observations, however, only showed the presence of *H. pylori* in liver tissues. HCC is usually accompanied by liver fibrosis, and in these circumstances, the intrahepatic immune status and hemodynamics may be changed to permit the inflow of *H. pylori* and escape from immunity in the liver, as noted above. Therefore, the presence of *H. pylori* only in HCC tissues does not provide strong support for an association with HCC.

Some *in vitro* studies have presented the possible mechanism underlying the association between *H. pylori* and hepatocarcinogenesis. As shown in the study by Zhang *et al*[102], *H. pylori* causes pathological effects on HepG2 hepatoma cells by upregulating the expression of some proteins related to gene transcription and signal transduction. Virulent type *H. pylori* cause cell cycle arrest and apoptosis of Huh7 cells, another hepatoma cell line[103]. According to Liu *et al*[104], histidine-rich protein

(Hpn), a small histidine-rich cytoplasmic protein from *H. pylori*, induces apoptosis by suppressing ubiquitin-specific peptidase 5 (USP5) expressions and activating the P14-P53 signaling pathway. However, these data are only indirect findings obtained from *in vitro* studies using cancer cell lines. Indeed, to the best of our knowledge, direct evidence for the tumorigenic effect of *H. pylori* on the liver has not been obtained. Ki *et al*[105] postulated that *H. pylori* infection might promote the transforming growth factor (TGF)-β1-dependent oncogenic pathway, disturbing the balance between hepatocyte apoptosis and proliferation in a murine model of CCl_4-induced fibrosis, but in this study, the development of HCC itself was not observed. Furthermore, in transgenic mice expressing HCV proteins, *H. pylori* infection did not promote the development of HCC[106]. Based on these findings, *H. pylori* infection is currently presumed to be unlikely to contribute to HCC development.

In summary, although *H. pylori* genes are frequently detected in HCC samples, possible correlations between *H. pylori* and hepatocarcinogenesis seem to be doubtful. Further studies showing the direct contribution *in vivo* using infectious animal models or mice transgenic for *H. pylori* genes are necessary to confirm this relationship.

CONCLUSION

H. pylori have a high prevalence, and its roles in liver diseases, as well as its well-known contribution to the pathogenesis of gastric disorders, have been discussed. As described in this review, several correlations between *H. pylori* and liver diseases, particularly NAFLD, have been reported in some clinical and experimental studies, but these correlations remain controversial. Further analyses are required to elucidate the associations. In addition, since only a few studies have examined the effect of *H. pylori* eradication on the pathogenesis of NAFLD, histopathological confirmation that *H. pylori* eradication specifically prevents or improves disease progression is necessary. Concerning chronic viral hepatitis and HCC, some observational studies suggested positive correlations. But, we have to recognize possibilities of the publication bias and confounding factors such as hygienic environments and contaminations resulting from the presence of cirrhosis. Actually, few studies have definitively confirmed the pathogenic contribution of *H. pylori* to increase of inflammation, progression of fibrosis, or acceleration of hepatocarcinogenesis. *H. pylori* infection and liver diseases still have high prevalences worldwide and significant impact on patients' prognosis. There is a room to discuss whether *H. pylori* are really involved in pathogenesis of each liver disease. To demonstrate the actual involvement of *H. pylori* in these processes, *H. pylori* itself or its gene product must be shown to accelerate the pathogenesis of these diseases using well-established animal models.

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MINIREVIEWS

Expansion of the hepatocellular carcinoma Milan criteria in liver transplantation: Future directions

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Abstract

Milan criteria are currently the benchmark related to liver transplantation (LT) for hepatocellular carcinoma. However, several groups have proposed different expanded criteria with acceptable results. In this article, we review the current status of LT beyond the Milan criteria in three different scenarios-expanded criteria with cadaveric LT, downstaging to Milan criteria before LT, and expansion in the context of adult living donor LT. The review focuses on three main questions: what would the impact of the expansion beyond Milan criteria be on the patients on the waiting list; whether the dichotomous criteria (yes/no) currently used are appropriate for LT or continuous survival estimations, such as the one of "Metroticket" and whether it should enter into the clinical practice; and, whether the use of living donor LT in the context of expansion beyond Milan criteria is justified.

Key words: Hepatocellular carcinoma; Milan criteria; Liver transplantation; Living donor liver transplantation; Expanded criteria; Downstaging

Core tip: After more than 20 years since their first description, the Milan criteria still represent the benchmark in liver transplantation for hepatocellular carcinoma. This review focuses on three unresolved issues, those being: the impact of expansion beyond Milan criteria for patients on the liver transplant waiting list; whether the dichotomous criteria (yes/no) currently used are appropriate for liver transplantation or continuous survival estimations, such as the one of "Metroticket" and whether

it should enter into the clinical practice; and, whether the use of living donor liver transplantation in the context of expansion beyond Milan criteria is justified.

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INTRODUCTION

Nowadays, hepatocellular carcinoma (HCC) represents the second cause of cancer-related death in the world[1]. Liver transplantation (LT) is an attractive option for treatment of HCC, giving that it simultaneously addresses the HCC and the cirrhotic liver, which is at risk for development of new tumors.

Since the introduction of the so-called Milan criteria (MC; single lesion ⩽ 5 cm or up to three separate lesions, none larger than 3 cm)[2] into clinical use, survival rates after LT for HCC have improved significantly. Today, the 5-year overall survival (OS) of patients within the MC reaches similar rates as those of nontumoral indications (65%-70% for HCC patients)[3,4]. As a result, the MC have been included in the Barcelona-Clínic Liver Cancer (BCLC) pretransplant staging, and the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver-European Organisation for Research and Treatment of Cancer (EASL-EORTC) practice guidelines[5-8].

However, the MC may seem too restrictive. Several groups have proposed different expansions of these classic criteria, with reasonable life expectancy after LT[9-14]. The rationale behind the expansion is that approximately 25% of the patients classified as Milan-in before LT present a Milan-out HCC in the explant histology[2,15,16]. The 5-year OS of these patients are better than the minimum acceptable rate of 50% proposed by some authors[17,18]. Despite this, the majority of the transplant centers are still using the MC. Currently, the EASL-EORTC guidelines on management of the HCC do not recommend the expansion of criteria outside of prospective research studies[8].

The International Consensus Conference regarding LT for HCC that was held in Zurich in 2010 states that the MC represent the benchmark for selection of patients for transplantation and the basis for comparison with other suggested criteria. However, according to the same group, modest expansion may be considered giving the favorable results of several studies[19]. Theoretically speaking, at least three different scenarios may be planned for the expansion of the HCC criteria - transplantation with deceased donor grafts in Milan-out HCC patients, living donor (LD)LT for patients beyond MC, and successful downstaging to MC before LT in patients initially Milan-out. Regarding the first of these three scenarios, the definition of a "time 0" (the moment when the patients with expanded criteria are included on the waiting list) will be very important, allowing for study of the expansion from an "intention-to-treat" point of view[20].

Several important issues should be discussed in order to evaluate the impact of the expansion beyond MC.

The first issue to be considered is what the effect of transplanting Milan-out patients on the waiting list for LT will be[21], by balancing the survival benefit for the patients beyond MC against the harm caused by delaying the LT for the other patients on the waiting list. According to the data published by United Network for Organ Sharing (known as UNOS) and the European Liver Transplant Registry (known as ELTR), the most important problem facing LT remains the scarcity of donors[3,4]. Theoretically, the expansion of criteria could lead to an overload of an already-large waiting list by adding patients that, until this moment, were deemed to not benefit from this treatment. The decision on whether to expand the criteria depends on what number would be considered as acceptable lowest survival after LT by each transplant community[22].

The second issue to be considered is if the decision to transplant an HCC patient should only depend on rigid criteria, like "size and number" or should be a dynamic decision in which expansion beyond the MC could be an option, depending on other characteristics such as the waiting list times and donor availability in each geographic region that performs LT.

The third issue to be considered is the strategy of using LDLT in patients with HCC, which is still questioned by some authors. Despite the advantage of transplanting patients beyond MC without affecting the conventional waiting list, at least two important problems have to be analyzed. One of them is the risk to the donor, especially in the context of expanding the criteria. The second one is that there are reports that describe significantly worse results with LDLT, as compared with conventional LT[23].

The objectives of this article are to review the current literature related to the expansion beyond MC in the three described scenarios and to evaluate the relevant data linked to the issues presented above. We believe that the transplant with deceased donor grafts and the LDLT are marked by different characteristics, therefore we will discuss each one separately.

LT with deceased donor grafts in patients with HCC beyond MC

In the last years, it has become evident that the conventional LT (with cadaveric donors) for HCC beyond MC is not necessarily associated with worse results. Several authors have described modest expansions of the MC with acceptable OS and recurrence rates (see Tables 1 and 2). Giving all these results, Mazzaferro[24] suggests that the tumor size and number used as criteria for transplantation should be defined at a regional

Table 1 Expanded criteria used for liver transplantation

Criteria	Type of donor	Detailed criteria
UCSF[9,27]	Cadaveric	Solitary tumor ⩽ 6.5 cm or ⩽ 3 tumors with the largest ⩽ 4.5 cm
Up-to-seven[10]	Cadaveric/LDLT	Seven: sum of tumor number and size of the largest tumor without microvascular invasion
Clinica Universidad de Navara (CUN)[12]	Cadaveric	1 tumor ⩽ 6 cm or ⩽ 3 tumors with the largest ⩽ 5 cm
Toso[29]	Cadaveric	Total tumor volume ⩽ 115 cm^3 and AFP ⩽ 400 ng/mL
Hangzhou University[13]	Cadaveric	One of the following: Total tumor diameter ⩽ 8 cm Total tumor diameter > 8 cm with histological grade Ⅰ or Ⅱ and AFP ⩽ 400 ng/mL
Onaca (ITR)[32]	Cadaveric	Solitary tumor, ⩽ 6 cm 2-4 tumors, ⩽ 5 cm
Tokyo (5-5 rule)[53]	LDLT	Maximum 5 tumors ⩽ 5 cm
Kyoto[55]	LDLT	⩽ 10 tumors, ⩽ 5 cm, DCP§ ⩽ 400 mAU/mL
Kyushu University[57]	LDLT	Any number of tumors with diameter ⩽ 5 cm or DCP§ ⩽ 300 mAU/mL
Asan[58]	LDLT	⩽ 6 tumors, diameter ⩽ 5 cm
Samsung[59]	LDLT/cadaveric	⩽ 7 tumors, diameter ⩽ 6 cm, AFP ⩽ 1000 ng/mL
BCLC[14]	LDLT	1 tumor, ⩽ 7 cm 3 tumors, ⩽ 5 cm 5 tumors, ⩽ 3 cm Maintained response within Milan criteria during 6 mo after downstaging

AFP: Alpha-fetoprotein; BCLC: Barcelona-Clínic Liver Cancer; DCP: Des-gamma-carboxy prothrombin; LDLT: Living donor liver transplantation; LT: Liver transplantation.

Table 2 Results after liver transplantation with expanded criteria

Ref.	Type	Patients, *n* (type)	Criteria (findings)	Survival, time (%)	Recurrence, time (%)	Factors for survival	Factors for recurrence
Yao *et al*[9], 2001	R	14 (MO)	UCSF (Histol)	5 yr (84.6)	-	pT4, total tumor diameter	-
Yao *et al*[27], 2007	P	38 (MO)	UCSF (Radiol)		5 yr DFS (93.6)		UCSF Vascular invasion AFP > 1000 ng/mL
Onaca *et al*[32], 2007	R	129 (MO)	Onaca		5 yr DFS (63.9)		Tumor > 6 cm AFP > 200 ng/mL Tumors > 4
Herrero *et al*[28], 2008	P	26 (MO)	CUN (Radiol)	5 yr (73) 5 yr I-to-T (68)			Vascular invasion
Zheng *et al*[13], 2008	R	99 (MI and MO), 26 (MO)	Hangzhou (Histol)	5 yr (70.7)	5 yr DFS (62.4)	Macrovascular invasion Tumor size > 8 cm AFP > 400 ng/mL Histological grading (Ⅲ)	Macrovascular invasion Tumor size > 8 cm AFP > 400 ng/mL Histological grading (Ⅲ)
Mazzaferro *et al*[10], 2009	R	283 (MI and MO)	Up-to-seven (Histol)	5 yr (71.2)	-	Microvascular invasion Tumor grade	-
Toso *et al*[29], 2015	P	38 (MO)	Toso (Radiol)	4 yr (74.6) 4 yr I-to-T (53.8)	4 yr DFS (68)	-	-
Togashi *et al*[54], 2016	R	14 (MO)	Tokyo	-	5 yr (8)	-	Tokyo criteria AFP ⩾ 400 ng/mL DCP ⩾ 200 mAU/mL
Kaido *et al*[56], 2013	R	42 (MO)	Kyoto	5 yr (80)	5 yr (7)		Kyoto criteria Pretreatment of the HCC
Shirabe *et al*[57], 2011	R	48 (MI and MO)	Kyushu (Histol)		5 yr DFS (80)		Kyushu criteria
Lee *et al*[58], 2008	R	174 (MI and MO)	Asan (Histol)	5 yr (81.6)	5 yr (15)	Largest tumor > 5 cm Number > 6 Gross vascular invasion	Largest tumor > 5 cm Number > 6 Gross vascular invasion
Kim *et al*[59], 2014	R	180 (in the whole study, including Samsung-out)	Samsung (Histol)		5 yr DFS -89.6		Tumors ⩽ 7 Diameter ⩽ 6 cm AFP ⩽ 1000 ng/mL
Llovet *et al*[14], 2018	P	22	BCLC (Radiol)	5 yr (80.2)	5 yr (23.8)	MI after locoregional therapies	

AFP: Alpha-fetoprotein; BCLC: Barcelona-Clínic Liver Cancer; DFS: Disease-free survival; Histol: Histology; I-to-T: Intention-to-treat; LT: Liver transplantation; MI: Milan-in; MO: Milan-out; P: Prospective; R: Retrospective; Radiol: Radiology; UCSF: University of California San Francisco.

level depending on the dynamics of the waiting list, the proportion of patients with and without HCC on the waiting list, the harm to the patients remaining on the waiting list, and the donor availability.

The San Francisco group published, in 2001, an expansion based on explant histological characteristics (solitary tumor ⩽ 6.5 cm or up to three tumors ⩽ 4.5 cm)[9]. The reported 5-year OS was 75.2% for all the patients meeting the University of California San Francisco (UCSF) criteria (including Milan-in) and was 84.6% for the 14 patients classified as Milan-out UCSF-in. However, it is expected that the pretransplantation radiological evaluation underestimates, with up to 25%-30% for the HCC stage, when it is compared to posttransplant histology findings[25,26]. For this reason, the same group published, 6 years later, the results of a prospective study using the same criteria applied to the pretransplant radiology exam. The 5-year disease-free survival (DFS) was of 91.1% for Milan-in patients *vs* 93.6% for Milan-out UCSF-in patients[27]. However, the application of these criteria was questioned by other authors. Decaens *et al*[20] analyzed the results of the UCSF criteria according to the intention-to-treat principle in a group with a relatively reduced waiting list time, of only 4 mo. When the UCSF criteria were applied at the "time 0" of inclusion on the waiting list, the 5-year OS of the Milan-out UCSF-in patients was 45.6% and of the Milan-in patients was 60.1%.

In 2009, Mazzaferro *et al*[10] published the results of a large, multicentric, retrospective study and identified a combination of tumor maximum size and number of nodules as a predictive factor for survival. The "up-to-seven" criteria (see Table 1) in patients without microvascular invasion was found to be associated with 5-year OS rate of 71.2%, which was comparable with that of the Milan-in patients. However, when the up-to-seven criteria was associated with microvascular invasion, the survival was significantly worse (48.1%). It is important to mention that the presence of microvascular invasion represents a variable not possible to identify before LT and that expansion beyond the MC is usually associated with higher rates of microvascular invasion[20].

The group of Pamplona, Spain reported the results of LT with the Clinic of Universidad of Navarra (CUN) criteria[12,28]. The 5-year OS was 68% when the analysis was performed from an intention-to-treat point of view, being statistically comparable to that for the patients with Milan-in tumors. Although none of the patients with Milan-out CUN-in HCC developed tumor recurrence in the posttransplant follow-up period, 12 of the patients recruited for that study progressed beyond the CUN criteria on the waiting list and were deemed to not benefit from LT[28].

Toso *et al*[29] published the results of a prospective study with criteria which included total tumor volume and alpha-fetoprotein (AFP). Survival and recurrence rates of the Milan-out patients meeting the criteria were acceptable, even though the "intention-to-treat analysis" showed statistically inferior results due to the waiting list drop-out rates. The criteria of the University of Hangzhou, China also took AFP levels into account[13]. Two conclusions could be drawn from that study: first, the application of this criteria did not yield worse results when compared with MC; second, even the patients exceeding the MC but fulfilling the Hangzhou criteria presented improved prognosis when compared with the Hangzhou-out patients. It has to be mentioned that, currently, the AFP level is included in the selection criteria in France and Canada, where patients with values ⩾ 1000 ng/dL are excluded for LT[30,31].

Onaca *et al*[32] analyzed the results of the International Registry of Hepatic Tumors in Liver Transplantation and concluded, similarly, that a modest expansion beyond MC could still offer favorable results (see Table 1). When patients presented in the explant analysis with one tumor of ⩽ 6 cm or 2-4 tumors of ⩽ 5 cm, the 5-year DFS was 64%.

Downstaging to Milan-in HCC before LT

In the context of HCC, there is a clear difference between the "bridge treatments" (referring to patients already on the waiting list for LT and submitted to locoregional therapies in order to diminish the drop-out rates) and the "downstaging" (defined as the treatment applied to patients initially outside of the established criteria). The latter is mainly used as a selection tool for the patients with better prognosis that could benefit from LT[33]. The strategy of downstaging to MC before LT by using locoregional therapies has been the subject of debate. In this review we will only be referring to the prospective studies related to the subject.

Roayaie *et al*[11] describes the results of the protocol of Mount Sinai Medical Center, which consisted of arterial chemoembolization with mitomycin C, doxorubicin and cisplatin at the time of diagnosis, LT with single systemic intraoperative dose of doxorubicin before revascularization of the new liver, and systemic doxorubicin for a total of six cycles, beginning on the sixth postoperative week. This protocol was applied to patients with unresectable HCC larger than 5 cm. The 5-year DFS of a subgroup of patients with tumors of 5-7 cm was considered acceptable (55%).

Yao *et al*[34] published, in 2015, an intention-to-treat study for a group of patients transplanted after downstaging and compared their results with the ones of Milan-in patients from an intention-to-treat point of view. Even though the cumulative risk for drop-out was higher in the downstage group (34.2% *vs* 25.6% at 2 years), the 5-year OS and the 5-year intention-to-treat OS were not statistically different between the groups. The factors related to the probability of drop-out were AFP > 1000 ng/mL and cirrhosis of Child B grade.

The group of Bologna also compared the results of downstaging and LT in 48 patients with those of 129 Milan-in patients, and concluded that the rates of transplantation, DFS and intention-to-treat OS were

Table 3 Prospective studies of downstaging of hepatocellular carcinoma before liver transplantation

Ref.	Criteria	Downstaging success rate (%)	LT rate (%)	Survival, time and rate (%)	HCC recurrence (%)
Roayaie *et al*[11], 2002	Mount Sinai protocol		53.75	5 yr OS (44) 5 yr DFS (48) 5 yr DFS, tumors < 7 cm (55)	
Yao *et al*[72], 2015	Beyond Milan: single tumor ⩽ 8 cm, 2–3 tumors (at least one > 3 and ⩽ 5 cm, total diameter ⩽ 8 cm), 4–5 tumors each ⩽ 3 cm and total diameter ⩽ 8 cm	To MC: 65.3	54.2	5 yr OS (77.8) 5 yr I-to-T (56.1)	7.8
Bologna criteria - Ravaioli *et al*[35], 2008	Beyond Milan: 1 lesion ⩽ 6 cm, 2 lesions ⩽ 5 cm, 3–5 lesions ⩽ 4 cm and total diameter ⩽ 12 cm	To MC: 72.9	66.7	3 yr DFS (71) 3 yr I-to-T (56.3)	18.8
Millonig *et al*[73], 2007	UCSF	RECIST	84.8	5 yr CR (66.6); PR (63.7); NR (25)	25
Graziadei *et al*[37], 2003	Beyond Milan, no upper limit	Partial response (> 50% of tumor size)	66.6	4 yr OS (41); 5 yr I-to-T (31)	30

CR: Complete response; HCC: Hepatocellular carcinoma; I-to-T: Intention-to-treat; LT: Liver transplantation; NR: No response; PR: Partial response; UCSF: University of California San Francisco.

comparable between the two groups[35]. On the other hand, Millonig *et al*[36] studied the effect of transarterial chemoembolization (TACE) on Milan-in and Milan-out UCSF-in patients. The response-to-treatment was evaluated according to RECIST criteria. Better intention-to treat OS and OS rates were observed for the Milan-in patients with complete or partial response to the treatment. Interestingly, the association of good response to TACE and good prognosis was not observed in the Milan-out patients, who were also more likely to drop-out or to present with recurrence after LT.

