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REVIEW

Locoregional therapies and their effects on the tumoral microenvironment of pancreatic ductal adenocarcinoma

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is expected to become the second leading cause of death from cancer by 2030. Despite intensive research in the field of therapeutics, the 5-year overall survival is approximately 8%, with only 20% of patients eligible for surgery at the time of diagnosis. The tumoral microenvironment (TME) of the PDAC is one of the main causes for resistance to antitumoral treatments due to the presence of tumor vasculature, stroma, and a modified immune response. The TME of PDAC is characterized by high stiffness due to fibrosis, with hypo microvascular perfusion, along with an immunosuppressive environment that constitutes a barrier to effective antitumoral treatment. While systemic therapies often produce severe side effects that can alter patients' quality of life, locoregional therapies have gained attention since their action is localized to the pancreas and can thus alleviate some of the barriers to effective antitumoral treatment due to their physical effects. Local hyperthermia using radiofrequency ablation and radiation therapy - most commonly using a local high single dose - are the two main modalities holding promise for clinical efficacy. Recently, irreversible electroporation and focused ultrasound-derived cavitation have gained increasing attention. To date, most of the data are limited to preclinical studies, but ongoing clinical trials may help better define the role of these locoregional therapies in the management of PDAC patients.

Key Words: Pancreatic ductal adenocarcinoma; Tumoral microenvironment; Stroma; Hyperthermia; Radiation therapy; High-intensity focused ultrasound

Core Tip: The prognosis of pancreatic ductal adenocarcinoma is poor, with a 5-year survival rate of approximately 8%. This is mainly due to an unfavorable tumoral microenvironment (tumor vasculature, stroma, and immune response). Locoregional therapies can alleviate some barriers to effective antitumoral treatment. This review explores the action of locoregional treatments on pancreatic cancer, with a specific focus on hyperthermia, radiation therapy, high-intensity focused ultrasound, and irreversible electroporation. After a description of the particularities of the tumoral microenvironment of pancreatic cancer, the effects of these treatments on the tumoral microenvironment and implications for future management of patients are discussed.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common types of pancreatic cancer[1]. Given the rapidly increasing incidence of PDAC in recent decades, it is expected to become the second leading cause of cancer death by 2030 in the United States and other industrialized countries[2]. The 5-year survival rate is approximately 8% following diagnosis[3], with only 20% of the patients eligible for surgery[4]. Among the patients who undergo surgery, the 5-year survival rate is only 20% due to rapid recurrence and metastasis development[5]. Systemic treatments are associated with various types of adverse events that result in a poor quality of life for patients[6] and are often inefficient due to the characteristics of the PDAC tumor microenvironment (TME), which protects tumor cells from chemotherapies and immunotherapies. The TME is increasingly being considered a potential target of choice to improve outcomes for PDAC. Locoregional treatments, such as hyperthermia (HT) with microwave ablation or radiofrequency ablation (RFA), radiation therapy (RT), irreversible electroporation (IRE), and high-intensity focused ultrasound (HIFU) therapy, are gaining increasing attention for their ability to specifically target the tumor while limiting deleterious systemic adverse events and may often be used in combination with anticancerous drugs. Among other effects, locoregional therapies can induce changes in the structure, components, and properties of the TME that may help alleviate some of the barriers to successful treatment. In this review, we will summarize the characteristics of the TME in PDAC and describe the effects of locoregional therapies on vasculature, stroma, and immune response. We will pay special attention on characterizing the TME for clinical applications (Table 1).

PDAC MICROENVIRONMENT

Although most solid tumors develop specific interactions with their host *via* neoangiogenesis, the creation of a supporting network of cells and extracellular matrix, and some form of immunomodulation, PDAC remains one of the most stroma-rich cancers, with 90% of its tumor mass composed of fibroblasts and their products[7]. The PDAC stroma is composed of an acellular and cellular compartment. The acellular compartment consists of an extracellular matrix rich in collagen, fibronectin, laminin, integrins, glycosaminoglycan, matrix metalloproteinase and secreted protein acids and is rich in cysteine[1,8] with cytokines and growth factors[9]. The cellular component is composed of pancreatic stellate cells and immune cells, such as macrophages, mast cells, lymphocytes, and plasma cells[1]. Pancreatic stellate cells, which are resident cells of the pancreas, acquire an activated myofibroblast-like phenotype when activated and are assumed to be the main regulators of TME extracellular matrix production. With the tumoral microvasculature, all these elements form the TME. Interactions between acellular and cellular components of the TME are key factors in PDAC progression[10]. In PDAC, the TME has been associated with tumoral progression, metastasis dissemination, and resistance to chemotherapy by various mechanisms[8]. The abundant extracellular matrix increases intratumoral interstitial pressure[11] and acts as a barrier for drug delivery with compression of blood vessels. Tumor stiffness can also have a direct effect on chemosensitivity: *in vitro*, mechanically compressed PDAC spheroids (displaying high stiffness) are less sensitive to gemcitabine than free spheroids (without any mechanical compression), whereas there is no difference in Hoechst dye penetration between compressed and free spheroids, suggesting a therapeutic effect of compression independent of the sole

Table 1 Locoregional therapies and their main effects on the tumoral microenvironment of pancreatic ductal adenocarcinoma

	Vasculature	Stroma	Immune response
Hyperthermia	Increased blood flow and vascular permeability. Recruitment of bradykinin and histamin. Increased iNOS.	Destructuration of collagen fibers. Reduction of CAF. Reduction of tumor stiffness.	Promotes APC activation. Increased infiltrating CD8+. Increased pro-inflammatory cytokines. Abscopal effect (RFA).
Radiation therapy	Reduced blood perfusion. Destructuration of microvessels with thickening vessel walls. Platelet aggregation. Microthrombus formation. Increased HIF-1 and VEGF. Increased vascular permeability.	Accumulation of extracellular matrix proteins. Increased stromal cells (fibroblasts). Thickened and stiffened tissue. Loss of hyaluronic acid. Collagen remodeling. Modification of CAF population.	Release of tumor antigens (DAMPs) ≥ APC presentation and CD8+ activation. Increased peptide availability and T cell repertoire. Release of inflammatory cytokines, CD8+, and CD4+ cells. Increased adhesion molecules (VCAM-1, ICAM-1). T cells homing. Increased PDL-1.
HIFU (mechanical effect)	Reduced blood perfusion and microvascular density.	Disruption of the collagen matrix.	Released DAMPs ≥ T cell activation. Induction of Th1 inflammation. Increased CD8+/Treg ratio.

iNOS: Inducible nitric oxide synthase; CAF: Carcinoma-associated fibroblasts; APC: Antigen presenting cell; RFA: Radiofrequency ablation; DAMPs: Damage-associated molecular patterns; HIFU: High-intensity focused ultrasound.

penetration of gemcitabine into the spheroid cells[12]. One hypothesis to explain this phenomenon is that mechanical stress decreases cell proliferation, which may alter the efficacy of chemotherapies targeting proliferating cells[12]. Intratumoral stiffness itself can modify intracellular signaling pathways and promote epithelial-mesenchymal transition, leading to tumoral progression and chemoresistance [13]. In PDAC, the microvascular density is generally low and leaky. Combined with mechanical forces caused by the dense stroma and tumoral growth that compresses the vessels, limited perfusion can result, which is responsible for hypoxia and low nutrient availability along with low anticancer drug delivery[1,14]. On the immune response side, the TME in PDAC is characterized by a reduced number of cytotoxic T cells, along with an increase in M2 macrophages, N2 neutrophils, and T-regulatory cells at the tumor site, which all contribute to an immunosuppressive environment[15].

Current studies suggest that the TME is an attractive target in the management of PDAC. Provenzano *et al*[11] showed that the administration of an enzymatic agent allowing the deletion of stromal hyaluronic acid (PEGPH20) in a murine model of PDAC led to normalized intratumoral interstitial pressure, expansion of the tumoral microvasculature, and increased survival *in vivo* when combined with gemcitabine compared to gemcitabine alone. However, caution should be taken since other studies have shown that the presence of some TME elements serves to prevent cancer progression and should therefore not be suppressed indistinctively[16,17]. In a mouse model of PDAC, as well as in patients with pancreatic cancer, a decreased bulk of aSMA + myofibroblasts was associated with poor prognosis and reduced overall survival[17].

HT

HT is a therapeutic procedure used to increase the intratumoral temperature. There are various ways to increase temperature: for superficial tumors, local HT can be applied by means of antennas or applicators that emit microwaves (microwave ablation) or radio waves (radiofrequency ablation or RFA) placed at the surface of the tumor with an intervening medium. Interstitial and endocavitary HT are used for small tumors in which an intratumoral implantation of the antenna can deliver various types of waves: microwaves, radiofrequency, ultrasound, heat sources, or laser fibers. Regional HT and partial body HT are more suitable for deep seated tumors, such as PDAC, that can be heated by antennas placed in rings around the patient. Whole-body HT is dedicated to the treatment of metastatic tumors[18]. HT can be generated by an external source, such as hot air or infrared radiation, or an internal source, such as magnetic nanoparticles, which can be deposited in the region of interest and then exposed to a magnetic field, leading to an increase in temperature and allowing localized heating [magnetic hyperthermia (MHT)][14]. Nanoparticles can also be used for photothermal therapy (PTT), during which a laser can activate nanophotoabsorbers. MHT and PTT are both nanoparticle-based HT treatments[19,20].

Hyperthermia and tumoral vasculature

HT is thought to increase blood flow and vascular permeability with an increase in antitumor effects. A study by Miyamoto *et al*[21] evaluated the effects of mild HT on the efficacy and accumulation of an anti-EGFR agent (cetuximab) in various xenograft mouse models of pancreatic cancer. Using a water bath, a temperature of 37 °C or 41 °C was applied to the tumor and allowed to decrease the tumoral volume compared to mice exposed to a standard ambient temperature of 25 °C (control). This was accompanied by an increase in cetuximab accumulation in cancer cells by 2.5- to 5-fold, depending on

the model studied. Of note, this effect was also observed when stromal dense tissue derived from surgically resected pancreatic cancer was transplanted into mice. This effect may be due to an increase in blood flow with an increase in tumor vessel permeability[22]. Indeed, another study showed that mild HT in a mouse model of pancreatic cancer could induce an 11-fold increase in blood perfusion in a reversible manner during heating and a 3-fold increase after the end of HT treatment along with an increase in vasculature permeability and an enhanced extravasation of macromolecules[23]. In a xenograft mouse model of breast cancer, non invasive radiofrequency (RF) produced increased transport and perfusion of fluorescent tracers into the tumors at temperatures below 41°C, whereas vessel deformation and blood coagulation were observed when the temperature reached 44°C[24]. The mechanism through which HT increases blood perfusion may be linked to a relaxation of smooth muscle following an increase in nitric oxide synthetized by endothelial cells. In a study by Song *et al* [25], the content of inducible nitric oxide synthase (iNOS) was evaluated in a murine model of fibrosarcoma following HT. No iNOS was detectable before HT treatment, while an increase in iNOS was observed 3 h after HT and remained detectable 24 h after treatment. Additionally, HT increases the recruitment of bradykinin and histamine molecules responsible for vessel dilation and the recruitment of capillaries[26].

Hyperthermia and stromal architecture

HT has been shown to disrupt the stromal architecture. In a study by Piehler *et al*[14], Achilles tendons (mainly composed of type I collagen) were exposed to various regimens of either extrinsic or ion-oxide nanoparticle-based MHT. The amount of intact collagen fibers decreased with the application of HT, with only 10% of collagen fibers intact after 1 h of the 42 °C regimen and almost complete degradation after 1 h of the 50 °C or 70 °C regimen. Mild HT applied to spheroids of pancreatic cancer cells (Panc1) and fibroblasts (WI 38) significantly decreased the amount of intact collagen fibers, with a coinciding decrease in spheroid volume and cell viability by apoptotic and necrotic processes[14]. Local HT through the use of a photothermal agent combined with a photothermal-chemotherapeutic agent (Abraxane@$Mose_2$) and subsequently irradiated by a laser beam could disrupt tissue architecture and reduce the number of carcinoma-associated fibroblasts (CAFs), subsequently enhancing the efficacy of Abraxane in a mouse model of PDAC[27]. Similarly, in a mouse model of cholangiocarcinoma, a nanoheater used for PTT (multifunctional iron oxide nanoflowers decorated with gold particles) with high uptake by CAFs produced a significant depletion of CAFs as well as a reduction in tumor stiffness followed by significant tumor regression[28]. In another study by Marangon *et al*[29], tumor stiffness was monitored following PTT in squamous cell carcinoma in mice. Shear wave elastrography revealed a transient and reversible increase in tumor stiffness after thermal ablation or mild HT, followed by a return to its initial value within 24 h of laser exposure in the case of thermal ablation or a reduced level for mild HT. Additionally, while increased tumor stiffness was observed in untreated mice, the stiffness in the treated group was stable over time. In the same study, second harmonic generation was used to evaluate the effect of PTT on collagen structure and revealed a destructuration of collagen fibers in the vicinity of heated carbon nanotubes.

In several mouse models of pancreatic cancer displaying various levels of stroma formation, the concomitant application of mild hyperthermia and cetuximab induced a more significant antitumoral effect on stroma-rich models[21]. *In vitro*, noninvasive RF has been shown to affect molecular transport in a 3D model of PDAC with increased diffusion of DAPI fluorescence in spheroids following RF compared to no treatment[30]. In a xenograft mouse model of squamous cell carcinoma, the combination of MHT and doxorubicin demonstrated a more efficient reduction in tumor growth than doxorubicin alone[31]. In the same study, the space between collagen fibers was determined following MHT: while there were no differences between the control group and the group injected with nanocubes without exposure to a magnetic field (no HT), there was an increase in the interfibrillar space between the group injected and exposed to the magnetic field compared to the injected group without exposure to the magnetic field[31].

Hyperthermia and immune response

Data on the effect of HT on the immune response in PDAC are scarce, but the literature is abundant for other types of cancer. HT promotes antigen presenting cell (APC) activation and antigen-specific naïve CD8+ T cell differentiation, allows CD4+ T cells to shift towards the Th1 phenotype, and transforms regulatory T cells (Tregs) into Th17 cells[32]. In a mouse model of PDAC, RFA induced an increase in infiltrating CD8+ T cells and a decrease in Treg cells but showed no difference in the proportion of infiltrating CD4+ lymphocytes[33]. HT has been shown to induce chemokine production, such as CCL21, combined with adhesion factors (selectin, integrin, ICAM-1), thus allowing an increase in the interactions between lymphocytes and endothelial cells and the homing of lymphocytes[34-37]. In parallel, HT induces the production of various proinflammatory cytokines, such as IL6[38]. HT induces immunogenic cell death through various mechanisms[32], such as triggering DNA damage that produces mutations in tumor cell genes, which generate neoantigens that stimulate the T cell-based immune response[32,39]. HT can also generate damage-associated molecular patterns (DAMPs), of which heat shock proteins (HSP) are the most important but also include molecules such as calreticulin, HMGB1 or ATP. HSPs are chaperones that participate in the presentation of the chaperoned antigen to

the MHC-1 complex of dendritic cells, thus allowing antigen-specific T-cell activation[34,35,40]. High levels of HSP are associated with poor prognosis in parallel to an enhanced immune response[32]. Membrane HSP has been found to be a tumor-specific target for natural killer cells, whereas extracellular HSP can be considered a potent adjuvant to facilitate tumor antigen presentation and the induction of antitumor immunity[32,41,42]. More specifically, HSP70 has been shown to induce tumor cell proliferation in a mouse model of PDAC by activating AKT-mTOR signaling[33]. HSP60 has been shown to induce IFNg secretion and T cell upregulation[43]. In a murine model of PDAC, a study by Lin *et al*[44] found that the maximum HSP synthesis was achieved at 43 °C, corresponding with an increased antitumor immune response. Beyond this temperature, both the release of HSP and the associated immune response decreased[34,44]. The accumulation of neoantigens, secondary to mutations and DAMPs, favors the activation of dendritic cells, allowing the transformation of the tumoral immunosuppressive microenvironment by inhibiting Treg cells and promoting tumor-infiltrating lymphocyte maturation[32].

The so-called "abscopal effect" has often been invoked to suggest that an immunomodulating mechanism had to take place when the local treatment of a malignant tumor - most commonly the use of RT - results in a response at a distant location[45,46]. In a PDAC mouse model implanted with tumors on both flanks, Fei *et al*[47] tried to determine whether RFA on one flank's tumor could affect the untreated tumor located on the other side. After RFA on one side, the immune response on the opposite side showed an increase in CD8+/PD-1+ T cells, along with suppression of immunosuppressive components of the tumor microenvironment (*i.e.*, Tregs, tumor-associated macrophages, and tumor-associated neutrophils). Additionally, immune checkpoints such as PD-1 and LAG3 were upregulated in distant (untreated) T cells after one-sided RFA. Similarly, Gameiro *et al*[48] found that RFA induced local immunogenic modulation at the tumor surface in a model of colon adenocarcinoma, and the combination of RFA with vaccine therapy eradicated both primary and secondary tumors. Finally, in a clinical study for 10 patients with locally advanced pancreatic cancer (LAPC) that evaluated the immune response following coagulation necrosis-inducing RFA ablation, an increase in CD4+, CD8+, and effector memory T cells along with IL 6 was seen[49].

RT

RT uses an ionizing radiation beam (X-rays) whose energy is deposited in water along its path, leading to the formation of free radicals (reactive oxygen species or reactive nitrogen species) that oxidize molecular targets, provoking a dysregulation of cellular functions. These free radicals target DNA, leading to single- or double-strand breaks[50]. Today, there is no consensus on the role of RT in PDAC. The LPA07 trial did not show any improvement to the tumor in a small number of fractions to minimize the impact on the surrounding organs.

Radiation therapy and tumor vasculature

The effect of RT on tumor vasculature has been widely explored in various types of cancers. RT has been shown to have many direct or indirect effects on endothelial cells[50], and these effects are dependent on the dose received and the radiation schedule[51]. High single doses of radiation have been shown to cause vascular damage with reduced blood perfusion and hypoxia[52]. RT induces changes in tumor vasculature by destructuring microvessels and thickening vessel walls, thus reducing vessel lumen, all of which favor atherosclerosis. RT also induces platelet aggregation and microthrombus formation with an increase in inflammatory cell adhesion to endothelial cells[53]. RT can regulate and stabilize the level of HIF-1, leading to the production of VEGF, which is responsible for endothelial cell proliferation and increased survival. RT can directly upregulate the expression of a_vb_3 integrins[54] and adhesion proteins [50]. In pancreatic cells, HIF-1 has been shown to induce the sonic hedgehog protein, leading to the formation of a stroma-rich microenvironment[55]. In a rodent model of pancreatic tumor, a single high dose (SHD) of radiation led to temporary vascular dysfunction along with enhanced expression of HIF-1, which could be restored after 14 d. However, vascular permeability was higher in irradiated tumors 14 d after RT[56]. Similarly, a study by Lee *et al*[57] evaluated the effect of an SHD of radiation *vs* a fractionated regimen of radiation, which showed increased perfusion ability of tumor vessels following SHD, whereas fractionated RT had no effect. Mechanisms were further studied and showed that vessels treated with SHD-RT had lower pericyte coverage; increased vessel perfusion could therefore be due to an increased leakage of immature vessels, and the surviving vessels after SHD-RT might favor the penetration of small molecule drugs.

RT and stroma

RT induces chronic inflammation, leading to fibrosis through the accumulation of extracellular matrix proteins and an increase in stromal cells such as fibroblasts[58], which thicken and stiffen the tissue[51, 59]. Fibrosis formation depends on the dose of radiation received. For example, in a 3D model of mammary cancer stroma, increasing RT doses resulted in a reduction in fibroblast proliferation and activation along with a modest increase in matrix stiffness[60]. RT induces a loss of hyaluronic acid

along with a remodeling of collagen and a modification of CAF population[50]. Protease activity is also altered with an upregulation of MMP2[61], which is responsible for an increase in tumor invasiveness [58]. *In vitro* studies showed that human lung fibroblasts develop an irreversible senescent phenotype after exposure to a radiation dose higher than 10 Gy, while lower doses induced reversible DNA damage without growth arrest[51,62,63]. Senescent fibroblasts can release proteolytic enzymes, cytokines, growth factors, and ROS, creating a protumorogenic environment[49,57,64]. Similarly, *in vitro*, the coculture of ionizing radiation-exposed CAFs with pancreatic cancer cells enhanced the invasion-promoting capacity of CAFs, induced a high secretion of CXCL12 (a chemokine implicated in hematopoietic stem cell maintenance and cell migration) by CAFs, and promoted pancreatic cell migration, invasion, and epithelial-mesenchymal transition[65].

RT and immune response

RT has been shown to modulate the immune response by various mechanisms, the first of which is the release of tumor antigens, whereby DAMPs allow APC presentation and CD8+ activation followed by cell death, called immunogenic cell death. RT can also increase peptide availability and activate mTOR, leading to an increase in the MHC-1 protein subunit and an increase in the T cell repertoire[66]. RT induces the release of inflammatory cytokines such as IFN *via* the cGAS-STING pathway, which is activated by DNA damage caused by RT[67]. Adhesion molecules are also upregulated, with an increase in VCAM-1 and ICAM-1 leading to increased infiltration of lymphocytes to tumor cells and affinity binding to CD3+ cells[68]. Finally, RT facilitates homing of T cells to the TME by upregulating chemokines such as CXCL16[69]. In a murine orthotopic pancreatic cancer model, irradiated tumors displayed increased CD8+ and CD4+ cells, with a high single dose of RT being more efficient in recruiting CD8+ T lymphocytes than fractionated RT. However, fractionated RT induced more infiltration of myeloid-derived suppressor cells than high-dose RT[57]. *In vitro*, RT increased the expression of PDL-1 in a Jack/stat1-dependent manner[70]. Evidence to date suggests that immunotherapy such as anti-CTLA-4 or anti-PD-1 in PDAC has disappointing results or displays efficacy only in patients with PDAC who test positive for mismatch repair deficiency or microsatellite instability-high (MSI-h) due to the poorly immunogenic nature of PDAC[71-74]. Some data suggest that the combination of RT with immunotherapy could be a future approach to overcome this limitation. In a study by Lee *et al*[57], the combination of SHD-RT with anti-PD1 increased the delivery of anti-PD1 in a murine orthotopic mouse model of PDAC (UN-KC-6141), which is consistent with the increased tumor perfusion observed *in vivo* following RT. The survival of mice receiving a combined treatment of anti-PD1/SHD-RT was significantly improved compared to that of mice receiving anti-PD1 or SHD-RT alone. Splenocytes isolated from mice treated with the combination therapy showed increased cytotoxicity specifically toward UN-KC-6141 cells. In addition, while the combined group was free of peritoneal tumors, all of the control, SHD-RT, and anti-PDL1 alone groups bore metastases. This encouraging result is in accordance with another *in vivo* PDAC mouse model study by Fujiwara *et al*[75] reporting increased survival following a combination of anti-PD1 therapy and RT.

HIFU

HIFU is a noninvasive therapeutic technique using a focused ultrasound beam to create either thermal effects or a mechanical effect called cavitation at the focal point. With respect to thermal effects, due to the focal concentration of energy delivery, HIFU is capable of producing rapid coagulation necrosis with limited inflammatory response and minimal damage to the TME outside the focal zone, inside of which the TME is destroyed. Otherwise, the effects of HT on the TME have been described above in a dedicated section. With respect to the mechanical effect of HIFU, acoustic cavitation can be defined as the initiation, growth, oscillation, and collapse of gas bubbles inside a medium due to high tensile acoustic pressures that exceed cohesion forces between molecules. When exposed to an acoustic field, a bubble will oscillate radially (regime of stable cavitation) and possibly collapse (regime of inertial cavitation). At the tissue level, stable cavitation can stretch tight junctions and allow the extravasation of molecules from the vascular to interstitial space, making the plasma cell membrane transiently permeable and allowing for the internalization of molecules. Comparatively, inertial cavitation is more violent and may induce irreversible membrane disruption and cell implosion or hemorrhage in tissues [76]. HIFU is regularly used in prostate cancer or in the management of uterine fibroids[77,78] but also in the management of PDAC, although it is much less common. HIFU has been suggested to improve quality of life and alleviate pain in patients with a metastatic course of their disease[79].

To date, few preclinical studies have evaluated cavitation as a potentiator of chemotherapy with promising results. A previous study from our group evaluated the impact of various inertial cavitation intensities combined with gemcitabine on the viability of PDAC spheroids composed of both KPC pancreatic cancer cells and activated fibroblasts designed to mimic the tumor stroma[80]. Even if this model was far from a PDAC tumor, it possessed some of its essential features, including the presence of activated fibroblasts, the production of extracellular matrix and a dense intercellular arrangement. This work demonstrated that inertial cavitation decreased the viability of spheroids exposed to cavitation

and gemcitabine compared to either cavitation alone or gemcitabine alone. Moreover, gemcitabine had no impact on fibroblast viability, whereas the effect of chemotherapy on the viability of PDAC cells was enhanced when combined with cavitation. Of note, the effects of gemcitabine toxicity were less evident in spheroids composed of both KPC cells and fibroblasts compared to those composed of KPC cells only, which is consistent with the protective effect of the TME and supports the benefit of the combination[80].

In 2015, Li *et al*[81]. showed in KPC mice that cavitation with pulsed HIFU enhanced the intratumoral concentration of doxorubicin by 4.5-fold compared to controls, with an increase in doxorubicin concentration when cavitation was high and sustained. Of note, there were no differences when pulsed HIFU was delivered during or before doxorubicin administration. On the pulsed HIFU-treated tumors, macroscopic evaluation revealed hemorrhagic areas, while microscopic evaluation showed disorientation and separation of the collagen matrix with fraying of collagen fibrils. A study by Huang *et al*[82] evaluated the impact of cavitation induced with an ultrasound contrast agent (microbubble) in a mouse model of pancreatic cancer. Blood perfusion evaluated by contrast-enhanced ultrasound imaging revealed a decrease in blood flow within the tumor after cavitation treatment compared to pretreatment measurements, whereas blood perfusion of nontumoral tissue was not impacted. Immunostaining of blood vessels also showed decreased expression of CD31 and reduced microvascular density in the cavitation group.

On the immunotherapy side, the mechanical effects of HIFU have been shown to induce subcellular fragmentation, leading to the release of DAMPs that are subsequently presented to dendritic cells[83] and trigger cytotoxic T cell activation[84]. Pulsed HIFU or low-intensity HIFU have been shown to drive Th1 inflammation, to stimulate localized cell recruitment factors and tumor cell surface immunogenic proteins, and increase the CD8+/Treg ratio[85]. However, these data come from non-PDAC tumor types.

IRREVERSIBLE ELECTROPORATION

IRE is a nonthermal ablative therapy using a direct high voltage current with a short pulse length to increase cell membrane permeability, resulting in permanent cell death with minimal thermal deposition[86-88]. IRE is applied by placing two or more electrodes in the tumor or around it[89] and can be used intraoperatively, laparoscopically, or percutaneously. IRE induces damage only to the cell membrane and has no effect on protein denaturation, blood flow, and connective tissue[88] and was first described for the treatment of human pancreatic cancer in 2012[90].

Studies on the specific effects of IRE on the PDAC stroma are scarce. One study by Bhutiani *et al*[91] described an increase in gemcitabine delivery to the tissue located in the electroporation area in mice treated by IRE compared to untreated mice. Even if mechanistic explanations were not explored, this effect may be attributable to IRE-related alteration of the stroma. TME modulation following IRE is also characterized by a transient increase in microvascular density and an increase in tumor blood vessel permeability along with a softening of the extracellular matrix can lead to an increase in T cell infiltration[92]. IRE can induce microvessel endothelial cell apoptosis with microvessel thrombosis *in vivo*[93]. In a xenograft mouse model of PDAC, alterations of tumor microstructure were described following IRE in which acute coagulative necrosis and thrombosis were visible throughout the treated tumor volume after IRE, whereas minimal thrombosis was observed in the control group (no treatment). Using transmission electron microscopy, microvessel endothelial apoptosis and microvessel thrombosis were visualized, and magnetic resonance imaging (MRI) analysis revealed a significant increase in water diffusion after IRE, with a reduction in diffusion-weighted MRI images reflecting an increase in diffusion (water mobility) in the tissue after IRE[93].

From an immunologic point of view, available data are limited: a mouse study by Yang *et al*[94] described an increase in calreticulin after IRE, suggesting an induction of immunogenic cell death, with an increase in the intratumoral expression of CD8+ cells and GrB (granules of enzymes expressed by cytotoxic lymphocytes) when IRE was combined with a dendritic cell vaccine. In the same study, stromal fibrosis formation was not modified following IRE. In another study by White *et al*[95], IRE was found to induce an increase in macrophage, T cell, and neutrophil infiltration within the tumor.

CLINICAL PERSPECTIVES

Hyperthermia

In accordance with mouse studies showing an increase in drug delivery with hyperthermia, a recent systematic review evaluated the clinical benefit of HT (regional, intraoperative, or whole-body HT) combined with chemotherapy, RT or both in 248 patients. Out of 14 studies, 6 showed a longer median overall survival in the HT group compared to the control group, with an 11.7 mo median survival *vs* 5.6 mo. The response rate was also higher in the HT groups[96]. These encouraging results have prompted

randomized clinical trials to more clearly demonstrate any benefit of this therapeutic approach. A phase II study (HEATPAC-NCT02439593) is currently recruiting to compare deep locoregional HT administered with a microwave system (Aim 40-43 °C for 60 min) with chemotherapy *vs* chemotherapy alone in LAPC. The results from this study could provide a practical assessment of the efficacy of HT in PDAC[97]. Other current studies are summarized in Table 2.

RFA in PDAC has been reported in small exploratory series for tumor debulking rather than for complete tumor ablation because safety margins are needed to avoid thermal damage to surrounding structures[98]. Following RFA combined with chemotherapy, overall survival ranges from 19 to 25.6 mo [98,99]. There is a lack of randomized studies assessing the place of RFA in the management of LAPC. The PELICAN trial (NCT03690323) is planned to evaluate whether the combination of chemotherapy and RFA improves overall survival compared to chemotherapy alone in patients with LAPC without any progression after 2 mo of systemic treatment[100] (Table 2).

Endoscopic application of RFA, which is already feasible, is an attractive approach because of its minimal invasiveness. The active component is a 19G needle that has a tip equipped with an electrode to be placed in the lesion under endoscopic ultrasound (EUS) guidance. This approach has been proven safe and feasible in small-sized studies of patients with unresectable PDAC[101-103]. Nevertheless, larger prospective studies are needed. EUS-RFA clinical trials are ongoing (Table 2), and it would be interesting to evaluate EUS-RFA as an alternative to RT in LAPC patients with an objective response to chemotherapy who retain criteria against surgical resection.

RT

Stereotactic body radiotherapy (SBRT) is increasingly being explored for the management of PDAC in combination with anticancerous drugs, especially for LAPC. An open-label phase 2 multicenter study by Herman *et al*. evaluated the combination of gemcitabine plus SBRT in patients with LAPC, showing a good safety profile[104]. Similarly, 39 patients who underwent FOLFIRINOX followed by SBRT seemed to have an increased chance of undergoing radical surgery[105]. SBRT in combination with immune therapy is also being studied. In a phase I study by Xie *et al*[106], a combination of immune therapy (durvalumab ± tremelimumab) with SBRT in metastatic PDAC showed a favorable safety profile but only a modest clinical efficacy. Of note, none of the responders were MSI-h. While these results are interesting, further exploration is required, and many clinical trials are underway to evaluate the combination of SBRT or RT with anticancerous drugs (Table 2). A challenge for the use of SBRT is the required placement of fiducials to facilitate the delivery of radiation, which can be made difficult by respiratory movements and the vicinity of other organs[107]. These fiducials can be placed percutaneously when not impeded by surrounding organs or in a more invasive fashion, surgically. EUS-guided placement of fiducial also appears to be a promising method with a high rate of technical success and a reasonable rate of adverse events[91,108], but randomized studies are needed.

HIFU

Cavitation generated by HIFU is a very attractive method with a high potential to disrupt the stroma, thus overcoming the barrier to efficient drug delivery and stimulating the immune response in preclinical works. To date, there are no published clinical trials. However, one upcoming clinical trial (NCT04146441) of HIFU combined with chemotherapy (FOLFIRINOX) will determine whether focused ultrasound can increase drug uptake and overcome chemoresistance (Table 2). In a minimalist approach of ultrasound-induced enhancement of chemotherapy, 10 patients were enrolled in a phase I clinical trial to receive gemcitabine combined with low intensity ultrasound and microbubbles as an ultrasound contrast agent programmed to favor sonoporation, with encouraging results in terms of the number of chemotherapy cycles tolerated and median overall survival when compared to 63 historical controls receiving only chemotherapy[109].

HIFU is also a very attractive approach to increase the intratumoral temperature and increase drug delivery. In a monocentric retrospective study among 523 patients, a combination of HIFU with gemcitabine appeared to produce better overall survival than standard CT in unresectable PDAC[110]. The PanDox study is a phase I study that plans to evaluate whether HIFU can increase the amount of drug delivery (doxorubicin or heat-sensitive doxorubicin) within the tumor in 18 patients with unresectable PDAC (NCT04852367, Table 2).

Challenges in the method of ultrasound delivery still need to be addressed, since extracorporeal delivery to the deeply seated pancreas with gas interposition could be challenging. We are currently working on an endoscopic device that could overcome these limitations and noninvasively deliver cavitation at any part of the pancreas. The endoscopic approach to HIFU delivery, foreseen by our team some time ago[111], has also been recently studied in a porcine model[112].

