<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1725</td>
<td>Gut homeostasis, injury, and healing: New therapeutic targets</td>
<td>Oncel S, Basson MD</td>
</tr>
<tr>
<td>1751</td>
<td>New insights in diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms</td>
<td>Yin F, Wu ZH, Lai JP</td>
</tr>
<tr>
<td>1768</td>
<td>Current status and future of targeted peptide receptor radionuclide positron emission tomography imaging and therapy of gastroenteropancreatic-neuroendocrine tumors</td>
<td>Grey N, Silosky M, Lieu CH, Chin BB</td>
</tr>
<tr>
<td>1781</td>
<td>Forkhead Box q1 promotes invasion and metastasis in colorectal cancer by activating the epidermal growth factor receptor pathway</td>
<td>Zhang JJ, Cao CX, Wan LL, Zhang W, Liu ZJ, Wang JL, Guo Q, Tang H</td>
</tr>
<tr>
<td>1798</td>
<td>Sirtuin1 attenuates acute liver failure by reducing reactive oxygen species via hypoxia inducible factor 1α</td>
<td>Cao P, Chen Q, Shi CX, Wang LW, Gong ZJ</td>
</tr>
</tbody>
</table>
LETTER TO THE EDITOR

1871  Could microbiome analysis be a new diagnostic tool in gastric carcinogenesis for high risk, *Helicobacter pylori* negative patients?

Turshudzhyan A, Rezaizadeh H
ABOUT COVER
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Gut homeostasis, injury, and healing: New therapeutic targets

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Abstract

The integrity of the gastrointestinal mucosa plays a crucial role in gut homeostasis, which depends upon the balance between mucosal injury by destructive factors and healing via protective factors. The persistence of noxious agents such as acid, pepsin, nonsteroidal anti-inflammatory drugs, or *Helicobacter pylori* breaks down the mucosal barrier and injury occurs. Depending upon the size and site of the wound, it is healed by complex and overlapping processes involving membrane resealing, cell spreading, purse-string contraction, restitution, differentiation, angiogenesis, and vasculogenesis, each modulated by extracellular regulators. Unfortunately, the gut does not always heal, leading to such pathology as peptic ulcers or inflammatory bowel disease. Currently available therapeutics such as proton pump inhibitors, histamine-2 receptor antagonists, sucralfate, 5-aminosalicylate, antibiotics, corticosteroids, and immunosuppressants all attempt to minimize or reduce injury to the gastrointestinal tract. More recent studies have focused on improving mucosal defense or directly promoting mucosal repair. Many investigations have sought to enhance mucosal defense by stimulating mucus secretion, mucosal blood flow, or tight junction function. Conversely, new attempts to directly promote mucosal repair target proteins that modulate cytoskeleton dynamics such as tubulin, talin, Ehm2, filamin-a, gelsolin, and flightless I or that proteins regulate focal adhesions dynamics such as focal adhesion kinase. This article summarizes the pathobiology of gastrointestinal mucosal healing and reviews potential new therapeutic targets.

Key Words: Intestine; Mucosa; Repair; Restitution; Sheet migration; Stomach ulcer

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The integrity of the gastrointestinal mucosa is crucial for gut homeostasis, which depends upon the balance between mucosal injury by destructive factors and healing via protective factors. An excess of destructive agents breaks down the mucosal barrier. Upon injury, under physiological conditions, gastrointestinal mucosa heals itself by complex processes. However, the gut may not heal under pathological conditions. Currently available drugs attempt to minimize or reduce injury to the gastrointestinal tract. Recent studies have focused on improving mucosal defense or directly promoting mucosal repair. This article summarizes the pathobiology of gastrointestinal mucosal healing and reviews potential new therapeutic targets.

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INTRODUCTION

Upon injury, gastrointestinal (GI) epithelial tissue is capable of renewing itself within hours to months by replacing damaged or dead cells, depending on the site and size of the wound. In order to appreciate potential new therapeutic targets, this review will first summarize the current understanding of the processes of mucosal healing and defense and describe their major extracellular regulators. Then, the importance of the quality of ulcer healing and novel approaches to promote such healing will be reviewed. This review focuses on mucosal injury and repair. Deeper injuries such as a deep ulcer, trauma, fistula, or surgical transection and anastomotic healing all require a complex interaction among endothelial cells, fibroblasts, and other cell types to reconstitute the submucosal and muscular layers of the bowel wall. This is beyond the scope of the current review but has been previously reviewed[1-5]. Angiogenesis is critical to these efforts, and requires a complex interaction between endothelial cells, the extracellular matrix, growth factors and cytokines, and other cell types[6,7].

PHYSIOLOGY OF MUCOSAL HEALING

The integrity of the gastrointestinal mucosa is crucial for gut homeostasis. The gut lining is continuously injured during normal gut function[8] by a variety of noxious luminal substances and abrasive interactions with luminal contents (Figure 1A). However, there is normally an equilibrium between gut injury, mucosal healing, and diverse factors that protect the mucosa[9]. This equilibrium favors healing in a healthy state. Under normal physiological conditions, GI epithelial cells migrate from the base of the crypt to the villi, where their interaction with each other and the extracellular matrix (ECM) is disrupted leading to epithelial cell shedding (anoikis)[9] (Figure 1B).

PROTECTIVE FACTORS FOR THE GASTROINTESTINAL MUCOSA

The gastrointestinal mucosa is protected at three levels: pre-epithelial, epithelial, and sub-epithelial defenses. Pre-epithelial protection, the first line of mucosal defense, is provided by the secretion of mucus, bicarbonate, phospholipids, prostaglandins, and trefoil peptides (Figure 1A and B). These factors not only neutralize the acid but also inactivate pepsin at the gastric mucosal surface. In addition, phospholipids secreted into mucus contribute to the hydrophobicity of mucus and prevent back-diffusion of hydrogen ions[10]. Prostaglandins are abundant in gastric juice. They inhibit acid secretion and stimulate mucus and bicarbonate secretion[11]. Bicarbonate-rich mucus is secreted throughout the GI tract, by mucoid cells in the stomach and goblet cells in the intestines, creating a near-neutral pH at the epithelial surfaces in the GI tract, thereby protecting the GI mucosa against autodigestion by the gastric juice and other noxious agents in the lumen[12,13].

Intestinal epithelial cells consist of four important cell types (Figure 1E and F). Enterocytes and colonocytes are most common in the surface epithelium. They are critical for the digestion and absorption of nutrients. Paneth cells are highly specialized cells located in small intestinal crypts. Paneth cells are essential for the secretion of antimicrobial peptides (AMP) such as defensins. These AMPs modulate the composition of the small intestinal microbiota. Goblet cells produce various types of mucin and are found throughout the GI tract. Similarly, enteroendocrine cells are scattered along with the epithelial cells of the GI tract. They produce and secrete more than 20 different hormones in response to nutrients in the lumen that regulate hunger, appetite, and satiety[14]. Intestinal epithelial
Figure 1 Normal gastrointestinal homeostasis, injury, and healing. A: Structure of gastric epithelium in healthy, injured, and repaired states. A healthy gastric barrier is essential to maintain gastric homeostasis. In a healthy state, there is an equilibrium between gastric injury and mucosal healing. An excess of destructive factors such as acid, pepsin, nonsteroidal anti-inflammatory drugs (NSAIDs), and \textit{H. pylori} leads to gastric barrier disruption. These noxious agents then diffuse deeper into the mucosa and create wounds. Epithelial cells at the edge of the injury redifferentiate to a migratory phenotype and collectively migrate as a sheet to close the wound. After successful restitution, the migrated cells redifferentiate to more specialized phenotypes. B: A diagram depicting the structure and cell types of gastric epithelium. C: In the injured state, epithelial cells at the edge of the wound spread and redifferentiate to a migratory phenotype, losing their classical apical brush border and assuming a more squamous morphology. Then, they migrate as a sheet to cover the injured area, with cells at the front of the migrating sheet transmitting traction forces to cells farther back via cell-cell contacts. Epithelial cells behind these migrating cells subsequently proliferate to provide more cells to fully cover larger wounds. D: Cells that have migrated across the defect may themselves then proliferate once the barrier has been reformed. In addition, following migration and proliferation, the migrated cells redifferentiate back to more specialized phenotypes. E: Structure of small intestinal epithelium in healthy and injured states. F: Structure of large intestinal epithelium in healthy and injured states. A healthy intestinal barrier is essential to maintain intestinal homeostasis. In the healthy state, there is an equilibrium between intestinal injury and mucosal healing. An excess of destructive factors such as NSAIDs, inflammation, bile acid, and toxic luminal substances leads to intestinal barrier disruption. These noxious agents then diffuse deeper into the mucosa and create wounds. Epithelial cells at the edge of the injury follow the processes described in the figure legends for in Figure 1C and D. IESC: Intestinal epithelial stem cells; EEC: Enteroendocrine cells; GC: Goblet cells; NSAIDs: Nonsteroidal anti-inflammatory drugs; \textit{H. pylori}: \textit{Helicobacter pylori}; PG: Prostaglandins; ECL cells: Enterochromaffin-like cells; PC: Paneth cells; IESC: Intestinal epithelial stem cells.
stem cells (IESCs) are crucial to maintaining intestinal epithelial function and homeostasis in both the small intestine and large intestine. IESCs divide for self-renewal and generate progenitor cells that undergo differentiation into enterocytes, colonocytes, Paneth cells, goblets cells, and enteroendocrine cells[15].

Epithelial protection represents the second line of defense. GI epithelial cells are connected to each other via tight junctions and act as a physical barrier against acid or toxic luminal agents. In addition to stimulating mucus and bicarbonate secretion, prostaglandins reduce the permeability of the epithelium by closing the apical spaces between the deeper cells, and thus decreasing the exposure of deeper layers along the GI tract to noxious agents by modulating these tight junctions[16]. Tight junction proteins are either transmembrane proteins such as occludin, claudins, and junction adhesion molecule proteins or cytoplasmic plaque proteins such as the zoneula occludens proteins. The dysregulation of these proteins via toxin exposure or autoimmune diseases such as celiac disease may lead to disruption of gastrointestinal barrier function[17]. For example, ulcerative colitis may alter the intestinal barrier function via changing the phosphorylation of colonic claudins[18]. The architecture and function of tight junctions are slightly divergent between the different regions of the GI tract and also between different epithelial cells. For example, disruption of occludin alters intestinal barrier function whereas occludin disruption does not cause barrier dysfunction in the stomach[19]. Moreover, the expression of tight junction proteins varies even among the different epithelial cells. For instance, IESCs and Paneth cells have high occludin levels whereas claudin-1, -2, and -7 expression is elevated in Paneth cells, IESCs, and enterocytes, respectively[20].

The final mucosal defense is sub-epithelial protection through augmentation of mucosal blood flow. Vascular flow not only removes acid rather than allowing it to diffuse deeper into the mucosa but also supplies necessary nutrients and oxygen to the epithelial cells for energy-consuming processes such as ion transport and secretion. Gut epithelial cells undergo continuous dynamic self-renewal in response to the damage caused by destructive factors under normal physiological conditions[21,22].

**DRIVERS OF MUCOSAL INJURY**

Although the gut epithelium can maintain normal homeostasis in the presence of modest or transient exposure to injurious stimuli, high level or extensive interactions with noxious factors such as excessive secretion of gastric acid and pepsinogen, the substantial inflammation caused by inflammatory bowel disease, or toxic luminal contents including ethanol or medication such as non-steroidal anti-inflammatory drugs (NSAIDs) can unbalance the equilibrium between mucosal injury and healing (Figure 1A).

Gastric juice includes mucus, hydrochloric acid (HCl), bicarbonate, pepsin, and intrinsic factor secreted by mucoid cells, parietal (oxyntic) cells, and chief (zymogenic) cells in the stomach (Figure 1B). *Helicobacter pylori* (*H. pylori*) infection, a gram-negative bacterium responsible for 90% of duodenal and gastric ulcers, impairs the bicarbonate secretion and promotes gastric acidity as well[23]. Such hyperacidity may injure the mucosa, causing gastritis, duodenits, peptic ulcer disease (PUD), or gastroesophageal reflux disease (GERD)[24].

The gastrointestinal mucosa is subjected to numerous physical forces such as strain and pressure during both normal gut function and illness. For instance, luminal chyme, peristalsis contractions, rhythmic villous motility, and some pathological conditions such as inflammatory bowel disease (IBD) may adversely impact GI mucosal healing by increasing luminal pressure[25-29]. Such pressure increases have been shown to inhibit mucosal healing, at least in mice, despite increased mucosal proliferation, and appear to act by inhibiting the cell motility required for restitution[25].

The balance between mucosal injury and healing may also be shifted by drugs such as NSAIDs, corticosteroids, biphosphonates, potassium chloride, steroids, and fluorouracil[23,30,31]. In particular, many studies have documented that NSAIDs decrease mucus hydrophobicity as measured by contact angle goniometry whereas prostaglandins, gastroprotective compounds, increase the contact angle of gastric mucus[32,33]. NSAIDs, the most commonly prescribed medications, increase the development of ulcers in the upper and lower GI tract by two distinct mechanisms (Figure 2)[34-37].

NSAIDs injure the upper GI mucosa mainly by cyclooxygenase (COX)-1 inhibition, resulting in a decrease in prostaglandins, mucus, and bicarbonate secretion. Moreover, NSAIDs also alter another important component of mucosal defense, the gastric microcirculatory system. Upon irritation, the gastric mucosa normally increases blood flow to remove any toxins, bacterial products, or back-diffusing acid. Impairment of this hyperemic reaction increases the vulnerability of gastric mucosa to damage[38]. Inhibition of prostaglandins, potent vasodilators, by NSAIDs leads to an increase in vascular tone and thus reduces gastric mucosal blood flow[39], consequently, increases ischemic tissue damage and exacerbating the mucosal injury[40]. NSAIDs may also induce local gastric mucosal injury independent of prostaglandin deficiency[41]. NSAIDs may lyse phospholipids from mucosal epithelial cells and may increase mucosal permeability, which then allows mucosal exposure to luminal aggressive factors such as bacteria and gastric acid[42].
Non-steroidal anti-inflammatory drugs induce mucosal injury in the upper and lower gastrointestinal tract by two distinct mechanisms. In addition to principal luminal aggressors such as acid, pepsin, Helicobacter pylori in the stomach and acid, bile, and pathogens in the small intestine, nonsteroidal anti-inflammatory drugs (NSAIDs) increase mucosal damage in both upper and lower GI by two different mechanisms. In the stomach, the inhibition of COX-1 by NSAIDs reduces prostaglandin secretion which in turn reduces mucus and bicarbonate secretion and increases acid secretion, resulting in increased permeability and eventually mucosal damage. In the small intestine, NSAIDs bind to bile in the enterohepatic circulation. This potentiates the mucosal damage caused by bile. NSAIDs also increase mucosal damage in the small intestine by altering the gut microbiota. The NSAID-associated increase in enteric gram-negative bacteria appears to contribute to intestinal lesions by increasing inflammation. NSAIDs: Nonsteroidal anti-inflammatory drugs.

The molecular and cellular mechanisms of NSAID-induced lower GI mucosal injury are clearly distinct from NSAID-induced upper GI injury[42,43]. As in the stomach, NSAIDs may inhibit COX-1 and contribute to mucosal damage. However, unlike gastric injury, the bile acid and intestinal microbiota play a crucial role in the pathophysiology of NSAID-induced intestinal injury[42,44]. NSAIDs and gut microbiota have complex and dynamic interactions. The gut microbiota can alter the efficacy and toxicity of NSAIDs either directly by biotransforming them into metabolites or indirectly by altering the host metabolism (e.g., interfering with hepatic function)[45]. On the other hand, NSAIDs themselves can directly change the composition and function of the gut microbiota or indirectly by altering the physiological functions of the host[45]. For instance, NSAIDs alter the intestinal microbiome by increasing the total number of bacteria and the proportion of gram-negative bacteria, which seems to be linked to the activation of toll-like receptor (TLR) 4 that increases inflammation and contributes to an intestinal injury[46-48].