Graziadei *et al*[37] published the results of a series of HCC patients without pretreatment criteria, with the only criteria for transplantability being a response of 50% or more of the total tumoral volume. With this type of protocol, the results were statistically inferior to those of Milan-in patients submitted to the same therapy (see Table 3).

Overall, the results of these studies and several other retrospective studies are positive and offer the possibility of identifying a group of patients that can obtain acceptable survival rates after LT, despite presenting with a tumoral stage beyond MC. The EASL-EORTC guidelines of 2012 did not recommend the downstaging outside of prospective trials[8], but the AASLD guidelines of 2018 not only recommend locoregional therapies for the Milan-in patients on the waiting list but also suggest that the patients beyond the MC should be considered for LT after successful downstaging to Milan[38] when this status is maintained at least 3 mo to 6 mo[39]. However, the level of evidence and the strength of the recommendation are still very low, probably because of the lack of intention-to-treat studies related to downstaging in the literature[39]. The same type of recommendation related to LT after successful downstaging has been included in the EASL Clinical Practice Guidelines of 2018[40].

Effect of expanding beyond the MC on the LT waiting list

The main problem facing LT remains the difference between the availability of organs and the number of patients on the waiting list. The last Organ Procurement and Transplantation Network (commonly known as OPTN) report[3], from 2012, describes an increase of the median pretransplant waiting time from 12.9 mo in 2009 to 18.5 mo in 2011. Similar data have been published by the European LT Registry[4]. It seems clear that by expanding the HCC transplant criteria, the number of possible candidates on the waiting list will rise. The two main questions related to expanding beyond the MC are: what is the minimal acceptable OS after LT for HCC patients; and, whether the expansion beyond MC would have a positive or a negative effect on the posttransplant survival of all the patients on the waiting list.

Initial reports suggested 50% as the minimal acceptable survival after LT for HCC patients[41], but the International Consensus Conference Report for LT for HCC from Zurich 2012 reported that the expansion beyond MC has to take into account the effect of delaying the LT for all potential liver recipients on the waiting list, including the ones with non-tumoral indications[19]. Therefore, this report recommends to reserve LT for patients who have an expected survival comparable to that of non-HCC patients.

Using a theoretical Markov model, the group from Michigan, United States compared the survival benefit of transplanting a patient with an HCC beyond the MC and the harm caused to the other patients on the waiting list[21]. The results of that study showed that the adoption of more liberal criteria would lead to an increase in risk of death (of 44%) among all patients on the waiting list. The adverse effect caused by expanding the criteria would outweigh its benefits when the expected 5-year OS of the transplanted Milan-out patients would be of

less than 61%. However, this result was very sensitive to the characteristics of donation and waiting list times of each geographical region, offering values between 25% and 72%.

Ten years after that publication, the analysis could be very different. Graft characteristics will have changed, with increased use of expanded criteria donors, such as aged donors, steatotic livers or donation after cardiac death (DCD) grafts[42]. On the other hand, factors related with the recipient's prognosis, like administration of direct-acting antiviral (DAA) treatment with 90% rates of hepatitis C virus (HCV) negativization, could change the characteristics of the waiting list[43]. In the last reports of the United States' transplant registry, the HCV was no longer the principal indication for LT, being overcome by HCC and alcohol intake[44]. Furthermore, recent published data have shown continuous improvement of the post-transplantation survival rates[44,45].

Related to the use of expanded criteria donors in LT for HCC, a theoretical model study from the University of Chicago, United States, from 2012, compared the effectiveness of DCD *vs* brain-dead donor LT in terms of costs, quality of life and beyond 1-year survival[46]. In the context of HCC, the use of DCD livers for LT, when compared with the alternative of waiting for a brain-dead donor liver, resulted in a survival benefit for patients without model for end-stage liver disease (commonly known as MELD) prioritization points. However, that study only referred to Milan-in patients. The inclusion of patients beyond the MC onto the waiting list could change the results of this analysis.

As described above, modest expansion of the HCC LT indications may offer results comparable to those of Milan-in patients and of non-HCC recipients. Since several expansion studies reported 5-year survival rates of more than 70%, it seems that LT can be an option for carefully selected patients beyond MC.

A different approach to separate the patients with good or bad prognosis after LT, including those beyond MC, would be the use of combined scores which take into account tumor characteristics (total tumor volume, rather than size and number) and AFP cut-off values (see above)[29,40,47]. In this way, both large HCC and small ones with potentially aggressive behavior as well as poor post-LT outcomes could be identified.

Regarding the effect of expanding criteria for the waiting list, the analysis is more complex, taking into account not only the recipient prognosis but also characteristics that depend of each geographic region that performs LT, like the number of patients on the waiting list, available donors, and their quality. Thus, the decision on whether to expand the HCC transplantation criteria should probably be made at a regional level after analyzing the impact of all these items.

Dichotomous vs continuous selection criteria

Despite the success of the MC in LT for HCC, one of the questions that has arisen is whether a dichotomous yes/no criteria is the best strategy to decide which patients should benefit from the transplant. Even inside the MC, there is a 10%-15% risk of recurrence after LT[48] and, as discussed above, several expanded criteria of LT are associated with OS and recurrence rates comparable to those of MC[10,27,28,32]. So, it is clear that not all the patients accepted for LT have a good prognosis and not all the patients discarded for LT based on MC have a dismal one.

In 2009, Mazzaferro *et al*[10] proposed a prognostic model, based on posttransplant estimation of survival probabilities related to the histological stage. This model, known as the "Metroticket", was recently validated by Raj *et al*[49]. In that retrospective analysis of a group of patients with a known 5-year OS of 74%, the model estimated a survival of 70%, statistically not different from the real one. By offering individualized survival predictions, the Metroticket could play a role in the regional organ allocation process. As described, if the expected survival of an individual is similar to that offered to transplanted Milan-in patients, then the LT could be justified depending on the characteristics of each individual region.

However, there are authors who have criticized that both dichotomous and continuous selection models only predict the posttransplant outcome, without taking into account the patient´s survival perspectives without transplantation, geographical differences in terms of donation or waiting list times, or the proportion of patients with and without HCC on the waiting list[50,51].

LDLT for expansion beyond MC

The strategy of LDLT in the context of HCC is different from the LT with deceased donor grafts because of, at least, two reasons. First of all, LDLT does not affect the conventional waiting list, therefore an expansion of the MC could be planned in this context without the fear of affecting other patients waiting for an organ. Second of all, LDLT is a complex procedure that involves not only the recipient, but also a living donor who is a healthy person submitted to a major surgery without a direct benefit. For this reason, the benefit of the recipient should always be evaluated in the context of the risk to the donor, a concept known as "double equipoise"[52].

The majority of LDLT studies regarding CHC expansion criteria have come from Asia, where, for cultural and religious reasons, the cadaveric donation is infrequent (see Tables 1 and 2).

The University of Tokyo published the "5-5 rule criteria" (see Table 1). Using these criteria, 5-year DFS was found to be 94%, while in the patients beyond Tokyo it was only 50%[53]. Two years ago, that same group published the results of their series after a large follow-up. The 5-year recurrence rates were 8% for Milan-out Tokyo-in patients and 6% for Milan-in patients. The OS and DFS rates were comparable between the two groups[54].

The group from Kyoto included dex-gamma-

carboxi prothrobine (DCP) in the criteria for LT (see Table 1)[55]. Applying these criteria, the 5-year OS and recurrence rates were 80% and 7%, respectively, when all the patients (Milan-out Kyoto-in and Milan-in) were considered[56]. The criteria of the University of Kyushu also took into account the DCP, but did not impose a limit on tumor number[57]. By using the Kyushu criteria, the 3- and 5-year DFS was 80%. In a multivariate analysis that considered UCSF, up-to-seven, Tokyo and Kyoto criteria, the Kyushu criteria was the only one statistically related to the DFS.

Another LDLT expanded criteria is the one of Asan Medical Center. The survival and recurrence rates of patients within these criteria were comparable with MC and UCSF survival rates, with the advantage that the Asan criteria can select more patients that can benefit from the transplant[58].

Kim *et al*[59] defined a set of expanded criteria based on reviewing the explant histology of 180 patients, the major portion of this population being submitted to LDLT. The results showed a DFS benefit when the number of tumors was lower than 7, the maximum diameter was smaller than 6 cm, and the AFP was less than or equal to 1000 ng/mL.

Our group also published, this year, the results of a prospective study of 22 patients with BCLC expanded criteria who had submitted to LDLT[14]. The criteria were related to the size and number of the tumors but also to the successful downstaging after locoregional therapies. The results were remarkable, with a 5-year OS of more than 80%. One of the factors that influenced the OS was a "Milan-in" status before the transplant and after performing locoregional therapies as downstaging or bridging therapy (see Table 1). As remarked by other authors, the results of this study seem to favor downstaging over expansion in the context of LDLT, even though the sample size is small[60].

All these studies demonstrate that the expansion of the MC in the context of LDLT does not necessarily associate with worse results. However, the majority of these articles are retrospective analyses of patients selected by the means of explant histological characteristics. Furthermore, some of them analyzed the survival and recurrence rates in Milan-in patients and with expanded criteria all together, which could have biased the results.

The report from the Vancouver Forum on the Care of the Living Donor from 2005 established that LDLT should be performed only if it offers an advantage to the recipient when compared to the alternative of waiting for a deceased donor graft and if the risk of the donor is justified by the expectation of an acceptable outcome of the recipient[61].

One of the main issues in LDLT is the safety of the donor. Clavien's group[62] analyzed the results of several important transplant centers throughout the world and published benchmark values related to acceptable complication rates for donors. That study described acceptable complication rates at discharge values below 26.9% for any complication and 6% for major complications (≥ ⅢA of Clavien-Dindo classification)[63]. Today, the reported donor mortality after LDLT is 0.15%-0.20%[52]. In the particular scenario of LDLT for HCC beyond MC, the concept of double-equipoise should be taken into account, it being unacceptable that a donor should take any risk if the benefit to the recipient is expected to be very low[52]. However, the living donor studies presented above report survival rates comparable to those of LT for MC and lead to optimism regarding the possibility of using LDLT for expanding HCC criteria.

The other important issue related to LDLT for HCC involves the reports of higher rates of recurrence than are related to the conventional LT[23,64]. One possible explanation of these results could be related to the reduced waiting time before LDLT compared with the usual waiting list time for conventional LT. It is possible that this reduced time did not permit drop-out of patients with aggressive HCC[64]. Theoretically, this concept can also apply to the expansion beyond MC. However, a meta-analysis published in 2012 by the group from Guangzhou, China showed no statistical differences between living and cadaveric LT in terms of 5-year OS or recurrence[65]. Of note, in our experience with the application of BCLC expanded criteria for LDLT, the 5-year recurrence rate was approximately 20%, but the OS rate was comparable to that published for Milan-in patients with cadaveric donors[3,4,14].

We believe that as long as the results in terms of survival of selected HCC patients beyond MC (*i.e.* up-to-seven, UCSF, extended criteria BCLC) submitted to LDLT are comparable to those obtained after conventional LT for HCC Milan-in, the utilization of LDLT in this context could be justified.

FUTURE DIRECTIONS

DAA treatment for HCV is one of the most important medical breakthroughs of the last decade. Its impact is already apparent on the United States' liver waiting list, where HCV is no longer the first indication for LT[44]. The liver grafts that are no longer needed for HCV patients could be used to explore the expansion beyond MC. On the other hand, since the association between HCV and HCC is well documented, the DAA treatment is expected to have an impact on the incidence of HCC as well[51]. However, further information is needed in order to explore these scenarios.

Some of the most intriguing future directions of research of HCC treatment are the molecular and genetic analyses and investigations into the relationship of tumor biology and recurrence (see Table 4). It is known today that complex genetic and epigenetic alterations, chromosomal mutations and changes in molecular pathways lead to HCC development[66-71]. Even though these insights have shown much promise in improving HCC treatments, one of their main issues is the retrospective character of the results themselves. In fact, the vast majority of the related studies analyzed

Table 4 Impact of genetic and molecular factors in post-liver transplantation outcome

Ref.	Criteria	Survival, time and rate (%)	Recurrence, time and rate (%)
Schwartz *et al*[68], 2008	FAI < 0.27		5 yr (10)
Dvorchick *et al*[69], 2008	FAI and macrovascular invasion (Pittsburg criteria)	Stage I (FAI ⩽ 20% and no macrovascular invasion) - 5 yr DFS (92.8)	
Miltiadous *et al*[67], 2015	Progenitor cell markers (CK19 or S2 signature)	5 yr (67)	5 yr (19)
Sugimachi *et al*[70], 2015	miR-718 and his target gene HOXB8	5 yr (≈ 80)	
Barry *et al*[71], 2012	67 miRNA		
Liese *et al*[72], 2016	miR-214, miR-3187 and MC	5 yr DFS (≈ 90)	

DFS: Disease-free survival; FAI: Fractional allelic imbalance; MC: Milan criteria; miRNA: MicroRNA.

the molecular and genetic characteristics in tumor samples of explanted tissues, which makes any kind of pretransplant selection of these patients based on the tumor biology virtually impossible. However, identification and measurement of genetic markers in serum before LT, like of microRNAs, could be a future direction of investigation[69]. However, more studies are necessary in order to confirm these results.

CONCLUSION

The current medical literature seems to support that modest expansions of HCC LT criteria beyond Milan offer results comparable to those of MC. However, these proposals require further prospective validation using radiological findings collected before LT as a selection tool. As summary, the three important questions cited at the beginning of this article will be addressed in the concluding remarks.

First of all, the effect of possible MC expansion on the waiting list is a variable depending not only on the stage of the HCC patients but also on regional characteristics of the waiting list itself and donors. Thus, we believe that the expansion of MC is a decision that will have to be analyzed carefully in each transplant region and according to the principle of survival benefit for all of the patients on the waiting list.

Second of all, in the Metroticket era, the use of a threshold of acceptable survival, rather than strict dichotomous yes/no criteria, could offer a flexibility to the HCC criteria and may help to expand LT indications beyond the MC in regions where the waiting list pressure permits.

Finally, in the real-life context of cadaveric donor shortage, the use of LDLT is generally accepted. As long as the expansion beyond MC in the context of LDLT offers survival rates comparable to those of accepted indications for LT, its use seems justified.

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MINIREVIEWS

Diagnosis and management of fibromuscular dysplasia and segmental arterial mediolysis in gastroenterology field: A mini-review

Masayoshi Ko, Kenya Kamimura, Kohei Ogawa, Kentaro Tominaga, Akira Sakamaki, Hiroteru Kamimura, Satoshi Abe, Kenichi Mizuno, Shuji Terai

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Abstract

The vascular diseases including aneurysm, occlusion, and thromboses in the mesenteric lesions could cause severe symptoms and appropriate diagnosis and treatment are essential for managing patients. With the development and improvement of imaging modalities, diagnostic frequency of these vascular diseases in abdominal lesions is increasing even with the small changes in the vasculatures. Among various vascular diseases, fibromuscular dysplasia (FMD) and segmental arterial mediolysis (SAM) are noninflammatory, nonatherosclerotic arterial diseases which need to be diagnosed urgently because these diseases could affect various organs and be lethal if the appropriate management is not provided. However, because FMD and SAM are rare, the cause, prevalence, clinical characteristics including the symptoms, findings in the imaging studies, pathological findings, management, and prognoses have not been systematically summarized. Therefore, there have been neither standard diagnostic criteria nor therapeutic methodologies established, to date. To systematically summarize the information and to compare these disease entities, we have summarized the characteristics of FMD and SAM in the gastroenterological regions by reviewing the cases reported thus far. The information summarized will be helpful for physicians treating these patients in an emergency care unit and for the differential diagnosis of other diseases showing severe abdominal pain.

Key words: Fibromuscular dysplasia; Segmental arterial mediolysis; Mesenteric lesion; diagnosis; Humans

Core tip: The vascular diseases in the abdominal lesions needs to be appropriately diagnosed and treated as it could be lethal if the appropriate management is not provided. Mesenteric ischemia caused by the atherosclerotic changes is rather famous however, fibromuscular dysplasia (FMD) and segmental arterial mediolysis (SAM) which are noninflammatory, nonatherosclerotic arterial diseases are rare and the cause, prevalence, clinical characteristics including the symptoms, findings in the imaging studies, pathological findings, management, and prognoses have not been systematically summarized. Therefore, we have summarized the characteristics of FMD and SAM in the gastroenterological regions and review the cases reported thus far. The information summarized will be helpful for physicians treating these patients in an emergency care unit and for the differential diagnosis of other diseases showing severe abdominal pain.

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INTRODUCTION

A literature search was conducted using PubMed and Ovid, with the term "fibromuscular dysplasia" or "segmental arterial mediolysis" and "mesenteric" to extract studies published in the last 55 years for fibromuscular dysplasia and in the last 21 years for segmental arterial mediolysis. We summarized the available information on demographics, clinical symptoms, image studies, histological findings, treatment, and clinical course.

FIBROMUSCULAR DYSPLASIA

Clinical characteristics

The detailed clinical and pathological classification of fibromuscular dysplasia (FMD) was first reported by Harrison and McCormack in 1971[1]. Since then, several studies regarding clinical course and histological data have been published, and recently, data from the first 447 patients from the United States Registry (US Registry) for FMD have been reported[1]. FMD is a noninflammatory, nonatherosclerotic arterial disease of the medium-sized arteries throughout the body, which could lead to arterial stenosis, occlusion, aneurysm, and dissection[2]. The details of the disease have not yet been clarified; however, it is typically found in the renal, extracranial, carotid, and vertebral arteries[2].

The disease is rare, with a frequency of 0.02%, predominantly occurring in women (91%) with a mean age of 55.7 ± 13.1 years especially in the Caucasian (95.4%)[2].

The mean patient age at first symptom or sign of FMD was 47.2 years (range, 5-83 years)[2]. The mechanisms underlying the pathogenesis of FMD are still poorly understood; however, smoking, hormones, HLA-DRw6 polymorphism, and physiological stimulation have been reported to be risk factors[3]. For example, a significant dose-response relationship between cigarette smoking and the presence of FMD has been reported, with an odds ratio of 8.6 when having smoked more than 10 pack-years of cigarettes[3]. The risk of HLA-DRw6 was reported with an odds ratio of 5.0, adjusted for the level of smoking[3].

FMD can occur in any medium-sized arteries throughout the body, and dissection and aneurysm have been identified in 19.7% and 17.0% of FMD patients, respectively. The three major sites affected with dissection are the carotid arteries (14.8% of all patients enrolled), followed by renal arteries (4.3%), and vertebral arteries (3.4%)[2,4]. The three major sites affected with aneurysm are the renal arteries (5.6% of all patients enrolled), followed by carotid arteries (3.6%), and the aorta (3.4%)[2]. FMD in abdominal lesions, classified as mesenteric FMD, which is caused by the celiac and mesenteric arteries, is a rare condition and often presents as an incidental diagnosis[2]. On the basis of the US Registry data, mesenteric ischemia was reported in only 1.3% of cases, with aneurysm and dissection in these vessels accounting for 6.8% and 22.3% of all cases reported, respectively[2].