Irreversible electroporation

After the landmark study by Martin *et al*[90] and subsequent large series of intraoperative applications [86], less invasive percutaneous IRE has shown promising results in terms of efficacy. A nonrandomized prospective single-center case series by Ma *et al*[113] evaluated the efficacy of a combination of percutaneous IRE with gemcitabine compared with gemcitabine alone. The combination increased the

Table 2 Ongoing studies in locoregional therapies used alone or in combination with chemotherapy or immunotherapy for pancreatic ductal adenocarcinoma

	Clinical trial number	Study name	Number of patients	Status	Country
Hyperthermia	NCT04858009	Hyperthermic intraperitoneal chemotherapy for the treatment of pancreatic cancer and peritoneal metastasis.	40	Not yet recruiting	United States
	NCT04889742	HT enhanced reirradiation of loco-regional recurrent tumors (HETERERO).	100	Recruiting	Germany
	NCT04310111	EUS-RFA for unresectable pancreatic cancer.	18	Recruiting	China
	NCT03218345	EUS-guided RFA for pancreatic neoplasms.	30	Recruiting	China
	NCT02439593	Concurrent HT and chemoradiotherapy in LAPC: phase II study (HEATPAC).	78	Recruiting	Switzerland
	NCT04164992	EUS-RFA of not-resectable pancreatic cancer.	15	Not yet recruiting	Italy
	NCT03690323	Pancreatic locally advanced irresectable cancer ablation (PELICAN).	228	Recruiting	Netherlands
	NCT04156087	Progression-free survival after microwave ablation plus durvalumab and tremelimumab for unresectable LAPC (MIMIPAC).	20	Recruiting	Belgium
Radiation therapy	NCT04361162	Nivolumab + ipilimumab + radiation in microsatellite stable pancreatic cancer.	30	Recruiting	United States
	NCT01972919	Magnetic resonance guided, dose-escalated RT + chemotherapy in pancreatic cancer.	23	Recruiting	United States
	NCT03374293	Combination of radiation therapy and anti-PD-1 antibody in treating patients with pancreatic cancer.	21	Recruiting	China
	NCT03492671	Testing the combination of two approved chemotherapy drugs and radiation prior to surgery in localized pancreatic cancer.	30	Recruiting	United States
	NCT04975516	Standard of care chemotherapy with or without SBRT for the treatment of oligometastatic pancreatic cancer.	50	Not yet recruiting	United States
	NCT04327986	Immune checkpoint inhibitor m7824 and the immunocytokine m9241 in combination with SBRT in adults with advanced pancreas cancer.	52	Recruiting	United States
	NCT03991962	Phase II study to evaluate modified folfirinox and SBRT in nonmetastatic unresectable pancreatic adenocarcinoma.	28	Recruiting	United States
	NCT02128100	Effects of folfirinox and SBRT for advanced pancreatic cancer.	28	Recruiting	United States
	NCT04089150	Mfolfirinox and SBRT for pancreatic cancer with high risk and locally advanced disease.	120	Not yet recruiting	Australia
	NCT04172532	Testing the addition of a new anticancer drug, m3814 (peposertib), to radiation therapy for localized pancreatic cancer.	24	Recruiting	United States
HIFU	NCT04146441	Ultrasound-enhanced uptake of chemotherapy in patients with inoperable PDAC.	30	Recruiting	Norway
	NCT04852367	PanDox: targeted doxorubicin in pancreatic tumors.	18	Not yet recruiting	England
Irreversible electroporation	NCT03257150	A study of the use of IRE in pancreatic ductal cancer.	47	Recruiting	Canada
	NCT03105921	IRE (nanoknife) for the treatment of pancreatic adenocarcinoma.	20	Recruiting	France
	NCT03899636	A pivotal study of safety and effectiveness of nanoknife IRE for stage 3 pancreatic cancer (direct).	528	Recruiting	United States
	NCT02822716	IRE for inoperable hepatic and pancreatic malignancy.	35	Recruiting	China
	NCT02343835	Antitumor immunity induced by IRE of unresectable pancreatic cancer.	20	Recruiting	China
	NCT03484299	Chemotherapy and IRE in the treatment of advanced pancreatic adenocarcinoma.	20	Recruiting	United States
	NCT04835402	Electroporation potentiated immunotherapy in cancer (EPIC-1).	16	Recruiting	Denmark

NCT04276857	Systemic therapy with a loco-regional treatment in patients with LAPC (smart).	27	Not yet recruiting	Canada
NCT04212026	IRE followed by nivolumab in patients with metastatic pancreatic cancer.	15	Recruiting	Switzerland
NCT04612530	Panfire-3 trial: assessing safety and efficacy of IRE + nivolumab + CpG for metastatic pancreatic cancer.	18	Recruiting	Netherlands
NCT02041936	Outcomes of ablation of unresectable pancreatic cancer using the nanoknife IRE system.	12	Recruiting	United States
NCT04310553	An open-label, multicenter, prospective study of IRE (nanoknife) combined with RT and chemotherapy in patients with LAPC.	240	Recruiting	China
NCT04093141	Chemotherapy followed by IRE in patients with unresectable LAPC (chemofire-2).	30	Recruiting	Denmark
NCT03614910	Ablation of unresectable LAPC with IRE system.	30	Recruiting	United States

HT: Hyperthermia; EUS: Endoscopic ultrasound; RFA: Radiofrequency ablation; LAPC: Locally advanced pancreatic cancer; RT: Radiation therapy; SBRT: Stereotactic body radiation therapy; PDAC: Pancreatic ductal adenocarcinoma; IRE: Irreversible electrotherapy.

overall survival from the time of diagnosis by 3-fold and nearly doubled the progression-free survival. In a *post hoc* comparison of data derived from a prospective IRE-FOLFIRINOX cohort and a retrospective FOLFIRINOX-only cohort, van Veldhuisen *et al*[114] found that the combination (30 LAPC patients) increased the time to progression compared to standard therapy (22 patients). Lin *et al*[115] showed promising results with IRE combined with allogenic natural killer cell immunotherapy with an increase in progression-free survival and overall survival. However, a multicenter prospective study by Ruarus *et al*[116] (PANFIRE II) described a high rate of adverse events in patients undergoing percutaneous IRE, with 29 out of 50 participants experiencing adverse events, 21 of which were major, and 2 deaths, including one clearly related to IRE. Thus, this procedure can be considered a high-risk procedure that requires the selection of patients who will benefit the most from the treatment. This high rate of adverse events, along with a relative cumbersomeness to set up, has limited the spread of this technique. New application methods are needed to overcome these issues. Many clinical trials are ongoing to better understand the benefits of combining IRE with chemotherapy or immunotherapy (Table 2).

CONCLUSION

The TME is one of the major causes of therapeutic resistance in PDAC. Fibrosis-related stiffness, hypomicrovascular perfusion, and an immune suppressive microenvironment are, within the limits of current knowledge, key determinants of this resistance. While systemic chemotherapies and immunotherapies have disappointing results and are responsible for adverse events resulting in poor quality of life, locoregional therapies can specifically target the tumor area with limited effects on surrounding tissues but significant impacts on the TME. Local HT using RFA and radiotherapy using local SHD are the two main modalities currently holding promise for clinical efficacy, but IRE and focused ultrasound-derived cavitation are also gaining increasing attention as treatments for PDAC. These techniques influence the tumor stroma, microvasculature, and immune environment and response (Table 1). To date, most of the data are preclinical with some promising results. Clinical trials are underway (Table 2) and will allow the scientific community to have a more precise idea of the interest in using these treatment options alone or in combination with systemic therapies.

FOOTNOTES

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MINIREVIEWS

Management of incidentally discovered appendiceal neuroendocrine tumors after an appendicectomy

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Abstract

Appendiceal neuroendocrine tumors (aNETs) are an uncommon neoplasm that is relatively indolent in most cases. They are typically diagnosed in younger patients than other neuroendocrine tumors and are often an incidental finding after an

appendectomy. Although there are numerous clinical practice guidelines on management of aNETs, there is continues to be a dearth of evidence on optimal treatment. Management of these tumors is stratified according to risk of locoregional and distant metastasis. However, there is a lack of consensus regarding tumors that measure 1-2 cm. In these cases, some histopathological features such as size, tumor grade, presence of lymphovascular invasion, or mesoappendix infiltration must also be considered. Computed tomography or magnetic resonance imaging scans are recommended for evaluating the presence of additional disease, except in the case of tumors smaller than 1 cm without additional risk factors. Somatostatin receptor scintigraphy or positron emission tomography with computed tomography should be considered in cases with suspected residual or distant disease. The main point of controversy is the indication for performing a completion right hemicolectomy after an initial appendectomy, based on the risk of lymph node metastases. The main factor considered is tumor size and 2 cm is the most common threshold for indicating a colectomy. Other factors such as mesoappendix infiltration, lymphovascular invasion, or tumor grade may also be considered. On the other hand, potential complications, and decreased quality of life after a hemicolectomy as well as the lack of evidence on benefits in terms of survival must be taken into consideration. In this review, we present data regarding the current indications, outcomes, and benefits of a colectomy.

Key Words: Neuroendocrine tumors; Carcinoid tumor; Appendiceal neoplasms; Colectomy; Neoplasm grading; Treatment outcome

Core Tip: In this review, we analyze the features to consider when appendiceal neuroendocrine tumor is found after an appendectomy. We critically analyze the main indications for a completion right hemicolectomy and the risk-benefit ratio.

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INTRODUCTION

More than 80% of appendiceal neuroendocrine tumors (aNETs) are diagnosed incidentally in appendectomy specimens and are found in approximately 0.5% to 1% of all appendectomies[1]. These neoplasms have several characteristic features that differ from other gastroenteropancreatic neuroendocrine tumors (GEP-NETs). They usually progress indolently and are diagnosed in younger patients other NETs; the majority are detected in the third or fourth decade of life while other NETs are usually diagnosed close to the sixth decade of life[2-5]. Nevertheless, these tumors do occasionally have an aggressive course with liver and mesenteric lymph node metastasis (LNM)[6]. In a recent review of patients included in the Surveillance, Epidemiology, and End Results database, the five-year survival rates for aNETs are 97.4%, 88.6%, and 27.4% for localized, regional, and distant disease, respectively. Non-metastatic aNETs had the highest overall survival rate of all GEP-NETs[7].

Surgery is curative in most cases[3]. However, controversy arises when deciding whether an appendectomy alone is sufficient or whether the patient will achieve better outcomes with a completion right hemicolectomy (CRH) after an initial appendectomy. The main purpose of a CRH is to complete the regional lymph node dissection. These nodes are involved in 6% to 9% of cases[8]. Both the North American Neuroendocrine Tumor Society[9] and European Neuroendocrine Tumor Society (ENETS)[10] guidelines suggest a tailored approach to these patients based on features such as tumor size, margin status, mesoappendix infiltration, vascular and lymphatic invasion, and tumor grade. In tumors smaller than 1 cm, an appendectomy is indicated whereas in those larger than 2 cm, a CRH is recommended. In tumors between 1 and 2 cm, CRH should be considered when there are affected margins in tumors located at the base, when there is invasion of the mesoappendix that measures greater than 3 mm, or when there are other risk factors. However, several studies have challenged these recommendations, mainly in tumors smaller than 2 cm, arguing that CRH offers no benefits in terms of survival in tumors smaller than 2 cm[8,11]. Furthermore, in addition to the potential postoperative complications, a colectomy could lead to a poorer quality of life for these patients.

This narrative review critically evaluates the management of these patients based on evidence in the current literature with a special focus on the indications for and outcomes of a CRH.

WHAT ARE THE FEATURES ANALYZED IN THE HISTOPATHOLOGICAL DIAGNOSIS?

A careful histopathological evaluation of the surgical specimen will provide crucial information for determining management. aNETs are epithelial neoplasms that likely arise from neuroendocrine cells, including enterochromaffin cell neuroendocrine tumors, L-cell NETs, and tubular NETs. Their pathogenesis is largely unknown. Their hypothetical origins include neuroendocrine cells within the mucosal crypts or subepithelial neuroendocrine cells, especially in enterochromaffin cell NETs[12,13].

Appendiceal neoplasms include well-differentiated aNETs (classified as low grade-G1, intermediate grade-G2, or high grade-G3 according to proliferative rate), poorly differentiated neuroendocrine carcinoma, and mixed neuroendocrine-non neuroendocrine neoplasms (MINEN). Between 70% to 75% of neuroendocrine neoplasms in the appendix are well-differentiated NETs. Goblet cell adenocarcinoma is no longer considered a subtype of aNET[13,14]. Goblet cell adenocarcinoma and MINEN will not be discussed further in this review.

Macroscopically, most of these cases are found incidentally on the tip of the appendix after an appendectomy for acute appendicitis. They are usually yellowish nodules and most are smaller than 2 cm in diameter (only 8% to 19% are larger than 2 cm)[15,16].

Microscopically, well-differentiated aNETs include:

Enterochromaffin cell appendiceal neuroendocrine tumors (EC-NETs): This is the most common subtype. EC-NETs usually appear as uniform polygonal cells arranged in nests or a glandular pattern with a fibrotic stromal response. Necrosis and mitosis are uncommon. Immunohistochemistry techniques demonstrate positivity to chromogranin A (CgA), synaptophysin, and serotonin production in EC cells[15,17] (Figure 1).

L-cell NETs: These tumors have trabecular or glandular growth patterns. L-cell NETs produce glucagon-like peptide 1 and proglucagon-derived peptides. L-cell NETs express chromogranin B rather than CgA[14].

Tubular NETs: These tumors represent < 10% of all aNETs[8] and must be distinguished from adenocarcinoma NOS and goblet cell adenocarcinoma[14].

Whether a tumor is an EC or L-cell NET is usually not specified on pathology reports as this distinction has no prognostic or therapeutic implications[14,18].

Poorly differentiated aNETs are rare and are microscopically similar to other intestinal neuroendocrine carcinomas[15,17,19].

The staging of aNETs is mainly based on tumor size and serosal or mesoappendix invasion. The pathology report should also include pTNM staging (according to either American Joint Committee on Cancer classification[20], ENETS classification[10], or both), margin status, and vascular and lymphatic vessel involvement (Table 1). Mesoappendix invasion is usually associated with a higher rate of vascular and lymphatic vessel involvement[10,15,17].

WHAT ADDITIONAL TESTS ARE RECOMMENDED IN CASES OF ANET?

After an incidental diagnosis of aNET, the main purpose of additional tests is to assess the presence of residual locoregional disease or distant metastasis in order to determine postoperative staging.

Biochemical tests

There are no clear benefits to measuring any specific biomarker in aNETs[10]. Although CgA is a widely used biomarker for evaluating and following-up on GEP-NETs, its role in aNETs is unclear. CgA levels are usually within normal range in NETs with a low proliferative potential, which are the majority of aNETs[21]. It has been suggested that its measurement may only be useful in advanced cases[10]. Carcinoid syndrome is uncommon at the time of diagnosis[2], but when it is suspected, determination of urinary 5-hydroxyindoleacetic acid (5-HIAA) could be useful[10].

Endoscopy

The usefulness of performing an endoscopy after an incidental diagnosis of aNETs seems to be negligible unless the tumor infiltrates the cecum[10].

Conventional imaging tests

After a complete resection of an incidentally diagnosed well-differentiated aNET measuring < 1 cm, no further diagnostic testing is required. However, controversy remains regarding tumors that measure between 1 and 2 cm, since these tumors rarely have LNM. To evaluate the presence of lymphatic involvement or distant metastasis, an abdominal computed tomography (CT) or magnetic resonance

Table 1 TNM staging for appendiceal neuroendocrine tumor according to European Neuroendocrine Tumor Society and American Joint Committee on Cancer classification

	ENETS	AJCC
T0	No evidence of primary tumor	No evidence of primary tumor
T1	Tumor ≤ 1 cm with infiltration of submucosa and muscularis propia	
T1a		Tumor ≤ 1 cm
T1b		Tumor 1-2 cm
T2	Tumor ≤ 2 cm with infiltration of the submucosa, muscularis propia and/or minimal (≤ 3 mm) infiltration of the subserosa and/or mesoappendix	Tumor 2-4 cm or with extension into the cecum
T3	Tumor > 2 cm and/or extensive (> 3 mm) infiltration of the subserosa and/or mesoappendix	Tumor > 4 cm or with extension into the ileum
T4	Tumor with infiltration of the peritoneum and/or other neighboring organs	Tumor with perforation of the peritoneum or invasion of other adjacent structures
N0	No regional lymph node metastasis	No regional lymph node metastasis
N1	Locorregional lymph node metastasis	Locorregional lymph node metastasis
M0	No distant metastasis	No distant metastasis
M1	Distant metastasis	Distant metastasis

ENETS: European Neuroendocrine Tumor Society; AJCC: American Joint Committee on Cancer.

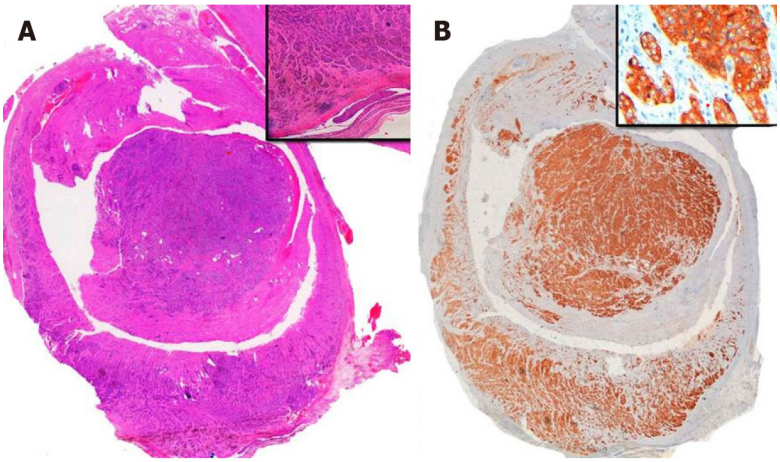

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Figure 1 Histological images. A: Well-differentiated aNET that infiltrate the entire wall of the appendix and focally infiltrate the adjacent fat, affecting the surgical margin; B: Immunohistochemical techniques reveal positivity for synaptophysin and CgA.

imaging (MRI) scan is recommended. These tests should also be considered for tumors > 2 cm or for those with mesoappendix infiltration or vascular and lymphatic vessel invasion. In these cases, somatostatin receptor scintigraphy (SRS) or a positron emission tomography with computed tomography (PET/CT) scan should be considered[10].

Nuclear medicine imaging

There are a multitude of articles on the applications of SRS in GEP-NETs, however, only sporadic cases of aNET have been described in this body of literature[22,23]. SRS using either indium-111 or technetium-99m (including single photon emission CT) or a PET scan using gallium-68-labeled somatostatin analogs (SSAs) in combination with a CT scan can be considered in cases in which curative resection is not completely assured or when distant metastasis is suspected[24]. In these cases, published studies suggest that SRS is useful for detecting residual disease[23,25] and that SRS results could modify management in 20% to 25% of patients[23,26], mainly for those with high proliferative activity.

Positron emitting radiopharmaceuticals such as gallium-68-labeled peptides or 18F-fluorodopamine are now preferred for the diagnosis of well-differentiated GEP-NETs, particularly those smaller than 1

cm[10]. These PET radiopharmaceuticals provide greater resolution, faster results, shorter imaging time, and 3D visualization. However, due to their cost and availability, their use is not yet widespread. 18F-2-fluoro-2-deoxy-glucose PET/CT is recommended for detecting poorly differentiated or heterogeneous NETs[27]. Furthermore, even though these radiopharmaceuticals have been widely studied in other GEP-NETs, to date no works have evaluated their usefulness specifically in aNETs.

INDICATIONS IN THE LITERATURE FOR A COMPLETION RIGHT COLECTOMY

Most cases of aNET are patients with a tumor < 1 cm in the distal third of the appendix. The presence of risk factors associated with more extensive disease, such as serosal or mesoappendix invasion, are usually associated with tumors greater than 2 cm[2].

After a CRH, residual disease or lymph node involvement is present in 0% to 40% of cases[2,8,28]. A recent review by Webb *et al*[11] of patients in the National Cancer Database treated with surgery found that, unlike other types of appendiceal neoplasms, in aNET, the presence of regional LNM does not reduce overall survival. Since most studies supporting the indication of a CRH are based on completing a lymph node dissection, the claim that this procedure improves patient survival is highly questionable.

The main indications for CRH are based on the following evidence:

Tumor size: Some recent studies suggest that size is the main factor related to the occurrence of LNM [1,8,15,29,30]. As stated above, controversy arises in aNETs that are greater than 2 cm and those from 1 to 2 cm, which represent from 3% to 7% and from 20% to 25% of cases, respectively[8,28]. The 10-year survival rate according to size is 100%, 92%, and 91% for < 1 cm, 1 to 2 cm, and > 2 cm, respectively[31]. Although the indication is most likely a continuum, it has been suggested that 2 cm may be the optimal cut-off point for presence of LNM[1]. Current guidelines recommend this size for considering a CRH after an appendectomy and to consider CRH for tumors from 1 to 2 cm when other risk factors are present. In a recent meta-analysis, the rate of LNM was 12.1%, 38.5%, and 61% for tumors measuring < 1 cm, 1 to 2 cm, and > 2 cm, respectively[32]. A reduction in the cut-off point for a colectomy from 15 mm to 13 mm regardless of other features has been proposed due to the possible presence of LNM, but no studies have found any benefit in terms of recurrence or survival[5,28].

Mesoappendix invasion: Its incidence ranges from 23% to 39%[5,8,28]. There is controversy in the literature regarding its prognostic relevance. Some studies have found no effects on the recurrence rate [33] or presence of LNM[1,8], while others suggest that the disease behaves more aggressively in these patients[17,31]. A recent publication found that presence of mesoappendix invasion entails a higher risk of LNM and suggests an optimal cut-off point of 1 mm for indicating a CRH, which is substantially smaller than what has been suggested in previous reports[28]. However, these data should be interpreted cautiously because they were calculated based on patients who underwent a right colectomy according to ENETS guidelines indications and thus, the sample is biased. Furthermore, no patients with mesoappendix invasion in that study had recurrence. A recent meta-analysis reported a LNM rate of 30.3% when mesoappendix invasion was present and 26.2% when it was absent (OR, 1.4; 95%CI: 0.8-2.4)[32]. Also, in some works, the increased risk of LNM found on the univariate analysis disappeared on the multivariate analysis, with only size remaining as a relevant factor[5,8].

Positive margins: Although this may seem uncommon, a French multicenter study that included patients treated in non-referral centers reported a rate of 8%[15]. Given the possibility that the tumor could remain in the cecum area, most authors recommend CRH in this scenario[8,28].

Vascular and lymphatic vessel involvement: This feature can be found in 11% to 18% of patients[5,8,28]. It could be particularly relevant in patients with tumors < 2 cm, in whom it could reflect a metastatic potential similar to tumors measuring > 2 cm[5,34]. On the contrary, some studies have not described a higher LNM rate, but the low incidence of this finding could limit statistical significance[1,8]. Perineural invasion has been reported in 18% of patients[8], but there is no data to suggest more aggressive disease when it is present[32].

Tumor grade: Almost all aNETs are G1. Less than 15% have been described as G2 and the existence of G3 and poorly differentiated aNETs is anecdotal[1,28]. Although some works do not show a higher LNM rate in patients with a grade higher than G1[8], many others describe a significant risk in up to 90% of G2 neoplasms[28,35].

SURGICAL TECHNIQUE

The main purpose of a right hemicolectomy after an appendectomy is not only to resect residual local tumor, but also to complete the regional lymph node dissection. Nodal spread of aNETs is usually through the ileocolic vessels, a territory with few anatomical variations. Laparoscopic dissection of this territory is quite a standard approach to treat right side colonic cancer. In the absence of gross central nodal involvement, a laparoscopic approach seems to be safe and could provide some benefits, namely a shorter recovery time.

OUTCOMES AFTER A COMPLETION RIGHT HEMICOLECTOMY

Postoperative complications

Patients who undergo CRH for aNETs are younger and have fewer comorbidities than typical patients with colon cancer. Nevertheless, the rate of major complications after a colectomy for aNETs ranges from 5% to 15%[28,36,37]. A systematic review and meta-analysis conducted by Ricci *et al*[30] found that when performing a CRH in aNETs > 2 cm, the number needed to treat was five, while the number needed to harm was six, suggesting that the risk was similar to the possible benefit.

Survival and recurrence

It is important to emphasize that in general, these patients have an excellent prognosis, with survival rates close to 100% at 10 years of follow-up. Table 2 summarizes the literature on recurrence and survival rates in this type of tumor[38-41].

Although several studies agree that 2 cm is a reliable cut-off point for a colectomy, others have shown that a simple appendectomy offers comparably good results in tumors > 2 cm[29]. For example, in the classic work by Moertel *et al*[42], only one patient treated with an appendectomy out of 12 experienced recurrence, and it occurred 29 years after the initial appendectomy. This patient then underwent a right hemicolectomy, after which he remained disease-free 17 years later. Such data could lead us to speculate whether there is a decrease in overall survival by waiting to confirm the presence of lymphadenopathy instead of performing "prophylactic" CRH. Similarly, Groth *et al*[43] performed an appendectomy alone on 34 of 122 patients with aNETs > 2 cm and there was no difference in survival compared to patients who underwent a right hemicolectomy. Controversy mainly arises in patients with aNETs between 1 to 2 cm. In these patients, all studies suggest that survival is similar after either an appendectomy or a CRH.

While most studies report features associated with LNM and recommend CRH when its presence is possible, there is a dearth of data regarding its influence on patient survival. A recent meta-analysis did not find any difference in disease-specific survival at five or ten years in patients with or without LNM (100% in both groups for five years and 95.6% *vs* 99.2% for ten years)[32]. It is clear the extremely long course of these tumors makes it difficult to evaluate overall survival.

An interesting study on how surgical technique may influence patient survival is a review of the National Cancer Database performed by Heller *et al*[44]. Their work described 3198 cases of aNETs and found that 32.4% of those smaller than 2 cm were treated with a right hemicolectomy and 31.5% of those larger than 2 cm were treated with a simple appendectomy. There were no differences in survival between the groups according to surgical procedure.

Quality of life

In addition to questions regarding a survival benefit after CRH in aNETs < 2 cm, its influence on patients' quality of life must also be considered. This topic has recently been evaluated in a multicenter study from five ENETS centers of excellence in which the health-related quality of life European Organization for Research and Treatment of Cancer-QLC-C30 was administered to 79 patients with aNET[29]. While the patient group did not have lower scores compared to matched healthy controls, patients who underwent CRH (30 patients) had worse scores on social functioning, diarrhea, and financial difficulties compared to patients treated only with an appendectomy (49 patients). Additionally, no benefit in disease-free survival was observed after CRH.

FOLLOW-UP

There are no specific recommendations based on randomized trial data on follow-up after resection of an aNET and no adjuvant therapy is recommended after complete resection of a well-differentiated midgut NET. Several international guidelines include strategies for follow-up based on tumor size and the surgery performed[9,10,45].

Patients with tumors < 1 cm or from 1 to 2 cm without poor prognostic factors do not generally require further routine surveillance and tests should only be ordered if they are clinically indicated[9,45]. ENETS guidelines extend this recommendation to patients with tumors treated with a hemicolectomy without evidence of lymph node involvement[10].

Patients with tumors from 1 to 2 cm with poor prognostic factors who do not undergo a hemicolectomy or those with tumors > 2 cm should be followed-up on every three to six months in the first year after resection and then every six to 12 mo for at least seven years. Due to the slow growth pattern of these tumors, lifelong monitoring for potential recurrence must be provided[9,10].

In the case of candidates for surveillance, follow-up should consist of a medical history and physical examination. In addition, tumor markers (including 5-HIAA and CgA) and abdominal imaging by means of a CT or MRI scan should be considered. The role of a colonoscopy or transabdominal ultrasound has not been established in these patients[9,10,45] (Table 3).

Table 2 Summary of published data on appendiceal neuroendocrine tumor recurrence and survival

Author (Yr)	*n*	Recurrence rate	Specific disease survival rate	Reported follow-up
Tsikitis *et al*[38], 2012	982	-	95.6%	5-yr rate
Volante *et al*[17], 2013	138	-	97.1%	86.5 mo (1 - 267)
Mosquera *et al*[39], 2017	418	-	95.7%	5-yr rate
Sarchekeh *et al*[40], 2017	118	-	97.5%	10-yr rate
Pawa *et al*[41], 2017	215	0	99.05%	10-yr rate
Alexandraki *et al*[32], 2020	136	2.2%	100%	10-yr rate
Brighi *et al*[5], 2020	435	0%	98.5%	Median follow-up not provided, but at least 20% longer than 10 yr
Alabraba *et al*[1], 2021	102	1%	99%	6.2 yr (0.8-27.8)
Holmager *et al*[28], 2021	335	0%	100%	66 mo (1-250)

Table 3 Follow-up recommendations according to European Neuroendocrine Tumor Society and North American Neuroendocrine Tumor Society

Tumor characteristics	Surgery	Follow-up
Size < 1 cm	Appendectomy	No
Any size	Hemicolectomy (no lymph node involvement)	No (consider follow-up if tumor size > 2 cm)
Size 1-2 cm with poor prognostic factors	Appendectomy	History and physical examination every three to six months for the first year and then every six to 12 months
Any size	Hemicolectomy (lymph node involvement)	Consider tumor markers and abdominal imaging tests

SRS imaging is not routinely recommended for restaging in the initial follow-up after resection with curative intent and should be performed only for restaging at the time of clinical or laboratory progression without progression on conventional imaging tests[46].

TREATMENT IN ADVANCED DISEASE

The initial evaluation for patients with metastatic relapse or progression should include conventional imaging tests (CT or MRI scan), functional imaging tests with SRS-PET, and an assessment of carcinoid syndrome. All patients with advanced aNETs should be referred to a center with experience in neuroendocrine neoplasms and evaluated by a multidisciplinary tumor board.

Systemic treatment strategies for advanced or metastatic disease are similar to what is indicated for other midgut NETs. SSAs are usually the first-choice systemic therapy for symptomatic control in functional tumors. Antiproliferative activity has been demonstrated in two placebo-controlled randomized trials: The PROMID study with octreotide LAR 30 mg/28 d[47] and the CLARINET study with lanreotide autogel 120 mg/28 d[48].

Patients with a positive SRS functional imaging test are potential candidates for peptide receptor radionuclide therapy after progression on somatostatin analogs. Currently, lutetium 177 is approved for GEP-NETs in light of data from the NETTER-1 trial on midgut NETs[49].

Treatment with targeted therapies is based on the mTOR inhibitor everolimus. The RADIANT-4 trial confirmed the efficacy of everolimus in non-functioning NETs of gastrointestinal and pulmonary origin, including midgut NETs[50].

In addition, for patients who need regional control of liver metastasis due to carcinoid syndrome or for disease control in cases with liver-limited disease, locoregional therapies should be evaluated. Liver-directed therapies include ablative techniques such as percutaneous radiofrequency ablation or particle embolization with or without cytotoxic agents or radioactive microspheres. Chemotherapy is not routinely recommended and is reserved only for patients with progressive disease along with other strategies or for patients with rapidly progressing disease[51].

DISCUSSION

A hemicolectomy after the incidental finding of an aNET during an appendectomy is currently indicated depending on the stratified risk of relapse. There is consensus on not performing a hemicolectomy in tumors that measure < 1 cm, as these patients are considered cured and do not even require follow-up. On the other hand, a hemicolectomy should always be considered for tumors that measure > 2 cm.

Indications for tumors between 1-2 cm are where the controversy lies. Current guidelines give recommendations based on risk factors beyond tumor size: mesoappendix invasion, positive margins, vascular and lymph node involvement, and tumor grade. However, these recommendations are not supported with enough high-quality evidence.

A decision regarding a hemicolectomy in an aNET between 1 and 2 cm should be discussed by a multidisciplinary tumor board in expert centers. The opinions of pathologists, surgeons, gastroenterologists, endocrinologists, radiologists, medical oncologists, and nuclear medicine specialists should be taken into consideration before making a recommendation. Long-term issues related to a hemicolectomy should be discussed with the patient, particularly with those who are younger.

This narrative review aims to examine current evidence that is mainly based on clinical guidelines, providing the framework for a multidisciplinary tumor board discussion. The main limitation is a lack of prospective studies, an unmet need that should be addressed in the future. Indeed, at present, the SurvivApp study aims to analyze distant metastasis and long-term outcomes in patients after complete resection of an aNET. It aims to recruit 700 participants over ten years and divide them into two cohorts of retrospective and prospective cases. Its primary objectives are related to tumors measuring 1-2 cm and include clinically relevant relapse, clinically relevant mortality, and frequency of distant metastasis [52].

CONCLUSION

Decisions related to the indication of a hemicolectomy and follow-up should be made by a multidisciplinary tumor board at expert centers in order to offer each patient an individualized treatment approach. The factors that should be discussed include tumor size, mesoappendix invasion, positive margins, vascular and lymphatic vessel involvement, and tumor grade. Prospective studies regarding optimal treatment for aNETs are an unmet need in the NET field and should be addressed in the future.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Jianpi Qingchang Bushen decoction improves inflammatory response and metabolic bone disorder in inflammatory bowel disease-induced bone loss

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Abstract

BACKGROUND

Bone loss and osteoporosis are commonly described as extra-intestinal manifestations of inflammatory bowel disease (IBD). Jianpi Qingchang Bushen decoction (JQBD) is a prescription used in clinical practice. However, further studies are needed to determine whether JQBD regulates the receptor activator of nuclear factor kappa B (NF-κB) (RANK)/receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) pathways and could play a role in treating IBD-induced bone loss.

AIM

To evaluate the therapeutic effect of JQBD in IBD-induced bone loss and explore the underlying mechanisms.

METHODS

An IBD-induced bone loss model was constructed by feeding 12 6-to-8-wk-old interleukin-10 (IL-10)-knockout mice with piroxicam for 10 d. The mice were randomly divided into model and JQBD groups. We used wild-type mice as a control. The JQBD group was administered the JQBD suspension for 2 wk by gavage, while the control and model groups were given normal saline at the corresponding time points. All mice were killed after the intervention. The effect

of JQBD on body weight, disease activity index (DAI), and colon length was analyzed. Histopathological examination, colon ultrastructure observation, and micro-computed tomographic scanning of the lumbar vertebrae were performed. The gene expression of NF-κB, tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8 in the colon was evaluated by real-time polymerase chain reaction. Colon samples were assessed by Western blot for the expression of RANKL, OPG, RANK, and NF-κB proteins.