NSAIDs make complexes with bile acids by glucuronidation in the liver. This interaction alters the stability and structure of bile acids and potentiates bile acid toxicity in the lower GI tract[42]. These NSAID-bile acid complexes are secreted into the duodenum and subsequently reabsorbed back in the ileum via the enterohepatic circulation. Within the intestinal lumen, particularly in the colon, conjugated primary bile acids are deconjugated into more toxic secondary bile acids, mainly by the gram-positive bacteria[49]. There is crosstalk between the microbiome and the bile acids because bile acids can control the composition of the intestinal microbiome, which in turn regulates the composition and size of the bile acid pool[50,51]. Alteration in the colonic microbiota may cause a shift towards to generation of more toxic secondary bile acids, which eventually increase intestinal permeability, particularly in the colon, bacterial translocation, and mucosal inflammation[52-54].
NSAID-induced ulcers are traditionally treated with proton pump inhibitors (PPIs) or histamine-2 receptor antagonists (H2-antagonists)[55,56], which permit ulcer healing by reducing gastric acid secretion without directly affecting mucosal restitution[5,57,58]. Although PPIs have historically been co-prescribed with NSAIDs to ameliorate gastroduodenal injury and are used to treat NSAID injury, such use may increase the risk of a different problem. There is no evidence that gastric acid plays a key role in the pathogenesis of NSAID-induced lower GI[42,59]. PPIs may worsen NSAID-induced enteropathy by increasing gastric pH and thus changing the enteric microbiome by increasing the number of gram-negative bacteria[35,42,60-62]. Thus, even though PPIs are still recommended to treat upper GI ulcers, their prophylactic use with NSAIDs to prevent upper GI injury is no longer recommended unless the patient has a moderate to high risk of peptic ulcer disease[62,63]. Similar concerns are likely to exist for H-2 blockers.

Inflammatory bowel disease is a broad term to describe disorders including Crohn’s disease (CD) and ulcerative colitis (UC) that are characterized by excessive activation of the mucosal immune system to normal microflora. This causes chronic inflammation and damages the gut mucosa[64,65]. Since the etiology of IBD is still unclear, the primary goal of treatment is centered on the elimination of inflammation with medical therapies such as 5-aminosalicylate, antibiotics, corticosteroids, immunosuppressants, and biological therapy[66-68]. Management of IBD with targeted therapies has been discussed in detail in a recent review[68]. However, none of these therapies is perfect, and even if patients achieve symptomatic remission, maintaining that remission can be challenging[69]. Recent evidence highlights the importance of mucosal healing over and above symptomatic remission in the quality of life and long-term prognosis of IBD patients[70-72].

MUCOSAL HEALING PROCESSES

Once an injury has occurred, diverse processes such as redifferentiation to a migratory phenotype[73-75], migration, proliferation, and eventual redifferentiation back to more specialized cells after healing are all regulated by various factors including growth factors, cytokines, physical forces, and the extracellular matrix itself. These coordinate healing of the injury (Figure 1). At the subcellular level, wounding of the apical plasma membrane is common in the epithelial cells of the intact, normal functioning stomach and intestines in vivo after mechanical and chemical stressors[8,76,77]. Since maintenance of plasma membrane integrity is essential for cell viability, the wounded cell rapidly repairs the injury to restore internal homeostasis and prevent cell death. Plasma repair processes such as tension reduction, budding, patch, endocytosis, and exocytosis may be triggered by the toxic level of Ca2+ influx through the plasma membrane wound to then reseal the injured plasma membrane[78,79].

Relatively small or superficial multicellular mucosal injury undergoes complex wound healing processes that quickly reconstitute the mucosal barrier, depending on the size and depth of the injury. Small wounds, less than eight cells in size, may close by the spreading of neighboring cells and formation of new cell-cell contacts[80,81] or by purse-string wound closure, which involves the formation of a multicellular actin cable purse string around the wound, with actin cables that parallel the wound edge. This then contracts, pulling the adjacent cells together[5,82].

Mucosal injury involving more than eight cells is generally too large for purse-string wound closure. This then requires restitution epithelial sheet migration to close the injury. Depending on the size and depth of these larger wounds, wound closure will require a longer healing time and may require one or more complex overlapping processes such as differentiation, proliferation, and angiogenesis for wound healing[5,83,84].

Restitution requires a phenotypic redifferentiation. Although some authors describe the initial steps of this process as dedifferentiation, it is the firm opinion of the senior author that this should rather be considered a redifferentiation toward a migratory phenotype. The gut epithelium normally consists of a monolayered layer of differentiated epithelial cells. At the edge of a mucosal wound, epithelial cells change their phenotype from differentiated columnar enterocytes or gastric cells to a migratory phenotype. They lose their typical morphology and (for enterocytes and parietal cells) their microvilli [85], disassemble their apical specialized membrane components[86], flatten out and extend lamellipodia toward the defect. Such migrating cells adopt a squamous phenotype with altered actin[87-89] and cytoskeletal organization[73,85] and specialized cell signaling pathways[90-93] that adapt these cells toward motility (Figure 1C)[73,85,94-96]. Moreover, it is worth noting that these signaling events are not only regulated by the activation of signaling proteins but also by the distribution and the amount of the signaling proteins within the migrating cells. For instance, both the actual amount of total adhesion kinase (FAK) and the amount of active FAK decrease while the ratio of activated to total FAK increases both in vitro[94] and in vivo[92] as the epithelial cells shift to the migratory phenotype[73]. Similarly, both paxillin protein and tyrosine-phosphorylated paxillin decrease in migrating cells compare to static cells[94]. (Paxillin is an adapter protein critical to focal adhesion complex assembly and disassembly in response to various stimuli)[97-100]. Total p38, ERK1, and ERK2 proteins do not show differences between migrating and static cells[94]. However, phosphorylated p38 increases, and phosphorylated ERK1 and ERK2 decreases in motile cells compared with nonmigrating
The transverse actin cables that drive purse-string closure for smaller wounds line up parallel to the wound edge at the migrating front, connected by cell-cell contacts, and unite the migrating front, so that these redifferentiated cells collectively migrate as a sheet, a.k.a., restitution, to close the wound (Figure 1C)[101-103]. Slightly deeper wounds that injure the basement membrane expose the cells to the interstitial extracellular matrix. While the basement membrane is predominantly laminin and type IV collagen, the deeper interstitial matrix is rich in type I collagen, across which the cells may migrate more rapidly[104,105].

After the closure of the wound by successful restitution, the migrating cells must redifferentiate back to the more specialized phenotypes required for the normal biology of the mucosa (Figure 1D)[102]. Tarnawski et al[106] have demonstrated the critical relationship between defective redifferentiation of these migratory cells and subsequent ulcer recurrence. This will be considered in more detail below.

If the wound surface area is extensive, restitution will likely be insufficient to seal the wound. In this situation, epithelial cell proliferation increases behind the migrating cells to create a larger pool of epithelial cells that can then migrate across and cover the defect (Figure 1C)[107]. However, if the wound extends into deeper layers such as the submucosa and muscularis, these must also be reconstructed for healing by processes beyond the scope of this review. In particular, the reconstitution of nutrient vessels in the submucosa is critical for mucosal wound healing because these provide oxygen and nutrients to the mucosa and remove waste products from the wound site[108,109]. This neovascularization can occur by two distinct processes called angiogenesis and vasculogenesis[110-114]. Angiogenesis refers to the process where new blood vessels are formed from preexisting blood vessels from the wound’s adjacent vasculature by sprouting and forming tube-like structures and networks. Vasculogenesis is the de novo formation of new blood vessels from the differentiation of bone marrow-derived progenitor stem cells.

RESTITUTION AND QUALITY OF ULCER HEALING AS THE SINE QUA NON FOR WOUND HEALING

GI ulcers have traditionally been assessed in clinical settings by a superficial visual endoscopic examination that cannot assess the histological and ultrastructural characteristics of the mucosa or deeper layers. Ulcer recurrence is, unfortunately, common, with rates exceeding 60% if the underlying problem has not been successfully addressed[23]. Recurrence of GI ulcers may be related to many factors including gastric acid secretion, H. pylori, NSAIDs, hormonal complications, size and depth of ulcers, anti-ulcer treatment, age, gender, comorbidity, alcohol consumption, and smoking[115-118]. In 1991, Tarnawski et al[119] drew attention to the relationship between recurrence of ulcers and ultrastructural abnormalities of deeper layers such as poor ‘redifferentiation, dilation of glands, reduced mucosal height, and disorganized microvascular network after ulcer healing and proposed the concept of the quality of ulcer healing (QOUH)[119-121]. QOUH is defined as ideal ulcer healing, demonstrating flat ulcer scar, high functional restoration, and histological maturity of the regenerated tissue[115,122]. Many patients treated with PPIs for GI ulcers still suffered from a recurrence of ulcers despite continuous anti-ulcer therapy[115,122-124]. It appears that acid inhibition by PPIs or H2-antagonists may be insufficient for successful high-quality gastroduodenal ulcer healing because low levels of prostaglandins and high levels of oxygen free radicals entail poor QOUH and thus potentiate ulcer recurrence[122,125,126].

Overall, cumulative data highlight the necessity of QOUH for successful and permanent ulcer healing and point out that contemporary treatments such as PPIs and H2-antagonists do not always provide such high-quality healing. Therefore, to improve QOUH and decrease the rate of recurrence of GI ulcers, new antiulcer drugs need to be developed to address this. Investigation of the endogenous biologic regulation of mucosal healing, suggests new therapeutic targets, both extracellular and intracellular.
REGULATORS OF MUCOSAL HEALING AND POTENTIAL NEW THERAPEUTIC TARGETS

Supplementing available therapeutic modalities that attempt to minimize or reduce injury, investigators have more recently focused on enhancing mucosal defense or promoting mucosal repair. Both mucosal defense and mucosal healing processes such as restitution, proliferation, angiogenesis, and vasculogenesis can be influenced by acid secretagogues, growth factors, trefoil peptides, cytokines, angiogenic factors, luminal nutrients, and the gastrointestinal microbiota[5,25,127]. In addition, physical forces like strain and pressure, engendered by peristalsis, villous motility, and interaction with luminal contents can influence intestinal epithelial migration and proliferation in a complex manner influenced by the deposition of fibronectin at the site of injury[25,128,129].

Acid secretagogues

Under physiological conditions, the stomach protects itself against various forms of endogenous and exogenous injury, primarily by gastric acid. Gastroprotective mechanisms could be triggered by acid secretagogues such as gastrin, histamine, and thyrotropin-releasing hormone (TRH)[130-132]. Pentagastrin, synthetic gastrin, stimulates gastroprotection in acidified aspirin-induced gastric injury in rats, likely through the activation of histamine-2 receptors, since this is abolished by ranitidine[133]. However, exogenous gastrin protects the rat gastric mucosa against ethanol-induced lesions but not against aspirin-induced gastric damage in rats[134]. Several studies have shown that exogenous histamine-stimulated acid secretion also exerts a protective effect on the gastric mucosa against erosions induced by exogenous HCl in rabbits and frogs by stimulating a greater alkaline tide[135,136]. The central vagal activation by intracisternal injection of the thyrotropin-releasing hormone analog RX77368 enhances mucosal resistance as well by stimulating mucosal blood flow via prostaglandin-independent manner which eventually results in the removal of diffused acid from the subepithelial interstitial space [157]. In addition, RX77368 increases the thickness of the mucus gel via prostaglandin-dependent manner which slows down the acidification of surface cells[137]. The potential therapeutic adaptation of molecules like RX77368 and other acid secretagogues awaits the further exploration of the disparities between results depending on how the ulcers are induced, as well as challenges with their pharmacologic delivery.

Growth factors, trefoil peptides, and cytokines

Growth factors have diverse pathophysiologic effects, including cytoprotection against destructive agents, epithelial wound healing in response to injury[102,138-140], and angiogenesis[141-144]. Epidermal growth factor (EGF) and transforming growth factor TGF-α are structurally related but different polypeptide growth factors[145]. They both bind to the same cell-surface EGF/TGF-α-receptor and induce generally similar effects[145].

EGF may act in a cytoprotective fashion against mucosal injury by increasing secretion of mucus and bicarbonate[146-148], enhancing blood flow[149-151], or releasing other cytoprotective agents such as prostaglandins[152]. Pretreatment of the stomach[153,154], small intestine[146,153], and colon[149,150] tissues, both in vivo and in vitro, with EGF decreases mucosal damage by various noxious agents. TGF-α is similarly cytoprotective against gastric injury by ethanol, acetic acid, or aspirin[156,157]. Pretreatment of Caco-2 cells with EGF prevents deoxylcholate-induced cellular damage, at least in part, by changes in intracellular calcium content[158], suggesting that EGF exerts direct cytoprotective effects on the epithelium in addition to its effects on blood flow and mucus secretion. Furthermore, because this EGF-induced cytoprotection was observed following only 30 minutes of pretreatment (insufficient for proliferation), these results also suggest that this protection is independent of the mitogenic effects of EGF[158]. Consistent with this idea, adding EGF to the basal surface of rabbit primary gastric epithelial cell monolayers cultured on collagen-coated inserts enhances cytoprotection against apical surface acid by opening the plasma membrane calcium channels and increasing intracellular calcium[159].

In addition to their cytoprotective effects, EGF and TGF-α also promote mucosal healing after injury, stimulating both cell motility and cell proliferation[104,140,154]. Indeed, part of the epithelial mucosal shift to a phenotype adapted to wound healing may be an increase in sensitivity to these growth factors. A recent study demonstrated a 75-fold increase in the number of cells expressing detectable EGF-receptors at the ulcer margin after gastric ulcer induction in rats[160]. Either parenteral or local submucosal intra-ulcer injection of EGF caused a comparable acceleration in the healing of acetic-acid-induced rat gastric ulcers, at least in part by increasing gastric blood flow, decreasing gastric acid secretion, and upregulating COX-2 expression[161]. This is in agreement with previous reports suggesting that COX-2 -influences mucosal healing by regulating both the hyperemic response and epithelial cell proliferation[162,163].

The trefoil peptides may also offer new opportunities for therapy because they are important both for mucosal defense[164-166] (by increasing the viscoelasticity of mucus[167,168]) and mucosal repair[169-171] (by influencing reepithelialization[171] and inflammation[172]). The trefoil factor (TFF) family includes TFF1 (also called pS2) expressed in gastric surface mucous cells, TFF2 (also called a spasmylocytic polypeptide or SP) produced by mucus-producing gastric mucous neck cells, antral gland cells, and duodenal Brunner’s glands, and TFF3 (also called intestinal trefoil factor or ITF), predominantly
produced by goblet cells of the small and large intestine and found abundantly within the mucus. A trefoil domain consists of three loops created by disulfide bonds and all TFFs are comprised of two trefoil domains[173]. Trefoil peptides have been detected in different forms including monomers, dimers, and complexes with other molecules. This influences the strength of their association with mucus[173]. In particular, TFF dimers tightly interact with mucus, increasing the viscosity and elasticity of mucus in comparison to the effect of TFF monomers[167,174].

The TFFs are mostly distributed to the basolateral domain of gastric neck cells and parietal cells in the stomach, the Paneth cells in the small intestine, and the crypt cells in the colon[175]. TFF interactions and specific functions have been discussed in detail in a recent review[176]. A specific TFF receptor has not yet been described. However, some binding and functional studies propose potential TFF receptors that may influence epithelial restitution. TFFs have been reported to bind to transmembrane proteins such as the β1 integrin subunit, CRP-ductin, CXC chemokine receptor (CXC) 4, CXC7, proteinase-activated receptor (PAR) 2, PAR4, leucine-rich repeat and Immunoglobulin-like domain-containing protein (LINGO) 2, LINGO3, and EGFR[177-181]. TFF3 enhances wound healing by activating EGFR and inducing MAPK[182] and PI3K/Akt signaling pathways in vitro[183] whereas TFF2 directly activates CXCR4 and enhances the phosphorylation of ERK1/2 and Akt in gastric epithelial cells[184]. Indeed, the CXCR4 antagonist AMD3100 blocks TFF2-dependent gastric epithelial repair[170]. TFFs, specifically TFF2 and TFF3, regulate epithelial motility via integrin-binding and activating focal adhesion kinase as well[175]. TFF2 also promotes cell migration via PAR4[185], while TFF3 activates PAR2[186]. Furthermore, TFF2 peptide may be required for optimum activity of EGFR and/or EGF signaling in the stomach because heparin-binding EGF and TGF-α do not induce EGF activation in the stomachs of Tff2 KO mice[177].

Oral administration of trefoil peptides, recombinant human SP, or rat ITF protects the gastric mucosa against ethanol or indomethacin-induced injury in a prostaglandin-independent manner[164]. Similarly, a more recent study has also shown that both parentally and topically applied trefoil peptides reduce ethanol-induced gastric damage, assessed by measurement of gastric mucosal Na+ leakage and area of macroscopic injury in rats[187]. Complementing these results, transgenic mice that overexpress human TFF1 display increased resistance to indomethacin-induced small intestinal damage[188] whereas ITF-deficient mice are more prone to ulceration and hemorrhage after oral administration of dextran sulfate sodium (DSS)[189], suggesting that trefoil peptides play an important role in GI mucosal protection. There are likely to be several mechanisms by which the trefoil peptides promote mucosal healing. For instance, exogenous recombinant TFF2 increases epithelial wound healing by decreasing inflammation by negatively regulating IL-12 production from macrophages and dendritic cells[172] whereas exogenous TFF3 activates epithelial wound healing via the Na+/H+ exchanger-2[171] and accelerates gastric repair via a mechanism that does not require cyclooxygenase activation[170].