Symptoms and imaging

The clinical symptoms depend on the vessels involved. When the renal arteries are affected, renovascular hypertension can be observed. Thus, when the carotid arteries are affected, headache, pulsatile tinnitus, and dizziness are the major symptoms[2]. Mesenteric FMD involves the celiac and mesenteric arteries; therefore, mesenteric ischemic symptoms occur, including unspecific abdominal pain. We reviewed the literature describing the cases and have presented the information in Table 1[5-37]. Our literature review summarized a total of 39 cases of mesenteric FMD, showing predominance in women, as reported, and the median age was 45.2 years (range: 19-78 years). Regarding the risk factors, four patients smoked (10%), two patients had smoking histories (5%), and one patient had taken oral contraceptive pills (2.6%). The most common presenting symptom was abdominal pain (62%), followed by hypertension, diarrhea, nausea or vomiting, and headache. Although approximately 80% of cases showed symptom improvement, eight patients (20%) died because of the severity of the intestinal

Table 1 Summary of mesenteric fibromuscular dysplasia reported to date

Case (*n*)	Ref.	Age (yr)	Gender (Male/ Female)	Risk factors	Symptoms	Vessels Involved	CT	Angiography	Pathology	Treatment	Anti-hypertensive drug	Anti-coagulants	Outcome
1	[5]	62	M	N/A	Upper abdominal pain, hemoperitoneum, shock	Celiac, SMA, IMA, RA	N/A	N/A	Intimal thickening in the branches of the SMA and IMA.	Laparotomy	None	None	Died
2	[6]	45	F	N/A	Abdominal pain	SMA, RA	N/A	N/A	N/A	Ileal resection	N/A	N/A	Improved
3	[6]	50	F	N/A	Hypertension, abdominal pain, diarrhea	SMA, RA, iliac	N/A	Stenosis and string-of-beads like Appearance in the SMA	N/A	SMA revascularization	N/A	N/A	Improved
4	[7]	73	F	N/A	N/A	Celiac, SMA, iliac	N/A	N/A	N/A	N/A	N/A	N/A	Improved
5	[7]	42	F	N/A	N/A	SMA, RA	N/A	N/A	N/A	N/A	N/A	N/A	Improved
6	[7]	50	F	N/A	Hypertension	Celiac, SMA, RAl	N/A	Minimal defects in the SMA and RA; stenosis of celiac artery	N/A	N/A	N/A	N/A	Improved
7	[7]	37	F	N/A	Visceral ischemic symptoms	Celiac, SMA, RA	N/A	N/A	N/A	Revascularization	N/A	N/A	Improved
8	[7]	47	F	N/A	Hypertension, abdominal pain	Celiac, SMA, RA	N/A	Defects in the SMA and RA	Medial hyperplasia	None	N/A	N/A	Died
9	[8]	41	F	N/A	Hypertension	SMA, internal carotid, RA, iliac	N/A	Corkscrew and string-of-beads like appearance in the RA, carotid, iliac artery	Replacement of the normal media with disorganized fibrous and muscular hyperplasia	Thromboendarerectomy on SMA	N/A	N/A	Died
10	[9]	64	F	N/A	Unconsciousness	SMA, circle of Willis	N/A	N/A	Medial hyperplasia	None	None	None	Died
11	[10]	21	M	N/A	Hypertension	Celiac, SMA, RA, carotid	N/A	Stenosis of celiac, SMA, RA	Intimal fibroplasia	Anti-hypertensive drug; revascularization of carotid artery; angioplasty of RA	Yes, N/A	N/A	Improved
12	[10]	20	F	N/A	Hypertension	SMA, IMA, RA, carotid	N/A	Stenosis of carotid, renal, SMA. Total occlusion of the IMA.	Intimal fibroplasia	Anti-hypertensive drug, subclavian-carotid bypass; vascular reconstruction of the kidney	Yes, N/A	N/A	Improved
13	[11]	55	M	N/A	N/A	SMA	N/A	N/A	Intimal hyperplasia in SMA	None	N/A	N/A	N/A
14	[12]	44	F	N/A	Asymptomatic bruit of the aortoiliac system	Celiac, SMA, RA, iliac	N/A	String-of-beads like appearance of iliac artery; aneurysms of SMA, RA	Intimal fibrosis with development of fibrosis	Resection and reconstruction	N/A	N/A	Improved
15	[13]	58	F	N/A	Body weight loss	Celiac, SMA, IMA, RA, iliac, aorta	N/A	Occlusion of celiac, SMA, IMA.	N/A	Open surgery	N/A	N/A	Improved
16	[14]	46	F	None (non-smoker)	Palpitations, headache, hypertension	Celiac, SMA	N/A	Aneurysms in the right RA, celiac; occlusion in the left gastric artery	Muscle hypertrophy and disorganisation of elastic tissue of the media in celiac artery.	Surgical ligation	N/A	N/A	Improved

17	[15]	60	M	None (non-smoker)	Left abdominal pain, diarrhea	SMA	Irregular nodular thickening in transverse colon.	Stenoses of the SMA	Intimal fibrosis and focal replacement of medial smooth-muscle fibers by fibrous tissue .	Splenic flexure resection and angioplasty	N/A	N/A	Improved
18	[16]	54	F	Smoking	Hypertension, headache, abdominal pain	SMA, RA, coronary	Liver cyst	Stenosis of the coronary arteries	Intimal hyperplasia in SMA, RA, coronary, splenic, intrahepatic artery	Anti-hypertensive drug	α,β-blocker → Ca blocker	N/A	Died
19	[17]	39	M	N/A	Melena, lower abdominal pain	Jejunal, Sigmoid	N/A	String-of-beads like appearance in the jejunal and sigmoid arteries.	Adventitia is thickened by fibroplasia	Resection of the jejunum	N/A	N/A	Improved
20	[18]	23	M	N/A	Hypertension	Celiac, SMA, RA, carotid, vertebral, opthalmic, superficial temporal, iliac, lumber, intercostal	Hematomasin the paraduodenal and right superior gluteral lesion and splenic infarction	Multiple saccular aneurysms in the celiac, SMA, RA< splenic, hepatic, iliac, lumber, and intercostal arteries	Mediolytic FMD with segmental dissection and thrombosis	Embolization of the gastroduodenal and right SMA to prevent hemorrhage	N/A	N/A	Improved
21	[19]	33	M	N/A	Abdominal pain	SMA	N/A	String-of-beads like appearance in the SMA	Thickening of the media due to hyperplasia in SMA	Ileal resection	N/A	N/A	Improved
22	[20]	78	F	N/A	Hypertension, abdominal pain, hemoperitoneum.	SMA, RA, colonary	Dilated loop of the small bowel and fluid in the peritoneal cavity.	N/A	Medial and perimedial fibrodysplasia, forms the characteristic petal-like appearance in SMA.	None	None	None	Died
23	[21]	43	M	None (non-smoker)	No symptoms	SMA, iliac	SMA aneurysm	Aneurysms in the SMA, hepatic artery, splenic artery, jejunal artery, iliac arteries.	Medial fibrodysplasia in the arteral walls	Aneurysm resection and arterial reconstruction	N/A	N/A	Improved
24	[22]	48	F	None (non-smoker)	Abdominal pain, hemoperitoneum	Celiac, SMA, RA	N/A	Multiple small aneurysms in celiac, SMA, RA	N/A	Surgical hemostasis and anti-hypertensive drugs	β-blocker	N/A	Improved
25	[23]	57	F	Smoking (40 packs/yr)	Abdominal pain, weight loss, anorexia, nausea, vomiting, diarrhea.	Celiac, SMA	Nothing particular	Stenosis of the celiac artery and SMA	Medial thickening, smooth muscle hyperplasia in SMA and celiac artery	Aortoiliac and aorto-SMA bypass	N/A	N/A	Died
26	[24]	48	F	Smoking (20 packs/yr)	Abdominal pain	Celiac, SMA, IMA	N/A	Occlusion of the celiac, SMA; enlarged hypertrophic IMA	Intimal fibroplasia and an increased deposition of fibrous tissue in the vessel wall media	Reimplantation of the SMA	N/A	N/A	Improved
27	[25]	38	M	Smoking	Gastrointestinal bleeding, anemia	SMA, IMA	N/A	Ectasia in IMA; string-of-beads like appearance in the SMA	Thickening and hyalinization of medium sized vessel walls, with intimal proliferation.	Ileal resection	N/A	N/A	Improved

28	[26]	Not provided	Not provided	None (non-smoker)	Abdominal pain, distension, constipation	SMA	N/A	N/A	Thick cuff (petal like) of smooth muscle proliferation with normal intima and media in mesenteric artery.	Right hemicolectomy	N/A	N/A	Improved
29	[27]	43	F	Smoking (10 cigarettes daily for 20 yr)	Hypertension, headache	SMA, RA	N/A	String-of-beads like appearance in the right RA and SMA; stenosis and multiple irregularities in the left RA	N/A	Angioplasty and anti-hypertensive drugs	Yes, N/A	N/A	Improved
30	[28]	38	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Improved
31	[29]	43	F	N/A	Hypertension, abdominal pain, headache	SMA, RA	Aneurysms in the left RA	Aneurysms in the left RA with sever fibrodysplastic stenosis; string-of-beads like appearance in the right RA; stenosis in SMA	Intimal fibroplasia, lost of internal elastic lamina, and massive destruction of the media in the aneurysm walls	Aneurysm resection and aortorenal bypass and percutaneous transluminal angioplasty	N/A	N/A	Improved
32	[30]	44	F	Oral contraceptive pills	Hypertension, abdominal pain, diarrhea, vomiting	SMA	Stenosis of SMA and nonspecific colitis	stenosis in SMA	N/A	Angioplasty	N/A	N/A	Improved
33	[31]	30	M	N/A	Abdominal pain, hypertension	Celiac, SMA, RA, iliac	Dissections of the celiac, SMA, left RA, and external iliac artery	stenosis in the right RA	N/A	Anti-platelet and anti-hypertensive therapy and angioplasty for right renal artery.	β-blocker, Ca blocker	warfarin, aspirin (100mg)	Improved
34	[32]	47	F	N/A	Abdominal pain, diarrhea, hypertension	All abdominal arteries	A partial occlusion of the celiac artery and a total occlusion of the SMA	N/A	Intimal and medial proliferation	Anti-hypertensive drug	Yes, N/A	N/A	Died
35	[33]	47	F	None (non-smoker)	Nausea, early satiety, abdominal pain	Celiac, SMA	N/A	Stenosis of the SMA, hypertrophy of the gastroduodenal artery and pancreaticoduodenal arteries	N/A	An aorto-superior mesenteric artery and an aorto-hepatic artery bypass.	N/A	N/A	Improved
36	[34]	19	F	N/A	Abdominal pain, vomiting	SMA, RA	N/A	Stenosis of the origin of the SMA and multiple aneurysms involving the proximal SMA. Right renal artery is mild irregularity.	N/A	Resection of the aneurysmal segment in the SMA; aorto-SMA interposition graft with polytetrafluoroethylene	N/A	N/A	Improved
37	[35]	52	M	Smoking	Abdominal pain	IMA	Stenosis of the IMA	N/A	Necrosis of the mucosa; fibrosis of the inima	Left hemicolectomy	N/A	N/A	Improved

38	[36]	20	F	N/A	Abdominal pain, hemorrhagic shock	SMA, right gastroepiploic, jejunal	Intraperitoneal bleeding in the omental bursa and mesentery of the transverse colon	String-of-beads like appearance in the jejunal artery	N/A	Transcatheter arterial embolization	N/A	N/A	Improved
39	[37]	61	F	N/A	Abdominal pain	SMA, IMA, RA	N/A	Multiple aneurysms and stenoses in SMA, IMA, RA	Multiple tears and dissections of the medial layer and fibrointimal thickening	Anti-coagulation	None	Yes, N/A	Improved

M: Male; F: Female; N/A: Data not applicable; SMA: Superior mesenteric artery; IMA: Inferior mesenteric artery; RA: Renal artery; CT: Computed tomography.

ischemia.

Reflecting the changes in stenosis, dissection, and aneurysm in the medium-sized arteries, FMD leads to the narrowing of the vasculature and shows a beaded appearance[38]. Therefore, catheter-based angiography has been considered to be the gold standard imaging modality; however, recent progress in imaging, such as computed tomography (CT) with high resolution, could support the diagnoses by determining the vessels affected by the disease. With the information obtained from imaging, the disease is classified into four types: multifocal, which comprises 62% of cases, showing multiple stenosis and string-of-beads; tubular, which comprises 14% of cases, showing long concentric stenosis; focal, which comprises 7% of cases, showing short stenosis of less than 1 cm; and mixed[39]. Our summary also showed aneurysms, stenosis, dissection, and occlusions in the cases for which information was available.

Histology

Histopathological findings are characteristics of the disease; thinned media and thickened fibromuscular ridges in which the arterial muscle is replaced by the fibroplasia can be observed. Based on this, the characteristic classification of FMD is essentially based on the arterial layer in which the dysplasia is predominant: intimal fibroplasia, medial fibroplasia, perimedial fibroplasia, medial hyperplasia, and adventitial fibroplasia[40-43]. Intimal fibroplasia, a relatively rare form of the disease, is characterized by focal eccentric or circumferential protuberant intimal proliferation. Medial fibroplasia, the most common type, accounts for more than 70% of this disorder, and angiography shows a typical "string of beads" appearance. Perimedial fibroplasia is the second-most common form of this disorder and is characterized by the accumulation of circumferential aggregations of elastic tissue between the media and the adventitia. Medial hyperplasia is an uncommon form and is characterized by apparent hyperplasia of normal medial smooth muscle with minimal architectural disorganization. Adventitial fibroplasia is characterized by collagenous fibroplasia encircling the adventitia and extending into the surrounding periarterial fibroadipose tissue[20]. In our case summary, fibromuscular change was confirmed histologically in the medial layer in 10 patients (26%), the intimal layer in 9 patients (23%), in both layers in 5 patients (13%), and in the adventitia layer in 1 patient (2.6%) (Table 1).

Treatment

The long-term outcomes of this disease entity have not been clarified to date, and no randomized clinical trials have been conducted to develop a standard treatment for this disease. Therapeutic options have been chosen on the basis of factors such as disease location, symptoms, prior history of symptoms, and the presence and size of aneurysms. Given that FMD often shows ischemic changes causing hypertension and stroke, most patients are treated with anti-platelet, anti-thrombotic, and anti-hypertensive therapy[44]. Anti-hypertensive medications are administered to 71.7% of patients. The median number of medications patients received was one, and 21.5% of patients received three or more anti-hypertensive medications. The most commonly used agents were beta-blockers (40.0%), diuretics (31.3%), and calcium channel antagonists (25.7%). A total of 21.0% of patients received an angiotensin-converting enzyme inhibitor, 21.6% received an angiotensin receptor blocking agent, and 0.8% received both[44]. The use of anti-hypertensive agents is related to the history of hypertension medication, body mass index, and renal function[44]. The use of anti-platelet treatment is associated with cerebrovascular involvement[44]; however, for this entity of medicines, further studies are necessary to determine the clinically meaningful patient outcomes. In our case

summary, insufficient information about medications was provided; thus, the actual number treated with antihypertensive therapy might be lower than 18% (Table 1). Anti-coagulation therapy was attempted for two patients (5%), including one patient each receiving warfarin and aspirin.

Vascular intervention and surgery for revascularization are considered with the appropriate clinical symptoms and are rarely performed other than for the renal artery[44]. For the renal artery, endovascular revascularization using the percutaneous transluminal angioplasty technique or surgical procedures are considered when the patients show hypertension resistant to a regimen of three anti-hypertension drugs, including diuretics, or in cases of renal artery aneurysm or renal artery dissection[2,4,38]. Thus far, no randomized clinical trials of revascularization *vs* medication have been conducted. For other arteries, including the carotid artery, given that FMD is not an atherosclerotic disease, stenting or surgical procedures are not the standard therapy, and medication with anti-platelet, anti-coagulant, and anti-hypertensive agents are the main treatment. However, when symptomatic, interventional radiology using the percutaneous transluminal angioplasty technique can be considered, although it is controversial[38]. In our case summary, open surgery was performed on 23 patients (59%) and endovascular intervention was performed on 9 patients (23%).

Prognosis

Though the prognosis is basically good, when FMD affects the cerebrovascular system, there is a risk of cerebral infarction and rupture. A larger number of cases are necessary to accumulate the information useful to conduct randomized clinical trials.

SEGMENTAL ARTERIAL MEDIOLYSIS

Clinical characteristics

Segmental arterial mediolysis (SAM) was first reported by Slavin and Gonzalez-Vitale in 1976[45] and is a rare disease entity for which 50 cases have been reported to date. It is defined as a nonatherosclerotic, noninflammatory disruption of the arterial medial layer of a medium- to large-sized artery. Histologically, it is characterized as vacuolization and lysis of the outer arterial media[45]. Because of its rarity and difficulty in differential diagnosis from the other vascular diseases, clinical information is insufficient, and little is known to date; however, no significant predominance of sex or age has been reported. The mechanisms underlying the pathogenesis of SAM that have been reported as risk factors are hypoxia, shock, aging, hypertension, circulatory disturbance, arteriospasm, and other vasoconstrictor stimuli[45-47].

Symptoms and imaging

For the abdominal lesion, the most common symptom is nonspecific abdominal and flank pain[46]; diarrhea, nausea, back pain, headache, hypertension, loss of consciousness, and hemiparesis have also been known to be symptoms, although not specific[47]. We reviewed the literature describing the cases and have summarized the information in Table 2[47-71]. The studies reported a total of 26 cases of mesenteric SAM, of which 17 were men and 9 were women, with a slight predominance in men. The median age was 53 years (range: 25-79 years). The most common presenting symptom was abdominal pain (78%), followed by various symptoms, including shock, diarrhea, nausea, back pain, headache, anorexia, hypertension, hemiparesis, and loss of consciousness (Table 2).

With the development of various imaging modalities, it has been reported that, in various combinations, SAM typically affects splenic, celiac, hepatic, mesenteric, and renal arteries in the abdominal lesion[47,72]. Because of the involvement of the celiac artery, splenic arterial aneurysm is frequently found, and its rupture could affect the prognosis. Angiography reveals aneurysms, dissections, occlusions, and stenosis; however, the findings could overlap with those found in collagen vascular diseases and FMD. Therefore, the differential diagnoses between the vascular diseases are based on the histopathological findings. SAM is difficult to distinguish from FMD, although FMD shows predominance in young women and affects renal arteries causing hypertension, whereas SAM commonly affects the celiac arteries. In addition, the clinical course shows ischemic changes in FMD, whereas SAM often causes profuse bleeding from the intestinal arteries. However, these findings often overlap each other; therefore, accumulation of more detailed information is necessary.

Histology

Although the suspicion of SAM is the basis of clinical and radiological features, the gold standard for diagnosis is a pathological finding involving injurious and reparative phases in the arterial lesions of the surgical specimens. These injurious states include mediolysis, separation of the outer media, and formation of arterial gaps; key is that there is no evidence of inflammation. These changes reflect the vascular aneurysms frequently found as angiographic features of this condition. Commonly, the inflammatory markers are negative and genetic diagnosis for collagen vascular disorders shows a normal pattern.

Treatment

The long-term prognosis is unclear, and no standard therapeutic strategy has been proposed, to date; however, given that some SAM cases showed sudden the onset of aneurysm rupture, the condition could be life threatening. Therefore, SAM treatment includes embolization, bypass, and resection of the injured arteries. In addition, anti-hypertensive therapy[28] could prevent further worsening of the arterial lesions. Anti-

Table 2 Summary of mesenteric segmental arterial mediolysis reported to date

Case (*n*)	Ref.	Age (yr)	Gender (Male/ Female)	Risk factors	Symptoms	Vessels Involved	CT	Angiography	Pathology	Treatment	Anti-hypertensive drug	Anti-coagulants	Outcome
1	[48]	65	F	N/A	Abdominal pain	SMA	N/A	Beaded appearance and stenosis of the MCA	Lysis and destruction in the media and intima	Resection of aneurysm in MCA	N/A	N/A	Improved
2	[49]	56	F	N/A	Abdominal pain	IMA	Intraabdominal hemorrhage	Aneurysm in IMA	N/A	Left hemicolectomy	N/A	N/A	Improved
3	[50]	78	M	N/A	Abdominal pain, diarrhea, shock	SMA	N/A	N/A	Destruction of the tunica interna and media in MCA	Emergent surgery (right hemicolectomy); a large hematoma and a ruptured aneurysm upon the surgery	N/A	N/A	Improved
4	[51]	35	F	N/A	Abdominal pain, perforation on transverse colon	SMA	Occlusion of the mesenteric vein and ischemic colitis	Unremarkable	Segmental vacuolar degeneration of smooth muscle with areas of wall thinning	Resection of terminal ileum	N/A	N/A	Died
5	[52]	52	M	N/A	Sudden hemiparesis, hypertension	Celiac, SMA, IMA hepatic artery	Aneurysm in the celiac, hepatic, SMA	Aneurysms in celiac, SMA, ICA, hepatic; stenoses in celiac and SMA	Multiple segmental mediolysis lesions of the muscular and elastic fibers of the media	Reconstruction of hepatic and celiac artery using autologous saphenous vein graft	N/A	N/A	Improved
6	[53]	49	M	N/A	Abdominal pain, shock	SMA	Large hematoma surrounding a high-density aneurysm	Beaded appearance in SMA	Multifocal fragmentation of the elastic fibers of the media	Right hemicolectomy	N/A	N/A	Improved
7	[54]	57	M	N/A	Abdominal pain	SMA, hepatic	Small aneurysm at the middle colic artery and mesenteric hematoma	Aneurysm and stenosis of the celiac, SMA, hepatic artery	N/A	Embolization with N-butyl cyanoacrylate for aneurysm in the SMA	N/A	N/A	Improved
8	[54]	76	F	N/A	Abdominal pain, nausea	IMA	Mesenteric hematoma	Aneurysm in IMA	N/A	Embolization with coil	N/A	N/A	Died
9	[55]	59	M	N/A	Abdominal pain, shock	SMA, RA, gastroepiploic, splenic	SMA dissection, aneurysm in RA, gastroepiploic, splenic artery; rupture of the splenic aneurysm	Saccular aneurysms and multiple stenotic region in gastroepiploic artery	Medial island spared from mediolysis	Emergency embolization of the splenic artery, resection of aneurysm in the gastroepiploic	N/A	N/A	Improved
10	[56]	57	M	N/A	Abdominal pain, diarrhea	SMA	Ascites throughout the abdomen	Aneurysm in SMA	N/A	Transcatheter arterial embolization	N/A	N/A	Improved
11	[57]	60	M	N/A	N/A	SMA	Rupture of the aneurysm of the MCA	Multiple beaded patters and aneurysm in SMA	N/A	Surgical resection	N/A	N/A	Improved
12	[47]	25	F	N/A	Anorexia, abdominal pain, diarrhea	SMA, hepatic	Ischemic colitis of the splenic flexure	Occlusion of IMA; stenoses of the hepatic artery	Patchy, isolated destruction of the arterial media involving both the internal and external elastic laminae	Partial colectomy of the splenic flexure	N/A	N/A	Improved
13	[58]	53	M	N/A	None	Celiac, SMA, splenic	Aneurysm in splenic, celiac, SMA; dissection in the celiac.	Aneurysm in the celiac, splenic, and SMA	N/A	Embolization with coil and aortic stent graft	N/A	N/A	Improved