RESULTS

The model group lost body weight, had a shorter colon, and showed a dramatic increase in DAI score, whereas JQBD had protective and therapeutic effects. Treatment with JQBD significantly improved inflammatory cell infiltration and reduced crypt abscess and ulcer formation. Three-dimensional imaging of the vertebral centrum in the model group revealed a lower bone mass, loose trabeculae, and "rod-shaped" changes in the structure compared to the control group and JQBD groups. The bone volume/total volume ratio and bone mineral density were significantly lower in the model group than in the control group. JQBD intervention downregulated the NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA expression levels. The RANKL and OPG protein levels were also improved.

CONCLUSION

JQBD reduces inflammation of the colonic mucosa and inhibits activation of the RANK/RANKL/OPG signaling pathway, thereby reducing osteoclast activation and bone resorption and improving bone metabolism.

Key Words: Inflammatory bowel disease; Osteoporosis; Jianpi Qingchang Bushen decoction; Inflammation; Bone metabolism

Core Tip: Bone loss and osteoporosis are commonly described as extra-intestinal manifestations of inflammatory bowel disease (IBD). Jianpi Qingchang Bushen decoction (JQBD) is a prescription used in clinical practice. This study aimed to evaluate the therapeutic effect of JQBD in IBD-induced bone loss and explore the underlying mechanisms. An IBD-induced bone loss model was constructed by feeding interleukin-10-knockout mice with piroxicam. JQBD reduced inflammation of the colonic mucosa and inhibited activation of the receptor activator of nuclear factor kappa B (NF-κB)/receptor activator of NF-κB ligand/osteoprotegerin signaling pathway, thereby reducing osteoclast activation and bone resorption and improving bone metabolism.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing immune-mediated inflammatory disease of the gastrointestinal tract, which includes Crohn's disease (CD) and ulcerative colitis (UC)[1,2]. Its main clinical manifestations are diarrhea, abdominal pain, bloody stool, and various degrees of systemic symptoms[3,4]. Nearly 50% of IBD patients show at least one kind of extra-intestinal symptom during their lifetime, among which musculoskeletal damage is the most common[5-7]. In recent years, increasing attention has been paid to the study of osteopenia and osteoporosis caused by IBD. Osteoporosis is a systemic bone disease characterized by low bone mass and the destruction of bone microstructure. It increases bone fragility, making it prone to fractures. Osteoporosis is one of the more common extraintestinal manifestations in IBD patients[8]. Studies have shown that the risk of osteoporosis is significantly higher in IBD patients than in healthy controls[9,10], exposing them to a higher risk of fracture. The risk of fracture in IBD patients is 40% higher than that in healthy controls, seriously affecting their quality of life[11]. The search for a suitable treatment to reduce pain and the economic burden has become a priority of international medical and social establishments.

The pathogenesis of osteoporosis in IBD patients has not been fully elucidated. Many factors affect bone metabolism. Recent studies have shown that intestinal inflammation is one of the most important factors leading to osteoporosis in IBD patients. Inflammation is involved in the pathophysiological process of bone loss in patients with IBD[12-14]. Therefore, controlling intestinal inflammation could help to prevent the occurrence and progression of osteoporosis. The receptor activator of nuclear factor kappa B (NF-κB; RANK), its ligand (receptor activator of NF-κB ligand, RANKL), and the soluble decoy receptor osteoprotegerin (OPG) orchestrate resorption of osteoclastic bone and play a key role in the common pathogenic pathway between gut inflammation and bone loss[15]. RANK/RANKL/OPG signaling is a key regulator of osteoclast biology and bone metabolism and an important pathway regulating osteoclast differentiation[16-18]. Stanisławowski *et al*[19] reported that patients with UC have higher local expression of serum OPG mRNA and protein than healthy participants[19]. Krela-Kaź mierczak *et al*[20] reported an increase in the OPG/RANKL ratio in CD patients[20]. According to a recent study, elevated osteocyte tumor necrosis factor (TNF)-α, interleukin (IL)-6, RANKL, and OPG correspond to higher osteoclast surfaces and a lower bone formation rate in an IBD model induced with dextran sodium sulfate[21]. No effective preventive or treatment modality exists for osteoporosis in IBD patients. A great deal of interest has been generated to evaluate the mechanism by which natural products exert their beneficial effects in the gastrointestinal tract[22]. Traditional Chinese medicine (TCM) has good effects on IBD patients with osteoporosis. Spleen and kidney insufficiencies and dampness-heat are important components of IBD-associated osteoporosis pathogenesis. Treatment is aimed at invigorating the spleen, clearing the intestine, and tonifying the kidney. Thus, Jianpi Qingchang Bushen decoction (JQBD) was established under the guidance of TCM theory. JQBD is a prescription developed by our team to treat IBD patients with osteoporosis. Our previous study reported that JQBD-medicated serum promotes osteoclast apoptosis by downregulating Bcl-2 and upregulating the expression of the Bax protein[23]. According to TCM theory, the kidney stores the essence and this can be transformed into the bone marrow to nourish the bones and strengthen the skeleton. Thus, a kidney deficiency can cause osteoporosis. Tonifying the kidneys regulates bone metabolism to alleviate osteoporosis. Modern pharmacological studies have confirmed that *Psoralea corylifolia* Linn, *Alpinia oxyphylla* Miq, and their extracts improve bone metabolism to prevent bone loss and are commonly used to treat osteoporosis[24-27].

In this study, the potential pharmacodynamic mechanism of JQBD for treating IBD-induced osteoporosis was studied *in vivo* for the first time. An IBD-induced osteoporosis model was constructed by treating interleukin (IL)-10-knockout mice with piroxicam. JQBD was given as an intervention, and its effects on the inflammatory response and bone metabolism were observed to provide a theoretical basis for the clinical prevention and treatment of osteoporosis in IBD patients.

MATERIALS AND METHODS

TCM preparation and identification of the phytochemicals

JQBD was prepared by the TCM pharmacy of Longhua Hospital, Shanghai University of TCM. It was composed of eight herbal species, including *Astragalus mongholicus* Bunge 30 g, *Codonopsis pilosula* (Franch.) Nannf. 15 g, *Alpinia oxyphylla* Miq. 12 g, *Cullen corylifolium* (L.) Medik. 9 g, *Portulaca oleracea* L. 30 g, *Sanguisorba officinalis* L. 15 g, *Aucklandia costus* Falc. 6 g, and *Glycyrrhiza glabra* L. 6 g. The components were soaked, boiled twice, filtered, concentrated, freeze-dried into a powder, and stored at −20 °C. Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF/MS) was used to analyze the samples and identify 44 phytochemicals in JQBD. The MS chromatograms of the JQBD negative and positive ion modes are shown in Supplementary Figure 1. The chemical information of the identified phytochemicals is shown in Supplementary Table 1. Quality control measures were performed according to the guidelines of the Chinese State Food and Drug Administration.

Animal model and drug intervention

Six IL-10-knockout C57BL/6 mice were produced by the Shanghai Nanfang Research Center for Model Organisms (SNRCMO; Shanghai, China) and were maintained and bred in a specific-pathogen-free animal room at 23 ± 3 °C, 35%–45% relative humidity, and a 12 h/12 h light/dark regime at the Laboratory Animal Center of the Shanghai University of Traditional Chinese Medicine. Food and water were provided *ad libitum*. The Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine approved this study (PZSHUTCM191108004).

The IL-10-knockout mice were bred, and 70 F6-generation mice underwent gene detection using tail tip samples. IL-10 knockout was detected in 30 of these mice. We used wild-type mice as controls, which were the progeny of IL-10 +/- mating and bred at the same facility. The gene detection results are shown in Figure 1. The IBD-induced osteoporosis model was constructed by feeding the IL-10-knockout mice 200 ppm piroxicam for 10 d[28]. The mice were randomly divided into IL-10-knockout controls (model group) and IL-10-knockout mice with JQBD intervention (JQBD group). Six mice were included in each group (three males and three females). The JQBD group was administered 16.5 g/kg/d of the

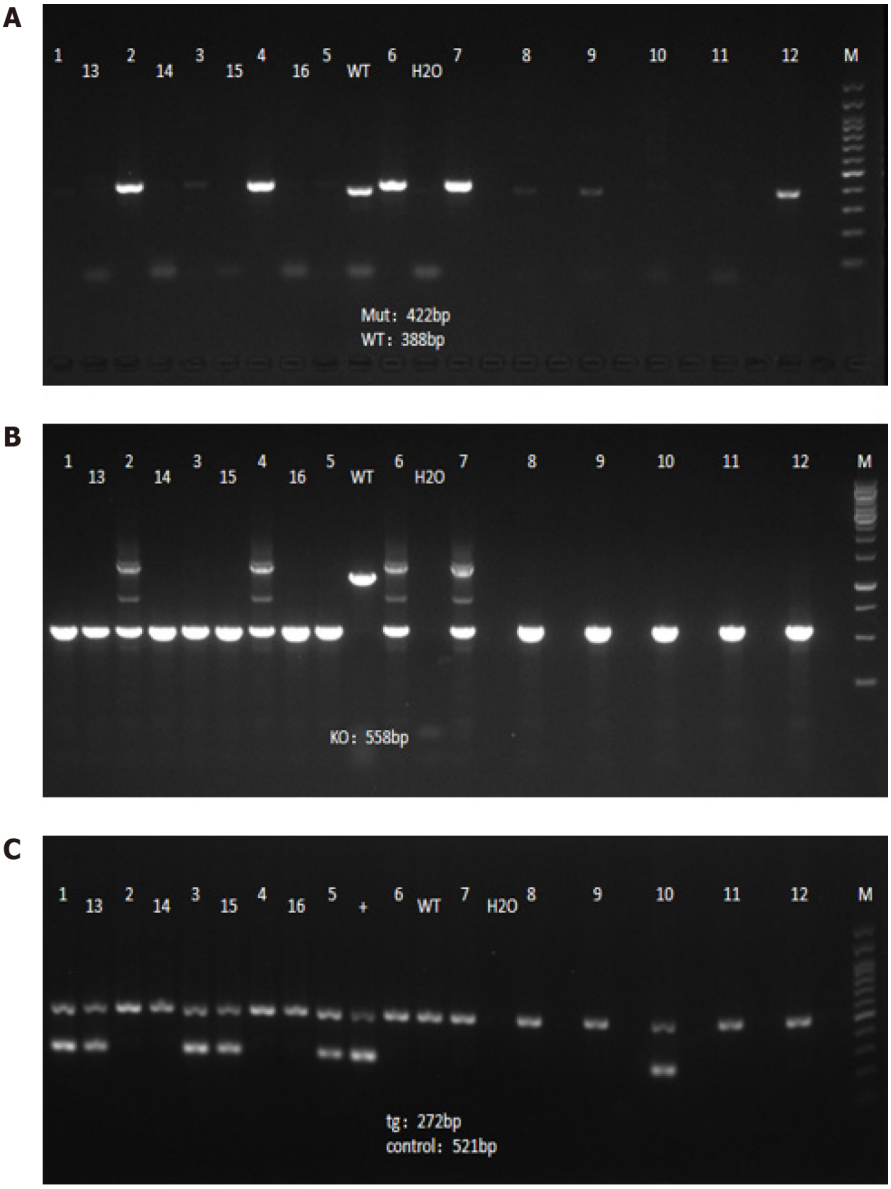

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Figure 1 Gene detection results in F6-generation mice. A: Interleukin (IL)-10 conditional knockout; B: IL-10 knockout; C: Dppa3-cre.

JQBD suspension by gavage, while the control and model groups were given the same volumes of normal saline at the corresponding time points. The intervention was conducted for 14 consecutive days. All mice were killed after the intervention, and blood samples and colon and bone tissues were harvested.

Daily record of observational parameters

The mental state, coat color, activity, body weight, diet, fecal traits, occult blood, and gross bloody stool were observed and recorded daily from the beginning of modeling. The disease activity index (DAI) score was used to evaluate colitis severity in the mice daily following the grading scheme presented in Table 1[29].

Measurement of colon length and colon histopathological examination

Dead mice were dissected to isolate the colon. The colons were placed on filter paper, and their length was measured with a ruler.

The colon samples were fixed in 4% paraformaldehyde solution, dehydrated, paraffin-embedded, sliced, and stained with hematoxylin and eosin (HE).

Ultrastructural observation of the colon

Part of each colon was fixed in 2.5% glutaraldehyde within 1 min, dehydrated, embedded, sliced, and stained with 3% uranium acetate and lead citrate. The colon ultrastructure was observed by

Table 1 Calculation of disease activity index score

Score	Weight loss	Stool consistency	Bleeding
0	Normal	Normal (well-formed pellets)	Not observed
1	1%-5%	Normal (well-formed pellets)	Not observed
2	6%-10%	Loose (pasty stools that do not stick to the anus)	Occult
3	11%-15%	Loose (pasty stools that do not stick to the anus)	Occult
4	> 15%	Diarrhea (liquid stools that stick to the anus)	Gross bleeding

transmission electron microscopy (TEM).

Specimen preparation and micro-computed tomography

After the mice were killed, the intact lumbar vertebrae were separated, and the residual soft tissue was removed. The bone tissue was fixed in a 75% ethanol solution pending the experiment. All specimen assessments were performed on the lumbar vertebra by μ-CT80 micro-computed tomography (CT) (SCANCO Medical AG, Bassersdorf, Switzerland). The scanning resolution was 18 μm *per* layer, followed by three-dimensional (3D) reconstruction. The morphometric analysis included bone volume/total volume (BV/TV) ratio, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), connectivity-density (Conn-Dens), and bone mineral density (BMD).

Real-time polymerase chain reaction detection of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA expression levels

Total RNA was extracted from the colon tissues with TRIzol, and the RNA concentration was determined. cDNA was reverse transcribed following the Takara kit instructions and was detected on the ABI StepOne Plus real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) instrument. The primers were designed and synthesized by Shanghai ShineGene Molecular Biotechnology Co., Ltd. (Shanghai, China) (Table 2) and verified using the BLAST program. A 20-μL RT-qPCR reaction system was configured, and the reaction amplification was carried out following the kit instructions. β-actin was used as the internal reference, fold-change was estimated by the $2^{-\Delta\Delta Ct}$ method, and the mRNA expression level was compared between the groups. The assay was repeated three times.

Detecting the effect of JQBD on RANKL, OPG, RANK, and NF-κB protein expression in colon tissue by Western blot

We added 1 mL of RIPA Lysis Buffer and 10 μL of a protease inhibitor mixture to a homogenization tube and precooled it on ice; 50 μg of colon tissue was weighed and homogenized repeatedly in the tubes six times until no obvious tissue was seen in the homogenate. The homogenate was centrifuged (Eppendorf 5417R, Eppendorf) at 4 °C and 15000 × *g* for 15 min, and the supernatant was placed in another centrifugation tube. A small volume of the supernatant was taken, and the protein concentration was determined by the bicinchoninic acid method. Samples of 30 μg were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a membrane, and sealed. TBST-diluted RANKL, OPG, RANK, and NF-κB antibodies (1:1000) were added, and the membrane was incubated overnight at 4 °C. After the incubation, the membrane was washed and incubated for 1 h with gentle shaking at room temperature with fluorescence-labeled secondary antibody diluted 1:3000. TBST was used to rinse the membranes. An enhanced chemiluminescence kit (reagents A and B were mixed at equal volumes) was placed on the surface of the polyvinylidene fluoride (PVDF) membrane and incubated in the dark at room temperature for 1 min. The PVDF membrane was placed on the operating table of a Western blot imager, and a strip image was acquired. The gray values of the bands were analyzed using ImageJ analysis software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 8.0 software (GraphPad Software Inc., La Jolla, CA, United States). Data are expressed as the mean ± SD. Differences between groups were assessed by one-way analysis of variance. A P value < 0.05 was considered significant.

RESULTS

General condition of the mice

The control mice showed a flexible response, agile movement, glossy fur, pink paws, active foraging, normal water intake, and mostly globular feces that were negative for occult blood. The mice in the

Table 2 List of primers used in this study

Gene	Primers(5'-3')	Primer length
NF-κB	Forward GTGGAGGCATGTTCGGTAGTG	195
	Reverse TCTTGGCACAATCTTTAGGGC	
TNF-α	Forward CCCTCCAGAAAAGACACCATG	183
	Reverse CACCCCGAAGTTCAGTAGACAG	
IL-1β	Forward GCTTCAGGCAGGCAGTATCA	196
	Reverse TGCAGTTGTCTAATGGGAACG	
IL-6	Forward AAATGATGGATGCTACCAAACTG	137
	Reverse CTCTGGCTTTGTCTTTCTTGTTATC	
IL-8	Forward TAGTGTTGAGCATGAAAAGCCTC	152
	Reverse TGACTTCACTGGAGTCCCGTAG	

NF-κB: Nuclear factor-kappaB; TNF-α: Tumor necrosis factor-α; IL: Interleukin.

model group were lazy, with a reduced appetite, dirty and sparsely erected fur, white paws, soft feces, and perianal body hair stained with loose stools. Some had bloody stools, occasional anal peeling, perianal blood, feces strongly positive for occult blood, and decreased body weight. The JQBD group mice displayed a better mental status and they had glossy hair, only slightly white paws, feces not as soft, only occasional bloody stools, lower positive occult blood rate, and less lost weight than the model group (Figure 2).

Weight change and DAI scores

The weights of the mice in the model and JQBD groups on day 10 of the experiment were significantly lower ($P < 0.05$) than that of the control group. The weight in the JQBD group was higher than that in the model group ($P < 0.05$). The weight in all groups on day 14 was higher than that at the start of the experiment, but the weight increase in the model and JQBD groups was less evident than that in the control group ($P < 0.05$). The weight of the model group was significantly lower than that of the other two groups ($P < 0.05$). Higher DAI scores were observed in the model and JQBD groups. The DAI score of mice in the model group was higher than that of mice in the control group ($P < 0.05$), which decreased significantly in response to JQBD treatment on day 14 ($P < 0.05$) (Figure 3).

Morphological changes in the colonic mucosa

The colon wall in the control group was smooth, red, and resilient. In the model group, it was thickened, stiff, hyperemic, edematous, and erosive, with some irregular ulcers and a brittle texture. The colonic mucosa hyperemia and edema in the JQBD group were less severe than those in the model group with significantly reduced ulcer formation. The colon in the model group was significantly shorter than that in the control ($P < 0.001$) and the JQBD ($P < 0.05$) groups.

Histological evaluation of the colonic mucosa

The colonic mucosal epithelium in the control group was intact and continuous, the glands were orderly arranged, the goblet cells were visible, the blood vessels and fibrous interstitium in the lamina propria and muscle layer were normal, and inflammatory cell infiltration was rarely observed. Necrosis, erosion, disordered gland arrangement, different degrees of inflammatory cell infiltration, a reduced number of goblet cells, and ulcer formation were observed in the colonic mucosa of mice in the model group, suggesting successful IBD modeling in these mice. JQBD provided partial protection to the mucosa, so it was less damaged, showing mild to moderate hyperemia and edema. Lower inflammatory cell infiltration and ulcer formation rates were noted, and the glands were arranged in an orderly manner (Figure 4).

Bone metabolic parameters on micro-computed tomography

The 3D imaging of the vertebral centrum in the model group revealed a lower bone mass, loose trabeculae, and "rod-shaped" changes in the structure compared to the control group. Treatment with JQBD resulted in a higher bone mass, tighter trabecular bone gaps, and higher connectivity than those in the model group. The quantitative micro-CT results showed that the BV/TV ratio and BMD decreased in the model group. The Conn-Dens and Tb.Th were similar in the normal and model groups. Although BV/TV and BMD were significantly lower in the model group than in the control group, these values

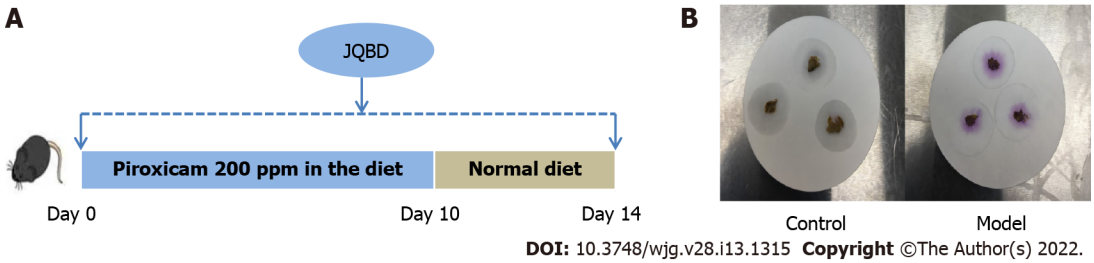

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Figure 2 Animal experimental flow and fecal occult blood test. A: An experimental bone loss inflammatory bowel disease model was induced by peroral administration of piroxicam for 10 d in interleukin-10$^{-/-}$ mice. Normal saline or Jianpi Qingchang Bushen decoction (JQBD; 16.5 g/kg/d) was given intragastrically to the control/model groups and JQBD group, respectively (n = 6, each); B: Fecal occult blood test of control and model groups: The control group was negative, and the model group was strongly positive for occult blood. JQBD: Jianpi Qingchang Bushen decoction Group.

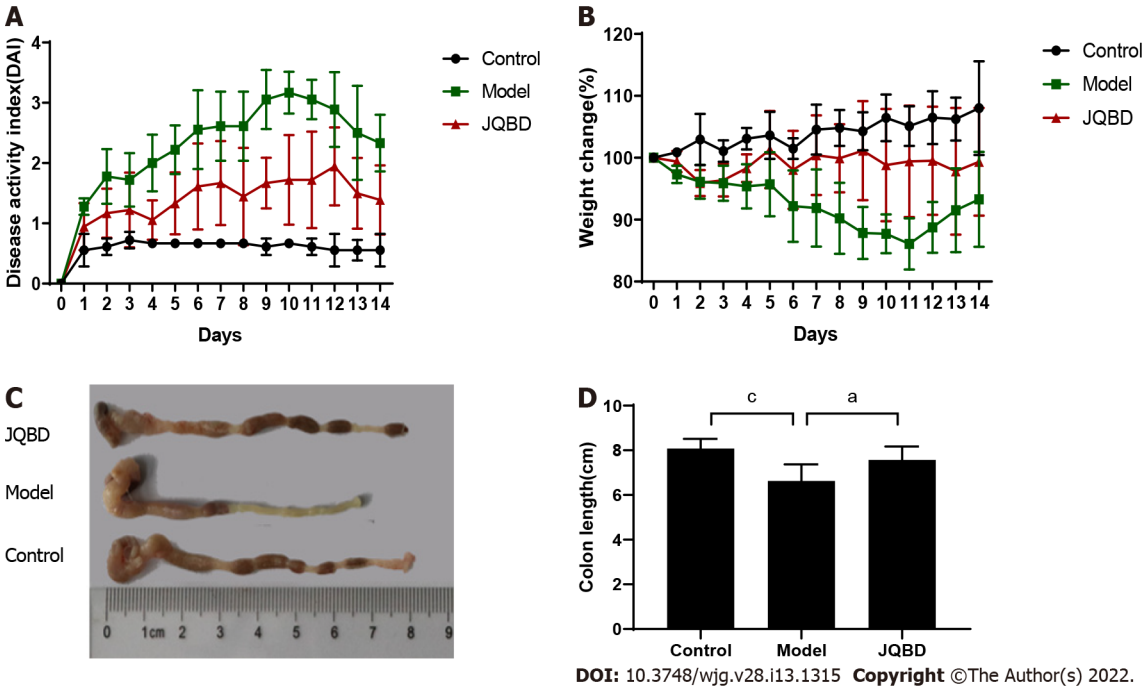

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Figure 3 General condition of the mice. A: Disease activity index scores gauged daily (n = 6 *per* group); B: Body weight measured daily (n = 6 *per* group); C and D: Colon length measurement and graph presenting the statistical analysis results. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ (n = 6 *per* group).

were similar in the JQBD, control, and model groups (Figure 5).

Structural changes in the colonic epithelial cells as observed by TEM

The TEM images illustrated intact colon cells in the control group, with no increase in intercellular space. The endoplasmic reticulum had a reticular structure and was bundled, the cavity was not expanded, many ribosomes were attached to it, and numerous mitochondria were observed. In the model group, the organelles were significantly swollen and the endoplasmic reticulum cavity was significantly expanded, attaining different shapes and sizes, with many vacuoles. Some of the organelles fused into clusters. A microvillar structure was partially observed in the JQBD group. The mitochondria were slightly swollen, the number of epithelial cells increased, and the cell structure was more intact than in the model group. The endoplasmic reticulum cavity was slightly expanded (Figure 4).

Effect of JQBD on NF-κB, TNF-α, IL-1β, IL-6, and IL-8 gene expression

The expression of the five genes was verified by RT-qPCR. The mRNA expression levels of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 were significantly upregulated in the colon of the model group compared to the control group. JQBD intervention downregulated the mRNA expression levels of these genes, suggesting that JQBD improved the mucosal inflammatory response (Figure 6).

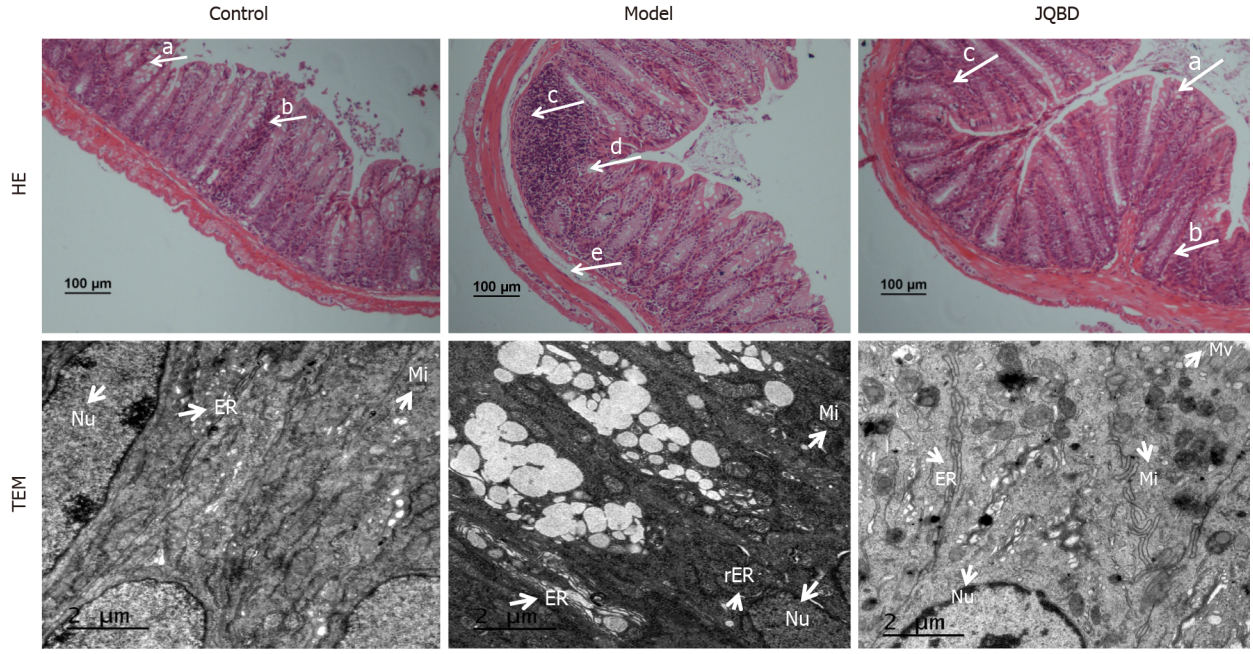

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Figure 4 Histological evaluation of the colonic mucosa following hematoxylin and eosin staining (× 100) and ultrastructure of the colonic epithelium by transmission electron microscopy (× 6000). Arrows indicate goblet cells (a), crypts (b), inflammatory cells infiltration (c), epithelium surface erosion (d), and submucosal oedema (e). Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; Nu: Nucleus; Mi: Mitochondrial; ER: Endoplasmic Reticulum; rER: Rough endoplasmic reticulum; Mv: Microvillus.

Effect of JQBD on RANKL, OPG, RANK, and NF-κB protein expression

The RANKL, OPG, RANK, and NF-κB protein levels were measured by Western blot. RANKL and OPG protein expression was significantly upregulated in the model group colon and was markedly downregulated following JQBD intervention. Although the RANK and NF-κB protein expression levels were significantly higher in the model group than in the control group, they were similar between the model and JQBD groups. Taken together, these data indicate that JQBD improved the mucosal inflammatory response and restrained the RANK/RANKL/OPG signaling pathway during the development of bone loss in IBD (Figure 7).

DISCUSSION

IBD is a chronic, non-specific, intestinal inflammatory disease with unclear etiology and common digestive system clinical presentation. The disease is protracted and difficult to cure, and the symptoms easily recur. IBD affects multiple systems, including the bones and calcium deposits. Studies have shown that IBD patients have a lower bone mass than healthy people[30-33]. The risk of osteoporosis and subsequent fractures is significantly higher in IBD patients[34,35], and it is the main cause of mobility problems and decreased quality of life in these patients[36]. Osteoporosis is a common and easily overlooked extra-intestinal manifestation in IBD patients. Early diagnosis and prevention of osteoporosis in IBD patients are very important. Therefore, we explored the possible pathogenesis of IBD-induced osteoporosis and evaluated the effectiveness of a promising drug in the present study. We propose that JQBD may have a protective effect against IBD-induced osteoporosis through the RANK/RANKL/OPG signaling pathway.

We established a bone loss IBD model in IL-10-knockout mice by peroral administration of piroxicam. IL-10-knockout mice are commonly used as an animal model to study IBD[37,38]. When exposed to non-steroidal anti-inflammatory drugs, such as piroxicam, these animals rapidly develop colitis that persists for a long time[39,40]. Holgersen *et al*[15] reported that they orally fed IL-10 knockout mice piroxicam for 12 d before the bone loss and trabecular bone structural changes occurred[15]. We report similar results in IL-10-knockout mice following 10 d of oral piroxicam administration. Our micro-CT data show that the BV/TV ratio and BMD were significantly lower in the model mice than in the controls. The 3D simulation map suggested that bone mass was significantly lower in the model group than in the control group and that the trabecular bone structure was lost, with visible fractures and destruction of the bone microstructure. The model group exhibited bone loss and significant colitis, similar to another report[41]. Our results preliminarily confirm the abnormal bone metabolism in the model group, suggesting successful modeling.

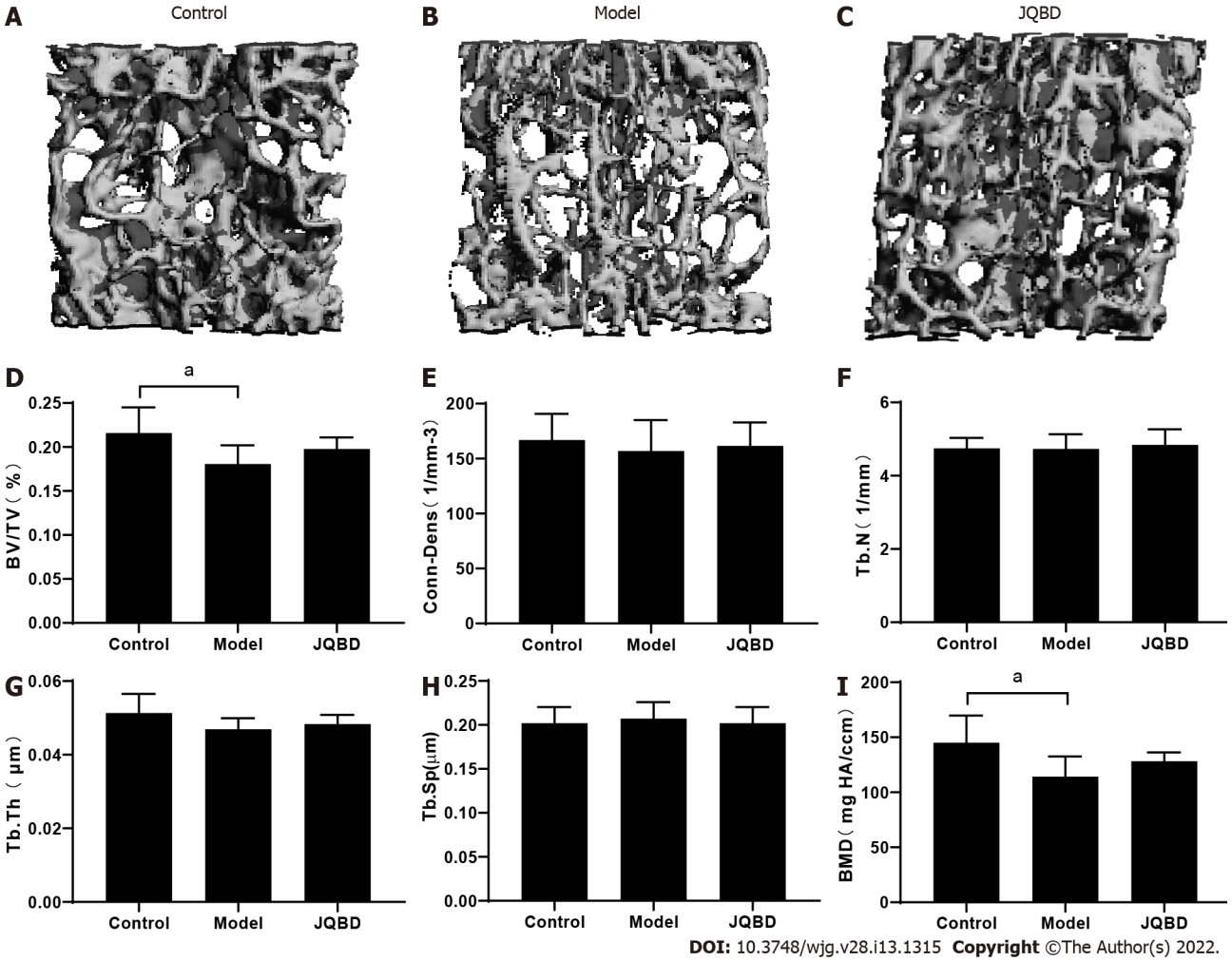

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Figure 5 Three-dimensional reconstruction of the lumbar spine trabecular structure in mice and micro-computed tomographic analyses of the lumbar vertebral metaphysis. Bone volume to total volume (BV/TV) ratio, Conn-Dens, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone mineral density (BMD) were obtained for all mice. A: Control; B: Model; C: JQBD; D: BV/TV; E: Conn-Dens; F: Trabecular number; G: Trabecular thickness; H: Trabecular separation; I: Bone mineral density. Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; BV/TV: Bone volume to total volume ratio; Conn-Dens: Connectivity-density; Tb.N: Trabecular number; Tb.Th: Trabecular thickness; Tb.Sp: Trabecular separation; BMD: Bone mineral density. Data are presented as the mean ± SD. $^{a}P < 0.05$. ($n = 6$ *per* group).