TGF-β expression increases in affected mucosa from patients with IBD[190], and at the edge of human gastric and colonic ulcers[92]. Intravenous administration of recombinant bone morphogenetic protein (BMP)-7, a subfamily of TGF-β superfamily, for five days significantly accelerates the healing of trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats by decreasing the expression of pro-inflammatory cytokines (IL-6, TNF-b, ICAM-1)[191]. It should be noted that all of these growth factors and cytokines mentioned in this section interact in a complex fashion, and TGF-β potentiates many of them [127,192,193]. TGF-β also stimulates the synthesis of FAK, a key intracellular signal protein for cell motility and proliferation[92].

Basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) stimulate the healing of acetic acid-induced gastric lesions in rats similarly when administered intraperitoneally or by local submucosal injection at the ulcer site, suggesting that these growth factors also accelerate mucosal repair[161]. The healing of gastric ulcers by bFGF and HGF may involve enhancement of gastric blood flow around the ulcer, suppression of gastric acid secretion, and upregulation of COX-2 expression [161].

When the mucus barrier fails due to overexposure to the noxious agents, acid-back diffusion occurs. In healthy mucosa, increased blood flow response rapidly increases the circulation of pH neutral or slightly alkaline blood through the mucosa to neutralize the diffused acid[103]. Moreover, new vasculature is needed to perfuse and support the newly forming tissue. Therefore, wounds deeper than the epithelial layer also require the formation of new blood vessels in granulation tissue for mucosal healing. Like restitution and proliferation, neovascularization is also modulated by growth factors. Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), fibroblast growth factor (FGF), and angiopoietin-1 (Ang1) are essential for blood vessel regeneration via angiogenesis and vasculogenesis following mucosal damage[141-144] and can therefore facilitate deep wound healing[112,193-195]. VEGF, the most potent angiogenic factor, is an indispensable regulator of angiogenesis, making it potentially an ideal candidate to induce angiogenesis/vasculogenesis in mucosal healing. Since VEGF is digested in the lumen by proteolytic enzymes, Jones et al.[141] used a single injection of a nonviral naked DNA plasmid encoding VEGF and Ang1 directly into the injured area to reduce such deleterious effects. They demonstrated that local gene therapy with a combination of VEGF and Ang1 cDNAs increases gastric ulcer healing and generates more mature vessels and a more complete epithelial structure in acetic acid-induced gastric injury in rats, suggesting that a combination of growth factors may have better therapeutic potency than the use
of any individual factor[141]. Similarly, local gene therapy with serum response factor (SRF) accelerates ulcer healing as well as muscle restoration in acetic acid-induced gastric ulcers in rats[196]. A recent study indicated that angiogenesis and vasculogenesis go hand in hand while forming new vessels in granulation tissue[197]. Delivering such naked genes to a damaged site at endoscopy may be a promising tool to treat such ulcers by increasing the bioavailability of essential GFs.

Cytokines are also involved in the regulation of mucosal barrier function at multiple levels including mucosal homeostasis and inflammation[198]. Proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-13 are upregulated in the inflamed mucosa of IBD patients. Anti-TNF therapy promotes mucosal healing in many patients, but not all patients respond to anti-TNF therapy. Many investigators have therefore focused on other cytokines to improve mucosal barrier function. Unfortunately, many of these attempts have ended disappointingly. For instance, experimental colitis in mice is controlled by using ROR-gamma null Th17 cells, which cannot produce IL17A/F and not induce colitis[199]. However, an anti-IL17A monoclonal antibody not only failed to improve CD in clinical trials but actually aggravated adverse symptoms[200]. IL-13 has seemed similarly promising in pre-clinical studies[201-204], but a trial of IL-13 blockade in UC failed[205]. Such failures of blockade of pro-inflammatory cytokines have recently prompted attempts to use anti-inflammatory IL-10 family cytokines to promote colonic mucosal healing. IL-10 is a major anti-inflammatory cytokine that targets hematopoietic cells in various autoimmune diseases[206]. Gene therapy with a single intravenous injection of an adenoviral vector encoding IL-10 (AdvmuIL-10) diminishes TNBS-induced colitis in mice, decreasing histological injury scores, weight loss, stool markers of inflammation (IL-1β and TNF-α), and serum amyloid protein in comparison to empty cassette virus (Adv0) or PBS treated mice in TNBS-induced colitis model[207]. Gelatin microspheres containing IL-10 have been developed to increase local bioavailability as a sustained release preparation[208]. Gelatin-microsphere-IL-10 treatment remarkably decreases colonic inflammation in IL-10(-/-) mice compared with IL-10 alone treatment, at least in part by decreasing IL-12 mRNA expression and down-regulating CD40 expression in macrophage-1 positive cells[208]. However, recombinant IL-10 did not improve clinical symptoms in Crohn’s disease[209]. Such disappointing results raise the possibility that manipulating a single cytokine may have unpredictable results due to its effects on the web of compensatory pro-inflammatory and anti-inflammatory cytokine pathways in the inflamed mucosa[198].

One cytokine that may be promising is IL-22. Even though it belongs to the IL-10 family of cytokines, IL-22 is unlike IL-10 in that it targets non-hematopoietic epithelial cells. IL-22 is produced by, apart from the adaptive T cell, innate cells including innate lymphoid cells (ILCs), specifically ILC3 cells in the GI tract. IL-22 has dual roles in inflammation. It can act as a protective (anti-inflammatory) cytokine or a pathological (pro-inflammatory) cytokine[210]. IL-22 influences various tissue epithelial functions such as inflammation[211,212], barrier integrity, regeneration, wound healing[213-215], and host defense against pathogens[216,217]. Beneficial effects of IL-22 have been demonstrated in various murine colitis models[210,218]. However, IL-22 actually appears to worsen the anti-CD40-induced colitis model, in that neutralization of IL-22 reduces the weight loss and colitis scores caused by the anti-CD40 injection and administration of IL-22 then recreates the colitis[219]. Thus, although most animal studies raise the possibility that recombinant human (rh) IL-22 might be a promising therapy for IBD, it remains unclear which effect will be seen in human disease. However, as for other cytokines, the short half-life of rhIL-22 (less than 2 h) limits its clinical applications. Several groups have sought to overcome this obstacle by engineering recombinant fusion proteins with a half-life of 1-2 wk to improve the cytokine’s pharmacokinetic properties. Currently, seven IL-22 clinical trials have been investigated for different indications. UTTR1147A is a human IL-22 fusion protein that links the human IL-22 with the Fc portion of human immunoglobulin (Ig) G4, which is prepared for IBD studies[220]. Extensive in vitro and in vivo studies suggest that UTTR1147A decreases histologic colitis severity by a pathway involving STAT3 activation[220]. These pre-clinical studies demonstrate that UTTR1147A is well tolerated and is not associated with increased inflammatory cytokines in mouse, rat, and monkey studies[220]. A randomized phase-I healthy volunteer study of UTTR1147A demonstrated satisfactory safety and pharmacokinetic profile[221]. A phase-II open-label extension study to evaluate the long-term safety and tolerability of UTTR1147A in patients with moderate to severe UC and CD continues with an estimated completion date in 2025[222].

Therapeutic use of growth factors (except BMP-7) may be limited by their low protein stability[144]. In addition, despite their beneficial effects on the GI tract, long-term or systemic use of any growth factors, trefoil peptides, or cytokines that stimulate cell proliferation, either for cytoprotection or for mucosal healing, may raise concerns about inducing hyperproliferative or dysplastic lesions and potential tumorigenesis. This remains an open issue for such mitogens.

**Luminal nutrients and GI microbiota**

Luminal nutrients and microbiota are also crucial for the maintenance and repair of the gut mucosa. Short-chain fatty acids (SCFAs) are produced by commensal microbiota, mostly by gram-positive anaerobic bacteria, and are essential for perpetuating intestinal health[223,224]. These SCFAs, especially butyrate, are a major energy source for enterocytes and support gut homeostasis[225-227]. SCFAs may stimulate the differentiation of epithelial cells and their proliferation in vivo[228-230], whereas they promote only differentiation in cell culture models but inhibit proliferation and migration[231-235].
Long known as an energy supply for colonocytes and enterocytes, SCFAs have attracted may also enhance gut barrier function. SCFAs decrease acid-back diffusion by dilating arterial walls and increasing blood flow in gut mucosa[236,237]. In addition, several studies have documented improved intestinal barrier function after SCFA supplementation[238-240]. The SCFAs activate 5’ adenosine monophosphate (AMP) kinase and therefore promote tight junction assembly, which in turn enhances intestinal barrier function[241,242]. However, a recent clinical study found no evidence that butyrate monotherapy or a combination of three SCFAs offered any advantage over placebo in improving the disease activity index in ulcerative colitis patients receiving maintenance oral anti-inflammatory medication[243].

Amino acids such as arginine, histidine, and glutamine promote enterocyte proliferation and decrease mucosal permeability by regulating tight junction proteins[244-247]. A recent study proposed that histidine and arginine play an important role in stimulating intestinal restitution, probably stimulating FAK via the TGF-β1/Smad2 signaling pathway[248]. Glutamine modulates the phenotype of gut epithelial cells by stimulating proliferation and decreasing differentiation in vitro[249]. Similarly, many studies have been shown that glutamine also promotes cell proliferation of intestinal epithelial cells in weaning mice[250] and weaning piglets[251], prevents mucosal injury, and regulates enterocyte restitution following acetic acid-induced intestinal injury in rats[252].

Biologically active phospholipids in milk, phosphatidylcholine (PC) and phosphatidic acid (PA), and their metabolites such as lysophosphatidic acid (LPA), all act to increase the barrier function of GI mucosa by increasing the hydrophobicity of the mucus[253]. This makes the tissue non-wettable[10] and provides mucosal protection against aspirin-induced gastric injury in mice[255]. Dietary essential omega-6 fatty acids can enhance the biosynthesis of prostaglandins and increase the GI mucosal barrier[255]. Milk fat globule-epidermal growth factor 8 (MFG-E8), a glycoprotein found in mammary epithelial cells but also produced by lamina propria macrophages, also plays a vital role in modulating enterocyte migration along the crypt-villus axis[255].

**Extracellular matrix**

Epithelial sheet migration during gut-healing requires crosstalk between focal adhesion (FA) complexes in the lamellipodium and the ECM. The extracellular matrix is an extremely dynamic meshwork comprised of proteins, glycosaminoglycans, and glycoconjugates. Its composition and organization differ between tissue types and with physiological and pathological conditions[256,257]. Besides its structural support, the ECM has a direct role in gastrointestinal wound healing by inducing extensive signaling cascades[258-260]. Plasma and tissue fibronectin accumulating in deeper wounds also help to shift the cells to a phenotype that responds to repetitive deformation by increased motility rather than by classical differentiation[129,261]. ECM remodeling is performed by matrix proteinases such as matrix metalloproteininas (MMPs), lysyl oxidases, and heparanases[262]. The gelatinases, a subgroup of MMPs, consist of two proteinases gelatinase A (MMP-2) and gelatinase B (MMP-9). In particular, MMP-9 is upregulated in the inflamed intestinal mucosa of IBD patients[263-267]. Furthermore, anti-gelatinase neutralizing antibodies have been reported effective in murine DSS-induced colitis[268]. However, a phase II, randomized, placebo-controlled study found that the MMP-9 inhibitor andecaliximab did not induce a significant symptomatic or endoscopic response in patients with active Crohn’s disease[269]. This lack of efficacy in Crohn’s disease prompted the termination of another clinical trial of the same medication[243].

**Regulation of cytoskeleton**

Epithelial restitution begins at the edge of the wound with the redifferentiation of epithelial cells. Reorganization of the actin cytoskeleton is controlled by the Rho family of GTPases including RhoA, Rac1, and Cdc42 (Figure 1C)[93,271-273]. Epithelial cells then form protrusions called lamellipodia with new focal adhesions (FAs) at the leading edge of the motile cells. The migrating cell increases its contractile forces and disassembles focal adhesions at the rear edge allowing the entire cell to move forward[274-276]. Cell-cell linkages[276] transmit this force to other cells behind the migrating front and stretch the epithelial layer across the wound as a sheet. This sheet migration is characteristic of epithelial cells and differs from the individual cell motility displayed by other cell types.

The cytoskeleton, a complex and dynamic network of actin filaments, microtubules, and intermediate filaments, is also an important factor in wound healing[277]. Epithelial restitution relies on the coordination of forward protrusions and retraction forces at the rear edge, which is orchestrated by the actin and microtubule cytoskeleton[278]. In the lamellipodium, the elongating actin filaments produce the driving forces for the protrusion while microtubules form a polarized network that permits organelle and protein transport throughout the cell during cell migration[279,280]. Intermediate filaments, however, are generally considered for the maintenance of the overall cell shape[280]. Alternatively, or in combination with therapy to reduce ongoing injury by improving mucus barrier function and promoting angiogenesis, one could consider attempting to directly stimulate restitution in order to accelerate barrier reconstitution. Thus, proteins that modulate cytoskeleton dynamics might be targeted for optimal wound repair. Fidgetin-like 2 (FL2), a microtubule-severing enzyme, regulates the
Oncel S et al. Gut homeostasis, injury, and healing

Figure 3 Focal adhesion kinase structure, phosphorylation sites, and its associated proteins. Focal adhesion kinase (FAK) contains an N-terminal band 4.1-ezrin-radixin-moesin (FERM) domain comprised of three lobes (F1, F2, and F3), a central kinase domain, a C-terminal FAT domain, and two linker domains with three PR regions that bind SH3 domain containing protein such as p130Cas. Y397 is the site of the FAK autophosphorylation, crucial for FAK activation, which interacts with proteins containing the SH2 domain such as Src and PI3K. Subsequently to the SH2 binding, Src binds to the PR1 SH3 domain (PXXP) and further phosphorylates the Y576/577 sites on FAK, which are crucial for the maximal catalytic activity of FAK. Further FAK phosphorylation at Y925 creates a binding site for Grb2. The phosphorylation of FAK-Y-925 and subsequent Grb2 binding dissociates paxillin from FAK, which results in FAK release from FAs, thus stimulating FA disassembly. The FERM domain regulates the interactions of FAK with growth factor receptors and integrins. The FAT domain recruits FAK to FAs by associating with paxillin. FERM: Band 4.1-ezrin-radixin-moesin; FAT: Focal adhesion targeting; PR: Proline-rich region; SH: Src homology; P: Phosphorylation.

organization of the microtubule cytoskeleton for faster and successful repair of murine wounds[281]. Actin remodeling proteins such as talin[282], Ehm[283], filamin-a[284], gelsolin[285], and flightless I (FliI)[286] have also been identified as potential new targets for improved wound healing. Unlike other members of the gelsolin family, FliI inhibits actin polymerization and FA turnover, thus decreasing migration[286,287]. FliI neutralizing antibodies (FnAb) decreased wound area with a quicker rate of healing in porcine and murine models of wound healing, respectively[288,289].

Regulation of FAs

Cell migration, and consequently wound healing, depend critically on the dynamics of assembly and disassembly of FAs. The subunit composition of integrin receptors and the downstream signaling pathways may vary in different scenarios[290-293]. Nevertheless, integrin binding to ECM triggers focal adhesion formation by recruiting many structural and signaling proteins including FAK, a non-receptor tyrosine kinase[294-298]. FAK regulates FA dynamics both by recruiting other FA proteins such as Src to FA sites and by phosphorylating other signaling and adapter FAs proteins such as paxillin and p130Cas[299-301]. FAK also influences the cytoskeletal remodeling essential for cell migration by regulating the Rho family of small GTPases such as Cdc42, Rac1, and RhoA[302-305]. Inhibition of FAK inhibits cell migration[306].

Although FAK appears to activate cell motility and promote restitution, and FAK is indeed activated during cell motility, levels of both activated FAK and total FAK protein (including both active and inactive FAK) actually decrease in migrating GI epithelial cells in vitro and at the edge of human gastric and colonic ulcers in vivo even though the proportion of activated FAK increases (at least in vitro)[92,94]. This reflects decreased FAK synthesis in cells that have adopted the migratory phenotype[306]. This apparently paradoxical reduction in this important protein makes FAK an attractive target for possible therapeutic intervention to promote mucosal healing.

FAK is a 125 kDa protein comprised of an N-terminal FERM (band 4.1-ezrin-radixin-moesin) domain, a central kinase domain, three proline-rich regions that are binding sites for Src homology 3 (SH3) domain-containing proteins, and a C-terminal focal adhesion targeting (FAT) domain (Figure 3).