14	[59]	51	M	N/A	Abdominal pain, shock	SMA, IMA	Abdominal hemorrhage	Active bleeding from SMA	N/A	Embolization and ligation of the branches of the SMA	N/A	Warfarin	Improved
15	[60]	29	F	N/A	Hypertension	SMA, RA, hepatic	Renal cortical nephrograms	Scattered microaneurysms in SMA, RA, hepatic artery	Segmental lesions of the media with loss of smooth muscle cells	Anti-coagulants	N/A	Warfarin	Improved
16	[61]	55	F	N/A	Abdominal pain	Celiac, SMA, hepatic, splenic	Unremarkable in vessels	Aneurysms in celiac, SMA, hepatic, splenic artery	N/A	Anti-coagulants	N/A	Warfarin followed by aspirin	Improved
17	[62]	56	M	N/A	Abdominal pain, shock	SMA	Aneurysm in MCA, SMA dissection	Saccular aneurysms in the MCA; dissections in the SMA	N/A	Embolization with coil	N/A	N/A	Improved
18	[63]	64	F	N/A	Abdominal pain, back pain, nausea	SMA, IMA, hepatic	Hematoma in the anterior pararenal space inferior to pancreatic tail; bleeding from aneurysm	Multiple aneurysms in the SMA, IMA, hepatic artery	N/A	Conservative	N/A	N/A	Improved
19	[64]	60	F	Hypoxia	Hypoxia, hypotension, cardiopulmonary arrest	SMA	Large hematoma in the retroperitoneal and intraperitoneal space; SMA aneurysm	Aneurysms and beaded appearance in the SMA	N/A	Conservative	N/A	N/A	Improved
20	[65]	36	M	N/A	Abdominal pain	Celiac, hepatic, anterior inferior pancreaticoduodenal artery	Stenosis and aneurysms in anterior inferior pancreatic duodenal artery	Aneurysms and beaded like appearance in the anterior inferior pancreaticoduodenal artery	N/A	Embolization with coil	N/A	N/A	Improved
21	[66]	47	M	N/A	Loss of consciousness, headache, abdominal pain	SMA	SAH, massive intraperitoneal hematoma	Beaded like appearance in SMA; dissection in VA	Medial islands and medial degenerations in SMA	Embolization with coil for VA and SMA. Surgical resection of part of middle colic artery and descending colon.	N/A	N/A	Improved
22	[67]	79	M	N/A	Abdominal pain, hypotension	IMA	Active bleeding from IMA and hemorrhage	N/A	Reduplication of the internal elastic lamina with arterial dissection within the tunica media and thrombus at the site of rupture	Surgical resection of left colic artery	N/A	N/A	Improved
23	[68]	40	M	N/A	Abdominal pain	Celiac, SMA	Extensive dissection of SMA with the thrombotic occlusion. stenosis and dilation of celiac artery	N/A	N/A	Conservative	N/A	N/A	Improved
24	[69]	32	M	N/A	Abdominal pain	IMA, RA	Aneurysm in renal and IMA, massive amount of hemorrhage	Stenosis and aneurysm in the RA	Media shows myxoid degeneration in the outer one-third adjacent to the adventitia	Surgical hemostasis and left hemicolectomy	Yes, N/A	N/A	Improved
25	[70]	58	M	N/A	Abdominal pain	SMA	Mesenteric hematoma and right inguinal hernia with unremarkable small bowel	Beaded like appearance in SMA	N/A	Immunosuppressive therapy	N/A	N/A	Improved

26	[71]	57	M	N/A	Hypertension, abdominal pain	SMA	Arterial dissection with luminal stenosis and aneurysm formation at the distal portion of the SMA	Segmental dilatation, aneurysm in the SMA	Vacuolization and decrease in the number of vascular smooth muscles	Aneurysmectomy and bowel resection	Ca-blocker	N/A	Improved

M: Male; F: Female; N/A: Data not applicable; SMA: Superior mesenteric artery; IMA: Inferior mesenteric artery; RA: Renal artery; CT: Computed tomography; MCA: Middle cerebral artery.

coagulation therapy is uncommon, and only a few cases have been reported to date[59-61]. In addition, given it is a noninflammatory disorder, no evidence of efficacy in use of anti-inflammatory agents or immunosuppressive agents has been reported. However, SAM has been treated with these agents when the differential diagnosis from the other arteritis was difficult[70].

For patients presenting acutely with intra-abdominal hemorrhage, patients are treated with emergent catheter angiography, endovascular intervention, or surgical treatment[73].

Shenouda reported that coil embolization was the most common endovascular intervention and was reported as successful in 88% of patients, with no mortality, whereas the open surgical approach was associated with a 9% mortality rate[72]. In our patient summary, open surgery was most commonly performed, and this was performed on 13 patients (50%). Endovascular intervention was performed on eight patients (31%), and anti-coagulation therapy was administered to two patients (7.7%), including warfarin and aspirin administration. Anti-hypertensive therapy was administered to one patient with Ca-blocker.

Prognosis

Although the prognosis of the disease is reported to be good when managed appropriately[72], SAM can be fatal when ruptured[49,73]. Therefore, a careful diagnosis and appropriate management are essential for this disease entity. Our case summary also showed that although 24 (92%) patients improved, 2 (7.7%) patients died, 1 having had a large hematoma and a ruptured aneurysm in the mesenteric lesion that was revealed upon the emergent surgery.

DISCUSSION

The inner wall of a normal artery is smooth and in the normal condition, blood flows through it without difficulty. The major cause of decreasing the blood flow is atherosclerosis which is due to the deposits of fatty materials, such as cholesterol, developing the thickened arterial walls and stenosis of the vasculatures. These changes cause ischemic changes in the organs fed by the vasculatures and if it occurred in the abdominal mesenteric lesions, the symptoms of severe abdominal pain, ischemic changes of the intestine could be observed leading to lethality. For other vascular diseases including aneurysm, occlusion, and thromboses in the mesenteric lesions could cause severe symptoms and appropriate diagnosis and treatment are essential for managing patients. With the development and improvement of imaging modalities, including CT and magnetic resonance imaging, the frequency of diagnosis of vascular disease in abdominal lesions is increasing. Among these, FMD and SAM are known as noninflammatory, nonatherosclerotic arterial diseases, difficult to be differentially diagnosed from each other. Although various arteries are involved in these diseases, we have focused on the mesenteric areas, reviewing cases in this study and summarizing the clinical characteristics of both disease entities (Table 3).

The histologic findings and the imaging findings of FMD and SAM are similar; for example, Lie proposed that SAM can represent a precursor of certain types of FMD[74]. Slavin and colleagues also proposed that SAM could represent a precursor of FMD, although a part of SAM might remain as unspecified aneurysms[46]. Although these similarities in radiological and histological diagnoses have been reported, the two entities exhibit a different clinical profile in terms of age of onset, sex, distribution of affected arteries, and clinical symptoms. Although FMD affects middle-aged women, there is no predilection for age or sex in SAM[2,73].

Considering the mesenteric lesions, as there are no specific symptoms, a greater knowledge and comprehensive understanding of these diseases are important for appropriate diagnosis and treatment. For example, FMD rarely shows significant symptoms and is frequently associated with symptoms of occlusive disease such as renovascular hypertension, headache, and pulsatile tinnitus. Although FMD does not rupture as often, SAM shows hemorrhages resulting from arterial rupture or dissection

Table 3 Clinical characteristic of the fibromuscular dysplasia and segmental arterial mediolysis

	Fibromuscular dysplasia	Segmental arterial mediolysis
Gender	Female (9:1)[2]	No presentation[74]
Age of presentation	Young to middle age[2]	No preference[74]
Laboratory findings	No serological markers[74]	No serological markers[74]
Risk factors	Smoking and extracranial arteries[4]	Hypoxia and shock or other vasoconstrictor stimuli[47]
Vascular distribution	Renal and extracranial arteries[4]	Celiac and mesenteric arteries[48]
CT	Alternating stenosis and aneurysms, less commonly dissections[38]	Dissections with alternating stenosis and aneurysms, dissecting aneurysms[48]
Angiography	Beaded aneurysmal appearance (string-of-beads)[38]	Beaded aneurysmal appearance (string-of-beads)[38]
Pathology	Fibrous or fibromuscular thickening of the arterial wall[38]	Vecuolization and lysis of the outer media[47]
Symptoms	Renovascular hypertension, Headache, Pulsatile tinnitus[4]	Acute abdominal pain, Intraperitoneal bleeding[47]
Treatment	Anti-platelet therapy and anti-hypertensive therapy. Balloon angioplasty and stenting[45]	Anti-hypertensive therapy and endovascular management, surgical management[74]

CT: Computed tomography.

from the weakened arterial wall[4,46] and is therefore symptomatic with acute abdominal and flank pain.

CONCLUSION

Mesenteric vascular diseases are rare compared with other disease entities in lesions; therefore, clinical information is insufficient and clinical trials to develop the standard therapy are lacking. Therefore, an accumulation of cases and a summary of the clinical characteristics of reported cases are important. For this purpose, we have summarized the characteristics of FMD and SAM in abdominal lesions. This information could help physicians to appropriately diagnose and treat cases, including consultation with interventional radiologists and surgeons.

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ORIGINAL ARTICLE

Basic Study

Abnormal expression of *HMGB-3* is significantly associated with malignant transformation of hepatocytes

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Abstract

AIM

To explore the relationship between dynamic expression of high mobility group box-3 (HMGB3) and malignant transformation of hepatocytes.

METHODS

Expression of HMGB family proteins were observed in rat hepatocarcinogenesis models induced with 2-acetylaminofluorene. Alterations of HMGB3 were analyzed at the mRNA level by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and at the protein level by immunohistochemistry or Western blotting. HMGB3 in human liver cancer tissues were evaluated using bioinformatics databases from GEO, TCGA, and Oncomine. A specific HMGB3-shRNA was used to knock

down HMGB3 expression in order to investigate its effects on proliferation and cell cycle *in vitro* and *in vivo*.

RESULTS

Elevated HMGB3 levels were first reported in hepatocarcinogenesis, with increasing expression from normal liver to cancer. Bioinformatic databases showed that HMGB3 expression in hepatocellular carcinoma tissues was significantly higher than that in normal liver tissues. Higher HMGB3 expression was discovered in liver cancer cells compared with LO2 cells *in vitro*. According to gene set enrichment analysis, HMGB3 mRNA levels were correlated with cell cycle and DNA replication pathways. Knocking down HMGB3 by specific shRNA significantly inhibited proliferation of HepG2 cells by cell cycle arrest and downregulating DNA replication related genes (cyclin B1, FEN1, and PCNA) at the mRNA and protein level. Furthermore, silencing HMGB3 significantly inhibited xenograft tumor growth (measured by Ki67) *in vivo*.

CONCLUSION

HMGB3 is involved in malignant transformation of hepatocytes and could be a useful biomarker for diagnosis and a potential target for therapy of liver cancer.

Key words: Liver cancer; *HMGB-3*; Hepatocarcinogenesis; Proliferation; Tumor growth

Core tip: High mobility group box (HMGB) family proteins were correlated with hepatocellular carcinoma (HCC) development and progression. This current study examined the effects of HMGB3 on HCC both *in vitro* and *in vivo*. Overexpression of HMGB3 was observed in hepatic malignant transformation and HCC tissues in bioinformatic databases. Knockdown of HMGB3 significantly inhibited proliferation, cell cycle, and tumor growth of HCC cells, providing a novel insight for HCC research.

Zheng WJ, Yao M, Fang M, Wang L, Dong ZZ, Yao DF. Abnormal expression of *HMGB-3* is significantly associated with malignant transformation of hepatocytes. *World J Gastroenterol* 2018; 24(32): 3650-3662 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i32/3650.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i32.3650

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and highly aggressive cancers worldwide, with the third most common cause of cancer related death[1]. Various factors can cause HCC, including cirrhosis, infections with Hepatitis B virus (HBV) or HCV, nonalcoholic fatty liver disease (NFALD), diabetes, aflatoxin B1, tobacco, and excessive alcohol consumption. Among them, chronic infections with HBV and HCV account for more than 60% of total HCC cases[2,3]. In past decades, although obvious improvement has been observed in therapeutic approaches, the prognosis of HCC remains poor because of aggressive invasiveness, frequent metastasis, and multi-drug resistance (MDR). Given that multiple genes and signaling pathways play crucial roles in the occurrence and development of HCC, target therapy is a promising approach for HCC treatment[4]. The Food and Drug Administration (FDA) has approved Sorafenib, a multi-kinase inhibitor, as a first-line treatment for advanced HCC. However, the overall effects are partially unsatisfying due to its low response rate and high frequency of adverse events[5,6]. Thus, it is of great importance to identify novel biomarkers for early diagnosis and potential targets against the progression of HCC.

The high mobility group (HMG)-box (HMGB) family belongs to the HMG protein superfamily[7] (HMGA[8], HMGB[9], and HMGN[10]). HMGB consists of four members (HMGB1, HMGB2, HMGB3, and HMGB4) with similar physiology and pathology features. It encodes proteins containing one or more DNA-binding motifs and participates in multiple cellular processes including cell differentiation, migration, and inflammatory-related activities. The HMGB family plays a complex role in carcinogenesis due to its diverse tumorigenic bioactivities in tumors. HBV functionally binds to HMG protein and activates it[11]. Mitochondrial biogenesis mediated by hypoxia promotes HCC growth through interaction between HMGB1 and Toll-like receptor 9[12]. HMGB1 secretion could be stimulated by HBX protein and subsequently enhance HCC metastasis[13]. HMGB1 signaling is also regulated by specific long noncoding RNA[14] or microRNA[15] showing pro- or anti- effects on invasion and metastasis of HCC[16]. Moreover, overexpression of HMGB2 is associated with aggressiveness and prognosis of HCC[17]. However, until now, there is rather less known about the HMGB3 or HMGB4 expression in HCC progression.

HMGB3 is a multifunctional protein with various roles in different cellular compartments and localized in the nucleus, chromosomes, and cytoplasm. It contributes to the balance between self-renewal and differentiation of hematopoietic stem cells, and enhances DNA flexibility to activate gene promoters[18]. Recently, abnormal HMGB3 has been characterized as pro-carcinogenic by promoting tumor growth, proliferation, invasion, and metastasis in several tumors including gastric[19], lung[20], esophageal[21], breast[22], colorectal[23], and urinary bladder[24]. However, the current knowledge concerning the positive and negative effects of HMGB3 on HCC development is not explicit. The aims of this study were to investigate the dynamic HMGB3 expression in hepatocarcinogenesis, bioinformatics databases, HCC cell lines, and a xenograft model, as well as to validate HMGB3 as a diagnostic marker or novel target gene for HCC.

MATERIALS AND METHODS

Rat hepatocarcinogenesis model

Forty Sprague-Dawley rats (4-6 wk old) were provided

by the Experimental Animal Center of Nantong University. Living conditions included a clean environment, 12 h light/dark cycle, and 55% humidity as previously described[25]. The control group (n = 10) were fed a normal diet, and the hepatocarcinogenesis group (n = 30) were fed a diet with 0.05% 2-acetylaminofluorene (2-AAF, Sigma, United States). The rats were sacrificed at different times depending on their condition. Rat livers were used for pathology, RNA extraction, and quantitative analysis of HMGB expression. Following the determination of morphological changes in the rat livers and hematoxylin and eosin (H&E) staining, the hepatocarcinogenesis group was divided into three subgroups: degeneration (n = 6), precancerous (n = 6), and HCC (n = 6). All procedures *in vivo* were performed according to the guidelines of Animal Care and Use Committee of Nantong University, China.

Cell culture and transfection

Human HCC cells HepG2, SMMC7721, HCCLM3, Huh7, BEL7404 and normal hepatocyte L02 were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). All cell lines were maintained in Dulbecco modified Eagle medium or RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37 ℃ in a humidified incubator with 5% CO_2. Three candidate shRNAs were designed by Genema (Shanghai, China). Cell transfection was conducted according to manufacturer's instructions. Briefly, once cells reached 80% confluence, plasmids were gently transfected into cells using a transfection regent kit. After incubation for 12 h, cells were treated with fresh complete medium. The transfection efficiency was observed using a fluorescence microscope after 24 h. shRNA sequences were as follows: shRNA-1, 5'-GGAAAGTTTGATGGTGCAAAG-3'; shRNA-2, 5'-CGATCATATTGTAGTCTCTCA-3'; shRNA-3, 5'-CCTCCCTATAAATGTGGTAGC-3'; and NC-shRNA, 5'-GGAAGACGATGTCCGGGAAAG-3'.

Histopathological examination

Sections of the formalin-fixed paraffin-embedded (FFPE) tissues were deparaffinized in xylene and rehydrated with a series of graded ethanol. After incubation in hematoxylin solution for 15 min, sections were counterstained in eosin solution using the Hematoxylin and Eosin (H&E) Staining Kit (Solarbio, China) according to the manufacturer's instructions. Samples were viewed under a light microscope.

Bioinformatics analysis

In order to analyze the mRNA expression of HMGB3 in more HCC samples, gene expression profiling data from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GSE-14520, GSE-5364, GSE-77314, and GSE-50579, United States), The Cancer Genome Atlas (TCGA) database, and Oncomine database (United States) were incorporated in this study. All data extracted from bioinformatics databases were presented as $\log_2$ value.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA v2.2) was performed to discover the differences in biological processes and signaling pathways in transcript levels between high and low HMGB3 expression in GSE-14520 and TCGA. Gene sets were obtained from the Molecular Signatures Database (MSigDB). Enrichment scores (ES) were calculated to estimate genes from a pre-defined gene set. The positive enrichment score indicated that the gene set was considered upregulated while negative score meant downregulated. The number of permutations was set to 1000, and $P < 0.05$ was considered significantly enriched.

MTT assay

Cell proliferation was detected with the MTT assays. HepG2 cells transfected with shRNA-1 and NC-shRNA were seeded in the 96-well plate at a concentration of 3000 cells/well. Then, MTT solution (0.5 mg/ml) was added to the appropriate wells for the 1st ,2nd, 3rd, and 4th d. Following a four hour incubation, DMSO was added. Then, the absorbance was detected at a wavelength of 490 nm.

Cell cycle

Cells were collected by trypsin and fixed in 70% methanol for 30 min. Then cells were resuspended in PBS containing 50 μg/mL propidium iodide (Invitrogen, United States) for one hour at room temperature. After that, samples were analyzed by a flow cytometer (BD Biosciences, United States). Percentage of each cycle phase was calculated by Modfit software.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described[25]. In brief, tissue samples were fixed with formalin, embedded in paraffin, and cut into 4 μm thick sections. Following incubation at 70 ℃ for one hour, slides were deparaffinized in xylene and rehydrated with gradient ethanol. Antigen retrieval was conducted using EDTA solution at pH 8.0. After being blocked for one hour, slides were incubated with primary antibody overnight at 4 ℃ and then with the secondary antibody for two hours at room temperature. After that, slides were visualized by DAB, and counterstained by hematoxylin.