Our data show that the JQBD was protective against IBD-induced bone loss by inhibiting inflammation. Evidence shows that intestinal inflammation is involved in the pathophysiology of osteoporosis in IBD[42-44]. Expression of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 mRNA in colonic tissue from the model group increased significantly, confirming that pro-inflammatory cytokines are involved in the occurrence of experimental colitis in piroxicam-induced IL-10-knockout mice with IBD. The administration of the JQBD reduced the levels of pro-inflammatory cytokines and decreased colonic inflammation. Several studies have evaluated the pathophysiology of IBD and its association with osteoporosis. Bone loss was initially attributed to the use of drugs, such as corticosteroids. However, it was later found that BMD decreases in IBD patients even without drug treatment, suggesting that IBD might be involved in the pathophysiology of bone loss, and chronic inflammation is the primary determinant[45-47].

We evaluated the curative effects of JQBD on the bone-loss IBD model mice and confirmed that the RANK/RANKL/OPG signaling pathway was involved in the process. The RANKL, OPG, RANK, and NF-κB protein levels increased significantly in colon tissue from the model group. Their expression levels were downregulated after JQBD intervention, suggesting that JQBD inhibits activation of the RANK/RANKL/OPG signaling pathway. Several pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-8 increase in IBD and have been shown to stimulate osteoclast differentiation[48]. Studies have suggested that the abnormal intestinal tract immune response in IBD leads to injury and inflammation of the intestinal mucosa, activation of the RANK /RANKL/OPG signaling pathway, changes in the bone conversion rate, osteoclast activation, and bone loss, leading to IBD-induced osteoporosis[21,49,50]. These results highlight the interactions between inflammation and the RANK/RANKL/OPG signaling pathway during the development of osteoporosis in IBD. JQBD reduced inflammation of the colonic mucosa and inhibited activation of the RANK/RANKL/OPG signaling pathway (Figure 8).

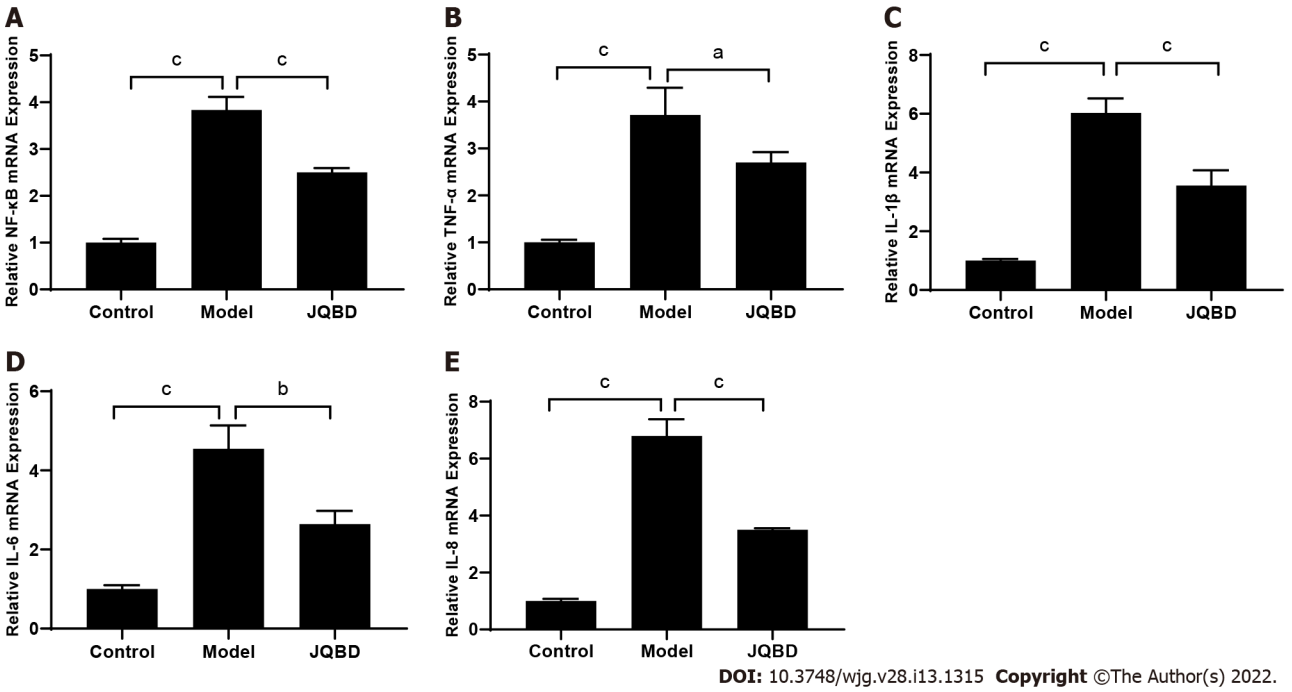

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Figure 6 Effect of Jianpi Qingchang Bushen decoction on nuclear factor-kappaB, tumor necrosis factor-α, interleukin-1β, interleukin-6, and interleukin-8 gene expression. The relative expression of these genes was quantified by real-time fluorescence quantitative polymerase chain reaction. A: NF-κB; B: TNF-α; C: IL-1β; D: IL-6; E: IL-8. Control: Control group; Model: Model group; JQBD: Jianpi Qingchang Bushen decoction Group; NF-κB: Nuclear factor-kappaB; TNF-α: Tumor necrosis factor-α; IL: Interleukin. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ ($n = 3$ *per* group).

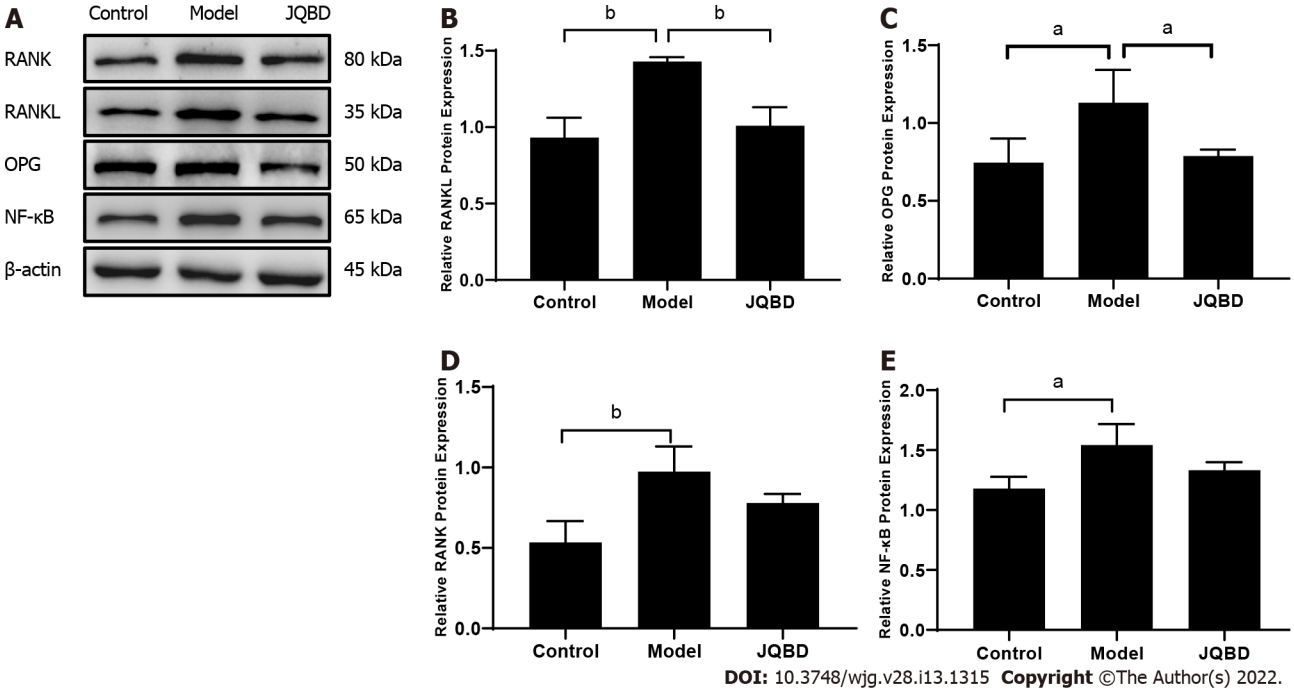

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Figure 7 Effect of Jianpi Qingchang Bushen decoction on receptor activator of nuclear factor κB ligand, osteoprotegerin, receptor activator of nuclear factor kappa B, and nuclear factor-kappaB protein expression. A: Protein expression quantified by Western blot; B: RANKL; C: OPG; D: RANK; E: NF-κB. Control: Control group; Model: Model group; RANK: Receptor activator of nuclear factor kappa B; RANKL: Receptor activator of nuclear factor κB ligand; JQBD: Jianpi Qingchang Bushen decoction Group. Data are presented as the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ ($n = 3$ *per* group).

Taken together, our present study data demonstrate that the JQBD improved IBD-induced osteoporosis *via* the RANK/RANKL/OPG signaling pathway.

This study primarily focused on the mechanism by which JQBD regulates the RANK/RANKL/OPG signaling pathway and improves IBD-induced osteoporosis. Our investigation has only preliminarily discussed the topic at the animal level and had several limitations. First, we considered using future

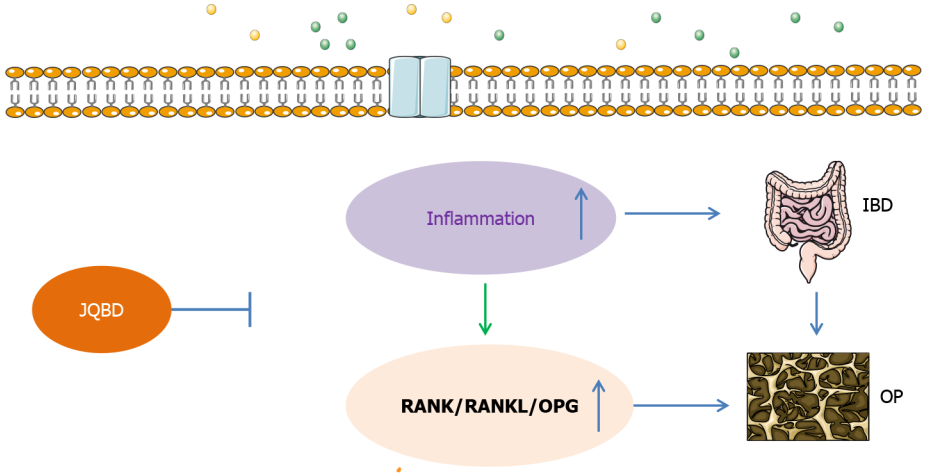

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Figure 8 Possible mechanisms of bone loss in inflammatory bowel disease. IBD: Inflammatory bowel disease; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor kappa B; RANKL: Receptor activator of nuclear factor κB ligand; JQBD: Jianpi Qingchang Bushen decoction Group.

emerging methods, such as systems pharmacology, to systematically explore the potential efficacy of JQBD in the treatment of IBD-induced osteoporosis. Such a study would include related experimental verification at the cell and animal levels. Second, the basic mechanism behind IBD-induced osteoporosis needs to be further explored. We will perform long-term observations in future experiments by extending the experimental period to 1 or 2 mo. We will also investigate the activation of the RANK/RANKL/OPG signaling pathway in bone tissues. Third, changes in intestinal microecology play an important role in the healing of the mucosa. Some studies have reported that an increase in the intestinal microbial bifidobacteria promotes healing of the colonic mucosa, increases the IL-10 level, and reduces the IL-6 and TNF-α levels[51,52]. The intestinal flora was not determined in this study. Thus, whether JQBD regulates intestinal microecology is worthy of further study.

CONCLUSION

JQBD plays a role in treating IBD-related bone metabolic abnormalities by inhibiting colonic mucosal inflammation, promoting mucosal healing, and inhibiting activation of the RANK/RANKL/OPG signaling pathway, osteoclast formation, and bone resorption.

ARTICLE HIGHLIGHTS

Research background

In recent years, increasing attention has been paid to the study of osteopenia and osteoporosis caused by inflammatory bowel disease (IBD). Osteoporosis is one of the more common extra-intestinal manifestations in IBD patients. No effective preventive or treatment modality exists for osteoporosis in IBD patients. Therefore, we explored the possible pathogenesis of IBD-induced osteoporosis and evaluated the effectiveness of a promising drug in the present study.

Research motivation

Jianpi Qingchang Bushen decoction (JQBD) is a prescription developed by our team to treat IBD patients with osteoporosis. In this study, the potential pharmacodynamic mechanism of JQBD for treating IBD-induced osteoporosis was studied *in vivo*.

Research objectives

The effects of JQBD on the inflammatory response and bone metabolism were observed to provide a theoretical basis for the clinical prevention and treatment of osteoporosis in IBD patients.

Research methods

An IBD-induced osteoporosis model was constructed by treating interleukin-10-knockout mice with piroxicam. JQBD was given as an intervention, and its effect on the inflammatory response and bone metabolism was observed.

Research results

Our data show that JQBD was protective against IBD-induced bone loss by inhibiting inflammation. The receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), osteoprotegerin (OPG), receptor activator of NF-κB (RANK), and NF-κB protein levels increased significantly in colon tissue from the model group. Their expression levels were downregulated after JQBD intervention.

Research conclusions

We evaluated the curative effects of JQBD on the bone-loss IBD model mice and confirmed that the RANK/RANKL/OPG signaling pathway is involved in the process.

Research perspectives

This study primarily focused on the mechanism by which JQBD regulates the RANK/RANKL/OPG signaling pathway and improves IBD-induced osteoporosis.

FOOTNOTES

Author contributions: Zhang YL and Chen Q contributed equally to this work, and both performed the majority of research; Dai YC, Zheng L, Zhang ZW, and Chen YJ performed the research and analyzed the data; Tang ZP designed and coordinated the research; Zhang YL and Chen Q wrote and revised the paper; all authors read and approved the final manuscript.

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Institutional review board statement: The study was reviewed and approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine, No. PZSHUTCM191108004.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: The datasets generated during and/or analyzed during the current study will be available upon request from the principle investigator. The shared data will only be allowed to be used by the applicant for scientific studies. No commercial activities are allowed.

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

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ORIGINAL ARTICLE

Basic Study

Comparison of the performance of MS enteroscope series and Japanese double- and single-balloon enteroscopes

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Abstract

BACKGROUND

Small intestine disease endangers human health and is not easy to locate and diagnose.

AIM

To observe the effect of the MS series of small intestine endoscopes on the gastrointestinal tract, the changes in serum gastrin levels and intestinal tissue, and the time required for the examination.

METHODS

In vivo experiments in 20 Living pigs were conducted, Bowel preparation was routinely performed, Intravenous anesthesia with propofol and ketamine was applied, the condition of the small intestine was observed and the detection time of the MS series of small intestine endoscopes were recorded, The changes in intestinal tissue using the MS series of small intestine endoscopes observed and compared before and after the examination, Venous blood (3-5 mL) from pigs was collected before and after the experiment; changes in intestinal tissue after use of the MS series of small intestine endoscopes observed after examination. After

completion of each type of small intestine endoscope experiment, the pigs were allowed to rest and the next type of small intestine endoscope experiment was performed after 15 days of normal feeding. The detection time data of the single-balloon small intestine endoscope and double-balloon small intestine endoscope were collected from four hospitals.

RESULTS

One case of *Ascarislumbricoides*, one of suspected Crohn's disease, one small intestinal diverticulum and one anesthesia accident were observed in pigs. The small intestine showed no differences in the MS series of small intestine endoscopes and there were no differences in serum gastrin between the groups ($P > 0.05$). The time required for inspection was recorded, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

CONCLUSION

Intestinal ascariasis is a common disease in pigs. Some pigs have abnormal intestinal variation. After continuous upgrade and improvement, the MS-3 and MS-4 small intestine endoscope appear superior in terms of detection time.

Key Words: Intestinal ascariasis; Intestinal variation; Crohn's disease; Serum gastrin; Small intestine endoscope

Core Tip: There are many types of hormones and their contents are extremely small (mostly in the range of nanograms and even picograms).They are neither the energy source of the body nor structural substances of the human body. However, they play essential roles in coordinating physiological processes such as metabolism and growth/development by transmitting information. Research on the changes in gastrin before and after the use of MS series of small intestine endoscope can provide theoretical support for the safe application of small intestine endoscopes.

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INTRODUCTION

The small intestine is the longest organ in the digestive system, and the average length varies among different species[1-3]. Thus, the precise treatment of small intestine disorders remains a challenge for clinicians[4-6]. In 2003, Yamamoto *et al*[7] from Jichi Medical University (Tokyo, Japan), together with Fuji Photo Optical Co., Ltd., developed a double-balloon endoscope and applied it in clinical practice[8, 9]. The double-balloon small intestine endoscope was developed by Yamamoto *et al*[10-12] in Japan. During double-balloon small intestine endoscopy, an over tube with a balloon on top is added to the original push-type endoscope, along with the addition of a balloon at the top of the master camera. Small intestine endoscopy can be completed by alternately pushing-pulling the balloons at the overtube and the master camera. The single-balloon small intestine endoscope is a small bowel endoscope designed by Olympus, Japan, in an attempt to avoid patent infringement[13]. The hook-pull action of the front-end of its master camera replaces the master camera of the double-balloon small intestine endoscope.

In 2005, we also initiated research and development on the MS series of small intestine endoscopes. The past ten years have witnessed the evolution of five generations of our small intestine endoscope. The MS-1 small intestine endoscope is a negative-pressure device that is advanced into the small intestine in a paralleled alternating manner, and has a long detection time. Based on the design of the MS-1 small intestine endoscope, the MS-2 small intestine endoscope was designed with two delivery devices, along with fixing rings and a metal slide. The MS-3 small intestine endoscope abandoned the fixing rings and metal slide and fixed the power and transmission parts together. The MS-4 small intestine endoscopeused a different fixation method to avoid the risk of bowel perforation under extreme working conditions. This new type of small intestine endoscopehad increased stability and speed during the examination, with fewer slippage events. The detection time was not significantly different between the MS-4 small intestine endoscope and MS-3 small intestine endoscope. The design of our latest MS-5 small intestine endoscope has been finalized, and its development is in progress.

The Japanese double-balloon small intestine endoscope has gradually been accepted by patients with the development of painless anesthesia. In this study, we aimed to analyze the detection time of the MS series of small intestine endoscopes and the detection time of the single-balloon small intestine endoscope (SBE) and double-balloon small intestine endoscope (DBE), investigated the possible reasons for the prolonged detection time of small intestine endoscopy.

MATERIALS AND METHODS

Experimental study

In vivo experiments were conducted in 20 Living adult pigs weighing 100 ± 20 kg. The animals were fed with water for two days and fasted for four hours before each experiment. Bowel preparation was routinely performed and each type of MS small intestine endoscope was checked before the experiment. Intravenous anesthesia with propofol and ketamine was applied. Following intravenous anesthesia, oxygen was provided to the pig *via* a nasal catheter at 3 L/min, and the vital signs were continuously monitored. The MS series of small intestine endoscopes was performed by a single endoscopist. When the auxiliary power tube was lubricated with paraffin oil, the camera was inserted into the main auxiliary power tube. Whether the endoscope could slide smoothly through the power tube was examined. After entering the upper part of the small intestine at 20 cm, the auxiliary power system was opened, the MS-1 small intestine endoscope used a double drive power pipe, the auxiliary power catheters were worked alternately; MS-2 to MS-4 small intestine endoscopes used four drive power pipes, MS-1 to MS-3 small intestine endoscopes worked with negative pressure power and MS-4 small intestine endoscope worked with positive pressure power, the auxiliary power catheters of group 1 and group 3 were pushed 40-50 cm to the distal end, group 1 and group 3 were given power catheter pressure or balloon dilatation, then the power catheters of groups 1 and 3 were pulled back to the starting position of groups 2 and 4. When the auxiliary power catheters of groups 2 and 4 were pushed 40-50 cm to the distal end, the auxiliary power catheters of groups 2 and 4 were injected with pressure, and then the power catheters of groups 2 and 4 were pulled back to the proximal end of groups 1 and 3. At the same time, the catheter was pulled back to the back of the catheter in groups 1 and 3, and then the catheter in groups 1 and 3 was pushed forward again. When the tube was folded or if the camera was difficult to insert, the extension distance of the power arm and the operating frequency were adjusted according to the insertion depth. The condition of the small intestine was observed and the detection times of MS-2 to MS-4 small intestine endoscopes were recorded. The changes in intestinal tissue using the MS series of small intestine endoscopes were observed and compared before and after the MS series of small intestine endoscopes. Venous blood (3-5 mL) from the pigs was collected before and after the experiment, and the changes in intestinal tissue after the MS series of small intestine endoscopes were observed after the examination. The MS series of small intestine endoscopes was assisted by a nurse during camera insertion. Using the auxiliary power system, the endoscopist repeatedly pushed the power device to continuously push into the small intestine to the distal end of the camera, thus completing the examination of the entire small intestine. After the examination, venous blood was collected again when anesthesia had worn off. After the completion of each type of small intestine endoscopy experiment, the pigs were allowed to rest, the time to return to normal diet and the time to return to normal stool were recorded, and the next type of small intestine endoscope experiment was performed after 15 days of normal feeding. After the experiment, the live pigs returned to their normal living state.

Clinical data

The detection times of the MS series of small intestine endoscopes were measured. Data on the detection times of SBE and DBE in the following four hospitals were analyzed: (1) Guangdong Provincial People's Hospital: 40 patients including 26 males and 14 females underwent Japanese DBE; (2) Shengjing Hospital Affiliated to China Medical University: From January 2009 to November 2013, DBE (Fujino EN-450P5 Double-Balloon small intestine endoscope, Japan) was performed in 78 patients (including 41 males and 37 females). No definite lesions were found before the procedure; (3) Affiliated Hospital of Zunyi Medical College: Small intestine endoscopy (SIF-Q260 type, Olympus, Japan) was performed in 17 patients with clinically suspected small bowel diseases from January to October 2010; (4) Zhongshan Hospital Affiliated to Fudan University: SBE (SIF-Q260, Olympus, Japan) was performed in 148 patients with suspected small bowel diseases from June 2009 to March 2011.

Observational indicators

To observe the effect of the MS series of small intestine endoscopes on gastrointestinal tract, the changes in serum gastrin levels and intestinal tissue were observed and compared before and after the MS series of small intestine endoscopes examination. The time required for the examination was recorded.

Statistical analysis

Statistical analysis was performed using the SPSS software package 21.0. The measurement data are expressed as mean ± standard deviation (X ± SD).

RESULTS

The experiment duration was 3 mo. One case of *Ascarislumbricoides* (Figure 1), one of suspected Crohn's disease (Figure 2), one small intestinal diverticulum (Figure 3) and one anesthesia accident were observed in pigs.

The serum gastrin level was 22.36 ± 2.88 pg/mL before the MS-1 small intestine endoscope examination, and was 22.33 ± 2.98 pg/mL after the examination. There was no difference in theselevels before and after the MS-1 small intestine endoscope experiment (P > 0.05), and there were no abnormalities in the intestinal tract. The time required for inspection was 2.30 ± 0.15 h. After completion of the MS-1 small intestine endoscope experiment, the pigs returned to their normal diet after 4 h and defecation returned to normal after one day.

The serum gastrin value was 21.88 ± 3.18 pg/mL before the MS-2 small intestine endoscope examination, and was 22.08 ± 2.96 pg/mL after the examination. There was no difference in these levels before and after the MS-2 small intestine endoscope experiment (P > 0.05), and there were no abnormalities in the intestinal tract. The time required for inspection was 1.50 ± 0.30 h. After completion of the MS-2 small intestine endoscope experiment, the pigs returned to their normal diet after 4 h and defecation returned to normal after one day.

The serum gastrin value was 22.28 ± 3.06 pg/mL before the MS-3 small intestine endoscope examination, and was 21.78 ± 3.76 pg/mL after the examination. There was no difference between these levels before and after the MS-3 small intestine endoscope experiment (P > 0.05). *Ascarislumbricoides* was found in one case, small intestinal diverticulum was found in one case and suspected Crohn's disease was found in one case. The time required for inspection was 0.67 ± 0.33 h. After completion of the MS-3 small intestine endoscope experiment, the pigs returned to their normal diet within 3 h and defecation returned to normal within one day. The pig with *Ascarislumbricoides* infection was isolated and treated with albendazole and ivermectin for one week. Small intestinal diverticulum and suspected Crohn's disease were not treated.

Serum gastrin level was 22.30 ± 3.08 pg/mL before the MS-4 small intestine endoscope examination, and was 21.66 ± 3.93 pg/mL after the examination. There was no difference in these levels before and after the MS-4 small intestine endoscope experiment (P > 0.05). The small intestinal diverticulum and the suspected Crohn's disease were as before. The time required for inspection was 1.00 ± 0.13 h. One experimental pig had an anesthesia accident due to excessive ketamine application in the later stage of the MS-4 small intestine endoscope experiment, and the other 19 pigs returned to their normal diet 3 h after the MS-4 small intestine endoscope experiment, and defecation returned to normal after 0.5 days. No *Ascarislumbricoides* was detected in the MS-4 small intestine endoscope group. All 20 experimental pigs completed the relevant experiments, including one case which developed cardiac arrest due to excessive infusion of anesthetics, and in the other 19 cases no abnormalities were found (Table 1).

There were no differences in serum gastrin between the groups (P > 0.05).

A total of 283 small intestine endoscopic procedures (including 165 SBE sessions and 118 DBE sessions) were performed in the four hospitals. The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

In Guangdong Provincial People's Hospital, the average detection time for DBE in 40 patients was 147 ± 28.5min. In Shengjing Hospital Affiliated to China Medical University, 78 patients successfully completed DBE, and the average examination time was 91.54 ± 22.59 min. In the Affiliated Hospital of Zunyi Medical College, the duration of SBE ranged from 30 to 80 min (mean: 45 min) in 17 patients, among whom one patient had an accidental bowel perforation, which improved after surgical repair. In Zhongshan Hospital Affiliated to Fudan University, 148 patients underwent 166 sessions of SBE, with an average duration of 83.4 min (range: 11–180min). The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time of the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h (Table 2).

DISCUSSION

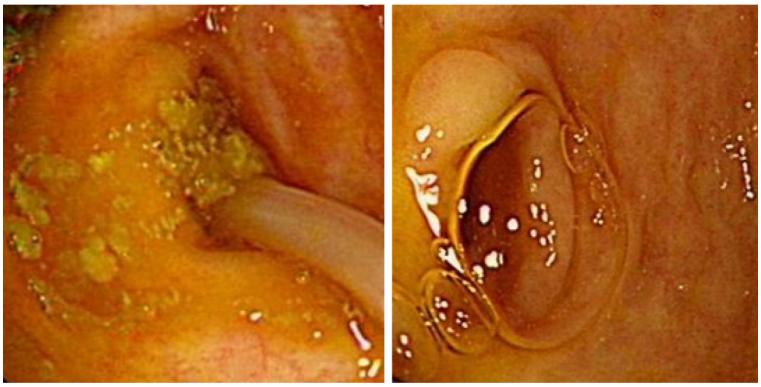
The small intestine includes the duodenum, jejunum and ileum, and each part has no clear boundary [14]. Microscopic observations can only be preliminarily judged by color, mucosal annular folds, small intestinal villus density, intestinal wall lymphatic follicles and so on[15].The main physiological function of the small intestine is digestion and absorption[16]. In addition to continuous digestion by gastric juice, pancreatic juice and bile in the small intestine, the mucosal glands of the small intestine can

Table 1 Detection time and serum gastrin level of MS series of small intestine endoscopes

Type	*n*	Serum gastrin level	Detection time (h)
MS-1 enteroscope	20	Before: 22.36 ± 2.88 pg/mL; After: 22.33 ± 2.98 pg/mL	2.30 ± 0.15
MS-2 enteroscope	20	Before: 21.88 ± 3.18 pg/mL; After: 22.08 ± 2.96 pg/mL	1.50 ± 0.30
MS-3 enteroscope	20	Before: 22.28 ± 3.06 pg/mL; After: 22.33 ± 2.98 pg/mL	0.67 ± 0.33
MS-4 enteroscope	19	Before: 21.30 ± 3.08 pg/mL; After: 21.66 ± 3.93 pg/mL	1.00 ± 0.13

Table 2 Detection time of the Japanese small intestine endoscopes

Type	*n*	Detection time (h)
EN-450P5/20 (double-balloon enteroscope)	118	2.01 ± 0.25
SIF-Q260 (single-balloon enteroscope)	165	1.36 ± 0.07
Overall	283	1.68 ± 0.16



DOI: 10.3748/wjg.v28.i13.1329

Figure 1 Ascarislumbricoides.

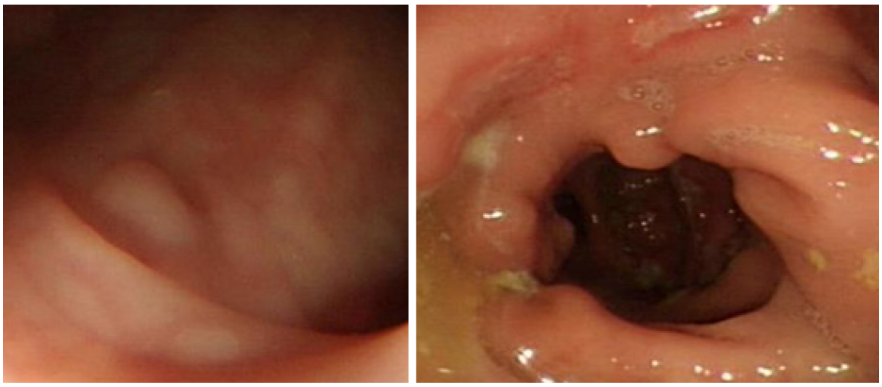


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Figure 2 Suspected Crohn's disease.

also secrete a variety of gastrointestinal hormones, including gastrin, trypsin and cholecystokinin. Hormones are chemical substances produced by endocrine cells and are highly efficient in transmitting information[17-20]. There are many types of hormones and their levels are extremely small (mostly in the range of nanograms and even picograms)[21]. They are neither the energy source of the body nor structural substances in the human body. However, they play essential roles in coordinating physiological processes such as metabolism and growth/development by transmitting information. Research on the changes of gastrin before and after the MS series of small intestine endoscopes examination can provide theoretical support for the safe application of small intestine endoscopes.

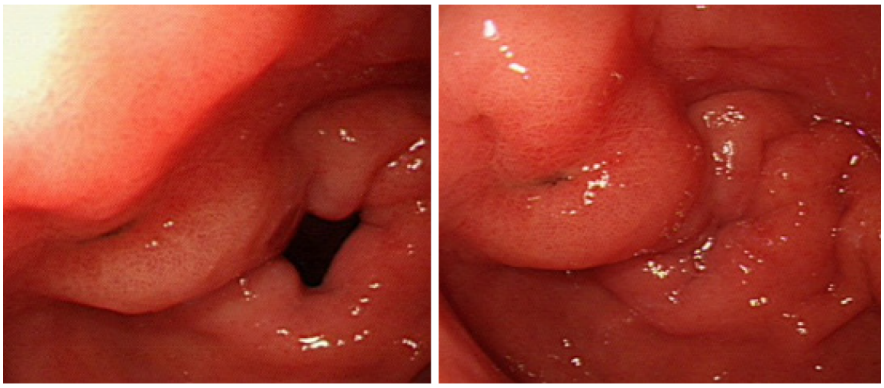

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Figure 3 Small intestinal diverticulum.

Analysis of the changes in gastrin may help determine the impact of small intestine endoscopes on the internal environment of the digestive system, thus research and development of small intestine endoscopes are important.

In the experiments performed in the present study, we found that the MS series of small intestine endoscopes had little effect on the gastrointestinal tract, and the experimental animals soon returned to normal. At the same time, we also found that diseases are often found in living organisms, such as *Ascarislumbricoides*, suspected Crohn's disease and small intestinal diverticulum[22-24]. *Ascaris-lumbricoides* can be cured quickly by the combined application of albendazole and ivermectin[25].At present, there isno good solution for small intestinal diverticulum and small intestinal tumor[26,27]. In the future, the precise treatment of lesions may be realized through small intestine endoscopes and laparoscopy.

The incidence rate of gastrointestinal diseases has reached 20% in China. The incidence rate of small intestinal diseases is approximately 2.5% of digestive system diseases. The number of people with small intestinal diseases in China is about 1.4 billion × 20% × 2.5% ≈ 7 million every year, these people were diagnosed by capsule endoscopy, double balloon small intestine endoscopy and single balloon small intestine endoscopy[28,29]. Each year, many patients attend tertiary general hospitals for hospital screening layer by layer to choose surgical treatment. However, due to the lack of small intestine endoscope locations, some patients have incomplete resection, intestinal leakage and intestinal obstruction. If these patients have the money to continue treatment, they could attend Nanjing Army General Hospital, which is a laminar flow ward. With regard to small intestinal tumors requiring surgery, although they only account for 5% of gastrointestinal tumors (the 7th edition of Huang Jiasi's Surgery), they cannot be ignored.