The FAT domain consists of a four-helix bundle[307] and is critical for targeting FAK to FAs via binding to paxillin[308]. In an inactive (autoinhibited) state there is an interaction between the FERM and kinase domains which prevents FAK autophosphorylation at Y397[309]. Upon competitive binding of candidate activating proteins such as the cytoplasmic regions of β-integrins or growth factor receptors on the F2 domain of FERM, the autoinhibited conformation of FAK is disassembled[310]. This conformational change allows Y397 phosphorylation, a key event in FAK activation[311]. In a subsequent step, Src is recruited and activated via SH2 binding to pY397 and SH3 binding to the PxxP sequence in the linker region, an essential step in promoting cell migration[311]. Then, Src phosphorylates the activation loop residues Y576 and Y577 of FAK and it acquires full catalytic activity after phosphorylation of the activation loop[312]. Phosphorylation of FAK at tyrosine 925 residue creates an SH2 binding site for the growth factor receptor-bound protein 2 (Grb2), adaptor protein[313]. The Grb2 binding site at FAK-Y-925 overlaps with one of the paxillin binding sites in the FAT domain of FAK[313]. The binding of Grb2 dissociates paxillin from FAK and potentiates the release of FAK from FAs[313]. On the other hand, paxillin acts as a scaffold protein for ERK signaling[305]. Subsequently, ERK may modulate FA turnover by further phosphorylating paxillin[305]. Therefore, Paxillin and Grb2 are critical FA proteins that interact with FAK and play an important role in FA turnover[97,313].
FAK has both a structural role as a scaffold for protein-protein interactions and a kinase function that phosphorylates many substrates in diverse signaling events\cite{314,315}. Its non-kinase scaffolding function allows several different proteins to bind its N-terminal FERM domain and C-terminal FAT domain, tethering them into complexes (Figure 3). For instance, FAK may regulate cell migration serving as a scaffold for Src phosphorylation of p130Cas\cite{316} in FAs. Similarly, nuclear FAK may promote cell survival functioning as a scaffold to stabilize p53-Mdm2 complexes, promoting p53 ubiquitination and proteasomal degradation\cite{317}. On the other hand, in its kinase signaling capacity, FAK triggers many downstream signals including the Ras/Raf/MAPK\cite{97,296,318-320}, p130Cas-Crk\cite{321-324}, and phosphatidylinositol 3-kinase (PI3K)-Akt pathways\cite{317}, which in turn coordinate to regulate cell proliferation, migration, and survival (Figure 4)\cite{313,325}.

Recent evidence suggests that direct modulation of FAK activity is possible, practical, and effective via small molecule FAK activators\cite{326}. A novel small molecule with drug-like properties, ZINC40099027 (ZN27), that mimics the FERM domain of FAK has been identified from the ZINC database and activates FAK in human intestinal epithelial cells without activating Pyk2, the closest paralogue of FAK, or Src, another canonical nonreceptor tyrosine kinase within focal adhesions\cite{327}. Indeed, ZN27 directly activates both full-length 125 kDa and its 35 kDa kinase domain, increasing the maximal activity ($V_{\text{max}}$) of FAK, suggesting that ZN27 is a highly potent and selective activator acting allosterically on the 35 kDa FAK kinase domain\cite{328}. ZN27 not only activates FAK but also stimulates intestinal epithelial migration \textit{in vitro} and mucosal healing in mice after ischemic injury or injury by indomethacin\cite{327}. ZN27 also activates FAK in gastric epithelial cells and promotes gastric mucosal healing in mice subjected to chronic ongoing injury by aspirin\cite{58}. Structure-activity-relationship studies have developed a library of novel FAK activators based on ZN27, that have drug-like properties, activate FAK, and stimulate epithelial sheet migration in \textit{in vitro}\cite{329}. At least one such molecule (dubbed compound 3) demonstrates reasonable drug-like properties based on \textit{in vitro}, \textit{in vivo}, and \textit{in silico} results with no obvious toxicity\cite{329}. Further development of this lead molecule may offer the potential for a new therapeutic approach to actually stimulate mucosal healing by activating FAK.
CONCLUSION

Given the enormous impact of GI mucosal healing on human health, there is certainly a need to expand therapeutic options in this regard. A new understanding of the biology of mucosal healing suggests several different possibilities (Figure 5). These include FAK activators, UTTR1147A, endoscopic gene therapy for angiogenic growth factors, mucus barrier enhancement via the thyrotropin-releasing hormone analog RX77368 or trefoil peptides, enhanced energy for the mucosa with butyrate, and attempts to increase the regenerative ability of the epithelium with growth factors, cytokines, or trefoil peptides. Future work will determine which of these potentially promising avenues will prove successful and will need to balance their effects against potential risks and issues, including bioavailability, mitogenicity, and tumorigenesis.

FOOTNOTES

Author contributions: Oncel S and Basson MD equally contributed to this manuscript with regards to the conception and structure of the manuscript in addition to the literature review; The final draft of the manuscript was read and approved by all the authors.

Conflict-of-interest statement: The senior author (Basson MD) is co-inventor on patents applied for by the University of North Dakota describing the use of small molecule FAK activators to promote mucosal healing. The authors have no other conflicts of interest.

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New insights in diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms

Feng Yin, Zi-Hao Wu, Jin-Ping Lai

Abstract

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are rare epithelial neoplasms derived from pluripotent endocrine cells along the gastrointestinal tract and pancreas. GEP-NENs are classified into well-differentiated neuroendocrine tumors and poorly differentiated neuroendocrine carcinomas. Despite overlapping morphological features, GEP-NENs vary in molecular biology, epigenetic, clinical behavior, treatment response, and prognosis features and remain an unmet clinical challenge. In this review, we introduce recent updates on the histopathologic classification, including the tumor grading and staging system, molecular genetics, and systemic evaluation of the diagnosis and treatment of GEP-NENs at different anatomic sites, together with some insights into the diagnosis of challenging and unusual cases. We also discuss the application of novel therapeutic approaches for GEP-NENs, including peptide receptor radionuclide therapy, targeted therapy, and immunotherapy with immune checkpoint inhibitors. These findings will help improve patient care with precise diagnosis and individualized treatment of patients with GEP-NENs.

Key Words: Gastroenteropancreatic neuroendocrine neoplasms; Neuroendocrine tumours; Neuroendocrine carcinoma; World Health Organization classification; Diagnosis; Treatment
Core Tip: Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are rare tumors, but their incidence rates have been steadily increasing over the past 3 decades. GEP-NENs consist of a genetically heterogeneous group of tumors ranging from slow-growing well-differentiated neuroendocrine tumor to aggressive, poorly differentiated neuroendocrine carcinoma. Surgery is the cornerstone for the clinical management of localized tumors. However, GEP-NENs have frequently been diagnosed at a later stage and, therefore, remain an unmet clinical challenge. In this review, we discuss recent updates on the histopathologic classification, molecular genetics, and systemic evaluation of diagnosis and treatment of GEP-NENs at different anatomic sites.

INTRODUCTION

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are epithelial neoplasms with neuroendocrine differentiation that occur inside the gastrointestinal (GI) tract and the pancreas[1]. They are a group of tumors with significant heterogeneity and complex clinical behavior, ranging from slowly growing well-differentiated gastroenteropancreatic neuroendocrine tumors (GEP-NETs) to highly aggressive, poorly differentiated gastroenteropancreatic neuroendocrine carcinomas (GEP-NECs)[1-3]. The traditional term for a NET is carcinoid, which has been largely discouraged in the updated disease classification published by the World Health Organization (WHO)[1].

GEN-NENs are relatively rare (1.0%-1.5% of all GEP neoplasms, 6.98 and 0.4 new cases per year per 100000 individuals in the United States for GEP-NETs and GEP-NECs, respectively)[4-7], although their incidence has significantly increased in the past 3 decades, largely due to the improved awareness and detection rate. The majority (>95%) of GEP-NENs are sporadic, although some (approximately 5%) could be part of syndromic presentations, including multiple endocrine neoplasm type 1 (MEN1), neurofibromatosis type 1 (NF1), and von Hippel–Lindau syndrome (VHL)[1-4].

Based on the embryological development of the GI tract, GEP-NENs can be divided into foregut (the esophagus to the proximal duodenum and pancreas), midgut (the distal duodenum to proximal two thirds of the transverse colon), and hindgut NENs (distal third of the transverse colon to the rectum)[5, 6], with the midgut (especially the small intestine) being the most common site for GEP-NENs. For example, Figure 1 shows the case of a 71-year-old Caucasian man who was initially identified to have multiple liver masses on computed tomography (CT) scans. CT-guided percutaneous liver needle core biopsy showed poorly differentiated neuroendocrine carcinoma and a small cell type, and the tumor cells were positive for neuroendocrine markers and GI tumor markers. A positron emission tomography (PET)/CT scan revealed the primary lesion in the ileum (Figure 1). In addition, compared with their foregut and hindgut counterparts, midgut NENs are more commonly associated with carcinoid syndrome[7]. In this review, we will discuss anatomic origin and pathologic feature-based classification systems for GEP-NETs as well as the diagnosis and current update of therapy. Based on our practice, we will also share some experience in the work-up of some unusual and challenging cases with diagnostic pitfalls.

HISTOPATHOLOGIC CLASSIFICATION

Tumor differentiation is closely associated with the clinical behavior of GEP-NENs and refers to how much the tumor tissue looks like the normal tissue that it was derived from. Based on tumor differentiation and histopathologic features, GEP-NENs can be classified into three major categories: Well-differentiated NETs, poorly differentiated NECs, and mixed neuroendocrine-nonneuroendocrine neoplasms (MiNENs)[8]. Well-differentiated GEP-NETs commonly present with a uniform population of tumor cells with round nuclei and finely stippled “salt-and-pepper” chromatin[9]. Their common growth patterns include nests, trabeculae, acini, and ribbons. On the other hand, poorly differentiated GEP-NECs could be further classified into small-cell NECs and large-cell NECs based on their cytological features and commonly grow in sheets or nests with frequent tumor necrosis[10]. Small-cell NECs have features that include blue cells with scant cytoplasm, finely dispersed chromatin, nuclear molding, smudging, no distinct nucleoli, high mitotic rate, and patterns of rosettes and/or peripheral palisading (Figure 1). Large-cell NECs also have a neuroendocrine architecture and features of large cells with abundant cytoplasm, round and vesicular nuclei, and prominent nucleoli. Some NECs have a concurrent adenocarcinoma component and are categorized as MiNENs[11,12].
Figure 1: Ileal small cell neuroendocrine carcinoma with liver metastases in a 71-year-old man. A: Computed tomography (CT) image (axial) showing ileal and multifocal liver lesions (arrow); B: Positron emission tomography-CT image showing ileal (arrow) and liver lesions (arrowheads) with hypermetabolic activity; C: Histopathologic features of small cell neuroendocrine carcinoma. Note the tumor cells with peripheral palisading, rosetting, scant cytoplasm, nuclear molding, finely granular chromatin, and lack of prominent nucleoli; D-F: Tumor cells with positive immunoreactivity for synaptophysin (D) and CDX2 (E) as well as a high Ki-67 proliferation index (70%) (F). (C-F: 400 ×).

Tumor grade is another important factor closely correlated with the clinical behavior of GEP-NENs. It refers to how abnormal the tumor cells look under a microscope, and in the case of GEP-NENs, the tumor grade is usually determined by the proliferation rate of the tumor cells that could be reported by the mitotic rate (number of mitoses per 2 mm²) and/or the Ki-67 proliferation index (average nuclear immunolabeling based on at least 500 tumor cells) (Table 1). The current guidelines use a 3-tier tumor grading system: Low-grade (grade 1, G1) tumors with a mitotic rate up to 2 per 2 mm² or a Ki-67 proliferation index up to 3%, intermediate-grade (grade 2, G2) tumors with a mitotic rate from 2 to 20 per 2 mm² or a Ki-67 proliferation index from 3% to 20%, and high-grade (grade 3, G3) tumors with a mitotic rate greater than 20 per 2 mm² or a Ki-67 proliferation index greater than 20% [13]. A suggestion has been made to use a Ki-67 proliferation index of 5% as the cutoff level, instead of 3% according to current guidelines [14], although additional large-scale studies are needed to validate this proposed cutoff value. Due to the heterogeneity among tumor tissues, a routine practice is to perform measurements in the most mitotically active tumor area. In cases with discrepancies between the mitotic rate and Ki-67 proliferation index, the tumor will be placed into the highest-grade category. A higher Ki-67 proliferation index is associated with a poorer prognosis [15].

In fact, the Ki-67 proliferation index appears to be a better prognostic marker than the mitotic rate for metastatic pancreatic and midgut NENs [16]. Of note, all NECs were high-grade carcinomas with a poorly differentiated morphology and a high Ki-67 proliferation index (> 20%), more than 50% in the majority of the cases (Figure 1) and high mitotic count (> 20 per 2 mm²). Historically, all G3 GEP-NENs were conceptually equal to NECs before 2017. However, recent studies have clearly demonstrated that G3 GEP-NETs and GEP-NECs are genetically different entities [17]. In general, G3 GEP-NETs are morphologically well differentiated and clinically less aggressive than GEP-NECs and have a poorer response to platinum-based chemotherapy [18].

All GEP-NENs are characterized by the expression of neuroendocrine markers, with or without secretion of biologically active substances. Immunohistochemical staining is often necessary, not only to confirm the diagnosis and to assign the tumor grade category but also to investigate the tumor origin in cases of metastasis. GEN-NENs are derived from the neuroendocrine epithelium and therefore normally express cytokeratin (CK), with CK8 and CK18 being the most common [19]. The expression of CK could separate GEP-NENs from their great mimics pheochromocytoma/paraganglioma. General neuroendocrine markers are also frequently used in routine practice to establish the diagnosis. Well-differentiated NETs usually express somatostatin receptors. In fact, the expression of somatostatin receptor subtype 2A (SSTR2A) is the basis of functional imaging (such as gallium Ga 68 dotatate) and somatostatin analog (SSA) therapy, including octreotide acetate and peptide receptor radionuclide therapy (PRRT) (such as lutetium Lu 177 dotatate) [20]. Some commonly used neuroendocrine immunohistochemical markers include chromograin A (CgA), synaptophysin (SYN), and CD56. Recent studies have demonstrated INSM1 (insulinoma-associated protein 1) as a novel and more specific marker of
neuroendocrine differentiation. In the case of poorly differentiated NECs, INSM1 appears much more sensitive (95%) than CgA (83%) and SYN (82%)[21]. Immunohistochemical staining could also be helpful to identify unknown primary tumors in cases of metastasis. Up to 20% of NETs originally present as liver or bone metastasis from unknown primary tumors, and identification of the primary tumor has significant therapeutic and prognostic implications. The jejunum, ileum, and pancreas appear to be the most common primary sites for patients with NET liver metastases of occult origin[22,23]. CDX2 immunoreactivity is present in the majority of jejuno-ileal NETs, and up to 24% of metastases are primarily pancreatic NETs[24]. The novel marker SATB2 (special AT-rich sequence-binding protein 2) is frequently and strongly expressed in NETs of the lower GI tract and has shown value in assigning NEC sites of origin[25]. For metastatic GEP-NENs, additional immunohistochemical panels include PDX-1 (pancreatic and duodenal homebox 1), PAX6 (paired box 6), PAX8 (paired box 8), ISL1 (islet 1), NESP55 (neuroendocrine secretory protein 55), PR (progesterone receptor), and PrAP (prostate acid phosphatase)[26,27]. We performed PAX6 and PAX8 immunohistochemical staining on 178 NETs, including 110 primary NETs (26 pancreatic, 10 gastric, 12 duodenal, 22 jejuno-ileo, 10 rectal, and 30 pulmonary) and 68 NETs metastatic to the liver (24 pancreatic, 1 duodenal, 37 jejuno-ileo, 1 rectal, and 5 pulmonary). Among primary GEP-NETs, PAX6 and PAX8 were positive in 65% (17/26) and 73% (19/26) of pancreatic, 0% (0/10) and 10% (1/10) of gastric, 92% (11/12) and 92% (11/12) of duodenal, 0% (0/22) and 0% (0/22) of jejuno-ileo, and 90% (9/10) and 80% (8/10) of rectal NETs, respectively. PAX6 and PAX8 positivity was seen in 46% (11/24) and 50% (12/24) of metastatic pancreatic NETs to the liver, respectively. None of the nonpancreatic NETs metastatic to the liver were immunoreactive for either PAX6 or PAX8[27].