Western blotting

Total protein was extracted from cell lysates using RIPA solution according to the manufacturers' instructions and separated by 10% SDS-PAGE. Then the samples were transferred onto a PVDF membrane. After blocking with PBS with 5% BSA, the membranes were incubated with primary antibodies at 1:1000 dilution (HMGB3, R&D, United States; Cyclin B1, FEN1, and PCNA, Abcam, United States; GAPDH, CST, United States) at 4 ℃ overnight. After secondary antibody incubation, the

Table 1 Primers for real-time polymerase chain reaction

Gene symbol	Species	Primer sequence (5'-3')	Location
HMGB1	Rat	F: GCTGACAAGGCTCGTTATGAA	186-205
		R: CCTTTGATTTTGGGGCGGTA	381-361
HMGB2	Rat	F: CGGGGCAAAATGTCCTCGTA	28-47
		R: ATGGTCTTCCATCTCTCGGAG	155-135
HMGB3	Rat	F: AGGTGACCCCAAGAAACCAAA	9-29
		R: TCAGCAAAATTGACGGGAACC	119-99
HMGB4	Rat	F: AGACCAGCTAAGGCCCAAG	12-30
		R: CCTTTTCGTGCTTTGAGATGGAT	172-150
HMGB3	Human	F: CCAAAGGGCAAGATGTCCG	25-43
		R: TTGACAGGGACCTCTGGGTTT	110-90
CCNB1	Human	F: AATAAGGCGAAGATCAACATGGC	43-65
		R: TTTGTTACCAATGTCCCCAAGAG	153-131
FEN1	Human	F: ATGACATCAAGAGCTACTTTGGC	62-84
		R: GGCGAACAGCAATCAGGAACT	142-122
PCNA	Human	F: CCTGCTGGGATATTAGCTCCA	77-97
		R: CAGCGGTAGGTGTCGAAGC	185-167

HMGB: High mobility group box; FEN1: Flap structure-specific endonuclease 1; PCNA: Proliferating cell nuclear antigen; F: Forward primer; R: Reverse primer.

samples were detected using the ECL detection system (Bio-Rad, United States).

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from tissues using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by using a reverse transcription kit (Invitrogen, CA, United States). Quantitative polymerase chain reaction (qPCR) was conducted by using SYBR Premix Ex Taq kit (Takara, Japan) according to the manufacturer's instructions. The relative mRNA expression (normalized to GAPDH) was assessed using the $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t=\Delta C_{t[\text{target gene}]}-\Delta C_{t[\text{GAPDH}]}$) analysis method. The primers in this study were presented in Table 1.

Xenograft assay

Male BALB/c nude mice (4-6 wk old) were subcutaneously injected with 2×10^6 HepG2 cells transfected with shRNA-1 or NC-shRNA. Tumor size was measured every four days and calculated according to the formula (Volume= length $\times$ width^2 $\times$ 1/2). Mice were sacrificed at the 30th d after injection. Tumors were weighed and fixed for further immunohistochemistry of HMGB3 and Ki67 (1:50, Abcam, United States). All procedures were approved by Animal care committee of Nantong University.

Statistics

The data in this study are presented as means ± standard deviation (SD) of at least three experiments. Comparisons between groups were performed using Two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Upregulating expression of HMGB3 in hepatocarcinogenesis

According to the morphological alteration and H&E staining, rats were divided into four groups: normal, degeneration, precancerous, and cancerous. The degeneration group was characterized as the granule-like degeneration in the cytoplasm. The precancerous group was characterized as dense nuclear chromatin and high ratio of nucleus to cytoplasm. The cancerous group showed denser nuclear chromatin, upper ratio of nucleus to cytoplasm, and loss of hepatic structure (Figure 1A). Then expression of the HMGB family (HMGB1, HMGB2, HMGB3, and HMGB4) were detected in liver tissues of each group above by RT-qPCR. No statistically significant changes of HMGB1 (Figure 1B) or HMGB2 (Figure 1C) were found among the four groups. Notably, hepatic HMGB3 expression had a significant increase during the transformation from normal hepatocytes to HCC (Figure 1D). HMGB4 expression was downregulated through the progression to HCC (Figure 1E). The upregulation of hepatic HMGB3 at protein level from normal to cancerous group (Figure 1F) were confirmed by IHC staining.

Validation of HMGB3 mRNA by bioinformatics databases

To further verify the HMGB3 expression in human HCC tissues, data from several bioinformatics databases (GEO, TCGA, and Oncomine) of normal livers (n = 359) and HCC tissues(n = 765) were analyzed. Compared with normal livers, HMGB3 had higher expression in HCC tissues according to the GSE14520 (fold change = 1.896, t = 11.270, P < 0.001, Figure 2A), GSE5364 (fold change = 1.720, t = 4.161, P = 0.002, Figure 2B), GSE77314 (fold change = 2.204, t = 4.473, P < 0.001, Figure 2C), and TCGA database (fold change = 1.709, t = 9.125, P < 0.001, Figure 2D). Meanwhile, GSE50597 presented upregulated HMGB3 expression at advance stage of HCC in comparison with that at early stage (fold change = 2.054, t = 3.046, P = 0.012, Figure 2E). Besides, Oncomine database elucidated that higher HMGB3 expression was detected in HCC tissues rather than liver cancer precursor (fold change = 1.469, t = 2.948, P = 0.005) or normal livers (fold change = 1.795, t = 3.380, P = 0.003, Figure 2F). Given the observation

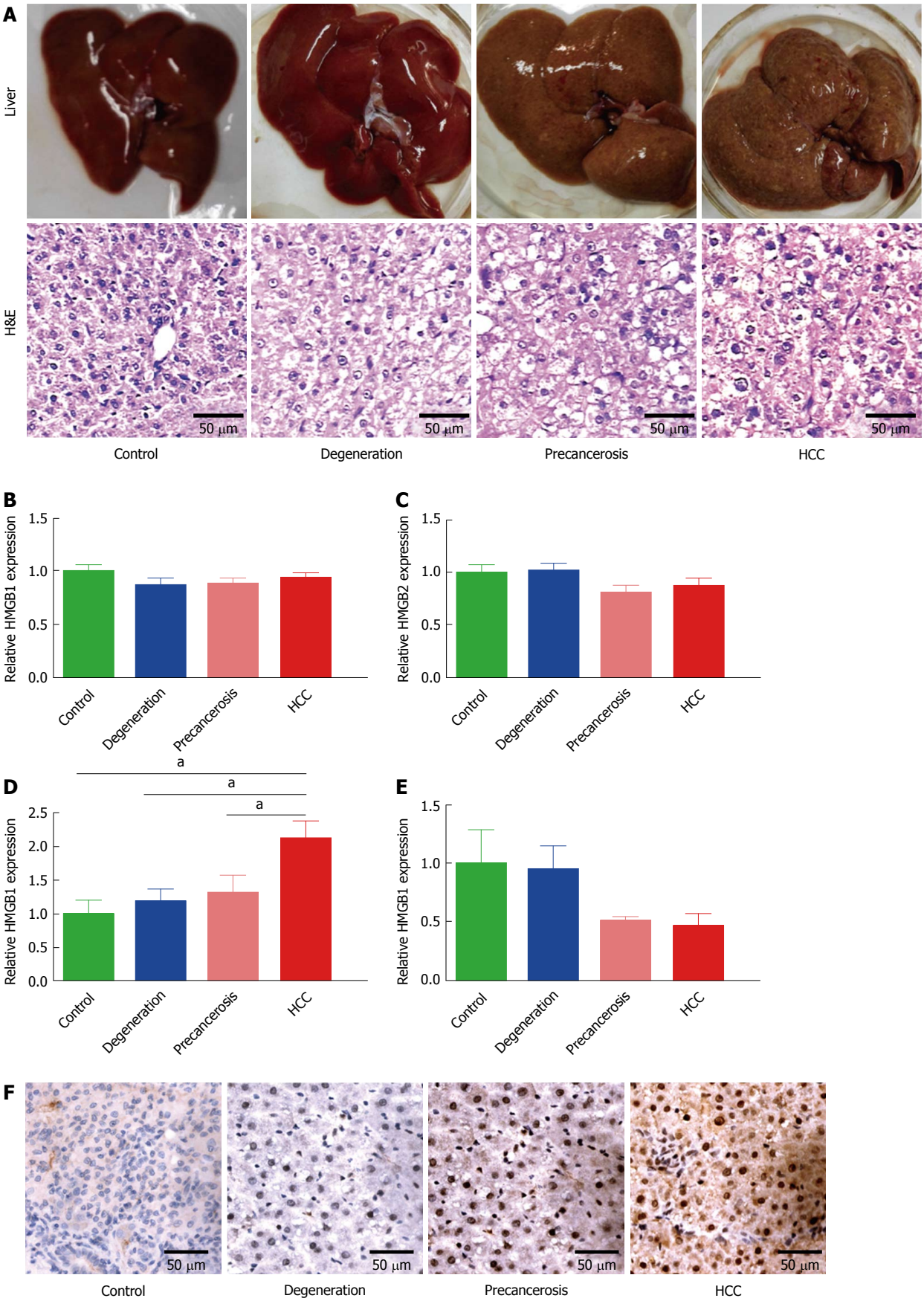


Figure 1 Dynamic upregulation of *HMGB3* in rat hepatocarcinogenesis. Rat hepatocarcinogenesis models were successfully made by consistent 2-AAF intake. A: The dynamic alterations of liver morphology (upper panel) and H&E staining (lower panel) of liver tissues in rat hepatocarcinogenesis. The livers of the rat model, according to the results of rat liver H&E staining, were divided into normal, degeneration, precancerous, and HCC group. B-E: the dynamic alterations of the HMGB family at the mRNA level in models were detected by RT-qPCR. B: HMGB1 mRNA. C: HMGB2 mRNA. D: HMGB3 mRNA. E: HMGB4 mRNA. Each band was presented as a relative value normalized to normal controls (n = 6). F: the immunohistochemical staining of rat HMGB3 expression in different groups. $^{a}P < 0.05$. 2-AAF: 2-acetylaminofluorene; H&E: Hematoxylin and eosin; HMGB: High mobility group-box; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

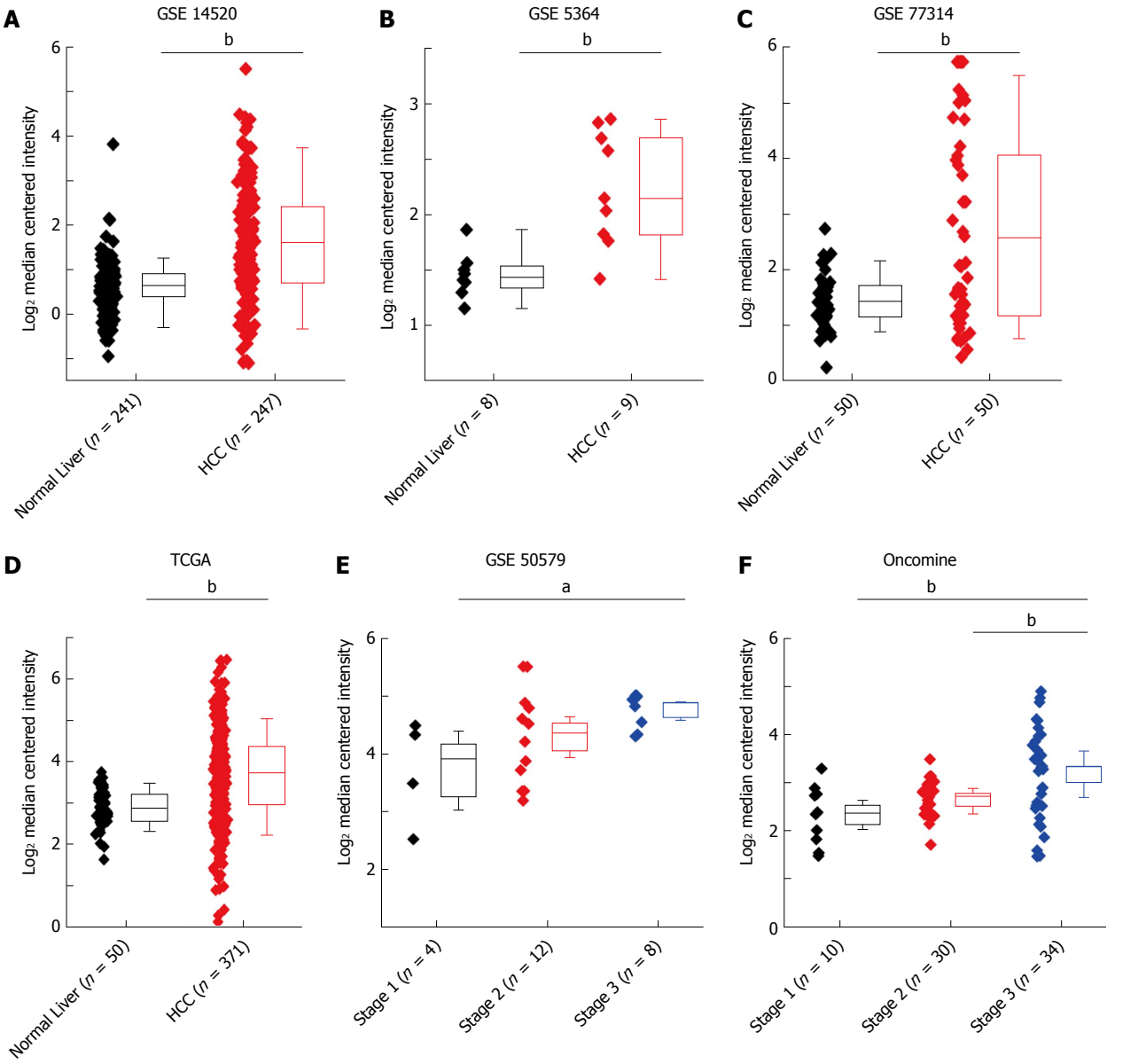


Figure 2 ***HMGB3*** **mRNA expression related to hepatocellular carcinoma by bioinformatic databases.** Comparative analysis of human normal livers (n = 359) and HCC (n = 765) tissues from bioinformatics databases suggested that the upregulation of hepatic HMGB3 mRNA might be involved in HCC progression. The data of HMGB3 mRNA in HCC or normal liver were extracted from A: GSE-14520, B: GSE-5364, C: GSE-77314, D: the HMGB3 mRNA in TCGA database, E: GSE-50579, and F: the HMGB3 mRNA in Oncomine database. Values were presented as Log2 median centered intensity. [a]P < 0.05; [b]P < 0.01.GSE and GEO Series; TCGA: The Cancer Genome Atlas; HCC: Hepatocellular carcinoma.

above, the data suggested that the upregulation of liver HMGB3 mRNA expression might be related to HCC progression.

Knockdown of HMGB3 in HCC cell lines

To further determine the role of HMGB3 in HCC progression, HMGB3 expression was detected and silenced in HCC cell lines. In contrast to normal hepatocyte L02, HMGB3 was overexpressed in HCC cell lines Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404 (Figure 3A and 3B). HepG2 had the highest expression of HMGB3 and was chosen to conduct RNAi using three specific shRNAs with GFP labeling (Figure 3C). Compared with the control and NC-shRNA group, HMGB3 expression was significantly (P < 0.001) downregulated with shRNA-1 at the mRNA (Figure 3D) and protein levels (Figure 3E and F).

Silencing HMGB3 inhibited proliferation and regulated cell cycle of HCC cells

Gene set enrichment analysis was performed to sort the pathways enriched in distinct phenotype labels according to HMGB3 levels. In both of GSE14520 and TCGA, high expression of HMGB3 was correlated with cell cycle and DNA replication (Figure 4A). Thus, the proliferation activity and cell cycle were studied in HMGB3-knockdown HepG2 cells. As shown in Figure 4B, knockdown of HMGB3 using shRNA-1 significantly inhibited the proliferation of HepG2 cells. Furthermore, silencing HMGB3 could lead to an obvious arrest of HepG2 cells in the G1

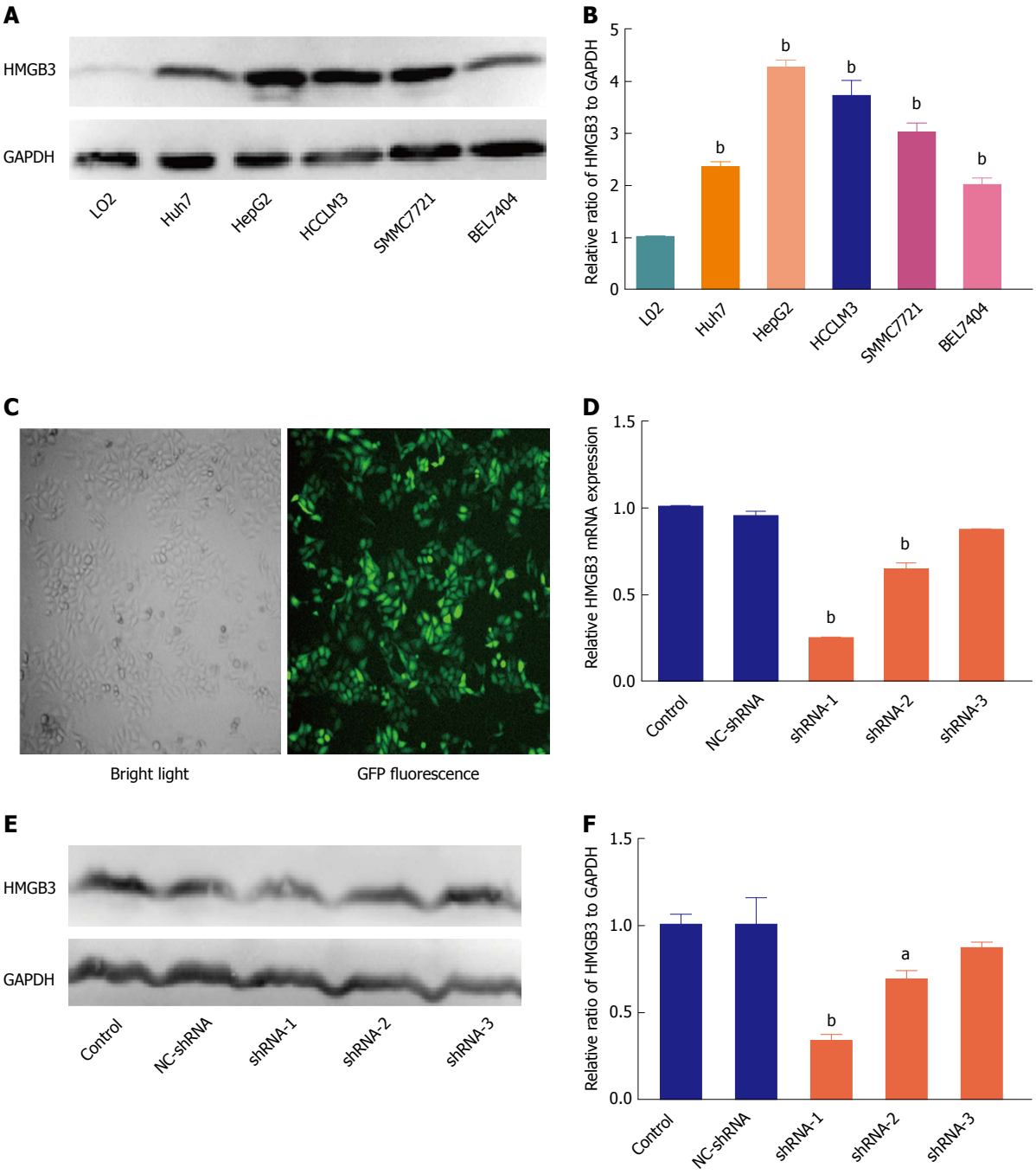
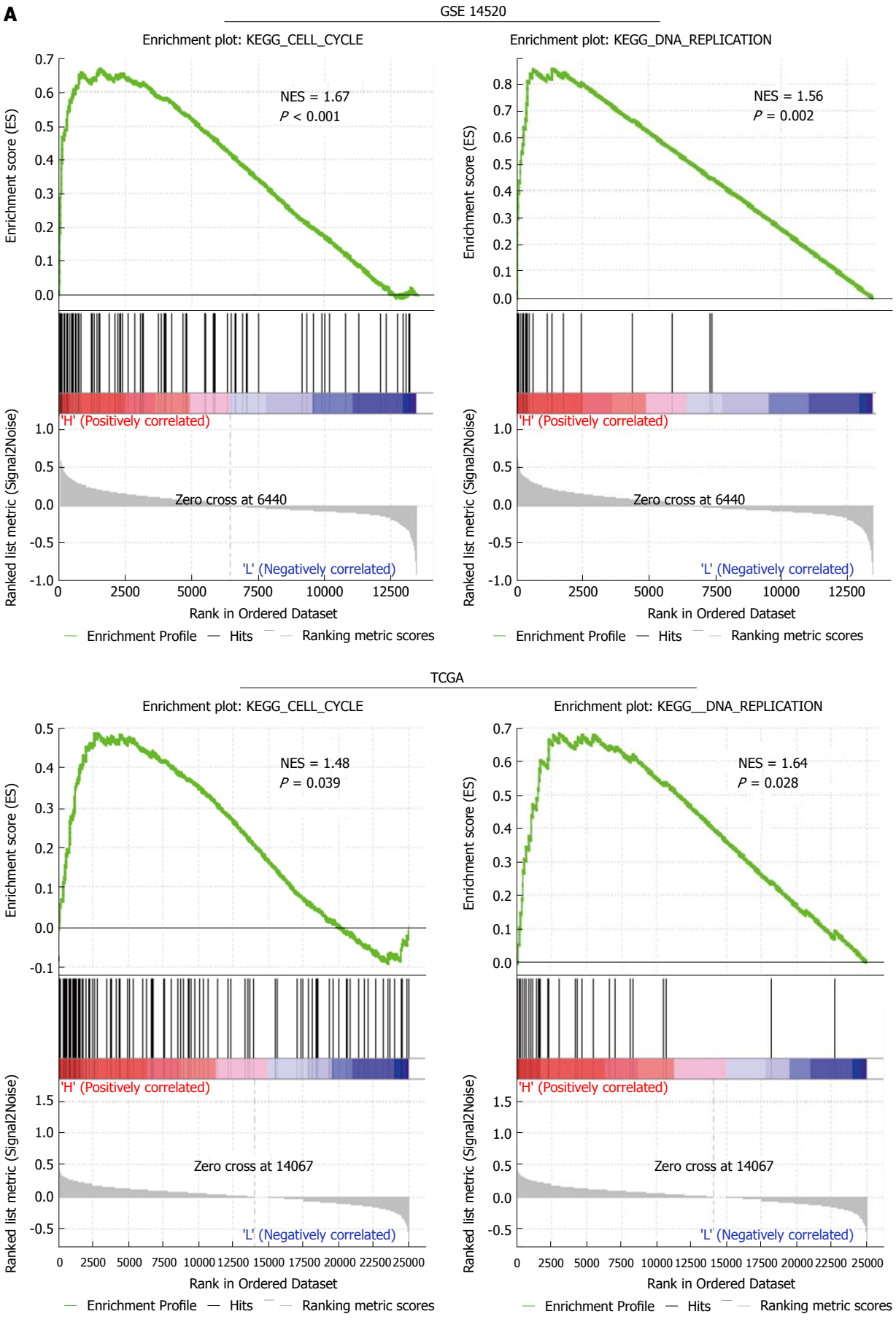


Figure 3 Silencing *HMGB3* in hepatocellular carcinoma cell lines. A: The protein expression of HMGB3 was detected in different HCC cell lines (Huh7, HepG2, HCCLM3, SMMC7721, and BEL7404), and normal hepatocytes L02 using western blotting. GAPDH was used as an internal reference. B: Each bar represents the corresponding intensity in A normalized to GAPDH. C: Representative morphology of HepG2 cells transfected with GFP-labeling shRNA in bright light and fluorescence. D: RT-qPCR was performed to detect HMGB3 mRNA levels in HepG2 cells transfected with different shRNAs and control. Relative value of HMGB3 was calculated according to the $2^{-\Delta\Delta Ct}$ method. E: Western blotting was conducted to analyze the HMGB3 protein expression in cells of the shRNA-transfected group and control group. F: Each bar represents the corresponding intensity in E normalized to GAPDH. $^{a}P < 0.05$; $^{b}P < 0.01$.

phase (Figure 4C and G). In addition, consistent with the results of GSEA, HMGB3 knockdown also inhibited the expression of cell cycle and DNA replication related genes Cyclin B1, proliferating cell nuclear antigen (PCNA) and flap structure-specific endonuclease 1 (FEN1), indicating that HMGB3 might promote the proliferation of HCC cells by regulating cell cycle and DNA replication pathway (Figure 4D-F).