The past ten years have witnessed our efforts in the research and development of the MS series of small intestine endoscopes. From the type MS-1 to MS-5, the performance of our devices has been constantly improved, and the detection time has been gradually shortened. The MS-1 small intestine endoscope is a negative-pressure device that is advanced into the small intestine in a paralleled alternating manner, and the detection time is long. In 2008, we adjusted the design to develop the MS-2 small intestine endoscope based on the patented drawings of the pipette small intestine endoscope, and its detection time was slightly shorter when compared with the MS-1 small intestine endoscope. However, slippage of the small intestine endoscope persisted due to the extremely poor stabilities of the fixing rings and the metal slide, making the MS-2 small intestine endoscope unfeasible for clinical applications. In 2010, we further optimized our design and developed the MS-3 small intestine endoscope based on the patented design of the built-in straw-type small intestine endoscope. This new-generation device had a shorter detection time than the MS-2 small intestine endoscope. It had higher stability and speed and fewer slippage events during small intestine endoscopy; however, negative-pressure perforation occurred in some experiments. Two articles describing the MS-3 small intestine endoscope were published in the Chinese Journal of Digestive Endoscopy and Chinese Journal of Gastrointestinal Surgery, respectively, and our research on this device also won the third prize of the Dalian Science and Technology Progress Award in 2012.In 2012, by adopting a new working principle, we developed the MS-4 type based on the patented design of the catheter balloon-type small intestine endoscope. The MS-4 small intestine endoscope has significantly higher stability and speed, along with fewer slippage events[30]. The detection time of the MS-4 small intestine endoscope did not decrease compared with the MS-3 small intestine endoscope. Notably, the MS-4 small intestine endoscope has obtained the production license from Liaoning Provincial Food and Drug Administration. In 2018, we completed the design of the MS-5 small intestine endoscope based on the latest and most practical design. In this new endoscope, we optimized the structure of the auxiliary power system using a four-drive design. Relevant studies have been carried out since then.

Our team is trying to further optimize the MS series of small intestine endoscopes, particularly to shorten the detection time of small intestine endoscopy. The clinical application of these endoscopes will facilitate the examination, diagnosis, and treatment of gastrointestinal diseases and solve existing problems. To perform new types of surgery, a small intestine endoscope is combined with laparoscopic resection of intestinal lesions. This reduces open surgery, avoids intestinal adhesions or intestinal obstruction caused by open surgery, increases the diagnostic accuracy and cure rate, and thus further improves the capability and quality of medical services.

CONCLUSION

Intestinal ascariasis is a common disease in pigs. Some pigs have abnormal intestinal variations. After continuous upgrade and improvement, the MS-3 and MS-4 small intestine endoscopes appear superior in terms of detection time.

ARTICLE HIGHLIGHTS

Research background

Small intestine disease endangers human health and is not easy to locate and determine. The fundamental reason for this is that there is no appropriate small intestine endoscopic equipment for treatment. The MS-5 small intestine endoscope should be introduced as soon as possible to benefit patients.

Research motivation

To alleviate the suffering of patients and reduce intestinal adhesions and intestinal obstruction.

Research objectives

To assess the data on the fifth generation small intestine endoscope should be fast-tracked because there is no blind spot in the examination, diagnosis and treatment of small intestinal diseases.

Research methods

Experimental animals and patients underwent routine bowel cleaning before the examination, which was performed using small intestine endoscopes.

Research results

The average detection time for the MS-1, MS-2, MS-3 and MS-4 small intestine endoscope was 2.30 ± 0.15 h, 1.50 ± 0.30 h, 0.67 ± 0.33 h and 1.00 ± 0.13 h, respectively. The detection time was 1.36 ± 0.07 h for SBE and 2.01 ± 0.25 h for DBE, and the overall detection time for the Japanese small intestine endoscopes was approximately 1.68 ± 0.16 h.

Research conclusions

After continuous upgrade and improvement, the MS-4 small intestine endoscope showed superior detection time.

Research perspectives

Improve the diagnosis and treatment of small intestinal diseases, solve practical problems, and reduce the number of cases of open surgery to improve the quality of medical treatment in the future.

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FOOTNOTES

Author contributions: Liu JH, Liu DY and Sun XJ designed the research; Liu JH, Liu DY, Yuan YF, Sun XJ and Shan SM performed the research; Liu JH, Liu DY and Sun XJ contributed new reagents/analytic tools; Liu JH analyzed the data; Liu JH and Sun XJ wrote the paper.

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

Conflict-of-interest statement: Xue-Jun Sun has received fees for serving as a speaker for The First Affiliated Hospital of Xi'an Jiaotong University; Jin-Hua Liu has received research funding from Liaoning Provincial Natural Science Foundation; Yong-Feng Yuan is an employee of Harbin Institute of Technology; Shu-Mei Shan owns stocks and/or shares in Dalian Mingsheng Technology Development Company; Jin-Hua Liu and Dan-Yang Liu owns patent Built-in Straw type small intestine endoscope: China, 200920351611.5 [P]. 2010-09-08; and Catheter balloon type small intestine endoscope: China, 200920351612.X [P]. 2010-09-08.

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ORIGINAL ARTICLE

Basic Study

c-MET immunohistochemical expression in sporadic and inflammatory bowel disease associated lesions

Grant Halliday, Ross J Porter, Catherine J Black, Mark J Arends, Shahida Din

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Abstract

BACKGROUND

Post-colonoscopy colorectal cancer (CRC) rates for patients with inflammatory bowel disease (IBD) are unacceptably high. During colonoscopy, an intravenous fluorescent anti-c-MET probe may improve endoscopic detection of lesions. However, c-MET expression in IBD lesions is poorly defined, limiting translational studies.

AIM

To comprehensively define c-MET expression in sporadic and IBD-associated colorectal carcinogenesis.

METHODS

c-MET expression was immunohistochemically assessed in 319 formalin-fixed paraffin-embedded tissue specimens, colonoscopically or surgically retrieved between 1994-2017. Tissue included: 30 normal colorectal biopsies, 30 hyperplastic polyps (HP), 31 sessile serrated lesions (SSL), 55 tubular/tubulovillous adenomas with low (TA-LGD, n = 32) or high grade dysplasia (TA-HGD, n = 23), 26 sporadic (s)-CRCs, 16 quiescent IBD biopsies, 11 active/inflamed IBD biopsies, 18 IBD-associated dysplastic lesions (IBD-dys), and 102 IBD-CRCs. Expression was scored by two independent observers as: 0 = absent, 1 = weak, 2 = moderate or 3 = strong. Mann-Whitney U and Kruskal-Wallis tests were used to assess significan-

ce.

RESULTS

Positive epithelial cytoplasmic and membranous c-MET expression was observed in all tissues, indicating there is ubiquitous expression in the colorectum. c-MET expression was weak in normal colonic epithelium compared with each of the sporadic colonic lesions, including TA-LGD (P < 0.001), TA-HGD (P = 0.004), HP (P < 0.001), SSL (P < 0.001), and s-CRC (P < 0.001). Specifically, in sporadic (non-IBD) lesions, expression was stronger in TA-LGD compared with normal mucosa (P < 0.001), and stronger in s-CRC compared with TA-HGD (P = 0.004). However, there was no significant difference between TA-LGD and TA-HGD (P = 0.852). Further, there was no difference in c-MET expression between HP and SSL (P = 0.065). In IBD, expression was weaker in quiescent colonic mucosa compared with inflamed colonic mucosa (P < 0.001). There was no difference between inflamed colonic mucosa and IBD-dys (P = 0.512) or IBD-CRC (P = 0.296). However, expression was stronger in IBD-dys (P < 0.001) and IBD-CRC (P < 0.001) compared with quiescent IBD colonic mucosa.

CONCLUSION

The characterisation of c-MET expression suggest that an intravenous probe may improve the endoscopic detection of lesions in both non-IBD patients and IBD patients with quiescent disease.

Key Words: Inflammatory bowel diseases; Colorectal cancer; Surveillance; Detection; c-MET; Immunohistochemistry

Core Tip: During colonoscopy, an intravenous fluorescent anti-c-MET probe may improve endoscopic detection of dysplasia and cancer. However, c-MET expression in inflammatory bowel disease (IBD) lesions is poorly defined, limiting translational studies. We demonstrate that stronger immunohistochemical c-MET expression is associated with dysplasia and cancer in both sporadic and IBD-associated lesions. Therefore, c-MET expression could be exploited clinically to enhance endoscopic detection of pre-malignant lesions and cancer, particularly in IBD where post-colonoscopy colorectal cancer rates are unacceptably high.

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INTRODUCTION

Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC), with poorer survival compared with the general population[1,2]. The British Society of Gastroenterology currently recommends high-definition surveillance ileocolonoscopy ± chromoendoscopy, with targeted biopsies starting 8-years after the onset of IBD symptoms[3]. Despite this, IBD post-colonoscopy CRC rates - defined as a diagnosis of cancer or high-grade dysplasia > 6 mo to 3 years following a colonoscopy that was negative for cancer - are unacceptably high, at 28%-45%[4,5]. One challenge in identifying dysplastic and malignant lesions in IBD is the morphological changes associated with flat lesions which are difficult to detect endoscopically. With an ageing population and rising global burden of IBD[6], there is an increasing requirement for endoscopic surveillance indicating an urgent clinical need to improve the endoscopic detection of IBD-associated dysplasia and cancer.

c-MET is a receptor tyrosine kinase (encoded by the *MET* gene on chromosome 7q21-31) overexpressed at a protein level in a variety of human primary tumours, including in the colorectum where it is associated with the sporadic adenoma-carcinoma pathway[7-11]. In 2015, Burggraaf and colleagues published a first-in-human study demonstrating that an intravenous injection of a fluorescently labelled peptide with a high affinity for c-MET was safe, well tolerated in humans, and could improve the detection of colonic polyps in a high-risk asymptomatic patient cohort[12]. Since then, translational data have emerged in support of this technology for sporadic CRC[13,14].

The original data from Burggraaf and colleagues stated that there were several lesions visible with fluorescence assisted colonoscopy that were not identified during first or second pass conventional

white light colonoscopy. These small lesions were mostly < 6 mm and non-polypoid[12]. Given this, we hypothesise that this technology may be especially useful for identifying IBD-associated lesions with similar flat morphology. However, the clinical utility of c-MET is unclear in the setting of chronic inflammation and injury to the colonic mucosa, as c-MET has been reported to be upregulated in tissue repair[15].

While the efficacy of an *in vivo* c-MET probe has never been investigated in the setting of human IBD, there are some murine data. The azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model is commonly used to simulate colitis-associated carcinogenesis in the laboratory[16]. Using this model, Tao and colleagues reported that there was an accumulation of fluorescence from their c-MET probe (Crizotinib and MPA, a water-soluble cyanine dye, covalently conjugate *via* PEG4) in colonic lesions, while there was minimal fluorescence in adjacent tissue[17]. While an acceptable surrogate model, AOM/DSS lesions are not identical to those seen in human disease. Further, Tao *et al*[17] resected colons for imaging *in vitro*, rather than assessment by *in situ* fluorescence colonoscopy. Nonetheless, these data are encouraging and warrant investigation in human IBD: there are a paucity of histopathological studies that define c-MET expression in human IBD and IBD-associated carcinogenesis.

To address this need, this study comprehensively defines the expression of c-MET in a large cohort of 319 paraffin-embedded tissue sections, representing the spectrum of both sporadic and IBD-associated colorectal carcinogenesis. Our results suggest that c-MET could be exploited clinically to enhance detection of potentially malignant lesions in IBD.

MATERIALS AND METHODS

Patient tissue selection

217 formalin fixed paraffin embedded (FFPE) human tissue specimens, colonoscopically or surgically retrieved between January 2000 to December 2017, were identified from the Edinburgh Pathology database. Tissue included: 30 normal colorectal biopsies, 30 hyperplastic polyps (HP), 31 sessile serrated lesions (SSL), 55 tubular/tubulovillous adenomas with low (TA-LGD, n = 32) or high (TA-HGD, n = 23) grade dysplasia, 26 sporadic colorectal adenocarcinomas (s-CRC), 16 quiescent IBD biopsies, 11 active/inflamed IBD biopsies, and 18 conventional IBD-associated dysplastic lesions (IBD-dys)-15 were considered low-grade and 3 were considered high-grade. A tissue microarray comprising 102 IBD-associated CRC (IBD-CRC) cores from 43 patient tumours, retrieved from surgical resection specimens between 1994 and 2011, was also used. For all tissue, the original haematoxylin and eosin (HE) diagnostic slide, case history and pathology report were reviewed by two expert gastrointestinal pathologists (MJA, CJB) to ensure consensual agreement and accurate tissue diagnosis. All selected cases were anonymised. Ethical approval was obtained from Lothian NRS Bioresource Research Tissue Bank (15/ES/0094; SR148, SR389, SR400 and SR588).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using an optimised protocol. In summary, 3μm tissue sections were cut by microtomy from each selected FFPE block and floated onto positively charged glass slides. Tissue was oven-dried overnight. Sections were deparaffinised and underwent heat-mediated antigen retrieval in pH6 citrate buffer for 15 min (BOND Epitope Retrieval Solution 1, Leica Biosystems, United Kingdom). After 5 min of peroxidase blocking, tissue was stained for 15 min with recombinant monoclonal anti-MET (c-MET) antibody (ab51067, clone EP1454Y, Abcam, United Kingdom) at a 1:200 dilution, on Leica Bond-III and BONDMAX autostainers (Leica Biosystems, United Kingdom). Primary antibody detection used the BOND Polymer Refine Detection Kit (Leica Biosystems, United Kingdom).

Interpretation of IHC staining

Two expert gastrointestinal pathologists (MJA, CJB) independently assessed c-MET expression as: 0=no staining, 1+ = weak intensity staining, 2+ = moderate intensity staining, and 3+ = strong intensity staining. Epithelial cytoplasmic and epithelial membranous immunopositivity was ubiquitously equivalent in this study which meant one representative score was assigned to each biopsy. Discrepant scores were resolved by discussion and consensus reached. Use of a histoscore was not considered appropriate in small dysplastic lesions. Scoring methodology and staining intensity agreement came from comparison with internal control normal tissue adjacent to the lesions, and previously published staining intensities[7,9-12,14,18]. Negative control slides were used to allow observers to account for background staining (which was negligible due to IHC optimisation).

Statistical analysis

Statistical analysis was performed using IBM®SPSS® V25.0.0.1 and GraphPad Prism software. Associations between ordinal and categorical variables were assessed using exact two-tailed Mann-Whitney U and Kruskal-Wallis tests. Scores of 0, 1+, 2+ and 3+ were thus compared and statistical significance was determined at $P \leq 0.05$.

RESULTS

c-MET is ubiquitously expressed in the colorectum, with increased expression in colonic dysplastic and other lesions

Positive epithelial cytoplasmic and membranous c-MET expression was observed in all tissues, indicating there is ubiquitous expression in the colorectum. As anticipated, c-MET expression was weak in normal colonic epithelium compared with each of the sporadic colonic lesions, including TA-LGD ($P < 0.001$), TA-HGD ($P = 0.004$), HP ($P < 0.001$), SSL ($P < 0.001$), and s-CRC ($P < 0.001$) (Figure 1).

Increased c-MET expression is associated with sporadic colonic dysplasia and adenocarcinoma

c-MET expression was stronger in TA-LGD compared with normal colonic mucosa ($P < 0.001$), and stronger in s-CRC compared with TA-HGD ($P = 0.004$). There was no significant difference in c-MET expression between TA-LGD and TA-HGD ($P = 0.852$).

There is no significant difference in c-MET expression between hyperplastic polyps vs sessile serrated lesions

Given the association between c-MET expression and malignancy, we investigated whether there was a difference between hyperplastic polyps (with low malignant potential) and sessile serrated lesions (with higher malignant potential). There was no statistically significant difference in c-MET expression between these lesions ($P = 0.065$).

c-MET shows increased expression in IBD-dysplasia and IBD-cancer compared with quiescent, but not actively inflamed, IBD mucosa

Given the association between c-MET expression and sporadic colorectal carcinogenesis, we assessed whether this was also true for IBD-associated dysplasia and cancer, where detection of dysplasia and cancer is more challenging. c-MET expression was weak in quiescent IBD mucosa compared with active / inflamed IBD ($P < 0.001$). There was no difference in c-MET expression between inflamed IBD mucosa and IBD-dys ($P = 0.512$) or IBD-CRC ($P = 0.296$). However, c-MET expression was stronger in IBD-dys ($P < 0.001$) and IBD-CRC ($P < 0.001$) compared with quiescent IBD mucosa. There was no difference between IBD-dys and IBD-CRC ($P = 0.673$) (Figure 2).

DISCUSSION

This immunohistochemistry study of 319 human tissue specimens provides a comprehensive overview of c-MET expression in both sporadic and IBD-associated colorectal carcinogenesis (Supplementary Table 1). We demonstrate c-MET expression in the colorectum, with an increase in expression in sporadic dysplasia and s-CRC. In IBD-associated lesions we report increased c-MET expression in IBD-dysplasia and IBD-CRC compared with quiescent, but not actively inflamed IBD mucosa.

Previous histopathological studies report overexpression of c-MET in sporadic CRC, associated with tumour invasion, metastasis, local recurrence, and poor overall survival[7-10]. Less data exist for pre-cancerous lesions. However, studies suggest that c-MET expression is increased across the adenoma-dysplasia-carcinoma sequence[11]. These data are in keeping with our comprehensive assessment of sporadic lesions; there is an increase in c-MET expression from normal colonic mucosa to dysplasia to colorectal adenocarcinoma.

Gayyed and colleagues reported increased c-MET expression in colonic polyps with HGD compared with LGD[11]. Our study reported no statistically significant difference between these LGD and HGD groups, using a rigorous approach to achieve full diagnostic agreement from two expert gastrointestinal pathologists, based upon review of the HE slide, and all available clinico-pathological data. The proposed use for c-MET is not necessarily to discriminate LGD from HGD; instead, it is as an *in vivo* probe to improve endoscopic detection of colonic lesions. Both lesions had stronger c-MET expression compared with normal mucosa suggesting that either lesion would be positively identified at colonoscopy allowing targeted biopsy for histopathological assessment.

Identification of SSL from HP is useful as the former have higher malignant potential which is not fully appreciated during endoscopic assessment[19]. Joshi and colleagues report increased c-MET expression in SSL compared with HP, using immunofluorescence on FFPE SSL ($n = 17$) and HP ($n = 10$) tissue. This was also observed by Wu *et al*[13]. In our study, there was no statistically significant difference between SSL ($n = 31$) and HP ($n = 30$) ($P = 0.065$). One reason could be that we used IHC whereas previous studies used immunofluorescence. There has been inconsistent reporting of SSLs; due to previous high inter-observer variability, lack of robust diagnostic criteria and prior misclassification of hyperplastic polyps[19-21]. As previously discussed, our study ensured accurate polyp sub-classification (through consensual agreement by two expert gastrointestinal pathologists) and it is reassuring both lesions had increased expression compared with normal mucosa, as this infers both lesions would

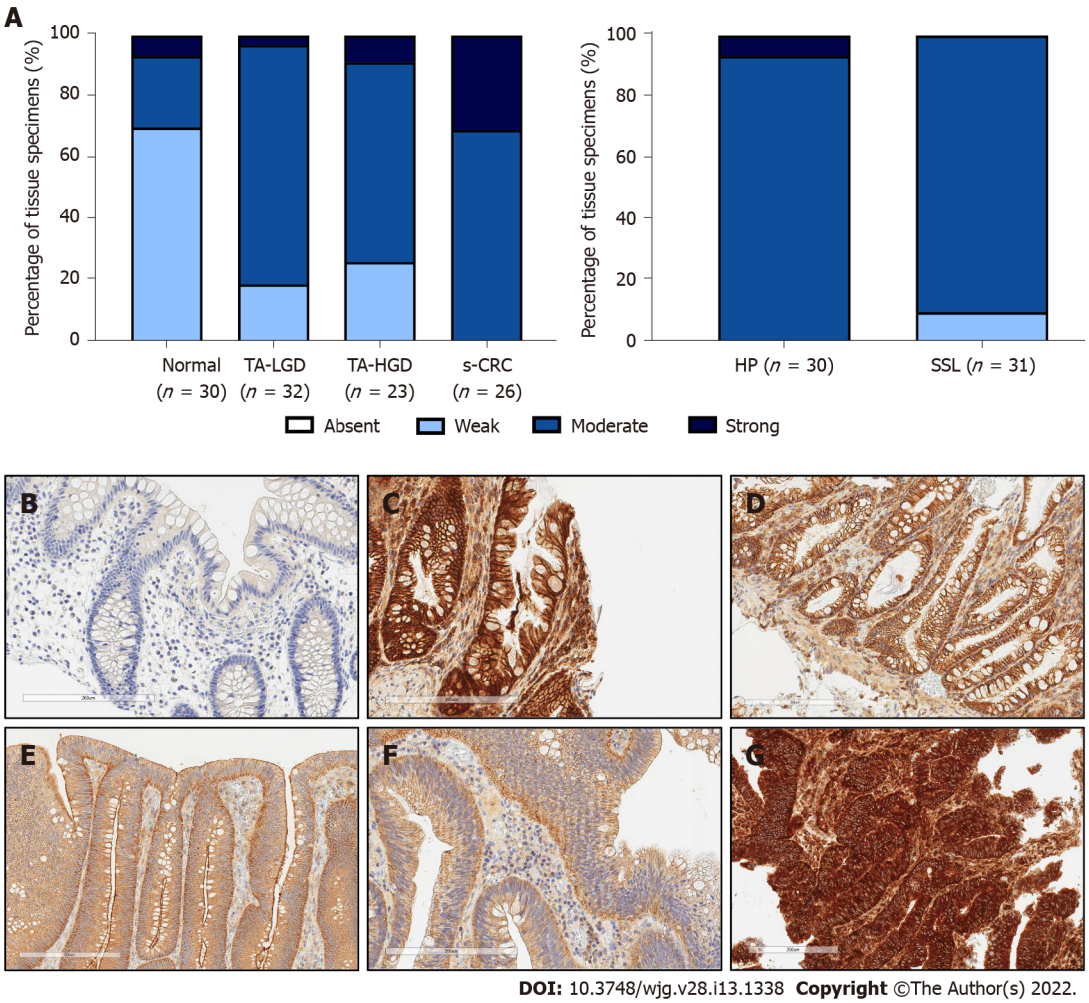

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Figure 1 Expression intensity of c-MET in sporadic colonic lesions. A: Semi-quantitative assessment of c-MET expression intensity throughout sporadic colorectal carcinogenesis; B: Representative photomicrograph of normal colonic mucosa; C: Representative photomicrograph of hyperplastic polyp; D: Representative photomicrograph of sessile serrated lesion; E: Representative photomicrograph of tubular/tubulovillous adenoma with low grade dysplasia; F: Representative photomicrograph of tubular/tubulovillous adenoma with high grade dysplasia; G: Representative photomicrograph of sporadic colorectal adenocarcinoma. Tissue provided by Lothian NRS Bioresource. Brightfield photomicrographs taken at X20 magnification. TA-LGD: Tubular/tubulovillous adenoma with low grade dysplasia; TA-HGD: Tubular/tubulovillous adenoma with high grade dysplasia; s-CRC: Sporadic colorectal adenocarcinoma; HP: Hyperplastic polyp; SSL: Sessile serrated lesion.

be positively identified for histopathological assessment and SSL would thus not be missed.

Our study found no difference in c-MET expression between inflamed IBD mucosa, IBD-dys and IBD-CRC. This is in agreement with the IHC study from Harpaz *et al*[18]. A key challenge is detecting dysplasia in the context of inflammation-both endoscopically and histopathologically. While this is a major priority, it is based upon the assumption that the post-colonoscopy CRC rate in IBD is related to active inflammation which reduces the ability to detect dysplasia. Therefore, we need to improve detection across the board. While an inability to distinguish between inflamed IBD mucosa, IBD-dys and IBD-CRC could thus be perceived as a barrier to clinical translation, our study reports lower expression of c-MET in quiescent IBD mucosa. Therefore, careful selection of patients could help identify a cohort which would benefit from the use of such an adjunct in detecting subtle lesions during surveillance colonoscopy. Objective biomarkers such as faecal calprotectin ± serum C-reactive protein levels could indicate whether a patient is more likely to have quiescent disease. Indeed, The British Society of Gastroenterology recommend surveillance colonoscopy in patients during quiescent phases of disease where possible[3]. Careful protocol optimisation will minimise any background fluorescence, due to microscopic histological inflammation, which may be present in endoscopically normal and/or quiescent mucosa in patients with IBD.

There are several practical questions that now need to be answered to determine the clinical viability of introducing a c-MET probe into IBD surveillance programs: (1) Is a c-MET probe safe and well tolerated in IBD patients; (2) Does inflammation confound endoscopic assessment of colonic lesions using a fluorescent c-MET probe; (3) Can a c-MET probe be optimised to differentiate between background quiescent/non-inflamed colonic mucosa and IBD-associated lesions, as assessed by fluorescence colonoscopy; (4) Can pre-screening patients using symptom questionnaires, faecal calpro-

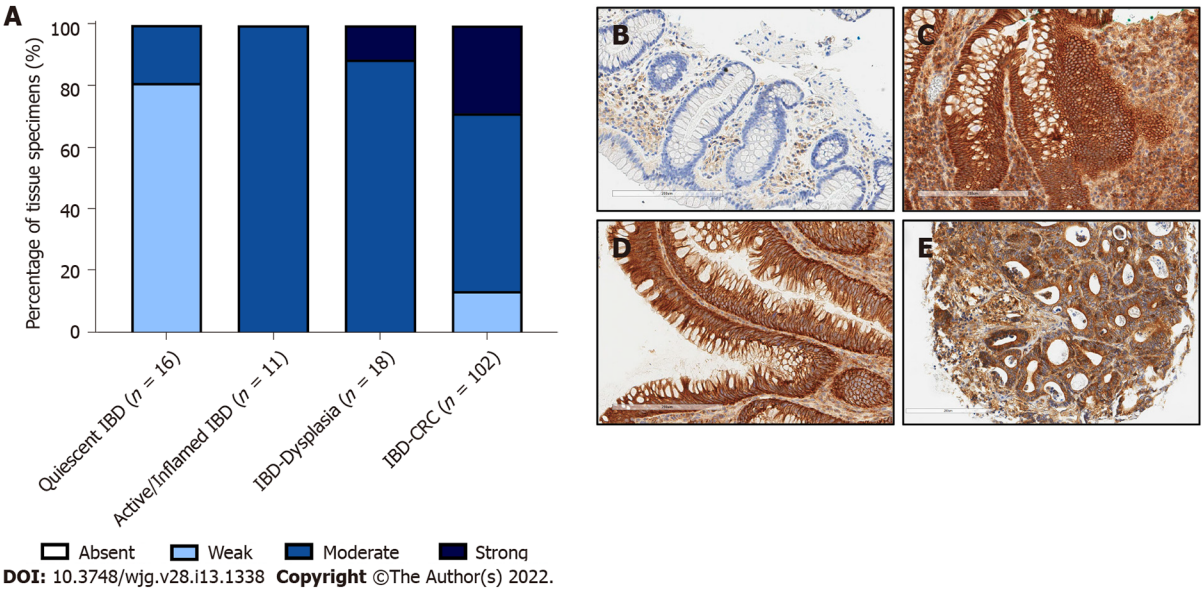

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Figure 2 Expression intensity of c-MET in inflammatory bowel disease-associated lesions. A: Semi-quantitative assessment of c-MET expression intensity throughout inflammatory bowel disease (IBD)-associated colorectal carcinogenesis; B: Representative photomicrograph of quiescent IBD mucosa; C: Representative photomicrograph of active/inflamed IBD mucosa; D: Representative photomicrograph of IBD-associated dysplastic lesions; E: Representative photomicrograph of IBD-associated colorectal cancer. Tissue provided by Lothian NRS Bioresource. Brightfield photomicrographs taken at X20 magnification. IBD: Inflammatory bowel disease; CRC: Colorectal cancer.

tectin ± serum C-reactive protein accurately identify quiescent disease, resulting in low false-positive fluorescence; and (5) Does a c-MET probe offer benefit compared with standard care or other advanced endoscopy techniques for the detection of IBD lesions: for example, can the probe detect endoscopically 'invisible' or flat dysplasia (*i.e.* dysplasia seen only histopathologically on a random biopsy of macroscopically normal mucosa)?

CONCLUSION

In this comprehensive study, we have robustly defined the expression of c-MET in both sporadic and IBD-associated colorectal carcinogenesis. These data provide a platform for clinical studies to investigate the efficacy of an *in vivo* c-MET probe to enhance the endoscopic detection of colonic lesions during IBD surveillance colonoscopy. Such an application would be especially important for identifying IBD-associated dysplasia, to reduce the high post-colonoscopy CRC rate within a pre-selected cohort of patients with quiescent disease.

ARTICLE HIGHLIGHTS

Research background

Patients with inflammatory bowel disease (IBD) are more likely to develop colorectal cancer (CRC) compared with the general population, and surveillance colonoscopy is therefore performed at defined intervals to identify pre-malignant lesions. Despite this, IBD post-colonoscopy CRC rates remain unacceptably high. One key challenge is endoscopically identifying the more subtle and flat lesions associated with dysplasia and cancer in IBD.

Research motivation

There is an urgent need to improve endoscopic detection of pre-malignant lesions, especially in patients with IBD. Recent studies have suggested that an intravenously administered fluorescent probe against c-MET protein may improve the detection of sporadic colorectal lesions-specifically small non-polypoid lesions of similar morphology to IBD-associated (pre-) malignant lesions. However, most data come from murine studies or sporadic disease, and there are lack of immunohistochemical data defining c-MET expression in IBD-associated colonic lesions. This is limiting translational studies.

Research objectives

This study was designed to systematically assess the immunohistochemical expression of c-MET in both sporadic and inflammatory bowel disease-associated colonic lesions.

Research methods

c-MET expression intensity was semi-quantitatively assessed after immunohistochemically staining formalin-fixed paraffin-embedded tissue specimens with an anti-c-MET antibody. Tissue had been colonoscopically or surgically retrieved from patients with and without IBD between 1994-2017, and included normal colonic mucosa, hyperplastic polyps, sessile serrated lesions, tubular/tubulovillous adenomas with low or high grade dysplasia, sporadic-CRC, quiescent IBD mucosa, inflamed IBD mucosa, IBD-associated dysplastic lesions, and IBD-associated CRC.

Research results

There was ubiquitous expression of c-MET in normal colonic mucosa, as well as in sporadic and IBD lesions. c-MET expression intensity was similar between low *vs* high grade dysplasia, and between hyperplastic polyps *vs* sessile serrated lesions. However, c-MET expression was stronger in sporadic dysplasia and cancer compared with normal colonic mucosa. Similarly, c-MET expression was stronger in IBD-associated dysplastic and malignant lesions compared with quiescent IBD mucosa. There was no difference in c-MET expression between inflamed IBD mucosa and IBD-associated dysplasia or malignant lesions.

Research conclusions

c-MET expression intensity is stronger in dysplastic and malignant lesions compared with normal colonic epithelium and quiescent IBD mucosa. These data provide a platform to allow future studies to investigate whether an intravenous anti-c-MET probe could help endoscopically identify dysplasia and malignancy, particularly within surveillance colonoscopy programmes for IBD patients where post-colonoscopy CRC rates are unacceptably high.

Research perspectives

Further study is needed to determine whether histopathological expression correlates with mucosal expression at endoscopy in the context of IBD. The ability of such a probe to improve the endoscopic detection of colorectal lesions and reduce the post-colonoscopy CRC rate should then be assessed, in patients with quiescent IBD.

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FOOTNOTES

Author contributions: Porter RJ and Halliday G contributed equally to this work; Porter RJ analysed data, wrote the first draft of the manuscript and reviewed and edited the final manuscript; Halliday G performed experiments, contributed to writing the first manuscript draft and reviewed and edited the manuscript; Arends MJ and Black CJ provided histopathological expertise, contributed to study design, performed experiments and reviewed and edited the final manuscript; Din S was the principal investigator for this study, conceptualised and designed the study, analyzed data and reviewed and edited the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: Ethical approval was obtained from Lothian NRS Bioresource Research Tissue Bank (15/ES/0094; SR148, SR389, SR400 and SR588).

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ORIGINAL ARTICLE

Retrospective Study

Increased prognostic value of clinical–reproductive model in Chinese female patients with esophageal squamous cell carcinoma

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Abstract

BACKGROUND

In China, it has been well recognized that some female patients with esophageal squamous cell carcinoma (ESCC) have different overall survival (OS) time, even with the same tumor-node-metastasis (TNM) stage, challenging the prognostic value of the TNM system alone. An effective predictive model is needed to accurately evaluate the prognosis of female ESCC patients.

AIM

To construct a novel prognostic model with clinical and reproductive data for Chinese female patients with ESCC, and to assess the incremental prognostic value of the full model compared with the clinical model and TNM stage.

METHODS
A new prognostic nomogram incorporating clinical and reproductive features was constructed based on univariatie and Cox proportional hazards survival analysis from a training cohort (n = 175). The results were recognized using the internal (n = 111) and independent external (n = 85) validation cohorts. The capability of the clinical–reproductive model was evaluated by Harrell's concordance index (C-index), Kaplan–Meier curve, time-dependent receiver operating characteristic (ROC), calibration curve and decision curve analysis. The correlations between estrogen response and immune-related pathways and some gene markers of immune cells were analyzed using the TIMER 2.0 database.

RESULTS
A clinical–reproductive model including incidence area, age, tumor differentiation, lymph node metastasis (N) stage, estrogen receptor alpha (ESR1) and beta (ESR2) expression, menopausal age, and pregnancy number was constructed to predict OS in female ESCC patients. Compared to the clinical model and TNM stage, the time-dependent ROC and C-index of the clinical–reproductive model showed a good discriminative ability for predicting 1-, 3-, and 5-years OS in the primary training, internal and external validation sets. Based on the optimal cut-off value of total prognostic scores, patients were classified into high- and low-risk groups with significantly different OS. The estrogen response was significantly associated with p53 and apoptosis pathways in esophageal cancer.

CONCLUSION
The clinical–reproductive prognostic nomogram has an incremental prognostic value compared with the clinical model and TNM stage in predicting OS in Chinese female ESCC patients.