### MOLECULAR GENETICS

GEP-NENs consist of a biologically distinct group of tumors with great genetic heterogeneity. Largely driven by high-throughput technologies and next-generation sequencing, significant progress has been made in recent years in understanding the key molecular drivers of tumorigenesis and progression, especially pancreatic and small bowel NENs[28-30]. Approximately 10%-20% of pancreatic NETs (P-NETs) are associated with hereditary genetic syndromes, including MEN-1, NF1, VHL, and tuberous sclerosis[31]. In cases with sporadic P-NETs, three types of major molecular alterations have been detected, including somatic mutations in MEN1 (44%), DAXX (death-domain associated protein)/ATRX (alpha thalassaeoma/mental retardation syndrome X-linked mutations) (43%), and laminar target of rapamycin (mTOR) pathway genes such as PTEN, TSC2, and PIK3CA (14%)[32]. Identification of mTOR pathway gene alterations has clinical relevance due to the available targeted therapy. Germline mutations in DNA repair genes, including MUTYH, CHEK2, and BRCA2, have been reported in sporadic P-NETs[33]. Chromosome 18 deletion is detected in 60%-90% of small intestinal NETs (SI-NETs), although its significance is still unclear at this time[34]. Chromosome 14 gain is also frequently detected in advanced and metastatic disease[35]. Approximately 8% of SI-NETs have somatic mutations in CDKN1B[36,37]. A recent study demonstrated that, as epigenetically dysregulated tumors, SI-NETs could be divided into three subgroups: (1) Chromosome 18 deletion with CDKN1B mutations and CpG island methylator phenotype (CIMP) negativity, the largest subgroup (55%) with the most favorable prognosis; (2) The absence of arm-level copy-number variation (CNV) with a high level of CIMP positivity, the subgroup (19%) with intermediate prognosis; and (3) The presence of multiple CNVs, the subgroup (26%) with a young age at onset and the worst prognosis[38]. In addition to P-NETs and SI-NETs, more studies are needed to understand the molecular genetics of GEP-NETs in other anatomic.

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**Table 1 World Health Organization classification for gastroenteropancreatic neuroendocrine neoplasms**

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Mitotic rate (%)</th>
<th>Ki-67 proliferation index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 NET</td>
<td>Well-differentiated</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>G2 NET</td>
<td>Well-differentiated</td>
<td>2-20</td>
</tr>
<tr>
<td>G3 NET</td>
<td>Well-differentiated</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>SCNEC</td>
<td>Poorly-differentiated</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>LCNEC</td>
<td>Poorly-differentiated</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>MiNEN</td>
<td>Well- or poorly-differentiated</td>
<td>Variable</td>
</tr>
</tbody>
</table>

NET: Neuroendocrine tumor; SCNEC: Small cell neuroendocrine carcinoma; LCNEC: Large cell neuroendocrine carcinoma; MiNEN: Mixed neuroendocrine-non-neuroendocrine neoplasm.

1Data derived from Klimstra et al[1].
sites.

From a molecular genetics point of view, pancreatic NECs (P-NECs) are entirely biologically different entities from P-NETs. The most common molecular alterations in P-NETs are somatic mutations in TP53, RB1, CDKN2A, and KRAS[39]. Notably, mutations in the TP53 and RB1 genes appear to be recurrent molecular events in GEP-NECs from different anatomic sites, including the stomach and colorectum [40]. KRAS mutations have also been detected in gastric and colorectal NECs, although BRAF mutations have only been reported in colorectal NECs[30]. Interestingly, somatic mutations in DAXX, ATRX, and MEN1 are almost exclusively detected in well-differentiated NETs but not poorly differentiated NECs [41].

The pathological diagnosis of grade-3 well-differentiated NETs and poorly differentiated NECs could be challenging in some cases because of the high mitotic rate (> 20 per 2 mm³) and high Ki-67 proliferation index (> 20%), particularly for the limited sample made from endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). The distinct molecular profile could help to separate these two entities. In practice, immunohistochemistry using antibodies against DAXX, ATRX, p53, and RB1 could be performed to surrogate for genetic status[42]. Poorly differentiated NECs frequently have absent RB1 and aberrant p53 protein expression, together with normal expression of DAXX and ATRX. On the other hand, normal RB1 and p53 protein expression is normally found in well-differentiated NETs.

CLINICAL PRESENTATION, DIAGNOSIS, AND TREATMENT

Gastric NENs

Gastric NENs (G-NENs) originate from different neuroendocrine cell types in the gastric mucosa, including enterochromaffin (EC) cells (serotonin producing), enterochromaffin-like (ECL) cells (histamine producing), D-cells (somatostatin producing), and G-cells (gastrin producing)[43]. The diagnosis of G-NENs is usually performed incidentally during upper GI endoscopy due to the lack of specific symptoms, although rare cases could be seen in systemic syndromes, especially Zollinger-Ellison syndrome. Gastric NETs (G-NETs) are commonly subclassified into three distinct types that are mostly derived from ECL cells (Table 2). NETs derived from D cells, G cells, and EC cells are extremely rare.

Type 1 G-NETs are the most common NETs (80%-90%) in the stomach and associated with advanced autoimmune metaplastic atrophic gastritis. It is more commonly seen in females that frequently have additional autoimmune disorders, such as type 1 diabetes mellitus and Hashimoto’s thyroiditis. The presence of autoimmune antibodies, including anti-parietal cell antibodies and anti-intrinsin factor antibodies, leads to the destruction of parietal cells and achlorhydria[44]. Laboratory testing shows elevated serum gastrin, decreased vitamin B12, and high gastric pH (> 7). Gastrin induces ECL cell hyperplasia (< 0.5 mm) and ultimately G-NETs when the lesions measure 0.5 mm or larger.

Type 1 G-NETs are usually diagnosed under upper GI endoscopy with biopsy. It usually presents with multiple small (< 1 cm) reddish polyps or nodules of the gastric body (Figure 2A) and fundus. Histologically, type 1 G-NETs show tumor cells with abundant eosinophilic cytoplasm, monotonous round nuclei, and characteristic chromatin arranged in trabecular or nested patterns (Figure 2B and C). Necrosis is not a feature for this type of tumor. The background gastric mucosa shows atrophic gastritis with frequent intestinal metaplasia (Figure 2B and C inset). In addition, the spindle cell morphology of type 1 G-NETs has been reported by us with histological features mimicking spindle cell gastrointestinal stromal tumors (GISTs) (Figure 2D)[45] and therefore represents a potential diagnostic pitfall.

Most type 1 G-NETs are small G1 tumors and are limited to the mucosa and rarely the submucosa. Imaging study is usually unnecessary. However, EUS is likely warranted if the tumor is greater than 1-2 cm due to a higher risk for lymph node metastasis (2%-9%), muscularis propria invasion, and angioinvasion[46]. The management of type 1 G-NET is generally conservative with endoscopic surveillance due to its favorable prognosis. Endoscopic resection could be performed on cases with larger lesions (> 5 mm) (Figure 3). Gastrectomy is only reserved for rare high-risk cases.

Type 2 G-NETs are rare and account for 5%-6% of G-NETs. They occur in Zollinger-Ellison syndrome in the setting of MEN-1 syndrome, and patients are younger. Type 2 G-NETs are usually caused by duodenal gastrinoma[47]. The common clinical presentation includes abdominal pain and watery diarrhea. Laboratory testing shows elevated serum gastrin and low gastric pH (< 2). Additional genetic testing is recommended to confirm MEN-1 syndrome in suspicious cases. Endoscopically, type 2 G-NENs present with multiple gastric polyps or nodules. Multiple gastric peptic ulcers are common findings. These polyloid lesions are usually larger but typically less than 2 cm. The microscopic features of type 2 G-NENs are similar to those of type 1 G-NENs, presenting as low-grade tumors (G1 tumors being the most common) in a background of ECL hyperplasia. However, background gastric mucosa in type 2 G-NETS demonstrates parietal cell hypertrophy/hyperplasia. Type 2 G-NETS are mostly limited to the mucosa and submucosa. It has a higher risk of lymph node metastases (up to 30%) and therefore a slightly worse prognosis than type 1 G-NETs.

The clinical management of type 2 G-NETs also includes endoscopic surveillance, endoscopic resection, and rarely surgery. It is of clinical importance to locate and resect the primary gastrinoma.
### Table 2: Clinicopathologic characteristics of gastric neuroendocrine tumor

<table>
<thead>
<tr>
<th>Type</th>
<th>Type 1 (%)</th>
<th>Type 2 (%)</th>
<th>Type 3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative frequency (%)</td>
<td>70-80</td>
<td>5-6</td>
<td>10-15</td>
</tr>
<tr>
<td>Gender</td>
<td>F &gt; M</td>
<td>F = M</td>
<td>M &gt; F</td>
</tr>
<tr>
<td>Cell of origin</td>
<td>ECL</td>
<td>ECL</td>
<td>ECL, EC, etc.</td>
</tr>
<tr>
<td>Associated disease</td>
<td>AMAG; Pernicious anemia</td>
<td>MEN1; ZES</td>
<td>None (Sporadic)</td>
</tr>
<tr>
<td>Site of tumors</td>
<td>Fundus and corpus</td>
<td>Fundus and corpus, occasionally antrum</td>
<td>Anywhere</td>
</tr>
<tr>
<td>Size of tumors</td>
<td>&lt; 1 cm</td>
<td>&lt; 2 cm</td>
<td>2-5 cm</td>
</tr>
<tr>
<td>Number of tumors</td>
<td>Multiple</td>
<td>Multiple</td>
<td>Single</td>
</tr>
<tr>
<td>Plasma gastrin level</td>
<td>High</td>
<td>High</td>
<td>Normal</td>
</tr>
<tr>
<td>Gastric acid output</td>
<td>Low or absent</td>
<td>High</td>
<td>Normal</td>
</tr>
<tr>
<td>Metastatic rate (%)</td>
<td>2-5</td>
<td>10-30</td>
<td>50-100</td>
</tr>
<tr>
<td>Tumor related death (%)</td>
<td>Approximately 0</td>
<td>&lt; 10</td>
<td>25-30</td>
</tr>
</tbody>
</table>

ECL: Enterochromaffin-like cell; EC: Enterochromaffin cell; F: Female; M: Male; AMAG: Autoimmune metaplastic atrophic gastritis; MEN1: Multiple endocrine neoplasm type 1; ZES: Zollinger-Ellison syndrome.

SSAs have been proposed in the treatment of type 2 G-NETs, although large-scale cohort studies are necessary for their validation.

Type 3 G-NETs are sporadic tumors and account for 10%-15% of all G-NETs. Laboratory testing showed normal serum gastrin and gastric pH. The clinical presentations are nonspecific, including abdominal pain, melena, and weight loss. Carcinoid syndrome could be seen in patients with liver metastases. Endoscopically, the tumor is usually a single large lesion (> 2 cm) arising in the normal background mucosa. These tumors commonly have aggressive clinical behavior, characterized by higher tumor grade, higher tumor stage, and frequent lymph node and distant metastases. The diagnosis involves upper GI endoscopy and biopsy. EUS is required to measure the depth of invasion and to evaluate lymph node status. Additional imaging studies, including computed tomography (CT), magnetic resonance imaging (MRI), and somatostatin receptor scintigraphy (SRS), are recommended for perioperative tumor staging.

Compared with the favorable prognosis in types 1 and 2 G-NETs, type 3 G-NETs frequently present as high-grade and deeply invasive tumors. Lymph node metastases are found in up to 71% of type 3 G-NETs measuring 2 cm or larger[48]. Although endoscopic resection could be applied for small and superficial lesions, radical surgical resection (total or subtotal gastrectomy) with lymphadenectomy is often the treatment of choice for type 3 G-NETs.

Gastric NECs (G-NECs) are poorly differentiated carcinomas with high mitotic counts and frequent necrosis. They have been further subclassified into small cell NECs (SCNECs) and large cell NECs (LCNECs)[49]. Histologically, SCNECs are similar to their counterparts in the lung, featuring small neoplastic cells with scant cytoplasm and hyperchromatic nuclei. Prominent nucleolus is uncommon. In contrast, LCNECs have large neoplastic cells with abundant eosinophilic cytoplasm, vesicular nuclei, and prominent nucleoli. G-NECs are high-grade neoplasms by definition, with a high Ki-67 proliferation index (> 20%, often more than 60%-70%) and a high mitotic rate (> 20 per 2 mm²). For G-NECs, surgical resection is often required, and postoperative chemotherapy is advised in cases with metastatic disease[50].

**Small intestinal NENs**

The incidence of small intestinal NENs (SI-NENs) has been increasing steadily over the past 3 decades to 1.05 new cases per 100000 individuals per year[51,52], and they are the most common GEP-NENs (29.5%) in the United States, followed by the rectum (29.2%) and pancreas (13.5%)[52]. The clinical presentations include nonspecific abdominal pain, mass effects (small bowel obstruction), and symptoms related to excess hormone secretion. Ampullary NENs could cause jaundice and acute or chronic pancreatitis. Duodenal gastrinoma is a cause of Zollinger-Ellison syndrome. Despite relatively slow growth, the small intestine is the most common primary NET site for metastatic disease along the GI tract[53].

CT scans are the most common imaging modalities for the diagnosis of SI-NENs. Other imaging studies include ultrasound, MRI, and SRS. Endoscopy with biopsy is the gold standard for the diagnosis of SI-NENs. Endoscopic examination of SI-NENs includes capsule endoscopy, colonoscopy, and double-balloon enteroscopy. Duodenal and periampullary NETs are usually single small (< 2 cm) polypoid or nodular lesions limited to the mucosa and submucosa. NETs at the jejunum and ileum are usually large
SI-NENs are morphologically similar to NENs at other sites. A few relatively specific histological features include psammoma bodies in somatostatin-producing D-cell NETs and nested growth patterns with peripheral palisading in serotonin-producing EC-cell NETs. Gangliocytic paraganglioma is a rare NET that is typically encountered in the second part of the duodenum and is characterized by the presence of three distinct components: A neuroendocrine epithelioid component, a Schwannian spindle cell component, and a ganglion cell-like component[55].

For localized SI-NETs, the standard of care is complete surgical resection of the primary tumor, regional lymph nodes, and mesenteric fibrotic tissue. A consensus has not been reached with routine administration of octreotide preoperatively or intraoperatively[56]. For metastatic disease, treatment options are surgical resection, liver-directed therapy (in cases predominantly with liver metastasis), and systemic therapy including SSA, PRRT, everolimus (mTOR inhibitor), and cytotoxic chemotherapy.

Appendiceal NENs

The appendix is a frequent primary site for GEP-NENs, with an incidence rate of approximately 0.15-0.6 new cases per 100000 individuals per year in the United States[52,57,58]. They frequently occur in children and young adults with a slight female predominance[59]. Appendiceal NENs (A-NENs) have the most favorable prognosis among all subgroups of GEP-NENs[52].

The common clinical presentations of A-NENs are similar to those of acute appendicitis. Carcinoid syndrome is extremely rare in A-NENs and is mostly associated with metastatic disease. Histopathologic evaluation is crucial to establish the diagnosis of A-NENs. The application of imaging studies, including CT and MRI, has limited value for the detection of small primary A-NENs. However, colonoscopy is recommended given that up to 18% of patients with A-NENs have concurrent GI neoplasms[60].
The majority (80%) of A-NENs are small and only incidentally found in appendectomy specimens [52]. Most A-NENs (60%-75%) are located at the appendiceal tip; therefore, the appendiceal tip should be examined carefully on all appendectomy specimens. The histological features of A-NENs are similar to those of NENs of other primary sites, with the exception of tubular NETs. Tubular NETs are rare benign neoplasms with a predominant tubular growth pattern, so it is important not to misdiagnose them as adenocarcinomas [61]. Of note, goblet cell adenocarcinoma (formerly goblet cell carcinoid) is no longer considered an A-NEN [62]. Currently, we believe that this is an unusual type of adenocarcinoma with neuroendocrine differentiation.

The management of A-NENs depends on the stage of the disease determined by the tumor size, location, and tumor extension. Simple appendectomy is considered adequate for tumors less than 10 mm. Right hemicolectomy is indicated for tumors larger than 20 mm. The implication of right hemicolectomy in A-NETs with a size of 10-20 mm is still controversial, likely depending on the presence of high-risk features (positive margin after appendectomy, base location, Ki-67 index of 3% or higher, > 3 mm mesoappendiceal invasion, angioinvasion, and perineural invasion) [63,64]. For patients with more advanced disease (stages III and IV), the treatment usually includes curative surgery and systemic therapy.