Silencing HMGB3 inhibited tumor growth

Compared with the NC-shRNA group, significantly decreased tumor volume and weight ($P < 0.001$) was observed in the shRNA-1 group at the 30th d after subcutaneous injection (Figure 5A and C). In addition, silencing HMGB3 by shRNA-1 significantly impeded the growth of xenograft tumors according to the growth curves (Figure 5B). Furthermore, IHC results showed


A
GSE 14520
Enrichment plot: KEGG_CELL_CYCLE
NES = 1.67
P < 0.001
Enrichment score (ES)
'H' (Positively correlated)
Zero cross at 6440
'L' (Negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment Profile
Hits
Ranking metric scores
Enrichment plot: KEGG_DNA_REPLICATION
NES = 1.56
P = 0.002
Enrichment score (ES)
'H' (Positively correlated)
Zero cross at 6440
'L' (Negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment Profile
Hits
Ranking metric scores
TCGA
Enrichment plot: KEGG_CELL_CYCLE
NES = 1.48
P = 0.039
Enrichment score (ES)
'H' (Positively correlated)
Zero cross at 14067
'L' (Negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment Profile
Hits
Ranking metric scores
Enrichment plot: KEGG__DNA_REPLICATION
NES = 1.64
P = 0.028
Enrichment score (ES)
'H' (Positively correlated)
Zero cross at 14067
'L' (Negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
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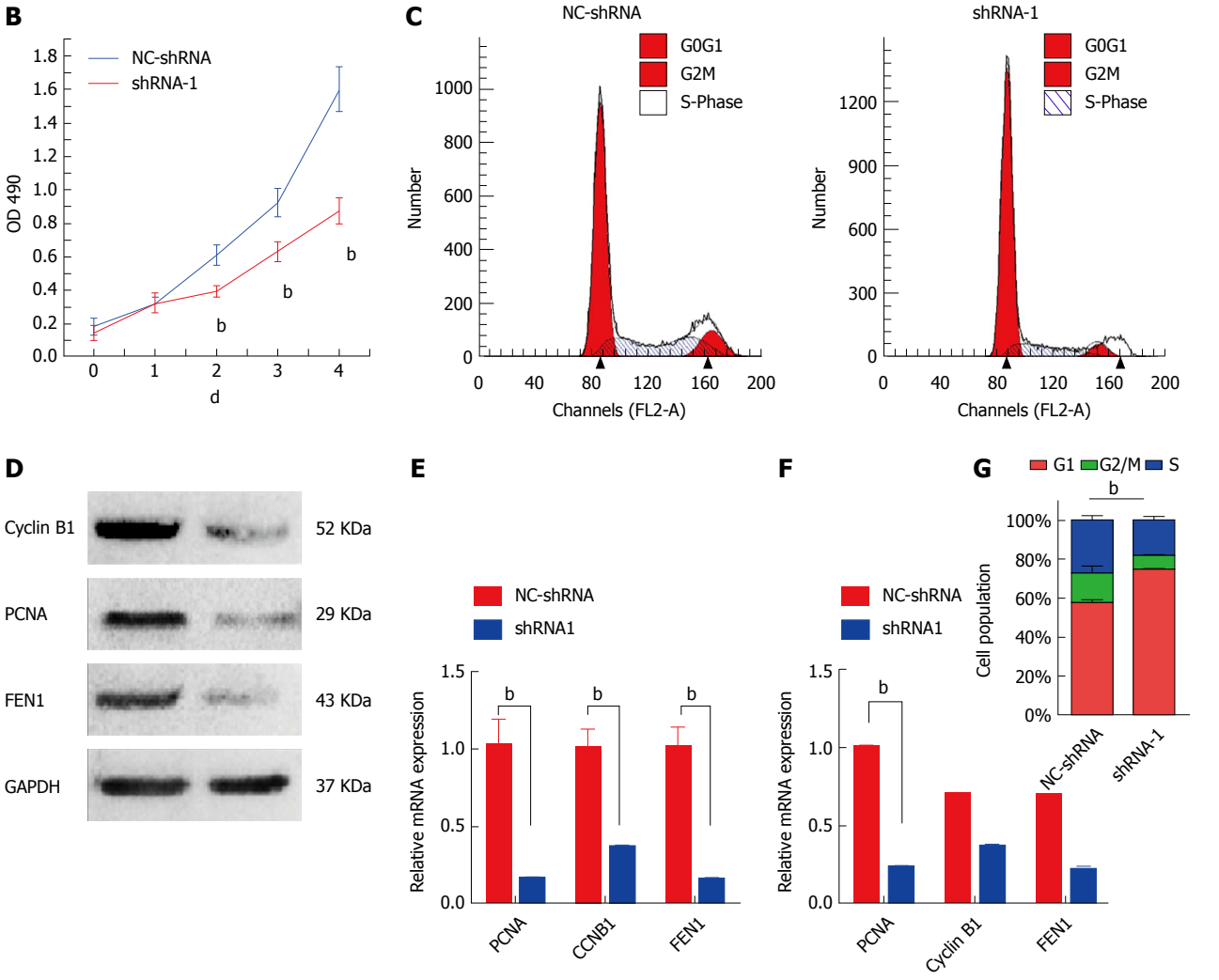

Figure 4 Knockdown *HMGB3* inhibited cell cycle and proliferation of hepatocellular carcinoma cells. A: Gene set enrichment analysis (GSEA) was conducted to sort the pathways according to HMGB3 expression in GSE14520 and TCGA. Cell cycle and DNA replication pathways were found to be significantly correlated with HMGB3 expression. B: Proliferation of HepG2 cells transfected with shRNA-1 and NC-shRNA was detected using the MTT method. C: Cell cycle of HepG2 cells was analyzed after transfection with shRNA using flow cytometry. D: According to the GSEA analysis, three genes (CCNB1, PCNA, and FEN1) involved in cell cycle and DNA replication were detected after shRNA transfection using RT-qPCR. E: Western blotting was conducted to observe the protein expression of cyclin B1, PCNA, and FEN1 in the NC-shRNA and shRNA-1 group. F: Each bar represents the corresponding intensity in E normalized to GAPDH. G: Percentage columns represent the distribution of the cell cycle in corresponding groups. $^{b}P < 0.01$.

that Ki67 expression in tumor tissues of the shRNA-1 group was significantly lower ($P < 0.01$) than that of the NC-shRNA group, indicating that HMGB3 might contribute to the proliferation of HCC cells *in vivo* (Figure 5D).

DISCUSSION

The HMGB family has been recognized as an important regulator in tumor progression[18]. Although the HMGB family with various physiological and pathological functions was previously associated with liver cancer, neither HMGB1 nor HMGB2 was reported to exhibit dynamic expression in tumorigenesis[26]. In this study, the rat hepatocarcinogenesis model was conducted to analyze the expression characteristics from HMGB1, HMGB2, HMGB3, and HMGB4. No statistical differences in HMGB1 or HMGB2 expression were observed. However, HMGB3 and HMGB4 expression were upregulated and downregulated, respectively through the malignant transformation *in vivo*. To further verify the HMGB3 expression in HCC, several HCC-related bioinformatics databases were assessed. Interestingly, consistent with the results of HCC model, analyses of normalized $\log_2$ transformed microarray expression data sets clearly confirmed the significant upregulation of HMGB3 mRNA in human HCC tissues, and especially in advanced HCC tissues, indicating that HMGB3 might be an oncogenic protein involved in the malignant transformation of hepatocytes.

HMGB proteins can assist in either activating or repressing transcription[27]. In adult vertebrates, HMGB1 is found in all cell types, whereas HMGB3 mRNA was reported to be absent in most adult tissues[7,28]. Indeed, overexpression of HMGB3 has been discovered in several cancer types and correlated with clinical features of cancer patients, including advanced tumor-node-

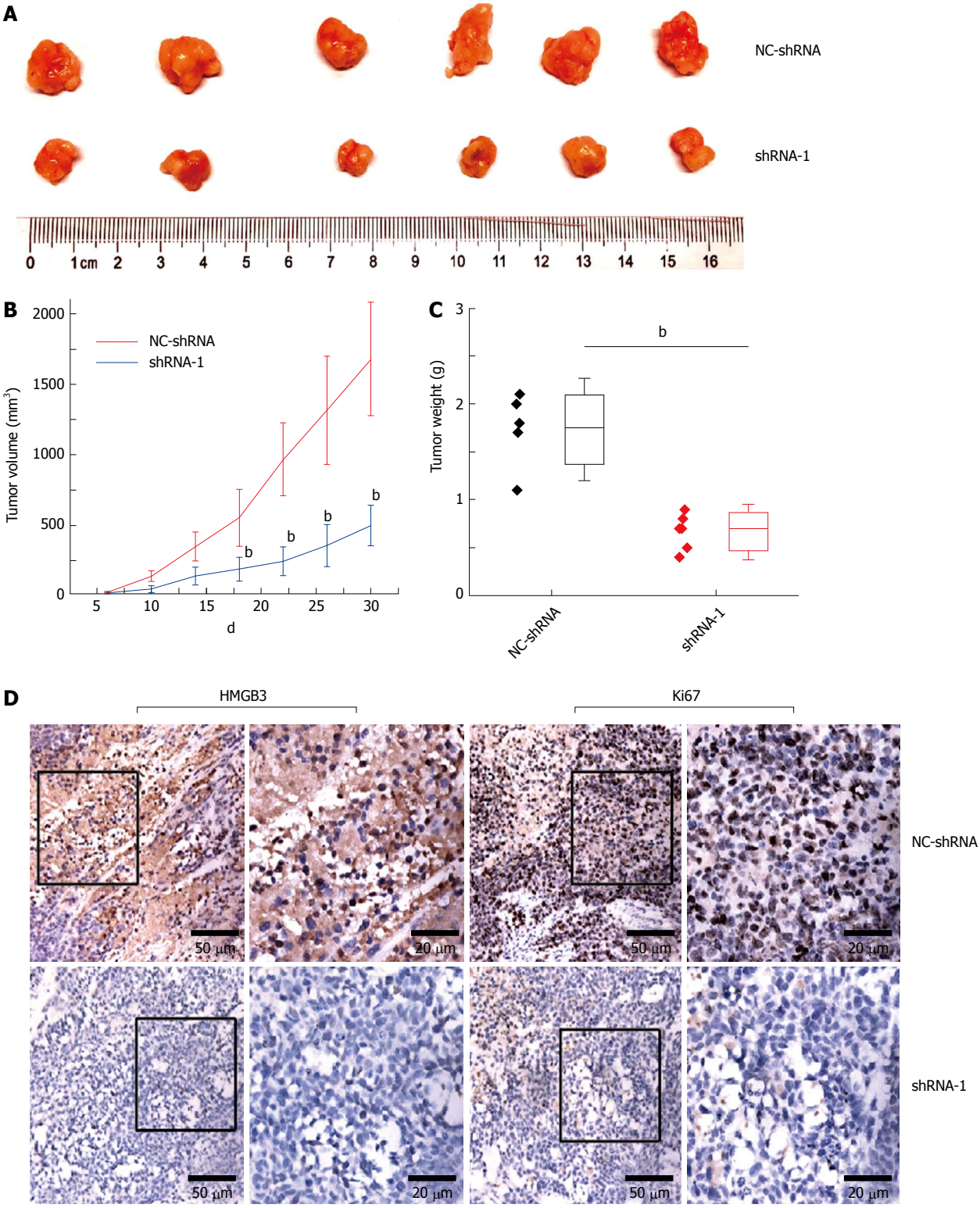

Figure 5 Silencing *HMGB3* suppressed growth of xenograft tumor. HepG2 cells transfected with NC-shRNA and shRNA-1 were subcutaneously injected into mice. A: The morphology of the xenograft tumor in the NC-shRNA or shRNA-1 group at 30th d. B: The growth curves of tumors derived from HepG2 cells in the NC-shRNA or shRNA-1 group. C: The weight of xenograft tumors in the NC-shRNA or shRNA-1 group. D: The immunochemical staining of HMGB3 and Ki67 in xenograft tumor tissues in the NC-shRNA or shRNA-1 group. $^{b}P < 0.01$.

metastasis (TNM) stage, serosal invasion, and overall survival[23,28]. The rat hepatocarcinogenesis model also indicated the potential role of HMGB3 in HCC progression. However, the expression features of HMGB3 and its roles in HCC are still unclear. Thus, the current study further investigated HMGB3 expression in HCC cell lines. Interestingly, overexpression of HMGB3 was observed in HCC cells rather than normal hepatocytes, which was consistent with the hepatocarcinogenesis model and bioinformatic analysis.

HMGB3 has been reported to play crucial roles in tumor progression by contributing to malignant behaviors and regulating oncogenic pathways. For instance, HMGB3 could enhance the migration and growth of gastric cancer cells *via* activation of the Wnt pathway[23]. It also could increase the proliferation and invasion of breast cancer cells as a target gene of miRNA-205[29]. However, for HCC, the malignant behaviors and mechanism mediated by HMGB3 still remain unclear. Thus, bioinformatic analysis was conducted to explore the underlying roles. Notably, GSEA based on GEO database and TCGA jointly indicated that overexpression of HMGB3 might be associated with cell cycle and DNA replication pathways. As expected, corresponding *in vitro* studies showed that silencing HMGB3 could significantly inhibit proliferation and induce cell cycle arrest in HCC cells. To further confirm potential mechanisms responsible for the anti-proliferation effects, we explored expression of Cyclin B1, PCNA, and FEN1, which were cell cycle and DNA replication related genes highly enriched in high-HMGB3-mediated pathways in GSEA. Consistent with this prediction, knockdown of HMGB3 obviously downregulated the three genes at the mRNA and protein levels, which have been reported to promote tumor progression[30-32]. Collectively, HMGB3 might promote proliferation of HCC cells by regulating cell cycle and DNA replication pathways.

Although HMGB3 has been correlated with proliferation, chemoresistance, and migration of cancer cells in previous studies[23,33,34], as far as we know, there was no xenograft assays to evaluate HMGB3 as a regulator of tumor growth *in vivo*. Given the interesting results from the rat model and *in vitro* study, our current study further evaluated HMGB3 as an important regulator of tumor growth *in vivo*. Xenograft tumors derived from HMGB3-silenced HCC cells grew slower than the control tumors, with an obvious reduction in the proliferation marker Ki67. It suggested that HMGB3 might contribute to the tumor growth *in vivo* via regulating proliferation of HCC cells.

In conclusion, to the best of our knowledge, this is the first report to investigate HMGB3 expression and indicate that it may be a novel diagnostic marker or therapeutic target for HCC. Here, the findings are promising, and the initial evidence confirmed that HMGB3 is one of the key molecules in HCC progression. Future studies should clarify the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis to elucidate how HMGB3 might promote proliferation of HCC cells and tumor growth by regulating cell cycle and DNA replication pathways.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is one of the most common and fatal malignancies worldwide with a multi-factorial, multistep, complex process, and poor prognosis. Early diagnosis of HCC at an early stage is of the utmost importance. This found a new molecular biomarker to monitor the malignant transformation of hepatocytes.

Research motivation

Although serum alpha fetoprotein (AFP) level is a useful tumor marker for the detection and monitoring of HCC, the false-negative rate with AFP level alone may be as high as 40% for patients with early stage HCC. Even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients. New specific markers, such as circulating HS-GGT, HS-AFP or AFP-L3, miRNA, GPC-3, and GP73, have been developed to improve the sensitivity, specificity, early detection, and prediction of prognosis. However, the overall results have been unsatisfactory.

Research objectives

The most urgent needs are to find sensitive markers for early diagnosis or monitor postoperative recurrence, and to give adequate treatment for HCC. It has many characteristics, such as fast infiltrating growth, metastasis in early stage, high-grade malignancy, and poorly therapeutic efficacy, thus the prognosis is poor and early detection is of the utmost importance. The present study focused on exploring the relationship between dynamic expression of HMGB-3 and malignant transformation of hepatocytes.

Research methods

Dynamic models of rat hepatocarcinogenesis were made to investigate the expression of the high mobility group box (HMGB) family. HMGB3 expression was measured at the protein level by immunohistochemistry or Western blotting and at the mRNA level by real time PCR. Human HMGB3 expression was evaluated using bioinformatics databases and its mechanisms were analyzed *in vitro*. Xenograft growth was also measured.

Research results

HMGB3 expression was upregulated through the malignant transformation of liver cells *in vivo*.

Research conclusions

The upregulation of liver HMGB3 expression was found by dynamic model of hepatocytes malignant transformation with the alterations of rat liver histopathology. This was confirmed by mining HMGB3 expression in human HCC tissues in bioinformatic databases. Further studies elucidating the role HMGB3 plays in regulating HCC progression, suggests that HMGB3 could be a novel marker for early diagnosis or a molecular therapy target.

Research perspectives

HMGB3 has been confirmed as one of the key molecules in the HMGB family with HCC development. However, the molecular mechanisms of the upregulation of HMGB3 expression and its important role in hepatocarcinogenesis should be clarified in promoting proliferation of HCC cells and tumor growth and regulating cell cycle and DNA replication pathways in the future.

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ORIGINAL ARTICLE

Retrospective Cohort Study

C-peptide as a key risk factor for non-alcoholic fatty liver disease in the United States population

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Author contributions: Atsawarungruangkit A contributed to the study concept and design, analysis and interpretation of data, and preparation of manuscript; Chenbhanich J and Dickstein G contributed to interpretation of data and preparation and manuscript; all authors read and approved the final manuscript.

Institutional review board statement: This study uses the publicly available data from the Third National Health and Nutrition Examination Survey (NHANES III), which is conducted by the National Center for Health Statistics (NCHS). The NHANES protocol was approved by the NCHS Research Ethics Review Board.

Informed consent statement: In NHANES Ⅲ, the consent form was signed by participants in the survey.

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Abstract

AIM

To determine whether fasting C-peptide is an independent predictor for non-alcoholic fatty liver disease (NAFLD) in United States population.