Key Words: Esophageal squamous cell carcinoma; Female; Nomogram; Prognosis; Estrogen receptor

Core Tip: In China, some female patients with esophageal squamous cell carcinoma (ESCC), even with the same tumor-node-metastasis (TNM) stage, have significantly different overall survival (OS) time. The prognostic value of the TNM system has been challenged due to its unsatisfactory discriminative ability. A new prognostic nomogram that combines clinical and reproductive features was developed and validated in this study. Compared with the clinical model and TNM stage, clinical–reproductive model has incremental prognostic value in predicting OS in Chinese female patients with ESCC, which can help clinicians to make individual treatment and medical decisions.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is highly invasive malignancy in China with a 5-year overall survival (OS) rate of only 10%-20% in patients with advanced disease[1]. In addition to pathological features, reproductive factors such as menopausal status, estrogen receptors, and pregnancy number are also correlated with clinical outcome in female ESCC patients[2,3]. The increased risk of esophageal cancer is associated with a decrease in estrogen level[4]. An epidemiological study of menopausal hormone therapy (MHT) confirmed that patients who have been using MHT have a reduced risk of ESCC[5]. Previous research also supports the protective effects of female hormones on the risk of ESCC[6].

The most common prognostic evaluation system for ESCC mainly depends on the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer, which has been adopted in the United States since 1959[7,8]. Recently, the prognostic value of the TNM system has been challenged due to its unsatisfactory discriminative ability[9]. Relevant studies have shown significant differences in OS in female ESCC patients even with the same TNM stage[3]. Combined with other clinical prognostic factors such as age and tumor differentiation, individual prognosis can be more

Table 1 Distribution of the clinical and reproductive factors for female patients with esophageal squamous cell carcinoma in primary training, internal and external validation cohorts (mean ± SD)

Characteristics	Primary training cohort (%)	Internal validation cohort (%)	External validation cohort (%)
Total	175 (61.2)	111 (38.8)	85 (100)
Diagnose age			
< 50	21 (12.0)	16 (14.4)	10 (11.8)
50-60	59 (33.7)	47 (42.3)	35 (41.2)
> 60	95 (54.3)	48 (43.2)	40 (47.1)
Age	61.35 ± 7.99	60.25 ± 7.97	60.15 ± 8.50
Incidence area			
High	138 (78.9)	89 (80.2)	64 (75.3)
Low	37 (21.1)	22 (19.8)	21 (24.7)
Tumor location			
Upper	34 (19.4)	19 (17.1)	11 (12.9)
Middle	113 (64.6)	79 (71.2)	66 (77.6)
Lower	28 (16.0)	13 (11.7)	8 (9.4)
Differentiation			
Higher	11 (6.3)	8 (7.2)	3 (3.5)
Moderate	101 (57.7)	67 (60.4)	51 (60.0)
Lower	63 (36.0)	36 (32.4)	31 (36.5)
T stage			
T1/T2	64 (36.6)	32 (28.8)	26 (30.6)
T3/T4	111 (63.4)	79 (71.2)	59 (69.4)
N stage			
N0	86 (49.1)	60 (54.1)	46 (54.1)
N1-N3	89 (50.9)	51 (45.9)	39 (45.9)
Therapy methods			
Operation	164 (93.7)	106 (95.5)	82 (96.5)
Others	11 (6.3)	5 (4.5)	3 (3.5)
Family history			
No	116 (66.3)	66 (59.5)	56 (65.9)
Yes	59 (33.7)	45 (40.5)	29 (34.1)
ESR1			
Negative	49 (28.0)	41 (36.9)	29 (34.1)
Positive	126 (72.0)	70 (63.1)	56 (65.9)
ESR2			
Negative	63 (36.0)	39 (35.1)	24 (28.2)
Positive	112 (64.0)	72 (64.9)	61 (71.8)
Menarche age			
< 14	28 (16.0)	23 (20.7)	12 (14.1)
14-16	85 (48.6)	45 (40.5)	41 (48.2)
> 16	62 (35.4)	43 (38.7)	32 (37.6)
Menopausal age			

< 46	48 (27.4)	19 (17.1)	23 (27.1)
46-55	47 (26.9)	36 (32.4)	20 (23.5)
> 55	80 (45.7)	56 (50.5)	42 (49.4)
Pregnancy number			
< 4	61 (34.9)	40 (36.0)	30 (35.3)
4-6	86 (49.1)	52 (46.8)	43 (50.6)
> 6	28 (16.0)	19 (17.1)	12 (14.1)

T: Tumor invasion depth; N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

accurately predicted[10,11]. An effective predictive model is needed for female ESCC to precisely assess the clinical outcome.

A nomogram is a graphical representation tool based on statistical predictive modeling[12]. So far, no nomogram including reproductive factors has been constructed to predict OS in female ESCC. The current study was designed to identify independent prognostic factors based on univariatie and Cox proportional hazards survival analysis, and then develop and validate a new prognostic nomogram incorporating clinical and reproductive characteristics to predict 1-, 3-, and 5-years OS in female ESCC. We also aimed to establish whether the nomogram model could provide more accurate prognostic prediction than the clinical model and TNM stage.

MATERIALS AND METHODS

Study population

This retrospective study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and Institutional Review Board of the First Affiliated Hospital of Nanyang Medical College. The documentation of informed consent was waived due to anonymity of the participants. In the multicenter study, the analysis was performed on the two independent cohorts of female patients with ESCC (Cohort 1, 500 000 esophageal and gastric cardiac carcinoma database of First Affiliated Hospital of Zhengzhou University[3]; Cohort 2, First Affiliated Hospital of Nanyang Medical College). Patients who were pathologically diagnosed with ESCC, underwent surgery, and had detailed follow-up information where eligible. Patients who received preoperative therapy (radiotherapy and/or chemotherapy) and lost clinical and reproductive information were excluded.

Baseline characteristics and clinical outcome

Clinicopathological factors, including age at diagnosis, incidence area, tumor location, tumor differentiation, tumor invasion depth (T), lymph node metastasis (N), metastasis, and therapy methods were collected from the medical records. Reproductive factors such as menarchal age, menopausal status, menopausal age, pregnancy number, and biomarker levels [estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2)] were also recorded. All eligible patients were followed-up every 3 mo *via* telephone interview or outpatient review. Patients were followed to the latest lost-to-follow-up time (December 31, 2020) or time of death. In our study, the primary clinical outcome was OS which was defined with reference to our previous study[3].

Construction and validation of the nomogram

Factors affecting OS in univariate analysis ($P < 0.20$) were included in a Cox proportional hazards regression for multivariable analysis[13]. Hazard ratio and 95% confidence interval were assessed. After testing for collinearity, a prognostic nomogram was constructed to predict 1-, 3-, and 5-years OS for female ESCC. The discrimination was evaluated using the time-dependent receiver operating characteristic (ROC) and concordance index (C-index) calculated by bootstrapping with 1000 resamples[14]. Calibration was examined *via* calibration plots. Decision curve analysis (DCA) was conducted to evaluate the clinical application and net benefit at different threshold probabilities[15]. Patients in the study were separated into high- or low-risk groups based on the best cut-off point of total prognostic score (TPS) that was decided by X-Tile software[16]. Survival curves were plotted using the Kaplan–Meier analysis. Sub-analysis was conducted to confirm the potential correlations between risk score and OS among different subgroups in the primary training cohort.

Correlations with immune-related pathways and gene markers of immune cells

We analyzed the correlations between estrogen response and immune-related pathways such as p53

Table 2 Comparison of three models in primary training, internal validation, and external validation sets

	Primary training set		Internal validation set		External validation set	
	C-index (95%CI)	*P* value	C-index (95%CI)	*P* value	C-index (95%CI)	*P* value
Full model	0.701 (0.655-0.746)	Ref.	0.684 (0.619-0.748)	Ref.	0.672 (0.609-0.734)	Ref.
Clinical-model	0.629 (0.578-0.681)	0.000	0.593 (0.531-0.655)	0.009	0.589 (0.514-0.663)	0.014
TNM stage	0.638 (0.576-0.699)	0.013	0.552 (0.462-0.641)	0.011	0.560 (0.462-0.657)	0.033

Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age+ incidence area; CI: Confidence interval; TNM stage: Tumor-node-metastasis stage; N: Lymph node metastasis.

pathway and apoptosis pathway in esophageal cancer from The Cancer Genome Atlas (TCGA) by R packages. We also assessed the associations of ESR1 and ESR2 levels with gene markers of tumor-infiltrating immune cells, including B cells, tumor-associated macrophages (TAMs), M1 macrophages, M2 macrophages, and neutrophils by TIMER 2.0 (http://timer.comp-genomics.org/).

Statistical analysis

All statistical analyses were conducted using R language (version 4.1.0). Baseline characteristics of female ESCC patients were summarized. Student's *t* test was used to analyze the continuous variables, and categorical variables were compared with the χ^2 test and Fisher's exact bilateral test. Survival curves were conducted by using the Kaplan–Meier method, and OS was analyzed with a log-rank test. The rms, Hmisc, survival, survcomp, and ggplot2 packages were used for analysis. $P < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Patient characteristics

In Cohort 1, all 286 eligible patients were randomly divided into two sets according by computer-generated random numbers: 175 (60%) (mean age, 61.35 years; range, 41–78 years) were randomly assigned to a primary training set; 111 (40%) (mean age, 60.25 years; range, 42–81 years) to an internal validation set. An independent dataset (n = 85) from Cohort 2 was established to be an external validation cohort. The mean age was 60.15 years with a range of 42–80 years. The median survival time in the primary training cohort was 47.59 mo and the 1-, 3-, and 5-years OS rates were 84.0%, 58.9% and 31.4%, respectively. In the internal validation cohort, the median survival time was 45.63 mo, and the 1-, 3-, and 5-years OS rates were 85.6%, 64.0% and 31.5%, respectively. The median survival time of the external validation cohort was 39.94 mo, and the 1-, 3-, and 5-years OS rates were 84.7%, 56.5% and 23.5%, respectively. The detailed distribution of baseline characteristics of the primary training, internal validation, and external validation datasets are shown in Table 1.

Prognostic roles of clinical and reproductive variables

The prognostic role of each variable in OS was tested in the training cohort (Figure 1). Incidence area (P = 0.014; high *vs* low area), N stage ($P < 0.001$; N0 *vs* N1–N3), therapy methods (P = 0.02; operation *vs* other methods), ESR1 expression level (P = 0.006; negative *vs* positive), ESR2 level ($P < 0.001$; negative *vs* positive), menarche age (P = 0.005; > 16 years *vs* < 13 years), and pregnancy number (P = 0.001; > 6 *vs* < 4–6) were significantly associated with OS in the univariate analysis (Figure 1A). Data were also represented using Kaplan–Meier curves (Figure 1C-I). Parameters associated with $P < 0.20$ based on univariate analysis and relevant clinical factors were entered into a Cox proportional hazards regression model, which included age, incidence area, tumor differentiation, N stage, therapy, ESR1, ESR2, menarche age, menopausal age, and pregnancy number (Figure 1B). No evidence of problematic multicollinearity was found.

Building and performance of prognostic nomogram

To address patient prognosis, we identified a nomogram model for the prognosis prediction of female ESCC patients at 1-, 3-, and 5-years OS in the primary training cohort. Incidence area, diagnose age, differentiation, N stage, ESR1 expression, ESR2 expression, menopausal age, and pregnancy number were finally included in the full model (Figure 2). Pregnancy number contributed to the highest points in the model, followed by menopausal age, N stage, differentiation, and ESR2 status. Incidence area, age and ESR1 Level had a minor impact on OS. To investigate whether the clinical–reproductive model had incremental prognostic value for individualized OS prediction, a clinical model was also constructed by

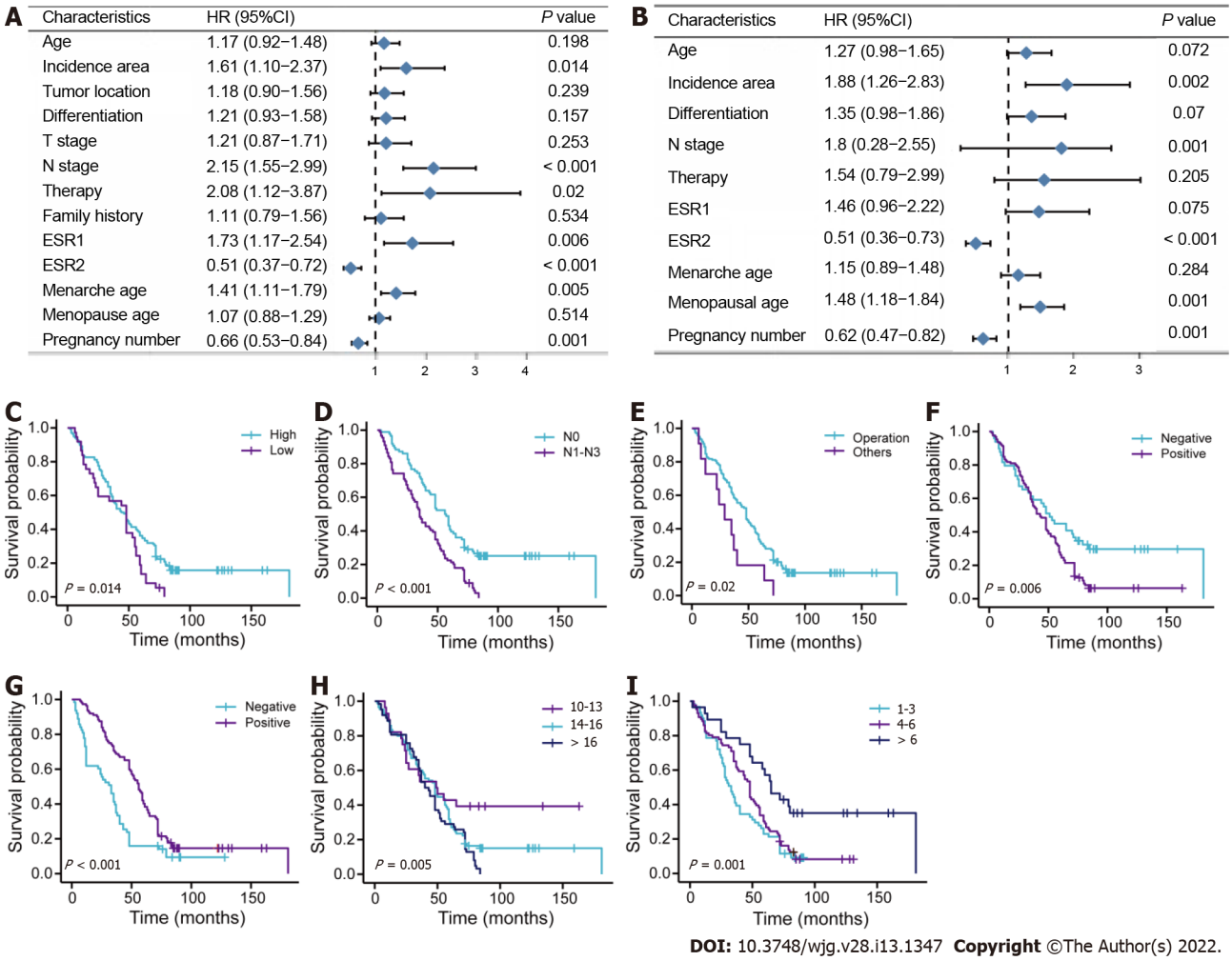

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Figure 1 Survival analysis for female patients with esophageal squamous cell carcinoma in primary training cohort. A: Univariate analysis; B: Multivariate analysis; C-I: Survival curves for patients with incidence area, lymph node metastasis, surgery methods, estrogen receptor alpha, estrogen receptor beta, menarche age, and pregnancy number, respectively. T: Tumor invasion depth; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta; N: Lymph node metastasis.

only including incidence area, age, differentiation, and N stage.

To further reveal the prognostic prediction of the full model, patients were categorized into high- and low-risk groups based on the optimal cut-off value of TPS in the primary training set. The relationships among risk scores and survival status of patients are shown in Figure 3A. Kaplan–Meier survival curves revealed that patients with higher risk scores had significantly poorer OS (0.28, 95%CI: 0.20–0.40, P < 0.001, Figure 3B). Using the same cut-off values, we identified the same risk groups in both internal and external validation sets with OS represented by Kaplan–Meier curves (0.47, 95%CI: 0.30–0.73, P = 0.001; 0.54, 95%CI: 0.34–0.85, P = 0.009, respectively, Figure 3C–F). In addition to highly differentiated patients and patients who received other treatments (surgery plus postoperative radiotherapy, surgery plus postoperative chemotherapy), the clinical–reproductive model also maintained a good and stable prediction performance among other subgroups (Figure 4).

Calibration and validation of clinical–reproductive model

The Harrell C-indexes are summarized in Table 2 for the full model, clinical model, and TNM stage in the training, internal and external validation sets. For OS prediction, the clinical model achieved a C-index of 0.629 (95%CI: 0.578–0.681) in the training set, with internal and external validation C-index of (0.593, 95%CI: 0.531–0.655, and 0.589, 95%CI: 0.514–0.633, respectively). After integrating the clinical with reproductive risk factors, the C-index of the full model increased to 0.701 (95%CI: 0.655–0.746, P = 0.000) in the training set, 0.684 (95%CI: 0.619–0.748, P = 0.009) in the internal validation, and 0.672 (95%CI: 0.609–0.734, P = 0.014) in the external validation set. The C-indexes for OS were also higher than those for TNM stage in all of the three cohorts (P = 0.013, 0.011 and 0.033, respectively) (Table 2).

The calibration suggested that the OS prediction at 1-, 3-, and 5-years was well matched to the actual outcomes in the primary training cohort (Figure 5A). The time-dependent ROC curve also demonstrated that the full model showed a good performance in predicting OS in the training set (Figure 5B). The AUC at 1-, 3-, and 5-years was 0.792, 0.738 and 0.789, respectively. These discoveries were verified in

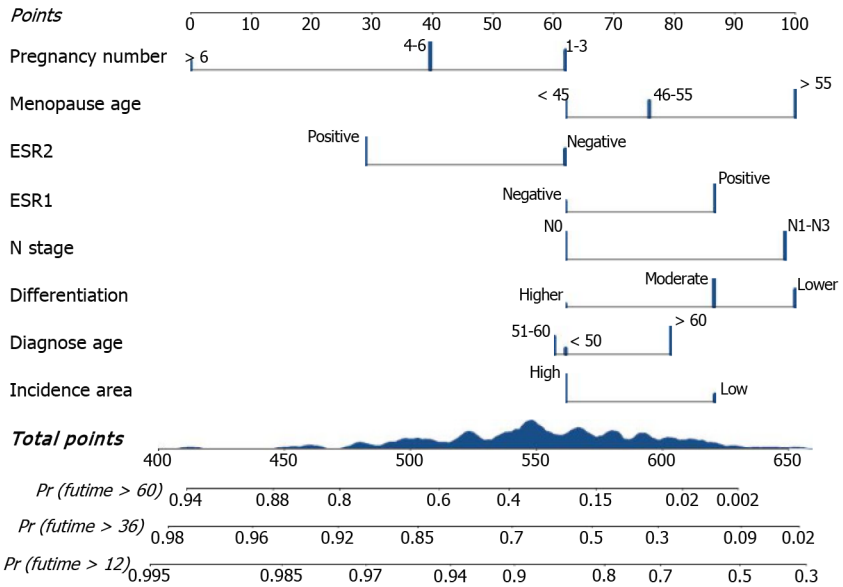

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Figure 2 The Clinical-reproductive model for predicting 1-, 3-, and 5-yr overall survival probability in female patients with esophageal squamous cell carcinoma. ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta; N: Lymph node metastasis.

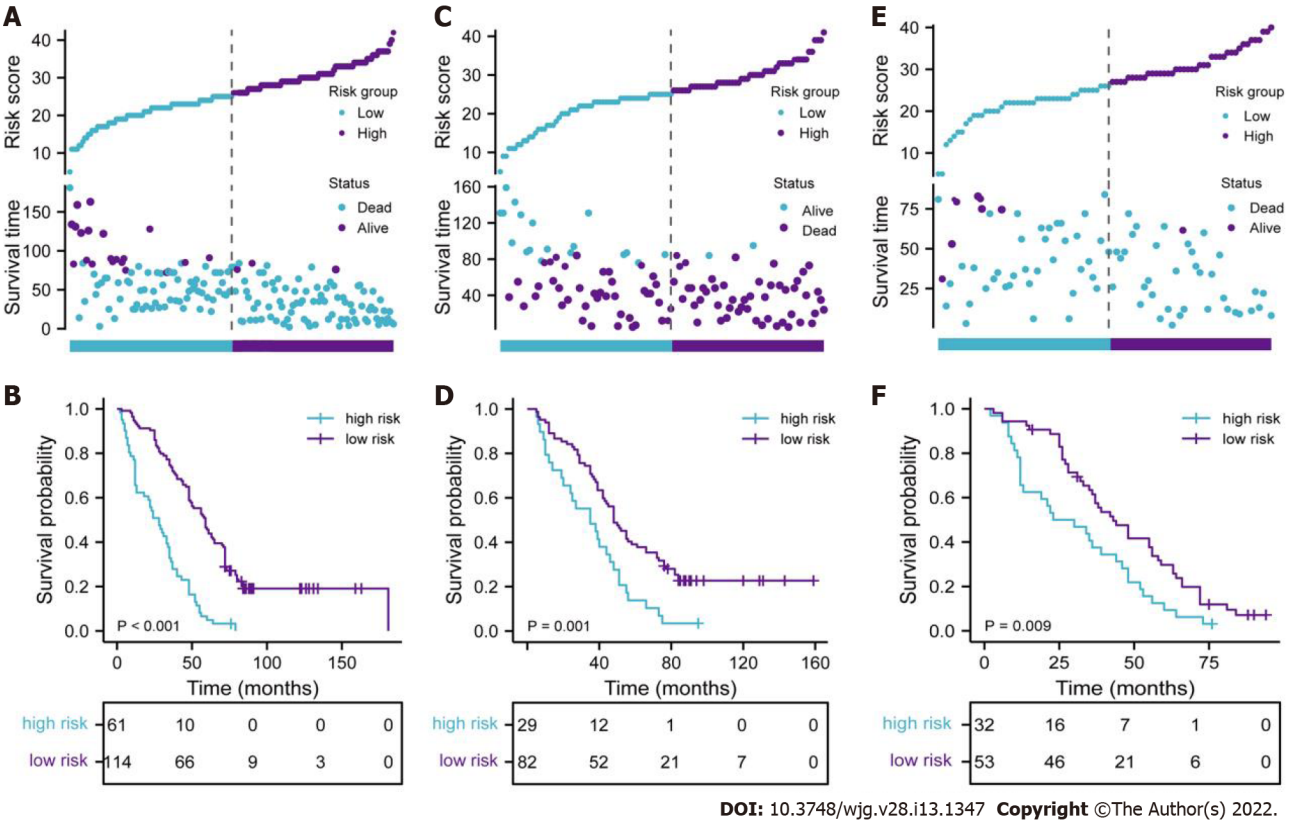

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Figure 3 Evaluation of full model performance in the primary training set and confirmation based on both internal and external validation sets. A, C, and E: Distribution of risk scores and survival status in primary training, internal and external validation sets, respectively; B, D, and F: Kaplan-Meier survival curves in primary training, internal and external validation sets, respectively.

two validation sets (Figure 5C–F). Compared with the clinical model and TNM stage, the predicted value of the full model was in good agreement with the OS at 1 year (Figure 6A), 3 years (Figure 6B), and 5 years (Figure 6C). The full model also confirmed the better discrimination for OS at 1 year (AUC: 0.792 *vs* 0.719 *vs* 0.744; Figure 6D), 3 years (AUC: 0.738 *vs* 0.631 *vs* 0.635; Figure 6E), and 5 years (AUC: 0.789 *vs* 0.629 *vs* 0.640; Figure 6F) OS in the primary training cohort. DCA for 18 mo OS prediction showed that the clinical-reproductive model yielded a larger net benefit than either clinical model or

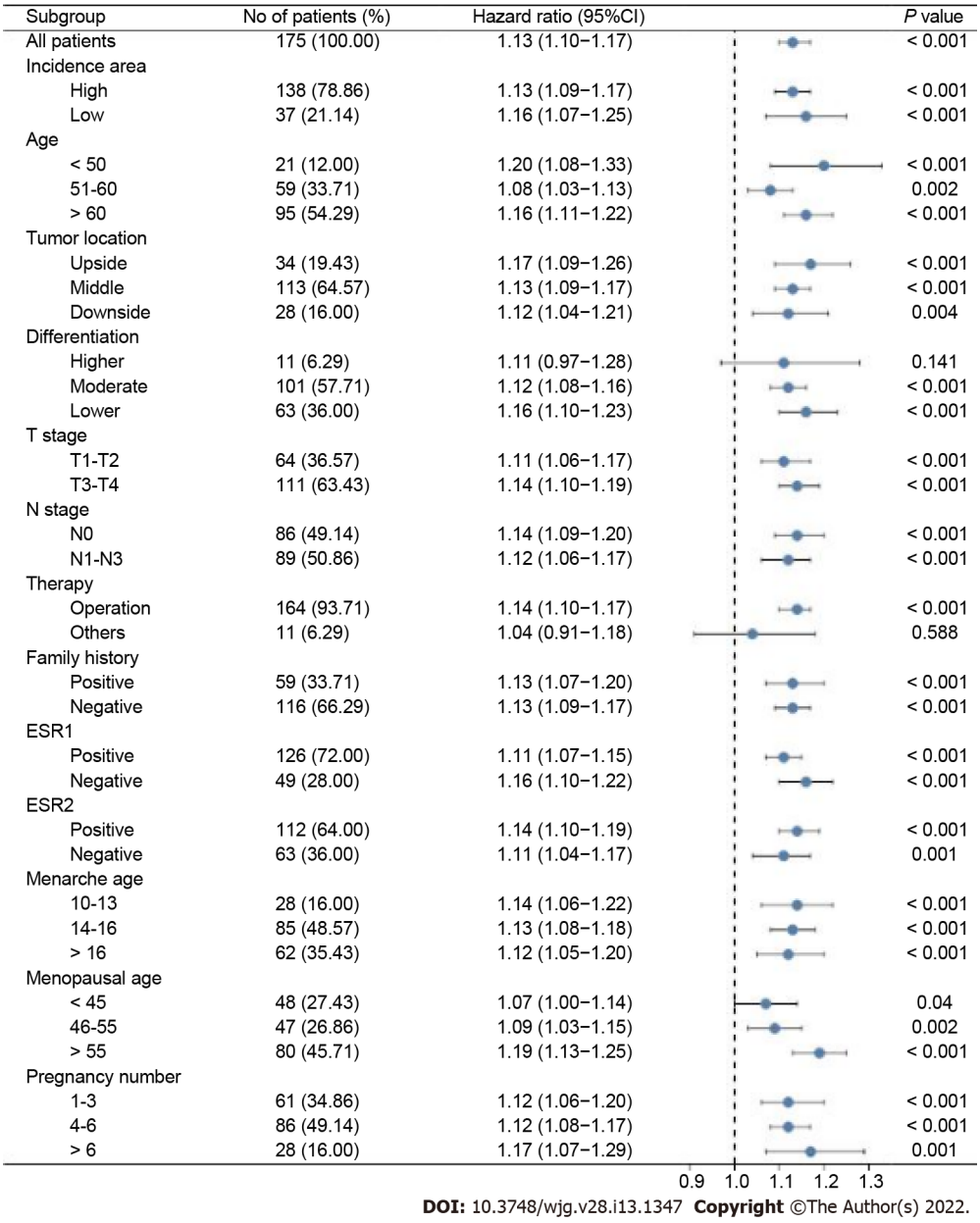

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Figure 4 Stratified analysis of the clinical-reproductive model in different subgroups. T: Tumor invasion depth, N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

TNM stage when the threshold probability was > 0.10 (Figure 7).

Correlations with immune-related pathways and gene markers of immune cells

The estrogen response was positively correlated with p53 pathway (r = 0.436, P < 0.001), and apoptosis pathway (r = 0.245, P < 0.001) (Supplementary Figure 1). ESR1 and ESR2 Levels were positively correlated with some biomarkers of immune cells or their subsets (Supplementary Table 1).

Clinical relevance of clinical–reproductive model

The relationships between the full model and clinical parameters are shown in Figure 8. In terms of TNM stage, female patients with III/IV stage ESCC had significantly higher risk scores than patients with I/II stage disease had (P < 0.001, Figure 8A). The risk scores were also higher for patients with than those without lymph node metastasis (P < 0.001, Figure 8B). No significant difference was found in patients with different T stages (P = 0.152, Figure 8C).

To improve the predictive performance and clinical utility of the full model, we transferred the data and formulae to a user-friendly website. Figure 9 shows a snapshot of web-based nomogram that is available on predictbcos.shaws.cn: https://female-escc-predictor.shinyapps.io/DynNomapp/. Visitors can select values from the drop-down list according to the circumstance of clinical and reproductive factors, and then click the "predict" button to predict the OS rates in Chinese female ESCC.

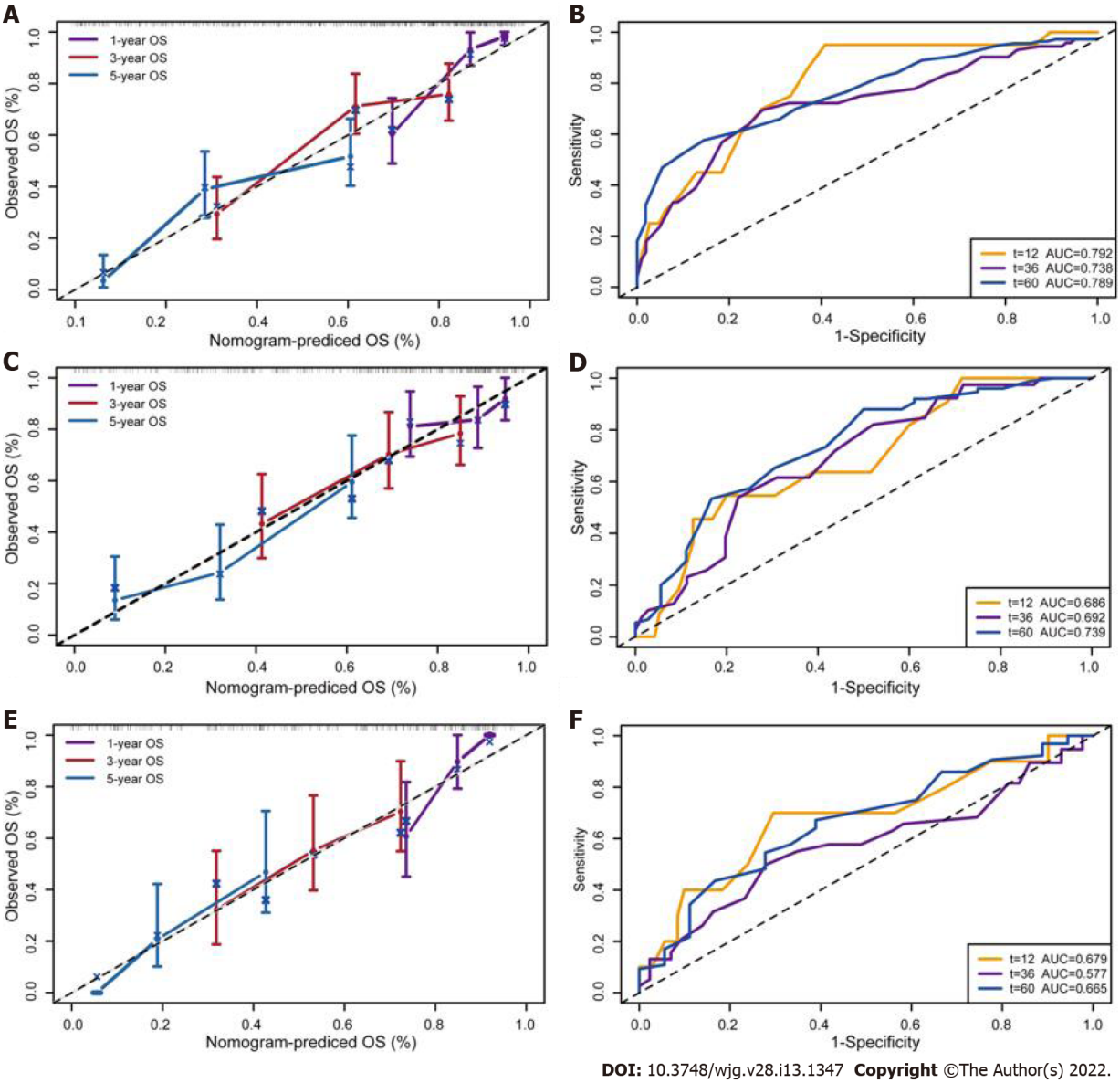

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Figure 5 Calibration curves and time-dependent receiver operating characteristic curves for validation of clinical-reproductive model in the primary training, internal and external validation cohorts. A and B: Calibration curves for predicting 1-, 3-, and 5-yr overall survival and time-dependent receiver operating characteristic (ROC) curves in primary training cohort; C and D: Calibration curves and time-dependent ROC curves in internal validation cohort; E and F: Calibration curves and time-dependent ROC curves in external validation cohort. OS: Overall survival.

DISCUSSION

Although advances have been achieved in the prevention and treatment of ESCC in the past few decades, the prognosis of ESCC is still poor with high mortality rates[17,18]. Traditionally, the TNM staging system has been used to predict the prognosis of many cancers. However, accumulated studies have reported that the prognostic predictive probability of clinical nomograms is more accurate than that of TNM stage due to the incorporation of all known significant prognostic factors of individual patients[19-21]. To date, nomograms have been widely applied in clinical prognostic evaluation for several cancers[22-24].

We constructed and validated a clinical–reproductive prognostic model for assessing the added value of reproductive factors over existing risk factors in female ESCC. Our results revealed that reproductive factors have independent prognostic value with respect to clinical parameters for individualized OS prediction in female ESCC. Subgroup analysis showed that the prognosis of female patients in the low-risk group was significantly better than that in the high-risk group. Our results of discrimination, calibration and DCA curves also showed that the clinical–reproductive model had enhanced prognostic value compared with clinical model and TNM stage.