Colorectal NENs

The incidence rates of colonic and rectal NENs are 0.2 and 1.2 new cases per 100000 individuals per year in the United States, respectively [52]. The mean age for colonic NENs is 65 years, which is significantly older than that for rectal NENs (56 years) due to late detection. The presentation of colorectal NENs is similar to that of colorectal adenocarcinoma with nonspecific mass-related effects, abdominal pain, and bleeding. Classic carcinoid symptoms could be seen in some cases, often with liver metastases.

The majority (70%) of colonic NETs (C-NETs) are located on the right side of the colon, especially the cecum [65]. C-NETs are usually larger, with an average size of 4.9 cm [66]. Approximately 30%-40% of C-NETs have local or distant metastasis at the time of presentation. Colonoscopy with biopsy is commonly performed to establish the diagnosis. C-NETs are usually derived from EC cells or Kulchitsky cells.
within the crypts of Lieberkühn. Therefore, C-NETs typically show EC cell features, including insular growth patterns and CDX2 immunoreactivity. Necrosis is usually absent. The preferred treatment is colectomy with lymphadenectomy.

Rectal NETs (R-NETs) are relatively smaller (< 1 cm), smooth, round polypoid lesions and generally have a better prognosis than their counterparts in the colon. R-NETs are subgrouped into the L-cell (glucagon-like peptide and pancreatic polypeptide producing) type and non-L-cell type according to their origin[67], with the L-cell type being the dominant type. L-cell R-NETs typically present with trabecular or tubular growth patterns. Of note, non-L-cell-type rectalNETs usually present as larger masses and have an increased risk of lymphovascular invasion and worse prognosis[88].

For the purpose of tumor staging, it is recommended to use EUS and MRI of the pelvis to determine the depth of invasion and lymph node status and to use SRS-based scans to determine distant metastases. Endoscopic mucosal resection and endoscopic submucosal dissection are indicated for small (1 cm or smaller) and superficial R-NETs if there is no evidence of muscularis propria invasion or lymph node metastases[69]. For R-NETs larger than 2 cm in size, low anterior resection or abdominal resection is recommended.

**Pancreatic NENs**

As rare neoplasms, the incidence rate for pancreatic NENs (P-NENs) is 1.0 new cases per 100000 individuals per year in the United States[70] and accounts for 2%-4% of all pancreatic neoplasms[71]. All P-NETs are considered to have malignant potential. These tumors are derived from pancreatic islet cells and could be subclassified into functioning and nonfunctioning subgroups. Functioning P-NETs, including insulinoma, gastrinoma, VIPoma, and glucagonoma, cause clinical hormone hypersecretion syndromes. The clinical presentations of functioning P-NETs are mostly related to hormone effects, such as hyperglycemia in insulinoma and large-volume secretory diarrhea in VIPoma. In contrast, nonfunctioning P-NETs are usually incidental findings on imaging studies for other causes or mass effects at late stages. With the increased use of imaging studies, nonfunctioning P-NENs have become more common, accounting for more than 60% of all P-NENs[72].

Insulinoma and gastrinoma are the two most common functioning P-NETs. In suspected cases of insulinoma, 72-h fasting tests for blood glucose, insulin, C-peptide, and proinsulin levels should be performed together with drug tests for sulfonylurea. In suspected cases of gastrinoma, laboratory testing includes fasting gastrin level and gastric pH. Laboratory testing for serum glucagon and VIP levels would be helpful for the diagnosis of glucagonoma and VIPoma. The circulating CgA level is also a sensitive and specific diagnostic marker for P-NETs, with the exception of insulinoma[73], and it has no added value for the diagnosis of nonfunctioning P-NETs.

The most common imaging studies for P-NENs include EUS, CT, MRI, and 68Ga-dotatate PET. Radiolabeled glucagon-like peptide-1 receptor (GLP-1R) scintigraphy is another sensitive tool to detect small insulinomas[74]. Based on our experience, P-NETs could appear as thin-walled cystic lesions with no communication with the pancreatic duct (Figure 4) that clinically and radiologically may mimic mucinous cystic neoplasms. Clinicians and pathologists should be aware of this unusual presentation to avoid misdiagnosis.

Microscopically, P-NETs are well-differentiated neoplasms that do not differ from NETs from other primary sites (Figure 5). One or more neuroendocrine markers (CgA, SYN, CD56, and neuron-specific enolase) and one epithelial marker (cytokeratin AE1/AE3 and CAM5.2) are indicated for the diagnosis of P-NENs. Ki-67 immunoreactivity is warranted to assign tumor grade. Insulin immunoreactivity is necessary in the diagnosis of insulinoma in cases of multifocal tumors or insulinomatosis. In the cases of metastatic disease, some markers (Pax 6, Pax8, ISL-1, PDX-1, and CDX2) could be helpful to determine pancreatic origin[75].

Surgical resection is the preferred and only curative therapy for P-NENs. Conservative management was suggested for small (< 2 cm) low-grade nonfunctioning P-NETs due to their excellent prognosis[76, 77]. However, even for small-sized nonfunctioning P-NETs, surgical resection is indicated in cases with high-risk features (55 years or older, grade 3 tumor, and distant metastases)[78]. Local resection or enucleation could be applied in localized and easily accessible disease to maximally preserve pancreatic tissue[79], especially if the tumor is located more than 2-3 mm from the pancreatic duct. Depending on the tumor location, surgical resection procedures for P-NENs include partial pancreaticoduodenectomy and distal pancreatectomy. Regional lymphadenectomy is recommended for surgical resection of P-NENs.

P-NENs commonly present with liver metastases. Surgical resection should be considered in metastatic disease for both nonfunctioning and functioning P-NENs. The presence of liver metastases is not a contraindication to surgical management for P-NEN patients[80]. Efforts should be made if surgical removal is feasible for both primary pancreatic tumors and metastatic liver lesions. However, it is still under debate whether to resect the primary tumor in cases of unresectable metastatic liver lesions. Surgical resection of metastatic liver lesions should be avoided in cases with unresectable primary P-NENs[81].
NOVEL THERAPEUTIC APPROACHES

The treatment options for advanced and metastatic GEP-NENs have significantly expanded during the past two decades[82,83]. Some important clinical studies, including the PROMID[84] and CLARINET[85] trials, have demonstrated a significant efficacy of SSA in the control of tumor growth in patients with metastatic GEP-NETs. A recent CLARINET FORTE phase 2 clinical trial further supports the clinical benefit of the SSA lanreotide autogel (LAN), which led to significantly improved progression-free survival (PFS) and disease control rate in patients with GEN-NETs, especially in cases with a Ki67 index ≤ 10%[86]. In addition to SSA[87], novel therapeutic approaches, including PRRT, targeted therapy, and immunotherapy, have demonstrated promising clinical benefits[88-90].

PRRT is a type of systemic radiotherapy specifically targeting tumor cells expressing SSTR[91]. In the phase 3 NETTER-1 trial, for patients with metastatic well-differentiated midgut NETs, treatment with $^{177}$Lu-dotatate led to a significantly improved PFS (median PFS not reached vs 8.4 mo in the control group with high-dose octreotide alone) and an improved radiographic response rate (18% vs 3% in the control group)[92]. The most common adverse effects for $^{177}$Lu-dotatate are nausea and vomiting. Based on this trial, PRRT with $^{177}$Lu-dotatate has been approved for patients with advanced GEP-NETs and SSTR expression on imaging.

Due to the hypervascularity in GEP-NETs, multiple clinical trials have investigated the therapeutic effects of targeted therapy against vascular endothelial growth factor (VEGF) receptors. In a phase 3 trial, patients with low- to intermediate-grade P-NETs received placebo vs sunitinib, a tyrosine kinase inhibitor targeting multiple receptors, including VEGF receptors-1, 2, and 3. Sunitinib led to a significantly longer median PFS [11.4 mo vs 5.5 mo in the control group; hazard ratio (HR) for progression or death, 0.42; $P < 0.001$][93]. Sunitinib has been approved for patients with advanced P-NETs.

mTOR is a multifunctional serine/threonine kinase related to NET growth. mTOR pathway genes, including PTEN, TSC2, and PIK3CA, are also frequently mutated in NETs. Multiple clinical trials have been conducted to test the treatment effect of the mTOR inhibitor everolimus in GEP-NETs. In the

Figure 4 Pancreatic neuroendocrine tumor. A: Computed tomography (CT) image showing a 3.5 cm distal pancreatic mass (arrow); B: Positron emission tomography-CT image showing a pancreatic mass with hypermetabolic activity (SUVmax = 4.3) (arrow); C: Endoscopic ultrasound-guided fine-needle aspiration showing clusters of neuroendocrine tumor cells with round nuclei and fine stippled “salt-and-pepper” chromatin (H&E stain, 400 ×); D: Distal pancreatectomy showing the gross cut surface of a firm fibrotic pancreatic neuroendocrine tumor (T) with focal hemorrhage.
RADIANT-3 trial, for patients with advanced P-NETs, everolimus treatment led to a significantly longer median PFS (11 mo vs 4.6 mo in the control group; HR: 0.35)\cite{94}. In the RADIANT-4 trial, patients with advanced nonfunctioning GI and lung NETs had a longer median PFS in the everolimus arm (11 mo vs 3.9 mo in the control group; HR: 0.48)\cite{95}. Everolimus is approved for patients with advanced P-NETs and nonfunctioning GI NETs.

Immune checkpoint inhibitors and antibodies targeting programmed cell death protein-1 (PD-1), programmed cell death protein ligand-1 (PD-L1), or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), have demonstrated promising therapeutic responses in various types of cancers\cite{96}. Based on the durable antitumor efficacy and favorable safety profile in patients with advanced metastatic Merkel-cell carcinoma, a high-grade cutaneous NEC\cite{97}, immunotherapy has been proposed to be potentially effective for advanced NENs with microsatellite instability, high tumor burden, and/or mutational load\cite{98}. Multiple clinical trials have been conducted to test the efficacy of immunotherapy in GEP-NENs\cite{99-101}. Currently, these studies only showed very limited therapeutic effects for GEP-NENs\cite{99-101}. Interestingly, in a phase 1b trial on toripalimab (an anti-PD-1 antibody) for patients with high-grade NENs, patients with PD-L1 expression greater than 10% and/or high tumor mutational burden (TMB) had a better objective response rate (ORR) than low PD-L1 (< 10%) (50.0% vs 10.7%, *P* = 0.019) and low TMB patients (75.0% vs 16.1%, *P* = 0.03)\cite{100}. Therefore, PD-L1 expression is a potential therapeutic and prognostic biomarker for GEP-NENs.

**CONCLUSION**

GEP-NENs are relatively rare tumors, although the incidence rates have been steadily increasing over the past three decades. GEP-NENs consist of a genetically heterogeneous group of tumors ranging from slow-growing, well-differentiated NETs to aggressive, poorly differentiated NECs. Great progress has
been toward understanding their unique molecular genetics and combating advanced disease through improved diagnostic tools and effective therapeutic regimens. A multidisciplinary and personalized treatment approach would be crucial to achieve optimal clinical outcomes for patients with GEP-NENs.

FOOTNOTES

Author contributions: Yin F wrote and finalized the manuscript; Wu ZH critically reviewed the manuscript; Lai JP collected and analyzed the data, made the figures, and finalized the manuscript; all authors have approved the final manuscript.

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Current status and future of targeted peptide receptor radionuclide positron emission tomography imaging and therapy of gastroenteropancreatic-neuroendocrine tumors

Neil Grey, Michael Silosky, Christopher H Lieu, Bennett B Chin

Abstract

Theranostics is the highly targeted molecular imaging and therapy of tumors. Targeted peptide receptor radionuclide therapy has taken the lead in demonstrating the safety and effectiveness of this molecular approach to treating cancers. Metastatic, well-differentiated gastroenteropancreatic neuroendocrine tumors may be most effectively imaged and treated with DOTATATE ligands. We review the current practice, safety, advantages, and limitations of DOTATATE based theranostics. Finally, we briefly describe the exciting new areas of development and future directions of gastroenteropancreatic neuroendocrine tumor theranostics.

Key Words: DOTATATE; Theranostics; Gastroenteropancreatic neuroendocrine tumors; $^{68}$Ga DOTATATE; $^{177}$Lu DOTATATE; Review

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Core Tip: “68Ga and “64Cu DOTATATE positron emission tomography imaging is the most sensitive and accurate method to identify well-differentiated gastroenteropancreatic neuroendocrine tumors (GEP-NETs). The paired therapeutic radiotracer, “177Lu DOTATATE, delivers targeted radiation which can prolong progression free survival. This is now established as the therapeutic best standard of care for patients with progressive, metastatic, or unresectable well-differentiated somatostatin receptors positive GEP-NETs. Ongoing investigations continue to expand the potential indications for DOTATATE theranostics. Additional novel ligands are also currently being developed for targeted imaging and therapy of GEP-NETs.

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INTRODUCTION

Neuroendocrine tumors (NETs) are a relatively rare and heterogeneous group of tumors arising from neuroendocrine cells throughout the body, which can cause a variety of symptoms based on the location and cell type. Midgut NETs are the most common, with the small bowel being the most frequent site of the primary lesion[1,2]. Although the incidence is relatively low, the majority are slow-growing, well-differentiated tumors which effectively contributes to a high prevalence. Improved clinical, laboratory, and imaging detection also likely contribute to an apparent increasing prevalence. NETs are often detected incidentally or after they metastasize and cause clinical symptoms either from their hormonal release and/or from mass effect.

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) comprise the majority of NETs (over 75%), with lung NETs comprising an additional approximately 15%[1]. GEP-NETs are characterized according to the WHO 2010 classification system based on the Ki-67 index, a widely used marker of cell proliferation. In this classification, GEP-NETs are scored as G1 (Ki-67 ≤ 2%), G2 (Ki-76 = 3%-20%), or G3 (Ki-67 > 20%), with G1 and G2 classified as well-differentiated tumors, and G3 tumors classified as poorly differentiated/de-differentiated carcinomas. Further stratification of G3 tumors can be assigned into well-differentiated (Ki-67 20%-50%) and poorly differentiated carcinomas (Ki-67 > 50%). This classification system, as well as the proposed subdivision of G3 tumors, has prognostic utility and helps direct appropriate diagnostic imaging and therapy.

The majority of well differentiated GEP-NETs (> 90%) express the G-coupled protein somatostatin receptors (SSTR)[3-5]. The WHO classification typically correlates with SSTR expression with well-differentiated tumors (G1 and G2) highly expressing SSTRs, and poorly differentiated tumors (G3) having lower SSTR expression. Diagnostic imaging and targeted radionuclide therapy take advantage of tumor SSTR expression by utilizing radiopharmaceuticals that bind to the same ligand. This elegant duality is emblematic of the expanding field of “theranostics”, a portmanteau of therapy and diagnostics.

In this paper, we review the current approaches to the diagnosis and treatment of GEP-NETs. We focus primarily on DOTATATE (“68Ga DOTATATE and “64Cu DOTATATE) positron emission tomography (PET)/computed tomography (CT) imaging and “177Lu DOTATATE peptide receptor radionuclide therapy (PRRT), and conclude with future directions of GEP-NET theranostics.

DOTATATE PET IMAGING

Somatostatin is an endogenously produced peptide hormone that binds to various SSTRs. In humans, there are 5 subtypes[6], with GEP-NETs predominantly expressing SSTR-2 both in the primary tumor and in their metastases[7].

The currently approved nuclear medicine DOTATATE PET imaging agents used to bind SSTR-2 are composed of three functional parts: (1) The radioactive PET imaging component (“68Ga or “64Cu; (2) The PET radiometal chelator and linker, DOTA (tetraazacyclododecanetetraacetic acid); and (3) The peptide binding part, TATE (tyrosine-3-octreotate)[8]. The earlier imaging agents used to visualize GEP-NETs was a single photon octreotide-based agent, [68Ga-Tyr³]octreotide[4], followed by “111In pentetreotide (Octreoscan, Mallinkrodt)[9]. “111In pentetreotide remained the mainstay of GEP-NET imaging until the approval of the PET imaging agents. Both “68Ga DOTATATE and the closely related “68Ga DOTATOC have received approvals in Europe and the United States[10,11], as has the more recently approved “64Cu
DOTATATE. Because of the extremely high binding affinity of $^{68}$Ga DOTATATE to SSTR-2 (approximately 100 times greater than $^{111}$In-pentetreotide)[12], the superior imaging characteristics of PET compared to SPECT, and the lower radiation delivered, DOTATATE PET imaging is now recommended in all cases over $^{111}$In-pentetreotide[13], including use in both adults and children[14].