METHODS

Using the National Health and Nutrition Examination Survey (NHANES) 1988-1994, NAFLD participants aged 20 or greater without any other liver diseases were included in this study. Excessive alcohol intake is defined as > 2 drinks per day for males and > 1 drink per day for females. C-peptide and 27 other factors known to be associated with NAFLD (*e.g.*, age, gender, body mass index, waist circumference, race/ethnicity, liver chemistries, and other diabetes tests) were tested in both univariate and multivariate level using logistic regression with a *P*-value 0.05.

RESULTS

Of 18825 participants aged ⩾ 20, 3235 participants (n = 3235) met inclusion criteria. There were 23 factors associated with NAFLD by univariate analysis. 9 factors, ranked by the highest change in pseudo R^2, were found to be significant predictors of NAFLD in multivariate model: waist circumference, fasting C-peptide, natural log of alanine aminotransferase (ALT), total protein, being

Mexican American, natural log of glycated hemoglobin, triglyceride level, being non-Hispanic white, and ferritin level.

CONCLUSION

Together with waist circumference and ALT, fasting C-peptide is among three most important predictors of NAFLD in United States population in the NHANES data set. Further study is needed to validate the clinical utility of fasting C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients.

Key words: Insulin resistance; Fatty liver; Hepatosteatosis; Metabolic syndrome; C-peptide

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a growing global epidemic and associated with many conditions and factors, including insulin resistance. However, C-peptide has not been used in practice to assess insulin resistance in NAFLD patients. Using a large national dataset, we demonstrated that three most important risk factors for NAFLD are waist circumference, fasting C-peptide, and alanine aminotransferase, respectively. Such results revealed that C-peptide superior to measurement of fasting insulin levels and can potentially be used for screening or monitoring the degree of insulin resistance in NAFLD.

Atsawarungruangkit A, Chenbhanich J, Dickstein G. C-peptide as a key risk factor for non-alcoholic fatty liver disease in the United States population. *World J Gastroenterol* 2018; 24(32): 3663-3670 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i32/3663.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i32.3663

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition in which hepatic steatosis exists in the absence of excessive alcohol consumption. NAFLD is the most common cause of chronic liver disease in the United States with estimated prevalence around 30%-40%[1-3]. Given the epidemic of obesity, NAFLD is increasingly prevalent and challenging[4,5]. NAFLD can progress to more severe liver diseases, such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma.

Obesity and insulin resistance are among important risk factors for NAFLD[6,7]. Many studies found that indicators of obesity [*i.e.*, body mass index (BMI) and waist circumference] and insulin resistance [*i.e.*, glycated hemoglobin (HbA1c), insulin level, fasting glucose, and diabetes mellitus] are independently associated with NAFLD and/or severity of liver fibrosis in NAFLD[8-11]. C-peptide levels can be used to measure insulin secretion[12]. However, there is limited evidence of the association between NAFLD and C-peptide at the multivariate level[13,14].

Both C-peptide and insulin are produced and released in equimolar amounts. C-peptide can therefore be used to assess endogenous insulin secretion. However, the level of C-peptide and insulin level in blood are typically different deriving from the differences in clearance mechanisms and half-life[15]. In addition to diabetes and insulin resistance, C-peptide has been associated with many risk factors for NAFLD including cardiovascular diseases and metabolic syndrome[16-18].

Therefore, our primary objective was to determine if fasting C-peptide is independently associated with NAFLD using multivariate analysis in the United States general population.

MATERIALS AND METHODS

Study population and study design

The Third National Health and Nutrition Examination Survey (NHANES Ⅲ) is a probability sample of 39695 persons aged 2 mo and older representing the United States population and conducted by the National Center for Health Statistics (NCHS) to evaluate health and nutritional status[19]. The survey collected multiple data sets, including demographic, interviews, physical examinations, and laboratory testing of biologic samples. NHANES Ⅲ was conducted from 1988 to 1994. The NHANES protocol was approved by the NCHS Research Ethics Review Board.

There were 18825 persons aged 20 years or older in NHANES Ⅲ that met inclusion criteria for this study. The exclusion criteria included: (1) Ungradable or missing ultrasound results for hepatic steatosis, (2) excessive alcohol consumption, (3) hepatitis B or hepatitis C infection (4) fasting period outside of 8-24 h (5) incomplete or missing data on physical examination and laboratory testing. Participants were divided into two groups: NAFLD participants (study group) and non-NAFLD participants (control group).

As presented in Table 1, we included 28 factors associated with NAFLD as independent variables in this study: Demographic (*i.e.*, age , gender, race/ethnicity), body measurement (*i.e.*, BMI and waist circumference), general biochemistry tests [*i.e.*, iron, total iron-binding capacity (TIBC), transferrin saturation, ferritin, cholesterol, triglyceride, HDL cholesterol, C-reactive protein, and uric acid], liver chemistry [aspartate aminotransferase (AST), Alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, total protein, and albumin], and diabetes testing profile [*i.e.*, HbA1c, fasting plasma glucose (FPG), fasting C-peptide, and fasting insulin]. Besides demographic variables, the above variables were selected as the risk factors based on the usage in clinical practice and the supporting evidence that demonstrated the association with NAFLD or its commonly accepted risk factors (*i.e.*, obesity, insulin resistance, and liver fibrosis).

Table 1 Baseline characteristics of participants with non-alcoholic fatty liver disease and controls *n* (%)

	NAFLD, *n* = 817	Controls, *n* = 2418
Demographic		
Age (yr)	48.47 ± 15.75	44.36 ± 16.20
Gender (male)	368 (45.04)	1004 (41.52)
Race/ethnicity		
White (non-Hispanic)	308 (37.70)	1046 (43.13)
Black (non-Hispanic)	193 (23.62)	705 (29.16)
Mexican American	280 (34.27)	550 (22.75)
Others	36 (4.41)	120 (4.96)
Body measurement		
Body mass index (kg/m^2)	30.38 ± 6.95	26.56 ± 5.42
Waist circumference (cm)	101.73 ± 16.32	90.84 ± 13.45
Biochemistry tests		
Iron (μg/dL)	75.35 ± 29.71	77.71 ± 32.75
TIBC (μg/dL)	364.79 ± 58.05	359.86 ± 56.59
Transferrin saturation (%)	21.13 ± 8.73	22.09 ± 9.65
Ferritin (ng/mL)	161.75 ± 152.55	110.16 ± 114.71
Cholesterol (mg/dL)	212.23 ± 44.95	202.73 ± 42.19
Triglyceride (mg/dL)	202.91 ± 137.97	136.58 ± 95.79
HDL cholesterol (mg/dL)	46.72 ± 16.80	52.10 ± 15.61
C-reactive protein (mg/dL)[1]	0.56 ± 0.80	0.45 ± 0.65
Uric acid (mg/dL)	5.62 ± 1.52	5.04 ± 1.42
Liver chemistry		
AST (U/L)[1]	24.76 ± 19.62	20.72 ± 14.71
ALT(U/L)[1]	22.78 ± 17.86	15.96 ± 12.14
GGT (U/L)[1]	42.87 ± 66.68	28.14 ± 41.69
ALP (U/L)[1]	93.72 ± 33.61	86.04 ± 36.29
Total bilirubin (mg/dL)	0.55 ± 0.30	0.54 ± 0.28
Total protein (g/dL)	7.49 ± 0.46	7.37 ± 0.45
Albumin (g/dL)	4.11 ± 0.35	4.12 ± 0.36
Diabetes testing profile		
HbA1c (%)[1]	6.02 ± 1.62	5.50 ± 1.09
FPG (mg/dL)[1]	114.50 ± 65.84	97.85 ± 35.68
Fasting C-peptide (pmol/mL)	1.11 ± 0.68	0.69 ± 0.53
Fasting insulin (μU/mL)[1]	21.85 ± 27.82	12.76 ± 19.24

[1]The distribution is positively skewed with skewness > 3. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FPG: Fasting plasma glucose; GGT: Gamma glutamyl transferase; HbA1c: Glycated hemoglobin; TIBC: Total iron-binding capacity.

Definitions

In this study, NAFLD is defined as: (1) Diagnosed with moderate to severe hepatic steatosis on ultrasound; (2) no history of excessive alcohol intake in the past 12 mo; (3) not infected with hepatitis B or hepatitis C.

To evaluate the presence and extent of hepatic steatosis, readers used five main criteria: (1) Parenchymal brightness, (2) liver to kidney contrast, (3) deep beam attenuation, (4) bright vessel walls, and (5) gallbladder wall definition. Based on the presence or absence of these five criteria, a main finding was categorized as normal, mild, moderate or severe[20]. It is worth nothing that participants aged above 74 were not eligible for ultrasound study in NHANES Ⅲ For this reason, patients age above 74 were excluded from this study.

Excessive alcohol intake is defined as more than 2 drinks per day for men or 1 drink per day for women in the past 12 mo, in which one drink of alcoholic beverage is equivalent to a 12 oz beer, a 5 oz glass of wine, or 1.5 oz of liquor. The average number of drinks per day is calculated from number of drinking days × number of drinks on drinking day/365 d. To qualify as hepatitis viral infection, participants must have tested positive for serum hepatitis B surface antigen or serum hepatitis C antibody HCP (anti-HCV).

Statistical analysis

Statistical analyses were performed using STATA Release 14 (StataCorp LP, TX, United States). Numbers are presented in mean ± SD or number (%). All continuous factors were first tested for skewness; if the distributions were extremely skewed to the right (herein defined as skewness > 3), the factors were log transformed before using them as predictors in regression models. Since the response variable is dichotomous variable (NAFLD or non-NAFLD), logistic model is an appropriate model for determining if predictors are significantly associated with the response variable. As a result, logistic regression was used to determine if NAFLD is associated with any predictor in univariate level. Then, the significant factors from univariate analysis were included as predictors in step-wise logistic regression to determine the significant predictors in multivariate level. The significance level is 0.05.

RESULTS

Out of 18825 participants aged ⩾ 20, there were 3235

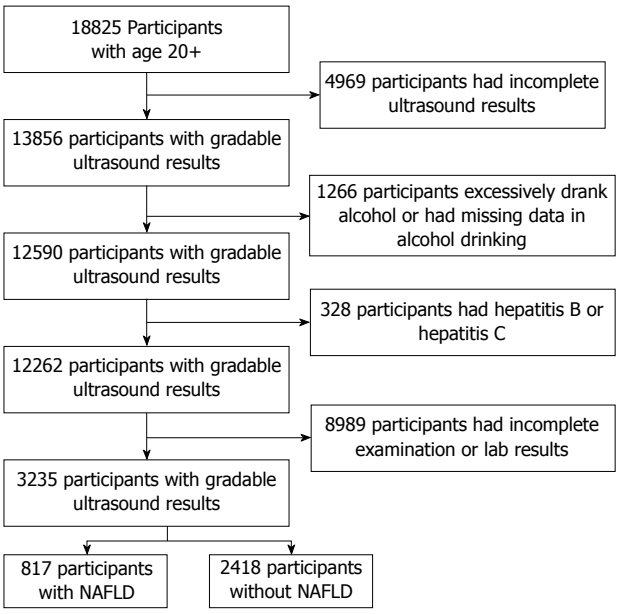

Figure 1 Study design and study population.

participants (n = 3235) that passed the exclusion criteria as shown in Figure 1. Based on ultrasound findings, 817 (25.26%) participants were classified as NAFLD. Baseline characteristics of participants in study group and control group are summarized in Table 1.

For continuous variables, there were 8 factors having skewness greater than 3. Subsequently, the log transformation was applied to these factors, including C-reactive protein, AST, ALT, GGT, alkaline phosphatase, glycated hemoglobin, plasma glucose, and insulin. As shown in Table 2, there are 24 variables significantly associated with NAFLD in univariate level; the P-value of these significant factors mostly below 0.001.

As presented in Table 3, the number of significant factors reduced from 24 to 9 in multivariate analysis. The top three factors ranked by the highest change in pseudo R^2 (ΔR^2) are waist circumference (OR = 1.03, ΔR^2 = 2.13%, $P < 0.001$), C-peptide level (OR = 1.82, ΔR^2 = 1.33%, $P < 0.001$), and $\log_e$ of ALT (OR = 1.76, ΔR^2 = 1.16%, $P < 0.001$). The pseudo R^2 of the multivariate model is 16.68%.

DISCUSSION

The most significant NAFLD risk factor in both univariate and multivariate levels is waist circumference. Since waist circumference and BMI are highly interrelated surrogate markers of obesity[21], it is not surprising to see one factor eliminated in multivariate level. Waist circumference-a measure of excess abdominal adiposity-has been identified as an independent risk factor for many obesity-related conditions, such as cardiovascular disease, type 2 diabetes, dyslipidemia, and hypertension, metabolic syndrome, polycystic ovary syndrome[22-27]. The results from this study support that waist circumference is also an independent and probably the most important risk factor for NAFLD in the United States population.

Insulin resistance is another well-known condition commonly found in NAFLD patients[28,29]. Indeed, all diabetes test profiles were positively correlated with NAFLD by univariate analysis. In fact, type 2 diabetes can be diagnosed directly from HbA1c level (⩾ 6.5%) or FPG (⩾ 126 mg/dL)[30], while C-peptide and insulin are not routinely used in clinical practice to diagnose type 2 diabetes. While there are situations where C-peptide or insulin levels are useful-the diagnosis of insulinoma[31], surreptitious use of insulin[32], the diagnosis of type 2 diabetes in the young[33], and the diagnosis of latent autoimmune diabetes in adults[34], direct measurement of insulin levels and not of C-peptide has been used to assess insulin resistance. Previous studies often found that C-peptide levels are raised in patients with NASH[35-37]. However, the application of C-peptide as a biomarker for interventions designed to improve insulin sensitivity remains to be determined. In case of NAFLD, there is only limited evidence; C-peptide was found to be associated with NAFLD in specific groups of population (i.e., obese adolescents and adults, latent autoimmune diabetes, and diabetes patients)[13,14,38]. Our results are the first to show that C-peptide has a role not only as an independent risk factor for NAFLD but can also be useful for screening or monitoring the degree of insulin resistance in NAFLD in the general population. Based on ΔR^2, we conclude that insulin resistance, as indicated by fasting C-peptide, is the second most important condition leading to NAFLD, second only to obesity as diagnosed by waist circumference, and is superior to measurement of fasting insulin levels.

Liver chemistries are used as an indicator of liver inflammation or liver cell damage. Commonly used liver chemistries include AST, ALT, ALP, GGT, total bilirubin, total protein, and albumin. For example, predominance of AST and ALT indicates hepatocellular injury; predominance of ALP and total bilirubin indicates cholestatic injury; an elevated ALP of hepatic origin may be confirmed GGT[39-41]. As shown in Table 2, total protein and the natural log of AST, ALT, ALP, and GGT were positively associated with NAFLD in univariate analysis. However, only total protein and natural log of ALT were positively correlated with NAFLD in multivariate level. AST and ALT are the most widely used liver chemistries. The fact that ALT is included in multivariate model is not unexpected since ALT is generally higher than AST level in NAFLD[40]. On the other hand, total protein is a non-specific marker of health, nutrition and liver synthetic capacity. Due to the fact that total protein consists of albumin and multiple subtypes of globulin, further investigation into the association between NAFLD and each subtype of globulin may provide a clearer explanation of our findings.

Ferritin is a protein that mainly stores iron in the body and serum ferritin level is the most accurate blood test to diagnose iron deficiency anemia[42]. Recently, the role of ferritin as a biomarker in inflammatory diseases has been increasingly recognized[43-45]. As an acute phase

Table 2 Univariate analysis for predictors of non-alcoholic fatty liver disease

	Beta	Standard error	Odds ratio	*P* value
Demographic				
Age (yr)	0.0157	0.0025	1.02	< 0.001[a]
Gender (male)	0.1435	0.0815	1.15	0.078
Race/ethnicity				
White (non-Hispanic)	-0.226	0.0831	0.80	0.007[a]
Black (non-Hispanic)	-0.2857	0.0937	0.75	0.002[a]
Mexican American	0.5715	0.0882	1.77	< 0.001[a]
Others	-0.1248	0.1945	0.88	0.521
Body measurement				
Body mass index (kg/m^2)	0.1004	0.0069	1.11	< 0.001[a]
Waist circumference (cm)	0.0506	0.003	1.05	< 0.001[a]
Biochemistry tests				
Iron (μg/dL)	-0.0024	0.0013	1.00	0.069
TIBC (μg/dL)	0.0015	0.0007	1.00	0.032[a]
Transferrin saturation (%)	-0.0111	0.0044	0.99	0.012[a]
Ferritin (ng/mL)	0.0029	0.0003	1.00	< 0.001[a]
Cholesterol (mg/dL)	0.005	0.0009	1.01	< 0.001[a]
Triglyceride (mg/dL)	0.0049	0.0004	1.00	< 0.001[a]
HDL cholesterol (mg/dL)	-0.0261	0.0031	0.97	< 0.001[a]
C-reactive protein (mg/dL)[1]	0.3398	0.0534	1.40	< 0.001[a]
Uric acid (mg/dL)	0.2649	0.0277	1.30	< 0.001[a]
Liver chemistry				
AST (U/L)[1]	1.004	0.1153	1.02	< 0.001[a]
ALT (U/L)[1]	1.0274	0.0777	1.04	< 0.001[a]
GGT (U/L)[1]	0.7441	0.0612	1.01	< 0.001[a]
ALP (U/L)[1]	0.9011	0.1303	1.01	< 0.001[a]
Total bilirubin (mg/dL)	0.0914	0.1395	1.09	0.512
Total protein (g/dL)	0.581	0.0896	1.79	< 0.001[a]
Albumin (g/dL)	-0.0871	0.1141	0.92	0.445
Diabetes testing profile				
HbA1c (%)[1]	2.2201	0.2184	1.34	< 0.001[a]
FPG (mg/dL)[1]	1.3125	0.1428	1.01	< 0.001[a]
Fasting C-peptide (pmol/mL)	1.1976	0.0753	3.31	< 0.001[a]
Fasting insulin (μU/mL)[1]	0.9646	0.059	1.02	< 0.001[a]

[1]Log transformation was applied to this factor before including in regression model. [a]*P* < 0.05. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FPG: Fasting plasma glucose; GGT: Gamma glutamyl transferase; HbA1c: Glycated hemoglobin; TIBC: Total iron-binding capacity. [a]*P* < 0.05.

Table 3 Risk factors of non-alcoholic fatty liver disease from step-wise logistic regression

	Beta	Standard error	Odds ratio	Change in pseudo R^2	*P* value
Demographic					
Race/ethnicity					
White (non-Hispanic)	0.2969	0.1162	1.35	0.18%	0.011
Mexican American	0.5495	0.1207	1.73	0.57%	0.020
Body measurement					
Waist circumference (cm)	0.0308	0.0035	1.03	2.13%	< 0.001
Biochemistry tests					
Ferritin (ng/mL)	0.0013	0.0004	1.00	0.15%	< 0.001
Triglyceride (mg/dL)	0.0008	0.0004	1.00	0.32%	< 0.001
Liver chemistry					
ALT (U/L)[1]	0.5658	0.0875	1.76	1.16%	< 0.001
Total protein (g/dL)	0.5319	0.1045	1.70	0.72%	< 0.001
Diabetes testing profile					
HbA1c (%)[1]	0.9266	0.2492	2.53	0.38%	< 0.001
Fasting C-peptide (pmol/mL)	0.6009	0.0877	1.82	1.33%	< 0.001

[1]Log transformation was applied to this factor before including in regression model. Note: Pseudo R^2 = 16.68%; constant (Y-intercept) = 0.0000153; change in Pseudo R^2 is an incremental increase in Pseudo R^2 resulting from adding variable to model last. ALT: Alanine aminotransferase; HbA1c: Glycated hemoglobin.

response protein, ferritin concentrations increase during inflammation and may not reflect the size of total body iron stores[44]. Moreover, ferritin was found be associated with histologic severity and advanced fibrosis in patients

with NAFLD[46-48].

Other factors significantly associated with NAFLD include race/ethnicity (non-Hispanic white and Mexican American), and triglyceride level. Race/ethnicity were often found associated with obesity-related diseases in United States based population[49,50] Triglyceride is an important biomarker of cardiovascular disease risk[51], another condition highly interrelated with NAFLD.

There are several limitations in this study. First, the diagnosis of NAFLD in this study is based on the hepatic ultrasound results although liver biopsy remains the gold standard for the diagnosis of NAFLD. Second, the statistical analysis used is logistic regression. Since the relationship among these factors are complex, inter-related, and non-linear, linearity assumptions embedded in logistic regression may not be able to address all aspects of NAFLD. Furthermore, given a pseudo R^2 of 16.68%, only 16.68% of variation can be explained by multivariate model in Table 3.

In conclusion, NAFLD is associated with many conditions and factors. Three most important factors from multivariate model in this study are waist circumference, fasting C-peptide, and ALT. Further study is needed to validate the clinical utility of C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States. Additionally, NAFLD can progress to more severe liver diseases, such as non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma. Many factors were found to be independently associated with NAFLD and/or severity of liver fibrosis in NAFLD. Nevertheless, there is limited evidence of the association between NAFLD and C-peptide.