To further explore the potential molecular mechanism of increased prognostic value of our clinical–reproductive model in female ESCC, we analyzed the correlations of estrogen response with

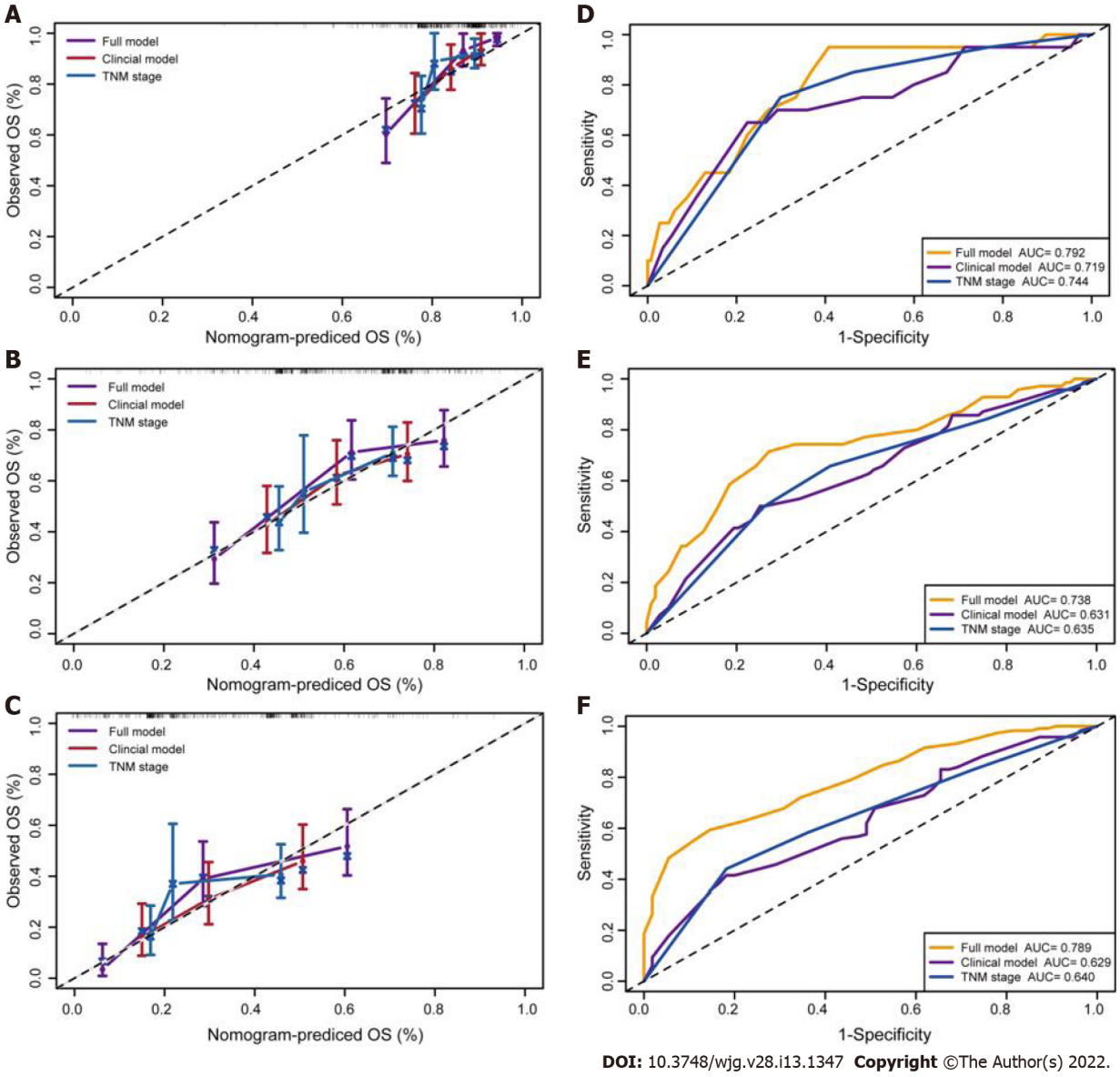

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Figure 6 Calibration curves and time-dependent receiver operating characteristic curves of 1-, 3-, and 5-yr overall survival prediction for the full model, clinical model and tumor-node-metastasis stage in the primary training set. A-C: Calibration curves for predicting 1-, 3-, and 5-yr overall survival (OS) in primary training set, respectively; D-F: Time-dependent receiver operating characteristic curves of 1-, 3-, and 5 yr OS prediction in primary training cohort, respectively. Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age + incidence area; TNM stage: Tumor-node-metastasis stage.

immune-related pathways and biomarker genes of immune cells in esophageal cancer. An in vitro study has found that estrogen mediates the apoptosis of esophageal cancer cells by interacting with ERs[25]. The p53 signaling pathway serves as a major barrier to prevent the occurrence and progression of cancer [26,27]. Our results revealed that estrogen response could prolong clinical outcome by promoting the p53 pathway and inducing apoptosis. ESCC expresses ERs and estrogen plays a protective role through ERs[28]. Overexpression of ERs is associated with prognosis and female ESCC patients with higher ESR2 expression have a better prognosis and those with higher ESR1 seem to have shorter survival[3]. In the present study, the expression levels of some biomarker genes in immune cells were positively correlated with ESR1 and ESR2 levels. Significant correlations were found between ESR2 and PTGS2 (Prostaglandin-Endoperoxide Synthase 2, biomarker of M1 macrophages), ESR1 and CD163, VSIG4 (V-Set And Immunoglobulin Domain Containing 4), MS4A4A (Membrane Spanning 4-Domains A4A, biomarker of M2 macrophages). It is well known that different cell subtypes may have distinct roles in the immune microenvironment. M1 macrophages showed an antitumor effect after being activated by Th1 cytokines, while M2 macrophages showed protumor activity[29]. This may partly explain the survival difference in female ESCC patients with different expression levels of ESR1 and ESR2.

Several studies have developed some prognostic nomograms to predict prognosis in ESCC. These models provide useful tools to stratify the risk and predict survival probability in ESCC. However, the patients in these studies were mainly extracted from the Surveillance, Epidemiology, and Final Results

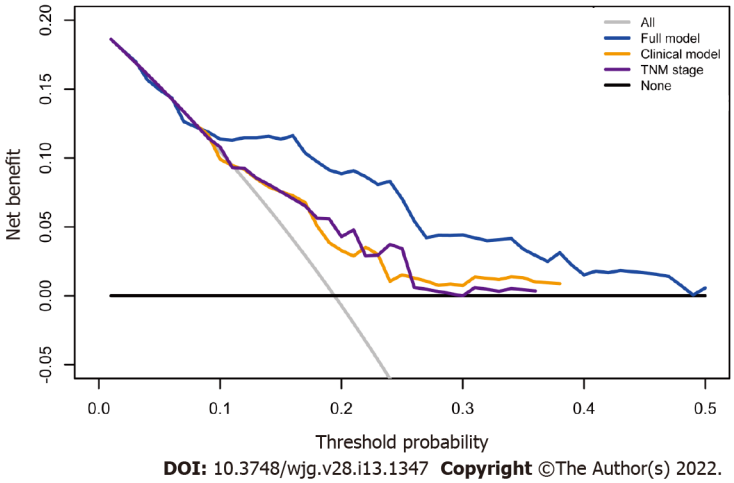

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Figure 7 Decision curve analysis for full model, clinical model, and tumor node metastasis stage. Black line: All patients were dead. Gray line: None of patients was dead. Full model: Pregnancy number + menopausal age + estrogen receptor alpha + estrogen receptor beta + N stage + differentiation + diagnose age + incidence area; Clinical model: N stage + differentiation + diagnose age+ incidence area; TNM stage: Tumor-node-metastasis stage.

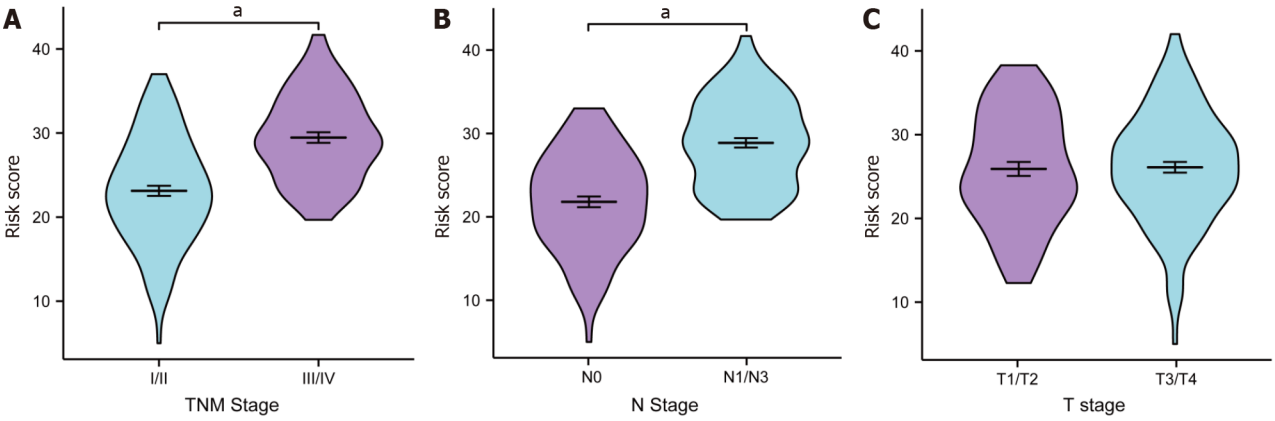

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Figure 8 Clinical relevance of full model. Distribution of the risk score based on different tumor-node-metastasis (TNM) stage, lymph node metastasis (N) stage, and tumor invasion depth (T) stage in the primary training cohort. A: TNM stage; B: N stage; C: T stage. [a]*P* < 0.001. T: Tumor invasion depth, N: Lymph node metastasis; TNM stage: Tumor-node-metastasis stage.

(SEER) database in the United States, and these models were not entirely suitable for predicting OS for Chinese female ESCC due to the ethnic difference. In our study, the internal C-index of our model was 0.701, which is higher than two previously released models (0.67 and 0.66 for both nomograms)[30,31]. Although the C-index of the Yu *et al*[32] model was higher (0.749), the nomogram can only be used to predict the clinical outcome of T1 esophageal cancer patients with positive lymph nodes. A recent study on breast cancer showed that user-friendly online prognostic tools could greatly improve patient care [33]. There is no such online tool available for female ESCC. We implemented our nomogram in an online webserver, which means that clinicians can easily use it to predict prognosis. For example, a 46-year-old menopausal ESCC patient with five pregnancies showed a poorly differentiated tumor with positive lymph node metastasis, and the tumor was ESR1 negative and ESR2 positive. The predicted results by the website show that the OS rates at 1-, 3-, and 5-years were 0.94, 0.81 and 0.58, respectively.

There were some limitations to our research. First, our analysis may have been affected by bias and loss of follow-up due to the retrospective nature of the study. Second, we must be careful to extrapolate our findings to patients of other races because most of our patients were of Han nationality. Finally, the in-depth molecular mechanisms of reproductive factors in progression and prognosis of female ESCC depend on further experimental studies to elucidate.

CONCLUSION

We developed and verified a prognostic nomogram with clinical and reproductive factors to improve

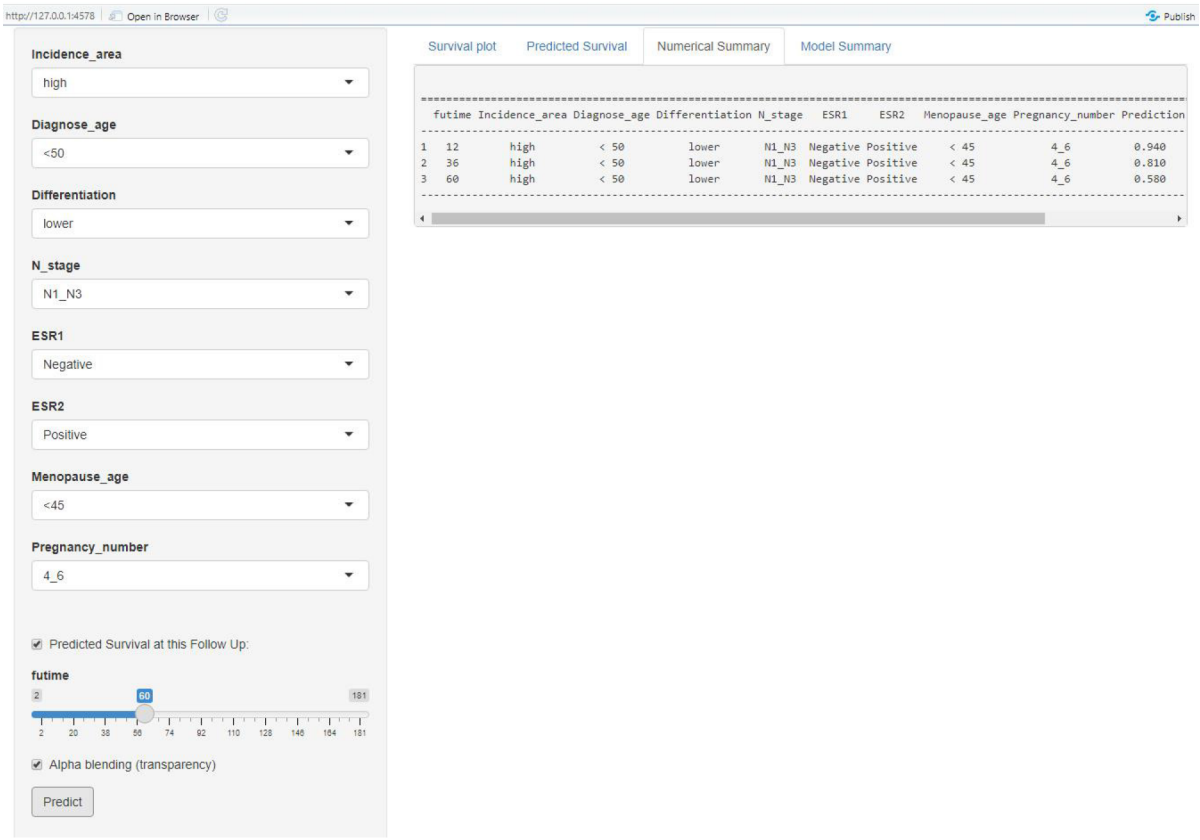

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Figure 9 Screenshot from the web-based nomogram for predicting 1-, 3-, and 5-yr overall survival for female patients with esophageal squamous cell carcinoma. N: Lymph node metastasis; ESR1: Estrogen receptor alpha; ESR2: Estrogen receptor beta.

the accuracy of prognostic prediction in Chinese female patients with ESCC. Compared with the clinical model and TNM stage, the full model has increased prognostic value and can help clinicians to make individual treatment and medical decisions.

ARTICLE HIGHLIGHTS

Research background

Nomogram has been widely used and proved to be more accurate than the tumor-node-metastasis (TNM) staging system for predicting prognosis in different cancers. In China, female patients with esophageal squamous cell carcinoma (ESCC), even with the same TNM stage, had distinct overall survival (OS) difference, which requires an effective prediction model to accurately evaluate the prognosis.

Research motivation

Several studies have developed some prognosis nomogram models, which provide useful tools for predicting OS probability in patients with esophageal cancer. The included patients were mainly extracted from the Surveillance, Epidemiology, and Final Results (SEER) database in United States. Due to the ethnic difference, the models are not entirely applicable to perform the OS prediction for Chinese female ESCC.

Research objectives

The purpose of the study was to develop and validate a clinical-reproductive model for predicting OS in Chinese female patients with ESCC, and to further explore whether the model had higher prognostic value than the clinical model and TNM stage.

Research methods

A new prognostic nomogram incorporating clinical and reproductive characteristics was constructed in the primary training cohort and verified in the internal and external validation cohorts. The

performance of the full model was evaluated by Kaplan-Meier curve, time-dependent receiver operating characteristic (ROC) curve, Harrell's concordance index (C-index), calibration curve and decision curve analysis.

Research results

The clinical-reproductive model incorporated incidence area, age, differentiation, N stage, estrogen receptor alpha expression, estrogen receptor beta expression, menopausal age, and pregnancy number. Compared to the clinical model and TNM stage, the ROC curve and C-index indicated good discriminative ability of the full model for predicting 1-, 3-, and 5-years OS in the primary, internal, and external validation sets.

Research conclusions

A clinical-reproductive nomogram for OS prediction in Chinese female ESCC was developed and validated in the present study, which showed superior survival prediction than the clinical model and TNM stage.

Research perspectives

The clinical–reproductive model has incremental prognostic predictive value in Chinese female ESCC, which may be beneficial to individualized treatment and medical decision-making.

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FOOTNOTES

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META-ANALYSIS

Generic and disease-specific health-related quality of life in patients with Hirschsprung disease: A systematic review and meta-analysis

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Abstract

BACKGROUND

Patients with Hirschsprung disease (HD) are at risk of persistent constipation, fecal incontinence or recurrent enterocolitis after surgical treatment, which in turn may impact physical and psychosocial functioning. Generic health-related quality of life (HRQoL) and disease-specific health-related quality of life are relevant outcome measures to assess the impact of HD on the QoL of these patients.

AIM

To summarize all available evidence on HRQoL of patients with HD after surgery and the impact of possible moderating factors.

METHODS

Pubmed, Web of Sciences, PsycInfo and Embase were searched with search terms related to 'Hirschsprung disease', 'Pediatrics' and 'Quality of life'. Mean and standard deviation of generic HRQoL overall and domain scores were extracted from each study, as well as data describing potential factors associated with QoL. Random effect models were used for meta-analytic aggregation of generic HRQoL

scores. Meta-regression was used to assess the relationship between patient and clinical characteristics and generic HRQoL. Disease-specific HRQoL outcomes of patients with HD were systematically reviewed.

RESULTS

Seventeen articles were included in the systematic review (n = 1137 patients) and 15 in the quantitative meta-analysis (n = 1024 patients). Four studies reported disease-specific HRQoL. Patient's age ranged between 0 and 21 years. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL (d = -0.168 [95%CI: -0.481; 0.145], P = 0.293, I^2 = 94.9) in patients with HD compared to healthy controls. Physical (d = -0.042 [95%CI: -0.419; 0.335], P = 0.829, I^2 = 95.1), psychosocial (d = -0.159 [95%CI: -0.458; 0.141], P = 0.299, I^2 = 93.6) and social HRQoL (d = -0.092 [95%CI: -0.642; 0.457], P = 0.742, I^2 = 92.3) were also not significantly lower compared to healthy controls. There was no relation between health-related outcomes and the sex of the patients and whether generic HRQoL was measured by parental proxy or self-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

CONCLUSION

In this systematic review and meta-analysis, no evidence was found for impaired generic HRQoL in patients with HD compared to healthy controls, neither for moderating effects of sex, parental proxy or self-report.

Key Words: Hirschsprung disease; Health-related quality of life; Meta-analysis; Systematic review; Pediatrics

Core Tip: Patients with Hirschsprung disease are at risk of constipation, fecal incontinence or enterocolitis after surgery, which in turn may negatively impact physical and psychosocial functioning. In this meta-analysis of 17 available studies representing 1137 patients, we found no evidence that patients with Hirschsprung disease have lower generic health-related quality of life (HRQoL) compared to healthy controls. Generic HRQoL was not dependent of sex of patients and not dependent of whether quality of life was measured by self-report or by parental proxy-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

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INTRODUCTION

Hirschsprung disease (HD) is a congenital anomaly with an incidence of approximately 1 in 5000 live births, characterized by a lack of ganglionic cells in the enteric nervous system of a distal segment of the gastrointestinal tract[1-3]. Affected neonates typically present with failure to pass meconium followed by obstructive defecation problems[2]. In most patients, the aganglionosis extends no further proximally than the rectum or rectosigmoid, which is defined as short segment disease[4]. In some patients aganglionosis is more severe and extends further proximal, with extension to the complete colon or small intestine in 5%-10%[5]. Definitive surgical management for HD involves resection of the affected bowel segment and restoration of bowel continuity with a straight anastomosis or a pouch[5]. Even after surgical resection of the aganglionic segment, it may take years for patients to acquire normal bowel function and continence[2]. In addition, patients are at risk of long-term disease-specific problems, including persistent constipation (11%-16%)[1,6-11], fecal soiling or incontinence (7%-48%)[1,6-11] or recurrent episodes of enterocolitis (0%-33%)[1,6-11]. HD can therefore be regarded as a chronic bowel condition, which may severely impact daily functioning.

According to the WHO, quality of life (QoL) can be described as a person's subjective evaluation of their position in life in the context of their culture and value systems[12]. Health-related QoL (HRQoL) describes a person's subjective evaluation of their physical and mental health[13]. HRQoL can be considered as a multidimensional construct and its evaluation generally relies on the patient's subjective

evaluation of well-being and functioning in different aspects and dimensions of well-being and functioning, together resulting in an overall construct[14]. Instruments that measure HRQoL can be generic and disease-specific[15]. Instruments that measure generic HRQoL can be used to measure HRQoL both in healthy and ill children and can be used for the comparison of HRQoL accros different conditions and settings, whereas disease-specific HRQoL instruments only measure HRQoL in patients with a certain condition, and are typically better in detecting changes in HRQoL over time[15]. For patients with HD the disease-specific questionnaire HAQL (Hirschsprung disease / Anorectal malformation Quality of Life) has been developed[4].

There is growing interest in HRQoL of patients with HD, as shown by the increasing number of studies reporting on generic and disease-specific HRQoL in these patients. The current body of evidence is inconsistent about whether HRQoL and functional outcomes are lower in patients with HD compared to healthy controls[6,7,11,15,16]. The available literature suggests evidence for several factors to be related to HRQoL and functional outcomes, including patient age[1,3,17], the use of parental proxy *vs* self-report[18], length of aganglionosis[19], type of surgical procedure and anastomosis technique used in surgery[20], postoperative complications[20-25], presence of a stoma[26-28] and syndromal anomalies [29]. Thus far the available evidence on generic HRQoL and disease-specific HRQoL among HD patients has not been systematically reviewed and aggregated precluding clear conclusions to be drawn.

The primary aim of this systematic review and meta-analysis was to summarize all available evidence on differences in generic health-related quality of life between patients with HD and normative data. Secondary aim was to summarize available evidence on disease-specific health-related quality of life of patients with HD. Third aim was to study patient and clinical factors that could explain differences in generic HRQoL between patients and normative data, including sex, age, the use of parental proxy *vs* self-report, length of aganglionosis, operation technique, postoperative complications, presence of stoma and syndromal anomalies using meta-regression.

MATERIALS AND METHODS

This meta-analysis was conducted according to the PRISMA guidelines[30].

Eligibility criteria

The following eligibility criteria were used: (1) The sample consisted of patients diagnosed with HD and a medical history of surgical resection of the affected bowel segment; (2) The majority of the patients included in the study had the age between 0 and 18 years old at assessment; (3) Generic HRQoL was measured as outcome; (4) Studies had an observational or case-control design and used HRQoL measures for which normative data is available, or compared patients with HD with normative data or a healthy control group; and (5) The study reported original data, with a minimum sample size arbitrarily set at $n = 10$. Studies were excluded if the full text was only available in a language other than English.

Search and selection

Pubmed, Web of Sciences, PsycInfo and Embase were searched using entry terms related to: 'Hirschsprung disease', 'Pediatrics' and 'Quality of life'. The full search strategy can be found in the Supplementary Material. Reference lists of included articles were checked for additional studies matching our eligibility criteria. Screening of title, abstract and full-text of the studies was performed by two independent researchers (N.W. and V.H.) using the online software Covidence[31]. Initial disagreements on study selection were discussed and in case of persistent conflicting judgement, a third party (D.R.) was consulted to reach consensus.

Data extraction

As primary outcome measurement, the mean scores and corresponding standard deviation of generic HRQoL were extracted by one researcher (V.H.). Additionally, the following characteristics were extracted from each study: domain scores (physical, psychosocial and social HRQoL), publication year, study design, sample size, type of questionnaire used to assess HRQoL, mean age of patients, percentage of male patients , percentage of measurements with parental proxy and self-report, percentage of patients with short aganglionic bowel segment, type of surgery (percentage of patients operated using the Duhamel technique and percentage of patients operated using Transanal Endorectal Pull-through [TEPT] technique), percentage of patients with postoperative complications, percentage of patients with a stoma present, and percentage of patients who had a syndromal comorbidity. If required data was not reported, authors were requested by email to provide additional data. In case of non-response of the author after one reminder and when available data was not sufficient for adequate analysis, the study was excluded from the analyses. In case a study reported median generic HRQoL scores, these were recalculated to mean scores[32,33], or else the median was considered to be the best approximation of the mean. For those studies that did not include a control group, questionnaire specific local age- and sex-matched normative values were used and compared with the reported outcomes of patients with HD[34-39].

Quality assessment

The Newcastle-Ottawa Scale (NOS) was used to assess quality of the studies in this meta-analysis[40]. According to the manual, we adapted the scoring system to our study design. A detailed description of the adjustment can be found in the Supplementary Material. The included studies were rated on a 9-point rating scale by two of the authors (N.W. and V.H.), based on aspects of participant selection (4 points), group comparability (2 points) and outcome assessment (3 points). Quality of studies was considered good, fair and poor using AHRQ (Agency for Health Research and Quality) criteria[41]. Rating discrepancies were resolved by consensus.

Statistical analysis

Statistical analysis was performed using CMA (Comprehensive Meta-Analysis)[42]. The standardized mean difference (expressed as Cohen's d) of generic HRQoL and domain scores between patients with HD and healthy control or normative populations were calculated for each study. Effect sizes d of 0.2, 0.5 and 0.8 were taken as thresholds to define small, medium and large effects, respectively[43]. For all outcomes, heterogeneity of effect sizes was quantified using I^2 statistic. Heterogeneity was regarded small ($I^2 \leq 0.25$), moderate ($0.25 < I^2 < 0.50$) or large ($I^2 \geq 0.50$), according to Higgins[44]. In case of moderate or large heterogeneity, random effects models were used.

Individual study findings were presented in forest plots and aggregated into summary estimates of standardized mean differences for: (1) Generic HRQoL; (2) Physical HRQoL; (3) Psychosocial HRQoL; and (4) Social HRQoL. In case a study reported on more than one outcome, within study findings were pooled into an weighted estimate on these predefined domains, using a built-in function in CMA. A detailed description of which items in different HRQoL-measurements were pooled into domain scores can be found in the Supplementary Material. In case a study reported no generic HRQoL score, the mean of at least two reported domain scores was considered as a reflection of the generic HRQoL.

Publication bias analysis included visual inspection of funnel plots and calculation of Egger's intercept. Robustness of the calculated aggregated effect sizes against the influence of publication bias was assessed with fail safe N. An effect was considered robust when fail safe N > 5k+10[45].

To explore sources of heterogeneity, subgroup analyses were performed to assess differences in each of the HRQoL outcomes between: (1) Studies reporting on different age groups ([< 12 years], [12-16 years] and [16+ years]); (2) Studies using different questionnaires to assess HRQoL (for example assessment by the CHQ *vs* PedsQL); (3) Studies that used a control group and studies that used normative reference data; and (4) Studies that reported generic HRQoL scores and studies in which a generic score was constructed from domain scores. Sensitivity analysis was used to explore the influence of study quality on summary estimates of all HRQoL outcomes. The moderating effect of sex and parental proxy *vs* self-report on generic HRQoL scores was explored using univariate meta-regression which was only conducted in case of at least 10 observations.

Narrative analysis of disease-specific HRQoL

Since the HAQL is a disease-specific questionnaire, a quantitative comparison with healthy controls could not be made. Designating one cohort as reference group is random and was therefore not considered to be meaningful in this context. Thus pooled summary estimates for the HAQL scores were made and findings within individual studies, in term of domains on which relatively lower disease-specific HRQoL scores were reported, were narratively described.

RESULTS

The flowchart of the study search and selection process is provided in Figure 1. Our search yielded 334 unique records, of which 17 studies (n = 1141 patients) were included in the current systematic review and of which 15 studies contributed data to the meta-analysis.

Study characteristics

Table 1 summarizes the characteristics of the 17 included studies. Of the 17 studies, 15 studies measured generic HRQoL and four studies measured disease-specific HRQoL. The PedsQL was most frequently used to assess generic HRQoL (in 10 of 15 studies). All four studies used the HAQL to assess disease-specific HRQoL. Four out of 17 studies used a controlled study design. Patients' ages ranged from 0 to 21 years.

Generic Health-related Quality of Life

Generic HRQoL: Fifteen (15) studies (n = 1024 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on generic HRQoL. A significantly lower generic HRQoL was found for patients with HD in 4 out of 15 studies, whereas 3 out of 15 studies found a significantly higher generic HRQoL in patients. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL (d = -0.168 [95%CI: -0.481; 0.145], P = 0.293, I^2 = 94.9) in patients with HD

Table 1 Characteristics of included studies

Ref.	Study design	Sample size	Self-report	Parental proxy report	HRQoL instrument	Age range (yr)	Mean age (yr)	Sex (% male)	Operation-technique	Segment-length (% short)	Syndromal comorbidity (%)	Stoma present (%)	Postoperative complications (%)
Amin *et al* [50], 2018	Cohort	46	0	46	PedsQL	0-18	4.2	71		80			
Baayen *et al*[70], 2017	Cohort	50	50	0	HAQL	12-16						6	
Cavusoglu *et al*[46], 2012	Case-control	12	6	12	PedsQL	2-12	4.7	84					
Collins *et al*[26], 2017	Cohort	60	0	60	PedsQL	2-10	6.4	82	13% Duhamel 80% Trans-anal PT	78	12	13	
Espeso *et al*[21], 2019	Cohort	63			HAQL	6-18	10.8	70	22% Duhamel 78% Trans-anal PT	79	0	0	44
Hartman *et al*[53], 2007	Cohort	121	121	0	HAQL / TACQoL	8-16	11.4	82		69	0	4	
Hartman *et al*[49], 2008	Cohort	152	152	0	TACQoL	8-16	11.6	80		68	0	3	
Khalil *et al* [47], 2015	Cohort	53	0	53	PedsQL	5-7	5.9	70					3
Lane *et al* [52], 2016	Cohort	118	0	118	PedsQL	0-17							
Meinds *et al*[17], 2019	Cohort	150	0	150	CHQ-CF87	8-17		79	62% Duhamel 16% Trans-anal PT	84		0	31
Mills *et al* [25], 2008	Cohort	51	37	44	PedsQL	3-21	9.8	82	15% Duhamel 77% Trans-anal PT	66	6		
Nah *et al* [33], 2018	Case-control	44	21	42	PedsQL	2-20	9.1	75		73			
Neuvonen *et al*[23], 2017	Case-control	39	27	12	PedsQL	0-17		75	96% Trans-anal PT	84	24	5	47
Roorda *et al*[61], 2018	Cohort	18 /14	7	7	HAQL / CHQ-CF87 / CHQ-PF50	4-17		77	67% Duhamel	0	13	6	
Sood *et al* [48], 2018	Cohort	58	38	58	PedsQL	11-18	14.5	84	7% Duhamel 86% Trans-anal PT	84	12	7	28
Townley *et al*[67], 2019	Cohort	56	0	56	PedsQL	0-13	5.4	80	84% Duhamel 16% Trans-anal PT	77		14	
Xi *et al* [50], 2018	Case-control	50	0	50	TACQoL	6	6.0	76	100% Trans- anal PT				

HRQoL: Health-related quality of life; HAQL: Hirschsprung disease/anorectal malformation quality of life.

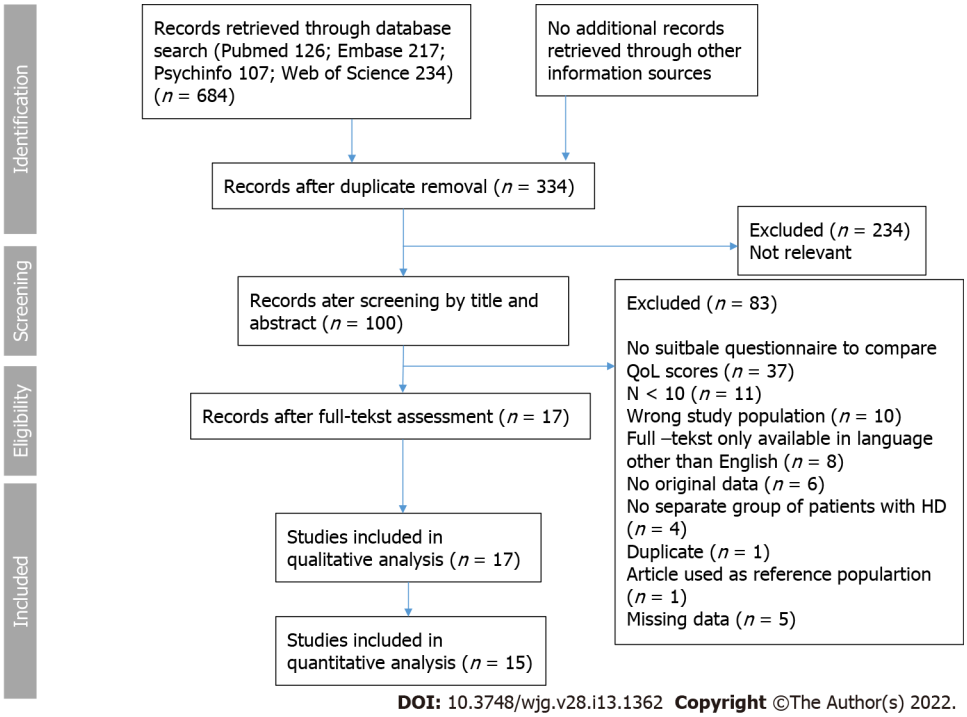

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Figure 1 PRISMA flowchart of study selection.

compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.841; P = 0.416) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 9 studies.