**Indications**

The appropriate use criteria for SSTR imaging in cases of known or suspected well-differentiated NETs with $^{68}$Ga- and $^{64}$Cu DOTATATE PET/CT imaging and $^{177}$Lu DOTATATE peptide receptor radionuclide have been recently summarized[13]. These include the following nine indications: (1) Initial staging after the histologic diagnosis of NET; (2) Evaluation of an unknown primary; (3) Evaluation of a mass suggestive of NET not amenable to endoscopic or percutaneous biopsy; (4) Staging of NET before planned surgery; (5) Monitoring of NET seen predominantly on SSTR PET; (6) Evaluation of patients with biochemical evidence and symptoms of a NET; (7) Evaluation of patients with biochemical evidence of a NET without evidence on conventional imaging or a prior histologic diagnosis; (8) Restaging at time of clinical or laboratory progression without progression on conventional imaging; and (9) New indeterminate lesion on conventional imaging with unclear progression[13].

In addition to its primary use in GEP-NETs, $^{68}$Ga DOTATATE PET has been used to image other SSTR positive tumors such as paragangliomas, pheochromocytomas, neuroblastomas, meningiomas, medullary thyroid cancers, Merkel cell carcinomas, small cell carcinomas, esthesioneuroblastomas, and tumor-induced oncogenic osteomalacia[8].

**Technique**

$^{68}$Ga DOTATATE is readily compounded from generator eluted $^{68}$Ga and a sterile vial kit[15]. Before intravenous administration, there is little patient preparation except for good hydration and frequent voiding to minimize radiation dose to the kidneys. An uptake phase between radiotracer administration and PET imaging is typically approximately 60 min, similar to $^{18}$F fluorodeoxyglucose (FDG) PET. This allows time for tumor uptake and for background washout clearance via the kidneys, which is analogous to the procedure used in FDG PET. Patients are typically imaged from the mid-thigh to the skull. In cases where tumor is known or suspected outside of this field of view, or in cases of unknown primary, longer imaging times may be needed to include the extremities. Intravenous contrast is not essential for accurate interpretation in most cases, but it can be helpful in some clinical settings. Additional procedural details are listed in the European Association of Nuclear Medicine guidelines[14].

Many patients with GEP-NETs will be on short- or long-acting somatostatin analogue (SSA) therapy which could interfere with $^{68}$Ga DOTATATE PET uptake due to competitive binding. Temporary discontinuation of short-acting SSAs is recommended for 24-48 h, and long-acting SSAs should be avoided for approximately 4-6 wk[8,16]. Long-acting release (LAR) SSAs are administered on 4 wk cycles, allowing $^{68}$Ga DOTATATE imaging to be scheduled for the end of the monthly cycle prior to redosing. Regardless of the specific approach, subsequent $^{68}$Ga DOTATATE scans are preferably performed with the exact timing between SSA injections.

**Normal biodistribution**

The normal biodistribution of $^{68}$Ga DOTATATE differs in several aspects compared to the most commonly used radiotracer, $^{18}$F FDG. For both $^{68}$Ga DOTATATE and $^{18}$F FDG, the genitourinary tract is the normal route of excretion for unbound radiotracer, and thus high radiotracer activity can be seen in the kidneys, ureters, and bladder. In $^{68}$Ga DOTATATE, the spleen has the highest normal activity, followed by the adrenal glands[17]. Unlike FDG, the pituitary is the only part of the central nervous system with high physiologic uptake. Salivary glands and the thyroid can have moderate uptake. Activity in the pancreas is variable and occasionally focal in the head and uncinate process[18]. Variable but typically lower-level diffuse uptake is seen in the small and large bowel[8]. Physiologic uptake in the liver can be quite variable[19], and uptake can be low in the spleen and liver if total body tumor burden is high (“sink effect”)[20]. $^{68}$Ga DOTATATE uptake is otherwise low throughout the muscles, adipose tissue, and bone.

**Dosimetry**

The recommended dose for $^{68}$Ga DOTATATE is 2 MBq/kg of body weight (0.054 mCi/kg) up to 200 MBq (5.4 mCi). The organ with the highest dose delivered is the spleen due to its high uptake. The whole body radiation effective dose equivalent (EDE) from $^{68}$Ga DOTATATE PET is 2-3 mSv[21,22], which is approximately only 10% of the 26 mSv received from a typical dose (222 MBq or 6 mCi) of $^{111}$In pentetreotide. Despite the addition of an EDE of 1-9 mSv from the CT component, which is needed for attenuation correction and anatomic localization, the total dose of $^{68}$Ga DOTATATE PET/CT (3-12 mSv) remains significantly lower than that of $^{111}$In pentetreotide.

**Imaging performance**

Due to the very high affinity of $^{68}$Ga DOTATATE for SSTR-2, target lesions typically show extremely
high uptake. With deficient (i.e., very low) background radiotracer uptake throughout most of the body including the thorax, head and neck, bone marrow, muscle, and brain (except the pituitary), the tumor to background ratio can be exceptionally high. In a large prospective study of GEP-NETs comparing accuracy of $^{68}$Ga DOTATE, conventional imaging (CT and magnetic resonance imaging (MRI)), and $^{111}$In pentetreotide, $^{68}$Ga DOTATE showed clear superiority in lesion detectability with mean tumor SUVmax values of over 65 [23]. Figure 1 shows an example of normal intense uptake in the spleen and lymph nodes and moderate $^{68}$Ga DOTATE uptake in the liver. Small liver lesions can also be visualized despite relatively high liver background activity. Extremely high radiotracer activity can be seen in larger lesions, as shown in Figure 2.

False positives are uncommon and do not typically present a diagnostic dilemma. The most common causes of false positives are inflammation or infection due to leukocytes and macrophages expressing SSTR-2[24]. For example, inflammatory prostatitis is relatively common and can show intense focal uptake within the prostate[25]. Other potential causes of false positive can arise from osteoblastic activity[26], such as in degenerative changes, fractures, fibrous dysplasia, vertebral hemangiomas, and epiphyses in pediatric patients[27,28].

False negative results may be due to loss of receptor expression. GEP-NETs that dedifferentiate and subsequently lose SSTR-expression may show lower $^{68}$Ga DOTATE avidity. Another cause of false negatives, as seen in PET imaging, may arise from small lesions below PET resolution. In our experience, however, the highly avid $^{68}$Ga DOTATE uptake in well differentiated GEP-NETs is commonly adequate to overcome the limitations of partial volume effect. If tumor uptake is sufficiently high, it can also overcome the detrimental effects of a relatively low administered dose and other physical limitations of $^{68}$Ga compared to $^{18}$F[29-32]. Small lesions can be readily visualized in phantom and patient clinical studies if the background activity is low. The non-contrast portion of the CT frequently does not reveal hepatic lesions; however, hepatic GEP-NETs as small 5 mm may be visualized despite high normal background liver activity[30].

**Role in evaluation and value in management**

$^{68}$Ga DOTATE PET is an excellent study for evaluating GEP-NETs and demonstrates very high sensitivity and specificity of 93% and 95%, respectively[33]. Its superior performance in detecting GEP-NETs compared to $^{111}$In pentetreotide and CT or MRI is well established[23]. This can result in a significant change in clinical management in up to a third of patients even when compared to $^{68}$Ga DOTATE uptake in well differentiated GEP-NETs[34-36]. $^{68}$Ga DOTATE has also shown increasing utility in detecting lesions not seen on other modalities. It can confirm suspicion of GEP-NET found on other modalities, determine the true extent of tumor, stage disease, identify otherwise occult tumors such as thoracic ACTH-secreting carcinoids, and guide treatment options. It may be used to confirm a clinical or biochemical suspicion of GEP-NET, identify the primary lesion in known metastatic disease, determine resectability, exclude other greater extent of disease prior to resection, and importantly, identify candidates for hormonal therapy or PRRT[8].

$^{64}$Cu DOTATATE

The primary practical advantage of $^{64}$Cu DOTATATE is its longer physical half-life ($^{64}$Cu $t_{1/2} = 12.7$ h). This allows for more flexibility in production, shipment to distant locations, and flexibility in the timing of PET imaging. $^{64}$Cu DOTATATE ($^{64}$Cu $t_{1/2} = 68$ minutes) is typically produced at a site close to the PET imaging center due to the short physical half-life. $^{64}$Cu DOTATATE allows flexibility for delayed imaging three hours after injection with no significant difference in lesion detectability[37]. Compared to the DOTATOC ligand, the $^{64}$Cu DOTATATE showed slightly higher lesion detectability than $^{68}$Ga DOTATOC[38]. Other differences between $^{64}$Cu compared to $^{68}$Ga do not appear to have a large clinical impact on lesion detectability. $^{64}$Cu compared to $^{68}$Ga has a disadvantage in a lower percentage of positrons emitted per decay (17.5% vs 88.9%). However, $^{68}$Cu has the physical advantage of shorter mean positron range prior to annihilation (0.7 mm vs 3.5 mm) which can improve spatial resolution[39]. Data are emerging to support quantitative imaging of $^{64}$Cu DOTATATE which correlates with prognosis[40, 41]. Finally, pre-clinical studies of new investigational agents, such as $^{64}$Cu DOTATATE, show the potential for further improvements in tumor uptake and image contrast when compared to currently approved agents[42].

**Role of FDG-PET**

FDG is a glucose analogue that enters cells through glucose transporters, undergoes phosphorylation, and then remains trapped within the cell as FDG-6-phosphate. This imaging metric of glycolysis is frequently upregulated in many cancers and generally associated with more aggressive, rapidly growing malignancies.

Well differentiated GEP-NETs, however, are typically slower growing, have lower mitotic rates, and have lower rates of proliferation including lower Ki-67 indices. The WHO classification of GEP-NETs relies on the Ki-67 index which reflects cellular proliferation. As GEP-NETs become more dedifferentiated, they tend to lose SSTR expression and decrease in $^{68}$Ga DOTATE avidity. These often exhibit a more aggressive and faster growing phenotype with increasing proliferation (Ki-67) and increasing
Figure 1 \(^{68}\)Ga DOTATATE positron emission tomography/computed tomography transaxial fusion and whole body projection positron emission tomography. A: A normal patient; B: A different patient with well-differentiated pancreatic neuroendocrine tumor with nodal, bone, and liver metastases. Small liver metastases can be seen SUVmax = 10.4 (arrow).

Figure 2 70 year old female with abdominal mass (arrow) showing intense \(^{68}\)Ga DOTATATE uptake. Pancreatectoduodenectomy showed a 4 cm well-differentiated (Ki-67 = 5\%) pancreatic neuroendocrine tumor. A: \(^{68}\)Ga DOTATATE positron emission tomography (PET) lesion SUVmax = 118; B: Computed tomography (CT); C: PET/CT fusion.

FDG PET may also be useful in identifying heterogeneity of tumors by directing biopsy of tumors suspicious for a more aggressive or higher-grade histology. When both \(^{68}\)Ga DOTATATE PET-CT and FDG PET-CT are performed, the differential imaging features may assist in prognosis and guide therapy options. FDG PET can therefore be complementary and aid in management decisions in which tumor dedifferentiation is suspected. An example of an FDG positive hepatic metastasis is shown in Figure 3.
Figure 3 73 year old female with well-differentiated neuroendocrine tumor (Ki-67 = 6%) and liver metastases with an unknown primary. A: \(^{68}\)Ga DOTATATE positron emission tomography (PET)/computed tomography (CT) fusion and whole-body PET projection confirms multifocal hepatic metastases SUV\textsubscript{max} 34.4 with an area of focal decreased DOTATATE avidity (arrow); B: Fluorodeoxyglucose (FDG) PET/CT fusion and whole-body PET shows mismatched focal intense increased FDG uptake SUV\textsubscript{max} 9.1 (arrow), suggestive of heterogeneous tumor phenotype with areas of dedifferentiation.

**THERAPY**

**Treatment options**

Partial or complete surgical resection of GEP-NET is the preferred approach when possible\[^{46}\]. Hormone therapy is another mainstay of GEP-NET treatment. Short- or long-acting SSAs bind to SSRTs and inhibit or slow tumor growth while simultaneously helping with hormone secretion related symptoms. Additional treatment options may include mTOR inhibitors, VEGF inhibitors, chemotherapy, radiation, and liver metastases directed embolization therapies\[^{47}\]. More recently, \(^{177}\)Lu DOTATATE PRRT has been established as a safe and effective treatment of metastatic GEP-NETs.

**PRRT**

PRRT is the logical extension of SSTR imaging into the treatment realm and comprises the therapy component of theranostics. The imaging radionuclide (\(^{68}\)Ga or \(^{64}\)Cu) is replaced with a beta emitter, \(^{177}\)Lu, which deposits lethal radiation precisely to the SSTR-2 positive cells, providing targeted radiotherapy to tumors. The resultant \(^{177}\)Lu DOTATATE radionuclide delivers local radiation specifically to tumor visualized on \(^{68}\)Ga DOTATATE imaging. \(^{177}\)Lu is primarily a beta emitter with a mean range of 2 mm in tissue, and a small fraction is gamma radiation (6.6\% at 113-keV and 11\% at 208-keV). This results in a relatively low exposure to individuals surrounding the patient, allowing therapies to be performed as an outpatient. The relatively long half-life of 6.7 d (160 h) delivers sustained radiotherapy for a prolonged period; however, this requires extended precautions to avoid exposure from urinary contamination. \(^{177}\)Lu DOTATATE (Lutathera) was approved by the EMA in 2017 and by the FDA in January 2018\[^{47}\].

**Patient selection**

\(^{177}\)Lu DOTATATE was approved specifically for treatment of SSTR-positive GEP-NETs that have progressed on SSA therapy\[^{47}\]. The most appropriate patients for therapy are based upon guidelines developed by the NETTER-1 trial\[^{48}\]. There are multiple considerations for patient selection for PRRT; however, patients with progressive metastatic low and intermediate grade GEP-NETs typically have highly positive SSTR scans and are most likely to benefit.

**Technique**

The standard protocol for \(^{177}\)Lu DOTATATE therapy is based upon the NETTER-1 trial\[^{48}\]. Patients are prescribed four doses of 7.4 GBq (200 mCi) eight weeks apart for a cumulative dose of 29.6 GBq (800 mCi). At least 30 min prior to therapy administration, an amino acid infusion is started for renal protection and lasts four hours. The two amino acids required for renal protection are arginine and lysine. Although other formulations of different amino acids exist, they do not provide any additional benefit and can cause significant nausea and vomiting.

Based on the typical dose of 7.4 GBq of \(^{177}\)Lu DOTATATE, the exposure rate at 1 m is 2 mR/h and decreases by 50\% within 24 h\[^{47}\]. If the patient is able to abide by standard radiation safety precautions, this can be performed as an outpatient. Precautions include bathroom hygiene, similar to radioiodine treatments, and appropriate distancing from others, specifically children and pregnant women, for approximately 3 d after therapy. Individualized safety instructions may be prepared by a radiation safety officer or radiation physicist, depending on the institution, and reviewed with the patient during the consent process.
Efficacy

¹⁷⁷Lu DOTATATE therapy in GEP-NETs has demonstrated efficacy in many studies over several years. The most notable large prospective randomized trial is the NETTER-1 trial[48]. This prospective randomized trial in adults with biopsy-proven low- and intermediate grade (G1 or G2, i.e., Ki-67 level ≤ 20%) GEP-NETs evaluated subjects treated with ¹⁷⁷Lu DOTATATE and SSA compared to a control group on high dose SSA alone. Inclusion criteria were metastatic disease or locally advanced and inoperable disease which was progressing on SSA[48].

The primary endpoint of the study was progression free survival (PFS) with secondary endpoints of objective response rate (ORR), overall survival (OS), safety, and the side-effect profile. Patients were judged to have failed treatment if there was tumor progression based on follow up imaging by CT or MRI according to RECIST 1.1 criteria[49].

Patients in the treatment arm experienced significantly better PFS at 20 mo of 65.2% (95%CI: 50.0-76.8) compared to 10.8% (95%CI: 3.5-23.0) in the control group. In other words, in the treatment group there was a 79% lower risk of disease progression or death and a 60% lower risk of death alone. The secondary endpoint of ORR was 18% in the treatment group and 3% in the control group. As noted in the NETTER-1 study, multiple large randomized trials with other systemic therapies, such as SSAs alone or in combination with other non-radionuclides, showed response rates of only 5% or less[50-53]. Median overall survival could not be calculated yet at the conclusion of NETTER-1, but there was a trend towards longer overall survival in the treatment group. An example of a patient with partial response to ¹⁷⁷Lu DOTATATE is shown in Figure 4.

Adverse side effects and overall safety

In NETTER-1, transient WHO grade 3 and 4 hematologic toxicity (thrombocytopenia 2%; lymphopenia 9%; neutropenia 1%) and no renal toxicity were reported after 14 mo of follow up[48]. Rare but serious side effects including acute leukemia and myelodysplastic syndrome have been reported, occurring in < 1% and < 1%-2% of patients, respectively[47,48,54]. Other studies have similarly shown limited side effects with ¹⁷⁷Lu DOTATATE therapy[54,55].