Research motivation

Among many risk factors that are associated with NAFLD, obesity and insulin resistance are probably the most well-known ones. C-peptide levels can be used to measure insulin secretion and a surrogate marker of insulin resistance. However, C-peptide is not routinely used in clinical practice to diagnose type 2 diabetes or monitor insulin resistance status in NAFLD.

Research objectives

The objective of this study was to determine if fasting C-peptide is independently associated with NAFLD using multivariate analysis in the United States general population.

Research methods

Using the National Health and Nutrition Examination Survey 1988-1994, NAFLD participants aged 20 or greater without any other liver diseases were included in this study. The participants with excessive alcohol intake (> 2 drinks per day for males and > 1 drink per day for female) were excluded from the study. C-peptide and 27 other factors known to be associated with NAFLD (*e.g.*, age, gender, body mass index, waist circumference, race/ethnicity, liver chemistries, and other diabetes tests) were selected as predictors in regression model. Univariate logistic regression and multivariate step-wise logistic regression were used to determine if the significant predictors of NAFLD, respectively.

Research results

There were 3235 participants (n = 3235) that passed the exclusion criteria. Based on ultrasound findings, 817 (25.26%) participants were classified as NAFLD. Twenty-four variables were significantly associated with NAFLD in univariate level; the P-value of these significant factors mostly below 0.001. Using multivariate analysis, we found 9 out of 24 factors to be significantly associated with NAFLD. Ranked by $\triangle R^2$, the top three factors ranked are waist circumference (OR = 1.03, $\triangle R^2$ = 2.13%, P < 0.001), C-peptide level (OR = 1.82, $\triangle R^2$ = 1.33%, P < 0.001), and $\log_e$ of ALT (OR = 1.76, $\triangle R^2$ = 1.16%, P < 0.001). The pseudo R^2 of the multivariate model is 16.68%.

Research conclusions

C-peptide is the second most important predictor of NAFLD in United States population after waist circumference.

Research perspectives

Further prospective research is needed to validate the clinical utility of fasting C-peptide in diagnosis or monitoring insulin resistance in NAFLD patients. Moreover, C-peptide should be considered as a potential factor for calculative liver scores to evaluate the fibrosis level.

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ORIGINAL ARTICLE

Clinical Trials Study

Vascular anatomy of inferior mesenteric artery in laparoscopic radical resection with the preservation of left colic artery for rectal cancer

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Author contributions: Wang KX and Bi DS designed the study and wrote the manuscript; Wang KX and Liu Z instructed the whole study and prepared the figures; Wang KX and Wang XY collected and analyzed the data; Wang KX, Cheng ZQ, Liu Z, and Bi DS performed the operations; all authors have approved the final version of the manuscript.

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Abstract

AIM

To investigate the vascular anatomy of inferior mesenteric artery (IMA) in laparoscopic radical resection with the preservation of left colic artery (LCA) for rectal cancer.

METHODS

A total of 110 patients with rectal cancer who underwent laparoscopic surgical resection with preservation of the LCA were retrospectively reviewed. A 3D vascular reconstruction was performed before each surgical procedure to assess the branches of the IMA. During surgery, the relationship among the IMA, LCA, sigmoid artery (SA) and

superior rectal artery (SRA) was evaluated, and the length from the origin of the IMA to the point of branching into the LCA or common trunk of LCA and SA was measured. The relationship between inferior mesenteric vein (IMV) and LCA was also evaluated.

RESULTS

Three vascular types were identified in this study. In type A, LCA arose independently from IMA (46.4%, *n* = 51); in type B, LCA and SA branched from a common trunk of the IMA (23.6%, *n* = 26); and in type C, LCA, SA, and SRA branched at the same location (30.0%, *n* = 33). The difference in the length from the origin of IMA to LCA was not statistically significant among the three types. LCA was located under the IMV in 61 cases and above the IMV in 49 cases.

CONCLUSION

The vascular anatomy of the IMA and IMV is essential for laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

Key words: Inferior mesenteric artery; Left colic artery; Rectal cancer; Laparoscopic

Core tip: One hundred and ten patients who underwent laparoscopic surgical resection with preservation of left colic artery (LCA) for rectal cancer were retrospectively reviewed. The 3D reconstruction of the vasculature was performed before surgical procedures. The types of branch vessels of inferior mesenteric artery (IMA) were classified. Furthermore, in the operations, relationships between the IMA, sigmoid artery (SA), LCA and superior rectal artery (SRA) were evaluated. The relationship between LCA and inferior mesenteric vein (IMV) was also evaluated. To recognize different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels during laparoscopic radical resection of rectal cancer.

Wang KX, Cheng ZQ, Liu Z, Wang XY, Bi DS. Vascular anatomy of inferior mesenteric artery in laparoscopic radical resection with the preservation of left colic artery for rectal cancer. *World J Gastroenterol* 2018; 24(32): 3671-3676 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i32/3671.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i32.3671

INTRODUCTION

In laparoscopic radical resection for rectal cancer, high-tie of the inferior mesenteric artery (IMA) at its origin is essential for *en bloc* lymph node dissection. Some researchers have demonstrated the clinical significance of lymph node dissection from the origin of the IMA for postoperative staging and prognosis[1]. However, high tie at the origin of the IMA may lead to postoperative poor anastomotic perfusion, which increases the incidence of anastomotic leakage[2,3]. Many surgeons prefer a low-tie technique, which ligates the IMA while preserving the left colic artery (LCA) after lymph nodes around the IMA were dissected[4,5]. A clear understanding of the vascular anatomy of the IMA and inferior mesenteric vein (IMV) is essential for this low-tie procedure. However, there are multiple types of branch vessels originating from the IMA that makes this surgical procedure technically demanding[6]. We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer.

MATERIALS AND METHODS

Patient data

Following approval of the Ethics Committee on Scientific Research of our hospital, the records of 110 patients, who underwent laparoscopic surgical resection with preservation of the LCA for rectal cancer from March 2016 to November 2017 were retrospectively analyzed. The research participants were recruited from the Department of General Surgery of Qilu Hospital, a teaching hospital of Shandong University in Shandong, China. The data of patient age, sex ratio, BMI, and histopathological stage were observed and compared.

Preoperative assessment of vascular anatomy by 3D reconstruction

The 3D reconstruction of the vascular anatomy was performed in all cases before surgery. The types of branch vessels of the IMA as well as the length from the origin of the IMA to the LCA were determined.

Surgical procedure

Laparoscopic surgery was performed by a single operating team consisting of two senior surgeons and three staff surgeons. During the operation, the patients were placed in lithotomy position. Multi-incision laparoscopic surgery was performed. That was, we placed five ports including the optical port. There were the first 10 mm trocar in the umbilicus as the optical port, another 12-mm trocar, and three 5-mm trocars. We established pneumoperitoneum and maintained abdominal pneumoperitoneum pressure at 12 mmHg. Sharp dissections were performed using a laparoscopic ultrasound knife. First, the small intestine was pulled on its cephalic side to allow for sufficient surgical space. Second, the sigmoid colon mesentery was mobilized with medial to lateral approach up to the origin of the IMA. Lymphous and adipic tissues were resected along the IMA down to the point of branching into the LCA or common trunk of LCA and sigmoid artery (SA). The dissection was then conducted from the LCA until the

Table 1 Patient characteristic data

Factors	Type A (n = 51)	Type B (n = 26)	Type C (n = 33)	Statistical value	P value
Age (yr, mean ± SD)	58.6 ± 14.1	60.4 ± 15.0	62.3 ± 12.9	F = 2.881	0.06
Male, n (%)	31 (60.8)	15 (57.7)	18 (54.5)	Pearson χ^2 = 0.324	0.85
BMI (kg/m^2)	25.3 ± 5.4	24.8 ± 4.9	25.1 ± 4.6	F = 0.331	0.72
Stage (UICC 8th)				Fisher = 1.510	0.84
Ⅰ	7	5	6		
Ⅱ	26	10	16		
Ⅲ	18	11	11		

P < 0.05. BMI: Body mass index; UICC: Union for International Cancer Control.

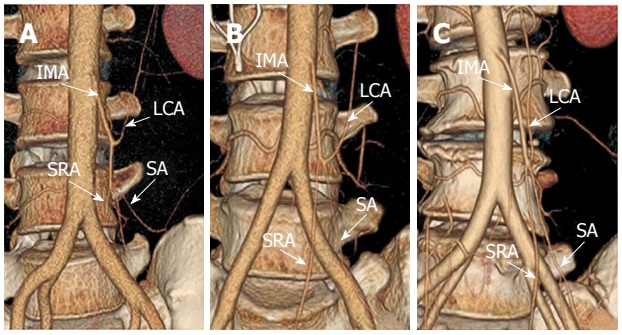

Figure 1 Preoperative 3D reconstruction of inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.

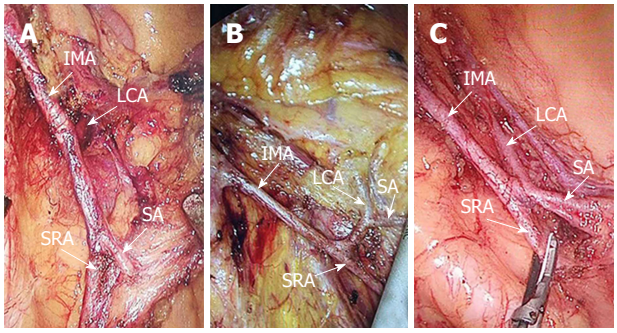

Figure 2 Inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery in laparoscopic operation. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.

IMV could be identified. The IMV was exposed to the plane of the origin of the IMA. After the dissection, the vessels were ligated and cut with the preservation of the LCA. The superior hypogastric nerve and left ureter were carefully preserved during the procedure.

During surgery, the relationship among the IMA, LCA, SA, and superior rectal artery (SRA) was evaluated, and the length from the origin of the IMA to the point of branching into the LCA or common trunk of LCA and SA was measured. In addition, the relationship between LCA and IMV was also evaluated. The number of resected lymph nodes and the incidence of metastasis to station 253 nodes were recorded. Furthermore, the incidence of anastomotic bleeding and anastomotic leakage, and the length of postoperative hospital stay were investigated.

Statistical analysis

All experimental data were analyzed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, United States). There were three groups of samples in this study. The three groups were compared by single factor analysis of variance (single factor ANOVA) and homogeneity of variance. The comparison between the two groups was used on the LSD method. The count data were represented by the value/percentage (%), and the three groups were compared with the chi square test and Fisher accurate test. When the theoretical number T < 5, the Fisher test value was used, if not, the Pearson chi square test was used.

RESULTS

Clinical characteristics of the patients

The data of patient age, sex ratio, BMI, and histopathological stage are shown in Table 1. There were no statistically significant differences among the three types.

Vascular study

IMA, LCA, SA, and SRA were studied in 110 cases by preoperative 3D reconstruction of the vascular anatomy (Figure 1A-C) and laparoscopic surgery (Figure 2A-C). Three vascular types were identified in the study: type A (Figures 1A, 2A, and 3A), LCA arose independently from the IMA (46.4%, n = 51); type B (Figures 1B, 2B, and 3B), LCA and SA branched from a common trunk of the IMA (23.6%, n = 26); and type C (Figures 1C, 2C, and 3C), LCA, SA, and SRA branched at the same location (30.0%, n = 33). The length from the origin of the IMA to the LCA is displayed in Table 2. There was no statistically significant difference among the three types.

In laparoscopic surgery, the relationship between the location of LCA and IMV was observed. The LCA was located under the IMV in 61 cases and above the IMV in 49 cases. The ratio regarding the location of the LCA under the IMV in the three types was similar (Table 2).

Surgical outcome

As shown in Table 3, the data of operating time, blood

Table 2 Results of vascular anatomy

Factors	Type A (n = 51)	Type B (n = 26)	Type C (n = 33)	Statistical value	P value
Length from the IMA to the LCA (mm) (mean ± SD)	35.2 ± 8.7	37.8 ± 9.4	41.3 ± 11.5	F = 1.976	0.144
No. of LCA under IMV	27 (52.9%)	14 (53.8%)	20 (60.6%)	Pearson χ^2 = 0.512	0.802

P < 0.05. IMA: Inferior mesenteric artery; IMV: Inferior mesenteric vein; LCA: Left colic artery.

Table 3 Surgical outcome of patients

	Type A (n = 51)	Type B (n = 26)	Type C (n = 33)	Statistical value	P value
Operation time (min)	153.4 ± 26.8	168.7 ± 31.6	161.4 ± 25.8	F = 1.618	0.20
Blood loss (g)	37.5 ± 18.4	42.1 ± 17.7	39.6 ± 20.1	F = 1.383	0.26
Lymph node numbers (n, mean ± SD)	15.7 ± 8.3	17.2 ± 8.1	16.8 ± 9.0	F = 0.620	0.54
Metastasis to station 253 lymph nodes (n)	2	1	1	Fisher = 0.368	1.00
Anastomotic bleeding	1		1	Fisher = 0.930	0.10
Anastomotic leakage	1	1		Fisher = 1.407	0.72
Postoperative hospitalized days	9 (7-21)	9 (6-18)	9 (7-13)	χ^2 = 0.863	0.65

P < 0.05.

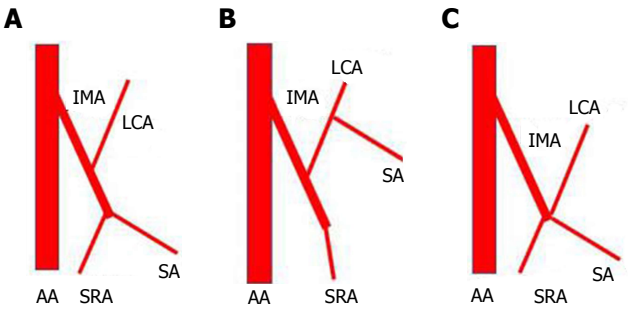

Figure 3 Vascular schematic diagram of inferior mesenteric artery, left colic artery, sigmoid artery and superior rectal artery. A: Type A, LCA arose independently from IMA. B: type B, LCA and SA branched from a common trunk from IMA. C: type C, LCA, SA, and SRA branched off at the same point. AA: Abdominal aorta; IMA: Inferior mesenteric artery; LCA: Left colic artery; SA: Sigmoid artery; SRA: Superior rectal artery.

loss, and the length of postoperative hospital stay were not statistically significant among the three groups. Additionally, there were no statistically significant differences in the dissected lymph node numbers. The incidence of metastasis to station 253 nodes was 4.5% (5 of 110). The postoperative complications included anastomotic bleeding in two cases and anastomotic leakage in two cases. No statistically significant difference was observed among the three groups.

DISCUSSION

In laparoscopic radical resection for rectal cancer, according to the location of the tie of the IMA, it is divided into the high-tie of the IMA at its origin and the low-tie of the IMA below the branch into the LCA with preservation of the LCA. Currently, there is still controversy regarding the indications for high-tie or low-tie approaches[7-9]. In traditional rectal cancer surgery, a high tie of the IMA is preferred. However, some anatomical studies suggest that anastomotic perfusion is diminished after the high-tie of the IMA[3]. Consequently, postoperative poor anastomotic perfusion increased the incidence of anastomotic leakage[10,11]. LCA can increase the blood supply of anastomotic and proximal colorectum. The Riolan artery arch is an anastomotic branch between ascending branches of the LCA and the left branches of the middle colonic artery. It is vital to the blood supply of the anastomotic and proximal colorectum. The Riolan arch exists in about 7.6% of the Chinese population[12]. In patients with absence of the Riolan arch, left hemicolon relies on blood supply from the IMA, and the high-tie technique causes ischemic changes of anastomotic stoma easily. Some Chinese surgeons have found that the absence of the Riolan arch is an independent risk factor for anastomotic leakage after laparoscopic radical resection of rectal cancer[13]. So the low-tie technique with preservation of the LCA to maintain the blood supply is recommended[14,15]. Furthermore, more and more research has indicated that the preservation of the LCA decreases the rate of anastomotic leakage[2,14,15].

A clear understanding of the vascular anatomy of the IMA and IMV is the essential knowledge required to conduct this surgical procedure. There are multiple types of branch vessels of the IMA that makes this surgery technically demanding. In our study, the branching patterns of the IMA can be divided into three groups by preoperative 3D vascular reconstruction and laparoscopic surgery (Figures 1 and 2). However, in other studies, there is a fourth pattern with the absence of the LCA[6,16]. Similarly, the relationship between the LCA and IMV was also observed. In 61 of the 110 cases, the LCA ran under the IMV. In these cases, during dissection of the LCA, extreme caution should be taken, and ligation of the SMV was performed first in order to avoid any damage to the IMV. Although there is still uncertainty with regard to the technical difficulty of the procedure, Sekimoto *et al*[17] have demonstrated that compared to the high-tie technique, the low-tie procedure did not prolong the

operating time and did not increase the amount of intraoperative bleeding. In the present study, compared with other previous studies on high ligation, the operating time was not prolonged significantly[17,18]. In addition, the incidence of postoperative complications was relatively low owing to the familiarity with anatomy and meticulous operation. According to our results, low-tie of the IMA with preservation of the LCA was safe and feasible.

Lymph node metastasis plays a crucial role in the prognosis of rectal cancer[18]. It was demonstrated that lymph node dissection around the IMA prolonged the survival of patients with lymph node metastasis. Low-tie of the IMA with preservation of the LCA was suspected to hinder the lymph nodes resection surrounding the IMA. A prospective study showed that the low-tie group had a similar number of lymph nodes dissected compared to the high-tie group[19]. Other studies compared the prognosis between high-tie and low-tie, and found that the overall survival (OS) and recurrence free survival (RFS) were similar between the two treatments, even in those with lymph node metastases[5]. In the present study, the number of lymph nodes dissected with the low-tie technique for rectal cancer (Table 1) was similar to other studies[1,6]. Nonetheless, our study did not include a high-tie control group, which is a major limitation to the results and prevents an effective discussion to be established to address these problems.

In conclusion, knowledge of the anatomy of the branch vessels originating from the IMA and the relationship between the IMA and IMV are essential in order to conduct a laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize the different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

ARTICLE HIGHLIGHTS

Research background

In laparoscopic radical resection for rectal cancer, according to the location of the tie of the inferior mesenteric artery (IMA), it is divided into the high-tie of the IMA at its origin and the low-tie of the IMA below the branch into the left colic artery (LCA) with preservation of the LCA. Currently, there is still controversy regarding the indications for high-tie or low-tie approaches.

Research motivation

In laparoscopic radical resection for rectal cancer, high-tie of the IMA at its origin is essential for *en bloc* lymph node dissection. We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer.

Research objectives

We aimed to investigate the vascular anatomy of IMA in laparoscopic radical resection with the preservation of LCA for rectal cancer.

Research methods

The records of 110 patients, who underwent laparoscopic surgical resection with preservation of the LCA for rectal cancer from March 2016 to November 2017 were retrospectively analyzed. The research participants were recruited from the Department of General Surgery of Qilu Hospital, a teaching hospital of Shandong University in Shandong, China. A 3D vascular reconstruction was performed before each surgical procedure to assess the branches of the IMA. During surgery, the relationship among the IMA, LCA, sigmoid artery (SA) and superior rectal artery (SRA) was evaluated.

Research results

IMA, LCA, SA, and SRA were studied in 110 cases by preoperative 3D reconstruction of the vascular anatomy and laparoscopic surgery. Three vascular types were identified in the study: type A, LCA arose independently from the IMA (46.4%, *n* = 51); type B, LCA and SA branched from a common trunk of the IMA (23.6%, *n* = 26); and type C, LCA, SA, and SRA branched at the same location (30.0%, *n* = 33). There was no statistically significant difference in the length from the origin of the IMA to the LCA among the three types. In laparoscopic surgery, the LCA was located under the IMV in 61 cases and above the IMV in 49 cases. The ratio regarding the location of the LCA under the IMV in the three types was similar. The data of operating time, blood loss, and the length of postoperative hospital stay were not statistically significant among the three types. Additionally, there were no statistically significant differences in the dissected lymph node numbers. The incidence of metastasis to station 253 nodes was 4.5% (5 of 110). The postoperative complications included anastomotic bleeding in two cases and anastomotic leakage in two cases.

Research conclusions

Knowledge of the anatomy of the branch vessels originating from the IMA and the relationship between the IMA and IMV are essential in order to conduct a laparoscopic radical resection with preservation of the LCA for rectal cancer. To recognize the different branches of the IMA is necessary for the resection of lymph nodes and dissection of vessels.

Research perspectives

We studied the vascular anatomy of the IMA to safely and effectively dissect the lymph nodes around the IMA while preserving the LCA in a laparoscopic procedure for rectal cancer. However, in the light of the limited evidence, the clinical benefit needs more high-quality RCT studies.

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