Physical HRQoL: Twelve (12) studies (n = 774 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on physical HRQoL. A significantly lower physical HRQoL was found for patients with HD in 3 out of 12 studies, whereas 3 out of 12 studies found a significantly higher physical HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower physical HRQoL (d = -0.042 [95%CI: -0.419; 0.335], P = 0.829, I^2 = 95.1) in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 1.391; P = 0.194) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 0 studies.

Psychosocial HRQoL: Thirteen (13) studies (n = 924 patients) were included in the meta-analysis of comparing patients with HD to normative data or controls on psychosocial HRQoL. A significantly lower psychosocial HRQoL was found for patients with HD in 6 out of 13 studies, whereas 4 out of 13 studies found a significantly higher psychosocial HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower psychosocial HRQoL (d = -0.159 [95%CI: -0.458; 0.141], P = 0.299, I^2 = 93.6) in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.476; P = 0.643) suggested that there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 18 studies.

Social HRQoL: Five (5) studies (n = 308 patients) were included in the meta-analysis comparing patients with HD to normative data or controls on social HRQoL. A significantly lower social HRQoL was found for patients with HD in 1 out of 5 studies, whereas 1 out of 5 studies found a significantly higher social HRQoL in patients. Meta-analytic aggregation showed a non-significantly lower social HRQoL (d = -0.092 [95%CI: -0.642; 0.457], P = 0.742, I^2 = 92.3), in patients with HD compared to healthy controls (Figure 2). Visual interpretation of the funnel plot and Egger's test (t = 0.554; P = 0.618) suggested there was no indication of publication bias, and the aggregated effect was also not robust with a fail safe N of 0 studies.

Patient and clinical factors explaining differences in generic HRQoL

Table 2 summarizes the results from the subgroup analyses. Meta-analytic effects for generic HRQoL did not significantly differ between age groups. There was an influence of the type of questionnaire used, as meta-analytic aggregation of studies using the CHQ showed a significant medium-sized impairment in generic HRQoL in patients with HD compared to normative data or controls, whereas meta-analytic aggregation of studies using the TACQoL or PedsQL showed no significant differences. Meta-analytic aggregation also showed no significant differences between studies comparing patients to

Table 2 Differences in health-related quality of life scores in subgroup analyses

Factor associated with HRQoL		Cohen's d [95%CI], *P* value[1]	*Q* value, *P* value[2]
Age	[0-12 yr]	*d* = -0.206 [-0.721, 0.310], *P* = 0.434	Q = 1.727, *P* = 0.422
	[12-16 yr]	*d* = 0.609 [-0.492, 1.711], *P* = 0.278	
	[16+ yr]	*d* = -0.033 [-1.170, 1.103], *P* = 0.954	
Type of questionnaire	PedsQL	*d* = -0.043 [-0.281, 0.195], *P* = 0.724	Q = 6.370, *P* = 0.041[a]
	TACQoL	*d* = -0.562 [-2.271, 1.147], *P* = 0.519	
	CHQ-CF87	*d* = -0.412 [-0.575,-0.249], *P* < 0.001[b]	
Reference data	Normative scores	*d* = 0.028 [-0.278, 0.334], *P* = 0.859	Q = 1.768, *P* = 0.184
	Selected controls	*d* = -0.793 [-1.965, 0.378], *P* = 0.184	
Overall HRQoL scores	Reported overall HRQoL scores	*d* = -0.440 [-0.817,-0.063], *P* = 0.022[a]	Q = 4.078, *P* = 0.043[a]
	Constructed overall HRQoL scores	*d* = 0.245 [-0.303, 0.792], *P* = 0.381	

[1]The difference in scores between patients with Hirschsprung disease and normative or control groups was calculated for each study and expressed as the standardized difference in means (Cohen's d) and aggregated across studies.
[2]A Q-test was done to test for significant heterogeneity between groups.
[a]*P* < 0.05.
[b]*P* < 0.01. HRQoL: Health-related quality of life.

normative reference data and studies comparing patients with constructed control groups. The complexity of the construct of generic HRQoL was expressed by the difference that was seen when comparing the effects sizes of studies reporting generic HRQoL scores and studies for which a generic HRQoL score was derived from the average of domain scores. Meta-analytic aggregation showed a significant medium-sized impairment in generic HRQoL when only studies were included that reported generic HRQoL scores, whereas meta-analytic aggregation showed no significant effect when only studies were included in which a generic HRQoL score was constructed from the average of the reported domain scores. There was no relationship between the percentage of male patients in studies and the individual study's effect sizes for generic HRQoL (b = 0.308, P = 0.631, R^2 = 0.00). Also the percentage of measurements with self-report in studies was not significantly associated with effect sizes for generic HRQoL (b = 0.331, P = 0.467, R^2 = 0.00). Based on the amount of observations, statistical power was too small to test the influence of age, length of aganglionosis, operation technique, postoperative complications, presence of stoma and syndromal anomalies on generic HRQoL of patients with HD.

Disease-specific health-related quality of life

Disease-specific HRQoL was reported in 4 studies (n = 252 patients). Hartman *et al*[49] only reported an overall QoL score, with 5 domain scores constructed into a single HAQL score (18.3 ± 1.7), which represented a good disease-specific HRQoL, but none of the other studies with the HAQL used this total disease-specific HRQoL score, making it impossible to compare these findings to those obtained in other studies. The domain scores of the remaining 3 studies were aggregated to construct summary estimates for the HAQL scores, of which an overview is presented in Table 3. Physical symptoms impacted disease-specific physical HRQoL negatively in all 3 studies, which is similar to the findings reported by Hanneman *et al*[4], where physical symptoms had the lowest mean rank score. Across all studies, diarrhea was the second factor to negatively impacted disease-specific HRQoL, and – to a lesser extent - fecal incontinence. The domains laxative diet, constipating diet, constipation, urinary continence, social functioning, emotional functioning and body image had no significant negative impact on disease-specific HRQoL. In summary, all studies showed an impairment on disease-specific physical HRQoL. Psychosocial and social HRQoL were not negatively influenced by disease-specific complaints in all four studies.

Quality of the evidence

The majority of the studies in the current systematic review were of good quality (10 out of 17 studies, 59%), whereas one study was of fair and 6 studies were of poor quality according to the NOS (Table 4). Meta-analytic estimates of generic HRQoL did not differ significantly between studies of good, fair and poor quality (Q = 2.220; P = 0.330), neither did meta-analytic estimates of physical HRQoL (Q = 4.391; P = 0.111) and psychosocial HRQoL (Q = 2.705; P = 0.259). However, meta-analytic estimates of social HRQoL did differ significantly between studies of good, fair and poor quality (Q = 9.285; P = 0.010). More specifically, social HRQoL was rated higher in the one study of low quality (d = 0.589 [95%CI:

Table 3 Summary estimates Hirschsprung disease / Anorectal malformation Quality of Life scores

Summary estimates HAQL scores	Aggregated HAQL domain scores[21,61,70][1]	Hanneman *et al*[4], 2001
Domain	Mean (SD)	Mean rank
Laxative diet	88.41 (19.63)	64.67
Constipating diet	91.67 (17.39)	63.77
Diarrhea	73.32 (26.86)	61.22
Constipation	83.33 (36.51)	64.75
Urinary continence	94.82 (13.57)	65.72
Fecal incontinence	78.70 (24.47)	68.58
Social functioning	93.77 (15.40)	65.33
Emotional functioning	84.42 (19.92)	65.11
Body image	84.70 (20.08)	65.48
Physical symptoms	69.33 (19.09)	58.52

[1]The average of mean scores was calculated across domain scores of each studies and aggregated into a weighted mean (SD). HAQL: Hirschsprung disease/anorectal malformation quality of life.

Table 4 Study quality assessment according to the Newcastle-Ottawa Scale criteria

Ref.	Selection	Comparability	Outcome	Assessment
Amin *et al*[50], 2018	2 points		2 points	Poor
Baayen *et al*[70], 2017	1 point		2 points	Poor
Cavusoglu *et al*[46], 2012	3 points	2 points	2 points	Good
Collins *et al*[26], 2017	3 points	1 point	3 points	Good
Espeso *et al*[21], 2019	1 point		2 points	Poor
Hartman *et al*[53], 2007	2 points		2 points	Poor
Hartman *et al*[49], 2008	3 points	1 point	3 points	Good
Khalil *et al*[47], 2015	2 points		3 points	Poor
Lane *et al*[52], 2016	2 points		3 points	Poor
Meinds *et al*[17], 2019	3 points	1 point	2 points	Good
Mills *et al*[25], 2008	3 points	1 point	2 points	Good
Nah *et al*[33], 2018	3 points	2 points	2 points	Good
Neuvonen *et al*[23], 2017	3 points	2 points	2 points	Good
Roorda *et al*[61], 2018	2 points	1 point	2 points	Fair
Sood *et al*[48], 2018	3 points	1 point	3 points	Good
Townley *et al*[67], 2019	3 points	1 point	3 points	Good
Xi *et al*[50], 2018	3 points	2 points	3 points	Good

0.317; 0.861], $P < 0.001$, $n = 1$), compared to those of fair and good quality.

When including only studies of good quality, meta-analytic aggregation showed a medium-sized significantly impaired generic HRQoL in patients compared to normative data or controls (d = -0.342 [95%CI: -0.665; -0.019], $P = 0.038$). When including only studies of good quality for psychosocial HRQoL, meta-analytic aggregation showed a medium-sized significantly impaired psychosocial HRQoL (d = -0.339 [95%CI: -0.656; -0.022], $P = 0.036$), whereas meta-analytic aggregation of only studies with good quality showed no significant differences between patients and normative data or controls for physical HRQoL (d = -0.199 [95%CI: -0.527; 0.129], $P = 0.235$) and social HRQoL (d = -0.322 [95%CI: -1.033; 0.390], $P = 0.376$).

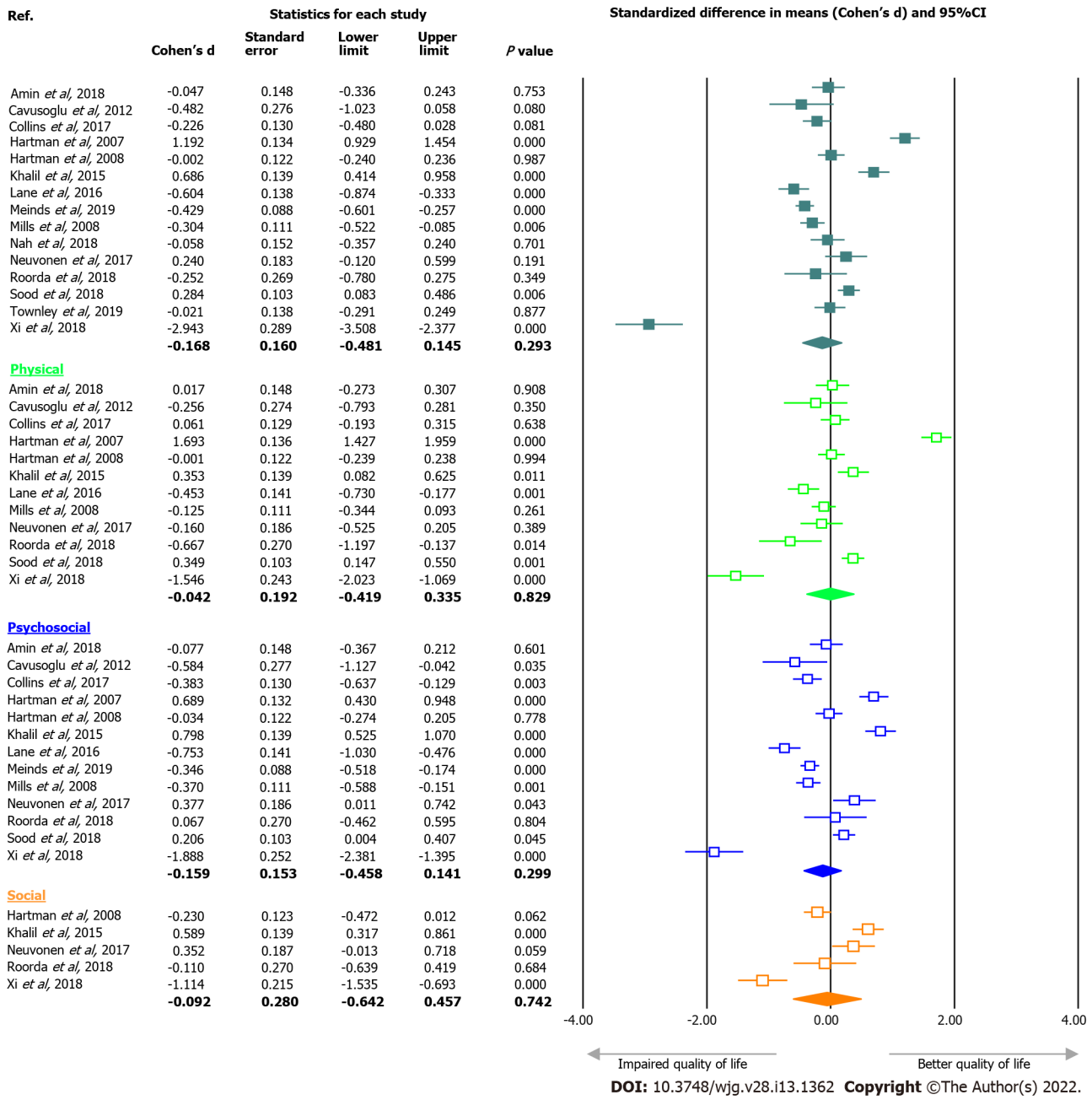

Figure 2 Forest plots of total health-related quality of life (HRQoL), physical HRQoL, psychosocial HRQoL and social HRQoL of patients with Hirschsprung disease compared to normative data.

DISCUSSION

The findings of this systematic review and meta-analysis suggest that generic HRQoL is not impaired in patients with HD compared to healthy controls and that physical HRQoL is most impaired as a result of disease-specific complaints. The high heterogeneity in HRQoL findings among the included studies implies that there are underlying factors moderating the HRQoL of patients with HD. Our findings indicated that generic HRQoL is not influenced by sex and type of respondent (parental proxy or self-report) and that generic HRQoL did not differ significantly between different age groups. The quantity of the available evidence did not allow to test for the moderating effect of length of aganglionosis, operation technique, postoperative complications, presence of a stoma and the presence of a syndromal anomalies on generic HRQoL.

Findings in several studies have shown no differences in HRQoL between male and female patients, which also corresponds to our findings[26,46-48]. Furthermore, there is evidence suggesting that HRQoL varies with increasing age and is influenced by factors like coping strategies[23,48,49] and psychological changes accompanying life events, including puberty[11,16,25,26,49-54]. Adolescents may have less ability to adapt their lifestyle because of the influence of peer pressure and the wish to adapt to their peers and not be different from their peers. Adult patients and parents of patients with HD are

more capable to adapt their lifestyle, which may result in better HRQoL in children and adults patients, compared to adolescent patients. Findings in previous studies have shown that parents tend to overestimate problems and impairments in patients with chronic diseases[41,55-58]. This in turn may be influenced by their cultural, social and educational background[39,46,53,59]. Also anxiety in parents of patients with HD may play an important role as well[54,60].

The severity of HD may also impact HRQoL. In this meta-analysis we could not test for the relation between length of disease and generic HRQoL. Findings from earlier studies are inconsistent[26,48,61,62], but suggest that HRQoL may not so much be dependent on length of disease itself, but with factors associated with differences in length of aganglionosis, including occurrence of obstructive defecation problems, postoperative complications and the operation technique that was used[49,53,61,63]. Also, current evidence is inconclusive on which type of the operation technique is associated with better HRQoL outcome[63-66]. Frequently reported disease-specific sequelae including obstipation[1,11,16,23,25,26,48,49,53,61,67,68], fecal incontinence[1,11,16,23,25,26,48,49,53,61,67,68] and the presence of a stoma [1,11,16,23,25,26,48,49,53,61,67] have shown to be associated with impaired HRQoL.

Previous studies have suggested that patients with HD who have an associated syndrome, may have lower HRQoL than patients with non-syndromal HD. In particular Down syndrome is known to be associated with lower QoL[29,69]. Patients with an associated syndrome unfortunately were underrepresented in the studies included in this meta-analysis, as these patients were often excluded from questionnaire surveys because of mental retardation that is associated with syndromes. Therefore, the influence of having an associated syndrome on HRQoL outcome could not be assessed in this study.

Our findings indicate that overall HRQoL estimates cannot simply be calculated by taking the average of domain scores, suggesting different weight of different aspects of health on the overall evaluation of HRQoL. This may be explained by differences in the items based on which the domain scores are constructed, which varies between questionnaires. But it may also reflect that HRQoL is a challenging multidimensional construct to measure. Some dimensions of health may have more influence on overall HRQoL than others, which may be based on the extent to which functioning in that domain is limited, but may also be under the influence of personal beliefs, goals and coping strategies.

The relationship between functional outcome and HRQoL outcome also remains subject to debate. Based on the available evidence in the current study, the relationship between functional outcome and HRQoL could not be tested. Our findings on disease-specific HRQoL suggest that some disease-specific symptoms, in particular diarrhea and fecal incontinence, may impair physical functioning of patients with HD rather than psychosocial and social functioning. Differences in outcome may be explained by differences in selection of cohorts between the few studies describing disease-specific HRQoL, as some cohorts included patients with Anorectal Malformation (ARM) and another cohort consisted only of patients with a severe form of HD: Total Colonic Aganglionosis (TCA)[61,70].

Heterogeneity in findings on generic HRQoL outcome may be explained by methodological aspects. Although we found no differences in HRQoL scores between studies that compared HRQoL findings of patients with HD to a normative cohort, and studies that compared HRQoL findings with a selected cohort of controls, sensitivity analysis showed that when aggregating only studies of good quality, overall and psychosocial HRQoL of patients with HD is significantly lower compared to healthy controls. This suggests our main findings on overall and psychosocial HRQoL may have overestimated overall and psychosocial HRQoL outcomes in patients with HD, resulting in a smaller difference compared to healthy controls.

Strengths and limitations

This is the first meta-analysis that summarizes the available evidence on HRQoL outcome of patients with HD. Our findings must be interpreted in the light of a few limitations. First, we could not test whether HRQoL is moderated by length of aganglionosis, operation technique, postoperative complications, presence of stoma and the presence of an associated syndrome, because of the small number of studies with complete data of study- and patient characteristics. Although for a few factors the moderation on HRQoL findings was tested in this study, this was done by meta-regression or group-comparison of meta-analytic findings. This type of analysis does not allow for the calculation of direct relations but calculates relations between studies' averages or proportions and studies effect sizes. Second, there was large heterogeneity in HRQoL findings among the small amount of included studies, resulting in non-robust effects in this study. Third, the evidence of this meta-analysis is based on small, cross-sectional studies, which makes it impossible to assess longitudinal trends in HRQoL, and to assess the influence of transitions in life including puberty and adolescence on HRQoL. Our findings showed no linear relationship between age and HRQoL, but this relation may not be linear. The subgroup analysis did not indicate significant differences between age groups, but is limited by the randomness of the cut-off values used to group the patients into age groups. A last limitation of this meta-analysis is that different instruments to measure HRQoL make different estimations, we aimed to correct for this by including the difference of patients with Hirschsprung disease with normative data in the meta-analysis, which is less vulnerable to a bias introduced by variance between instruments than directly including scores.

Risk of bias

There was a risk of selection bias, as some included studies consisted of a cohort with patients with anorectal malformations and patients with Hirschsprung disease, and also because patients with Down syndrome were underrepresented. Although we tried to limit the influence of information bias by including only studies that used questionnaires that had shown to have adequate construct validity to measure HRQoL, there was heterogeneity in HRQoL outcomes assessed by the different instruments. Differences in the design of the questionnaires, the amount of detail assess with the different items and constitution of domain scores may account for this. There was no evidence of an influence of publication bias on our findings based on Egger's intercept and visual inspection of funnelplots (presented in the Supplementary Material), although findings were not very robust with low fail safe N's.

CONCLUSION

In this systematic review and meta-analysis, no evidence was found for impaired HRQoL outcome in patients with HD compared to healthy controls, neither for the moderating effect of sex, parental proxy or self-report on HRQoL outcome. Physical functioning was most impaired by disease-specific complaints. To further study the longitudinal trends in HRQoL and determinants of HRQoL in patients with HD, we need longitudinal studies that assess the relationship between patient characteristics, functional outcomes and HRQoL.

ARTICLE HIGHLIGHTS

Research background

Patients with Hirschsprung disease are at risk for disease-specific sequelae, that in turn may impair health-related quality of life (HRQoL).

Research motivation

An increasing number of cohort studies describe health-related quality of life in patients with Hirschsprung disease, but no previous study has systematically reviewed and quantitatively analyzed all available evidence on HRQoL.

Research objectives

To summarize all available evidence on HRQoL in patients with Hirschsprung disease and to study moderating factors of HRQoL.

Research methods

A systematic review and meta-analysis in accordance with the PRISMA guidelines was conducted. After search in Pubmed, Web of Sciences, PsycInfo and Embase and selection, 17 studies met eligibility criteria and were included in this study. Random effect models were used for meta-analytic aggregation of generic HRQoL scores. Meta-regression was used to assess the relationship between patient and clinical characteristics and generic HRQoL. Disease-specific HRQoL outcomes of patients with HD were systematically reviewed.

Research results

Seventeen articles were included in the systematic review (n = 1137 patients) and 15 in the quantitative meta-analysis (n = 1024 patients). Patient's age ranged between 0 and 21 years. Meta-analytic aggregation showed a non-significantly impaired generic HRQoL in patients with HD compared to healthy controls. Physical, psychosocial and social HRQoL were also not significantly lower compared to healthy controls. There was no relation between health-related outcomes and the sex of the patients and whether generic HRQoL was measured by parental proxy or self-report. Disease-specific complaints of patients with HD impaired physical HRQoL, but not psychosocial and social HRQoL.

Research conclusions

In this systematic review and meta-analysis, no evidence was found for impaired generic HRQoL in patients with HD compared to healthy controls, neither for moderating effects of sex, parental proxy or self-report.

Research perspectives

Future studies should focus on longitudinal trends in HRQoL and on the relation between funtional outcome and HRQoL.

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FOOTNOTES

Author contributions: Huizer V, Roorda D, Derikx JPM and Rajindrajith S designed the study and designed data collection; Huizer V and Wijekoon N performed study selection, performed study quality assessment, collected data, carried out the initial analyses, drafted the initial manuscript and revised the manuscript; Roorda D supervised data collection, statistical analyses and critically reviewed the manuscript; Derikx JPM, Rajindrajith S, Benninga MA, van Heurn LWE and Oosterlaan J reviewed the manuscript; all authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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LETTER TO THE EDITOR

Endoscopic resection for early gastric cancer: Towards a global understanding

Alba Panarese

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Abstract

Gastric cancer is widespread globally, and disease diagnosis is accompanied by high mortality and morbidity rates. However, prognoses and survivability have improved following implementation of surveillance and screening programs, which have led to earlier diagnoses. Indeed, early diagnosis itself supports increased surgical curability, which is the main treatment goal and guides therapeutic choice. The most recent Japanese guidelines for endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer consider the degree of endoscopic curability in relation to the characteristics of the gastric lesions. In clinical practice, the management approach for both prevention and treatment should be similar to that of colon lesions; however, unlike the established practices for colorectal cancer, the diagnostic and therapeutic pathways are not shared nor widespread for gastric cancer. Ultimately, this negatively impacts the opportunity to perform an endoscopic resection with curative intent.

Key Words: Early gastric cancer; Artificial intelligence; Malignancy; *Helicobacter pylori*; Autoimmune gastritis; Dysplasia

Core Tip: Gastric cancer accounted for 5.6% of all new global cancer cases in 2020 and 7.7% of all cancer deaths. It's generally high mortality and morbidity rates highlight the need for early detection, to increase the curability of surgical treatment. In countries where gastric cancer screening programs exist, endoscopic curability is possible because gastroscopy with magnification and chromoendoscopy can detect gastric lesions at an early stage. It is necessary to support screening programs more widely to achieve the successful implementation of the common strategies of prevention, diagnosis and treatment, thereby reducing the incidence of advanced gastric cancer around the world.

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TO THE EDITOR

The review by Young *et al*[1], which offers a broad overview of endoscopic diagnosis and treatment of gastric dysplasia and early cancer with the prospect of future implications, was received with great interest. The authors reported that endoscopic treatment of precancerous lesions and early gastric cancer reduces rates of advanced carcinoma and avoids the requirement for surgery. However, there are relevant differences between the East and West for data related to the practice of endoscopic resection and the histological definitions of lesions[1].

Gastric cancer accounts for a significant proportion of cancer-related morbidity and mortality worldwide[2,3], and early detection is required to reduce the rates of each. The risk of progression from low-grade and high-grade dysplasias detected by biopsy to gastric cancer is high. For this reason, guidelines suggest endoscopic removal of any dysplastic lesion[4,5]. However, the incidence of early gastric cancer differs among countries, particularly for those in the Eastern and Western regions of the world[2,3,6]. In European countries, for example, early-stage gastric cancer accounts for less than 10% of diagnosed cases, which is much less than that in Asian countries where endoscopic screening is practiced. It has been reported that endoscopic screening may reduce the risk of death from gastric cancer without affecting gastric cancer incidence[7]. In fact, in the East, gastric cancer screening programs allow early diagnosis and consequently increase the curability of subsequent treatment by surgical resection. Likewise, endoscopic resection allows endoscopic curability, and through these collective clinical efforts, morbidity and mortality of gastric cancer can continue to decrease[3].

Therefore, advances in endoscopic imaging and resection techniques as well as improved endoscopist training are very important. Yet the maximal benefits will only be realized if these efforts are carried out alongside expanded implementation of screening and surveillance gastroscopies because gastric cancer originates from precancerous lesions. Intriguingly, this pathogenic pattern is similar to that of colon cancer, whereby the cancer arises from polypoid and non-polypoid lesions of the colon[8].

Our ongoing Italian multicenter prospective observational study on endoscopic submucosal dissections of early gastric cancer has, so far, enrolled 32 cases in 18 mo in 12 centers. A comparison with other studies is not possible at this time for several reasons, namely among them unavoidable alterations to the clinical routine caused by the coronavirus disease 2019 pandemic. However, it appears that in addition to technological and diagnostic advances and improved skills of endoscopists, the presence of screening programs could be important to further identify and enroll patients who are at-risk. This represents a similar trend to that experienced in the colon cancer screening programs.

The ongoing high incidence of gastric cancer, especially in the youngest generations, due to the persistent spread of *Helicobacter pylori* (*H. pylori*) infection, makes gastric cancer a major public health challenge. Currently, the mortality rate for total gastric cancer deaths is higher than that of either breast cancer or colon cancer[3,9]. Since it is important to achieve a common vision for eradicating *H. pylori* on a global scale, the additional application of both *H. pylori* antibody titer and pepsinogen levels together may further promote the effectiveness of gastric cancer screening programs[10].

The new Japanese Guidelines for Endoscopic Submucosal Dissection and Endoscopic Mucosal Resection for Early Gastric Cancer (Second Edition)[11] define the indications for endoscopic treatment in relation to curability and according to the risk of lymph node metastases, based on current scientific evidence. Absolute, expanded and relative indications for the endoscopic treatment of gastric lesions and levels of curability ("endoscopic curability A: curative resection," "endoscopic curability B" and "endoscopic curability C")[11] are the starting point for prospective confirmatory studies in both Eastern and Western countries. These studies should determine the 5-year survival when these screening programs are implemented.

FOOTNOTES

Author contributions: Panarese A conceived, designed, wrote and revised the manuscript.

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LETTER TO THE EDITOR

Therapeutic drug monitoring in inflammatory bowel disease: At the right time in the right place

Brindusa Truta

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Abstract

Therapeutic drug monitoring (TDM) was one of most sought-after objective tools to determine therapeutic efficiency of different biologics and its role in the management of patients with inflammatory bowel disease (IBD) was regarded with great anticipation. But implementation of the TDM in clinical practice was challenged by several factors including uncertainty of the optimal cut-off values, assay variable sensitivity in detecting drug levels and antibodies and, most importantly, individual pharmacokinetics. While reactive TDM was embraced in clinical practice as a useful tool in assessing lack of response to therapy, the utility of proactive TDM in managing IBD therapy is still challenged by the lack of consistency between evidence. Described here, there are four groups of IBD patients for whom proactive TDM has the potential to greatly impact their therapeutic outcomes: Patients with perianal Crohn's disease, patients with severe ulcerative colitis, pregnant women with IBD and children. As the future of IBD management moves towards personalizing treatment, TDM will be an important decision node in a machine learning based algorithm predicting the best strategy to maximize treatment results while minimizing the loss of response to therapy.

Key Words: Therapeutic drug monitoring; Inflammatory bowel disease; Biologics; Crohn's disease

Core Tip: While reactive therapeutic drug monitoring (TDM) was embraced in clinical practice as an important tool for assessing lack of response to biologics, existent evidence inconsistently supports the proactive use of TDM in managing inflammatory bowel disease (IBD) therapy. Exceptions are made for patients with severe ulcerative colitis and perianal Crohn's disease (fistula) for whom TDM has consistently shown to improve clinical outcome, pregnant women with IBD for whom TDM has the potential to play a decisive role in withholding therapy and for children, for whom proactive TDM was found to increase steroid free clinical remission. Future studies are needed to define the real value of TDM in management of IBD.

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TO THE EDITOR

The article presented by Albader *et al*[1] titled "Therapeutic drug monitoring in inflammatory bowel disease: The dawn of reactive monitoring" addresses a controversial topic in clinical practice: The role of therapeutic drug management in patients with inflammatory bowel disease (IBD).

Therapeutic drug monitoring (TDM) was as one of most sought-after objective tools to determine therapeutic efficiency of different biologics. Around one third of patients are primary non-responders and 25%-50% who respond, lose response over time [secondary loss of response (sLOR)][2,3]. Clinicians investigated different techniques to early detect, prevent and overcome sLOR in their patients including serologic and fecal biomarkers, capsule endoscopy and imaging. TDM was regarded with great hope. But, as recognized by Albader *et al*[1], implementation of TDM in clinical practice was challenged by few factors including uncertainty of the optimal cut-off values, assay variable sensitivity in detecting drug levels and antibodies and, most importantly, individual pharmacokinetics influenced by severity of the disease and body weight[4-6].

The studies presented in this review, of which the majority are retrospective and targeting anti-tumor necrosis factors (TNFs), have controversial results regarding the utility of TDM in management of IBD. This controversy arises in part due to the differences in study design including different outcomes: Clinical, endoscopic, histologic response and/or cost efficiency but also due to timing of TDM implementation proactive *vs* reactive to sLOR. The author concluded that it is "difficult to prove that proactive TDM is associated with better therapeutic outcomes" but it should be considered an addition to the other tools already routinely used in practice including biomarkers (calprotectine), imaging, capsule endoscopy[1].

There are few situations that should be discussed as exempt from this conclusion.

In patients with perianal Crohn's disease (CD), closure of the fistula have been consistently shown to require higher trough level of infliximab (≥ 10 μg/mL) (IFX) than the level considered optimal for luminal CD disease (3–7 μg/mL)[6]. This finding seems to be true for both induction and maintenance phase[7,8]. It needs to be recognized that most of the studies reporting on the anti-TNF levels in perianal CD are retrospective in design[7-9]. The results of the prospective randomized controlled trial of adults with perianal fistulizing CD and optimized therapeutic IFX levels (PROACTIVE Trial) currently evaluates the benefit on clinical, radiological, patient-reported outcomes and economic costs of a higher than standard IFX[10].

In patients with moderate to severe ulcerative colitis, a higher than 30 μg/mL IFX level after the induction phase and a detectable drug level at 54 wk has been associated with greater clinical and endoscopy improvement in the post-hoc analysis of 728 patients who participated to ACT-1 and ACT-2 clinical trials[11]. This higher level is also associated with lower colectomy rates and hospitalization (OR: 9.3, $P < 0.001$)[12] when compared with patients with standard IFX level. Patients with severe inflammation have lower tissue anti-TNF levels than those in remission[13] likely due to increased clearance, although drug clearance depends on other additional factors such as albumin level, body mass and gender[14,15]. For these patients, proactive TDM may represent the rescue technique for clinical improvement and colectomy sparing.

TDM may be useful in managing anti-TNF therapy in IBD pregnancy where concerns of intrauterine fetal exposure has been raised, as the data showed higher than therapeutic levels for children of mothers who continue biologics beyond second trimester than for those of mothers who stopped biologics early in pregnancy[16]. Since mother's IFX trough levels increased during pregnancy by 4.2 μg/mL *per* trimester ($P = 0.02$), it has been suggested that late second trimester trough level of biologic may determine timing and dose of biologic agent in the third trimester[17,18].

Although withholding biologic therapy in the third trimester has been associated with increased risk of flaring in pregnancy[19], this approach may be considered safe with TDM in a well-defined group of patients once there is a clear understanding of drug pharmacokinetics and determinants of flaring in pregnancy. TDM may be considered in children with intrauterine drug exposure to decide the timing of safe administration of life virus vaccines. Current guidelines recommend avoiding any live vaccinations for at least 6 mo following delivery unless serum levels in the infant are undetectable[20].

Pediatric IBD represents a special group of patients, where the limited therapeutic armamentarium and challenges in balancing drug safety and efficiency created a critical need for drug monitoring[21]. Pro-active TDM showed to increase corticoid-free clinical remission in children with CD treated with Adalimumab (ADL) compared with reactive monitoring (PAILOT study)[22] and sustained clinical remission in children with CD, ulcerative colitis or IBD-unclassified treated with either IFX or ADL therapy[23]. In addition, model outcomes indicated that proactive TDM *vs* reactive TDM for ADL may provide higher quality-adjusted life-years at lower cost in pediatric CD patients[24].

In comparison with proactive TDM, the utility of reactive TDM has received a greater consensus in guiding therapy for those patients who lost response and where either dose intensification or change to an alternative therapy may be necessary. The utility of reactive TDM have been extended to recently introduced biologics and oral small molecules[25].

As the future of IBD management moves towards personalizing treatment, TDM will play an important role in the algorithm of machine learning based models that predict best strategy to optimize treatment outcomes while minimizing the sLOR to therapy.

FOOTNOTES

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