Common mild side effects (Grade 1 or 2) include nausea (59%) and vomiting (47%)[48]; however, this has been primarily attributed to the specific amino acid infusion. Use of the simpler arginine and lysine infusion appears to have much lower incidence and severity of side effects. Fatigue (40%), decreased appetite (18%), headache (16%), and alopecia (11%) were significantly higher in the ¹⁷⁷Lu DOTATATE treatment group[48]. Although relatively frequent, abdominal pain (26%), and diarrhea (29%) were not statistically different compared to the control group. An uncommon side effect of treatment is hormone crisis, which in one study of 504 patients happened in only 6 (or 1.2%) and can be adequately managed in a brief hospital stay with complete recovery[54].

FUTURE DIRECTIONS

While ⁶⁸Ga- and ¹⁷⁷Lu DOTATATE have shown remarkable efficacy in imaging and treatment of GEP-NETs, many additional imaging and treatment options are currently under investigation. A comprehensive review is not possible in the context of the rapidly evolving landscape of theranostics. A few representative clinical trials are briefly mentioned to provide a perspective of the breadth of ongoing investigations.

Current clinical trials

Extending PRRT into higher grade GEP-NETs is an active area of investigation. The COMPOSE trial compares different-differentiated higher grade (G2 or G3, Ki-67 index between 10%-55%) GEP-NETs treated with ¹⁷⁷Lu Edotreotide (DOTATOC) compared to best standard of care chemotherapy regimens[56]. The COMPETE trial similarly evaluates advanced GEP-NETs for safety and efficacy of ¹⁷⁷Lu DOTATOC compared to Everolimus[57]. The NETTER-2 trial investigates higher proliferation index tumors (G2 or G3) as first line therapy with ¹⁷⁷Lu DOTATATE therapy and SSA compared to high dose SSR therapy alone[58].

¹⁷⁷Lu DOTATATE retreatment

While ¹⁷⁷Lu DOTATATE is now given as a four dose regimen, additional doses have been administered on an investigational basis. If a patient shows continued improvement in tumor burden and symptoms throughout the ¹⁷⁷Lu DOTATATE treatment course established by NETTER-1, (four cycles, 8 wk apart), they may benefit from continued treatment with additional doses of ¹⁷⁷Lu DOTATATE. A meta-analysis suggests that ¹⁷⁷Lu DOTATATE re-treatment in patients with advanced GEP-NETs is well tolerated with a safety profile similar to initial PRRT[59]. This provides an additional treatment strategy potentially to improve PFS, OS, and disease related survival.
Alternative PRRT agents

Either systemic or arterially-delivered PRRT with $^{90}$Y DOTATATE or -DOTATOC is an approach that is under further investigation. $^{90}$Y is beta emitter with a higher energy and longer mean free path in soft tissue than $^{177}$Lu. This theoretically favors treatment of larger tumors where high intratumor pressure limits blood flow and radiotracer delivery. However, a variety of factors mediate tumor killing including bystander effect\[60]. A major limiting drawback of $^{90}$Y is its greater toxic effects to surrounding tissues and bone marrow\[61]. Renal dose is also higher than $^{177}$Lu which poses a higher risk of nephrotoxicity\[62]. Arterially-administered $^{90}$Y DOTATATE is more focally directed but is operator intensive and requires a prolonged procedure typically performed in the interventional radiology suite.

Novel imaging and treatment agents

Antagonists: In contrast to the established SSTR agonists (e.g., DOTATATE), somatostatin antagonists are currently being investigated for imaging and therapy of GEP-NETs. Agonist-ligand complexes are internalized into the cell and entrapped, which is believed to generate higher contrast imaging and prolonged tumor targeted therapy. Antagonists were developed to evaluate the functions of receptors \[63] and are typically not internalized, but this may be overcome by binding to a higher number of receptor sites than agonists\[64]. The SSTR-2 antagonist JR11 has shown uptake in renal cell cancers, most breast cancers, non-Hodgkin lymphomas, and medullary thyroid cancers with binding comparable to NET targeting with SSTR-2 agonists\[65]. This study also showed that peritumoral vessels, lymphocytes, nerves, mucosa, and stroma were more strongly labeled with the antagonist than with the agonist. Antagonists, therefore, may show higher binding leading to improved detection and more avid tumor binding in targeted radiotherapy. $^{68}$Ga NODAGA-LM3, $^{68}$Ga DOTA-LM3, and $^{68}$Ga NODAGA-JR11 (OPS202) are three of the agents showing early promise\[66-68] with clinical trials underway\[69].

Similar to DOTATATE, therapeutic radionuclides can be attached to these antagonists for PRRT\[70,71].

Alpha emitters: PRRT for GEP-NETs is currently performed primarily by beta emitters ($^{177}$Lu and $^{90}$Y), but targeted alpha therapy (TAT) is potentially much more effective\[72,73]. Alpha particles travel a much shorter distance in tissue, typically on the order of only a few cell diameters, and deliver a dramatically higher damaging radiation effect to cells compared to beta emitters. In contrast to beta emission, which results primarily in single breaks in DNA, highly energetic alpha particles result in clusters of double stranded DNA breaks which are irreparable and highly lethal\[72]. Alpha particles also generate more ionization events and an immunogenic cell death which could generate an immunostimulatory environment and promote an abscopal effect\[73]. The dual effect of higher tumor cell death and limited radiation to non-target tissues increases the lethality to tumor cells and decreases the off-target adverse side effects.

The primary systemic alpha emitter under investigation is $^{225}$Ac which can be stably bound to DOTATATE or DOTATOC\[73]. Early studies have shown promising results\[74,75] with avoidance of severe renal and hematologic toxicity\[76]. In the future, this could be given as an initial treatment strategy, in sequence with $^{177}$Lu DOTATATE, or as a salvage therapy for patients progressing on $^{177}$Lu PRRT.
Additional therapies or combination therapies
Beyond radionuclides, there are other drugs and regimens being developed for treatment of GEP-NETS. These therapies may provide additional benefits to PRRT, particularly if they can be used to sensitize tumors to PRRT or be sequenced in such a way to deliver synergistic lethality. Alternative methods of administration may also allow higher local dose PRRT via intra-arterial rather than systemic delivery.[77, 78]. PRRT could also be used in a neoadjuvant fashion prior to surgery to make some patients operative candidates and increase the chances of curative resection.[54]. The sorting out of the milieu of therapies, their timing, and indications will require ongoing research.

CONCLUSION
Currently, the most sensitive and accurate established method to image well-differentiated GEP-NETs is with DOTATATE PET imaging. Due to the favorable uptake properties and biodistribution, targeted PRRT with $^{177}$Lu DOTATATE has been established as best standard of care for patients with progressive, metastatic, or unresectable well-differentiated SSTR positive GEP-NETs. $^{177}$Lu DOTATATE is well tolerated with a very mild toxicity profile and rare serious adverse events. Ongoing investigations are continuing to expand in both imaging and therapy applications for DOTATATE and novel ligand theranostics for GEP-NETs.

FOOTNOTES
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Basic Study
Forkhead Box q1 promotes invasion and metastasis in colorectal cancer by activating the epidermal growth factor receptor pathway

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Abstract
BACKGROUND
Colorectal cancer (CRC) is an extremely malignant tumor with a high mortality rate. Little is known about the mechanism by which forkhead Box q1 (FOXQ1) causes CRC invasion and metastasis through the epidermal growth factor receptor (EGFR) pathway.

AIM
To illuminate the mechanism by which FOXQ1 promotes the invasion and metastasis of CRC by activating the heparin binding epidermal growth factor (HB-EGF)/EGFR pathway.

METHODS
We investigated the differential expression and prognosis of FOXQ1 and HB-EGF in CRC using the Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/index.html). Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were used to detect the expression of FOXQ1 and HB-EGF in cell lines and tissues, and we constructed a stable low-expressing FOXQ1 cell line and verified it with the above method. The expression...
changes of membrane-bound HB-EGF (proHB-EGF) and soluble HB-EGF (sHB-EGF) in the low-expressing FOXQ1 cell line were detected by flow cytometry and ELISA. Western blotting was used to detect changes in the expression levels of HB-EGF and EGFR pathway-related down-stream genes when exogenous recombinant human HB-EGF was added to FOXQ1 knockdown cells. Proliferation experiments, transwell migration experiments, and scratch experiments were carried out to determine the mechanism by which FOXQ1 activates the EGFR signaling pathway through HB-EGF, and then to evaluate the clinical relevance of FOXQ1 and HB-EGF.

RESULTS
GEPIA showed that the expression of FOXQ1 in CRC tissues was relatively high and was related to a lower overall survival rate. PCR array results showed that FOXQ1 is related to the HB-EGF and EGFR pathways. Knockdown of FOXQ1 suppressed the expression of HB-EGF, and led to a decrease in EGFR and its downstream genes AKT, RAF, KRAS expression levels. After knockdown of FOXQ1 in CRC cell lines, cell proliferation, migration and invasion were attenuated. Adding HB-EGF restored the migration and invasion ability of CRC, but not the cell proliferation ability. Kaplan–Meier survival analysis results showed that the combination of FOXQ1 and HB-EGF may serve to predict CRC survival.

CONCLUSION
Based on these collective data, we propose that FOXQ1 promotes the invasion and metastasis of CRC via the HB-EGF/EGFR pathway.

Key Words: Colorectal cancer; Forkhead Box Q1; Heparin binding epidermal growth factor; Epidermal growth factor receptor pathway; Migration; Invasion

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Core Tip: Invasion and metastasis play important roles in tumorigenesis, resulting in the death of most colorectal cancer (CRC) patients. Forkhead Box q1 (FOXQ1) is a well-established oncogene in multiple tumors, including CRC. Our previous study suggested that FOXQ1 positively regulates the expression of heparin-binding epidermal growth factor (HB-EGF) and triggers the activation of the epidermal growth factor receptor (EGFR) pathway in CRC. However, the role and mechanism of how FOXQ1 promotes tumorigenesis in CRC by activating the HB-EGF/EGFR pathway remain unexplored. In the present study, our findings demonstrated that the essential role of FOXQ1-induced invasion and metastasis in CRC was related to activation of the HB-EGF/EGFR pathway.

INTRODUCTION
Colorectal cancer (CRC) is the third most common cancer in the world and the fourth most common cause of cancer death in the world[1]. Among all cancerous processes involved, local invasion and metastasis are the main factors related to cancer. The metastatic dissemination of primary tumors is directly related to the survival rate of patients, accounting for approximately 90% of all colon cancer deaths[2]. The median survival of metastatic CRC is less than 2 years[3]. Therefore, clarifying the mechanism of invasion and metastasis is the key to improving the survival rate of patients with CRC.

Forkhead Box q1 (FOXQ1) is a member of the forkhead transcription factor family[4], and it promotes tumorigenesis by activating cell proliferation, invasion and apoptosis[5]. Kaneda et al[6] found that compared to adjacent tissues, FOXQ1 is overexpressed in CRC. Overexpression of FOXQ1 reduces cell proliferation but increases cell tumorigenicity and tumor growth, and it inhibits apoptosis and promotes angiogenesis, thereby promoting CRC tumorigenesis. Liu et al[7] also demonstrated that the expression of FOXQ1 in either CRC tissue samples or cancer cell lines is higher than that in normal colorectal tissues and cell lines, and they reported that FOXQ1 promotes cancer metastasis by regulating PI3K/AKT signaling. Weng et al[8] verified that FOXQ1 can be used as an independent indicator of the prognosis of CRC patients.
The epidermal growth factor receptor (EGFR) signaling pathway plays an important role in physiological processes, such as cell growth, proliferation, and differentiation. The expression level of EGFR gradually increases from normal mucosa, adenomas with low-grade dysplasia, and adenomas with high-grade dysplasia to CRC, confirming that EGFR plays an important role in CRC\(^9\). Heparin-binding epidermal growth factor (HB-EGF) is one of the seven major ligands of EGFR. HB-EGF was originally identified as a secreted product from human macrophage U937 cells, and it induces cell proliferation and differentiation\(^10,11\). Soluble HB-EGF (sHB-EGF) is the main stimulator of cell proliferation. The affinity of sHB-EGF binding to target cells to promote proliferation of the target cells and activation of EGFR tyrosine kinase activity is 20-40 times higher than that of EGF. Thus, sHB-EGF is the most effective EGFR signaling pathway activator\(^12,13\). Membrane-bound HB-EGF (proHB-EGF) is affected by a variety of proteins, and it can be shed into active sHB-EGF to promote cell proliferation\(^14-16\).

There is evidence that poor prognosis and low survival rates for CRC are associated with abnormally activated signaling pathways, including the EGFR signaling pathway\(^17\). In advanced CRC the most commonly used targeted therapies are the monoclonal antibodies cetuximab and panitumab, which block EGFR activation\(^18\). Activation of EGFR signaling leads to resistance to chemotherapy in CRC cells and promotes cell survival, while inhibition of EGFR signaling significantly reduces proliferation in CRC cells\(^19\). In nasopharyngeal carcinoma, Luo et al\(^20\) reported that FOXQ1 induces vasculoendothelial mimicry through the EGFR signaling pathway, thereby promoting the metastasis of nasopharyngeal carcinoma cells. Our previous study suggested that FOXQ1 positively regulates the expression of HB-EGF and triggers the activation of the EGFR pathway in CRC\(^21\). However, the role and mechanism of how FOXQ1 promotes tumorigenesis in CRC by activating the HB-EGF/EGFR pathway remain largely unknown. Therefore, we analyzed the correlation between FOXQ1 and the HB-EGF/EGFR pathway by constructing FOXQ1 knockdown cells and using tissue microarrays, cell function experiments, quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting. Our findings elucidated the critical role of the FOXQ1 and HB-EGF/EGFR pathways in CRC, providing theoretical support for the clinical application of targeted FOXQ1 in the treatment of CRC.

**MATERIALS AND METHODS**

**Cell cultures**

The human CRC cell lines, DLD1 and SW480, as well as the human embryonic kidney 293 (HEK293) cell line were purchased and authenticated from the Cell Bank of the Chinese Academy of Science in Shanghai, China. DLD1 cells were cultured in RPMI 1640. SW480 and HEK293 cells were cultured in DMEM. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cells were maintained in a 5% CO\(_2\)-humidified atmosphere at 37 °C.

**Plasmid construction and transfection**

Three siRNAs targeting the human FOXQ1 sequence (NM_033260.3) were designed using siRNA Target Finder (InvivoGen, San Diego, CA, United States), and one scrambled siRNA was designed as a negative control, refer to our previous research\(^21\). The pSPAX2 packaging system from Addgene was used to construct a lentiviral PLKO.1 vector (PLKO.1-puro-shFOXQ1). After each group of recombinant plasmids was transfected by sequential, lentiviral vectors and packing vectors (pRSV-rev, pMDlg-pRRE and pCMV-VSV-G) were cotransfected into HEK293 cells using Lipofectamine® 2000 transfection reagent (Life Technologies). Lentivirus was collected to infect DLD1 and SW480 cells. Stable cells were generated after selection with puromycin (Solarbio, Beijing, China) (0.4 ng/μL for DLD1 cells and 0.1 ng/μL for SW480 cells) for 7-14 d after infection. The most effective knockdown cells were designated DLD1-shFOXQ1 and SW480-shFOXQ1, and the corresponding controls were named DLD1-shControl and SW480-shControl, respectively.

**Flow cytometry**

Cell surface HB-EGF was detected using APC-conjugated anti-human HB-EGF (eBioscience, San Diego, CA, United States). APC-conjugated mouse IgG2ak isotype was used as a control (eBioscience, San Diego, CA, United States) according to the manufacturer’s directions. Briefly, CRC cells were harvested and blocked with blocking buffer (PBS containing 2% BSA) for 10 min at 4 °C and then stained with 5 μL of monoclonal HB-EGF antibody (eBioscience, San Diego, CA, United States) for 30 min at 4 °C. After two washes, cells were resuspended in 100 μL of PBS. Samples were analyzed using a MoFlo flow cytometer (Beckman Coulter) and FlowJo software (Becton, Dickinson and Company).

**Western blotting**

Protein extracts were isolated from treated cells using mammalian cell lysis reagent containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States) and phosphatase inhibitors (Roche, United States) following the manufacturer’s directions. Equal amounts of protein (30 μg) were resolved on a 10% sodium dodecyl sulfate (SDS)-precast polyacrylamide gel (Bio–Rad Laboratories) and...
Table 1 Oligonucleotide sequences used for real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXQ1</td>
<td>TGACTTCAACAGCGACACCCA</td>
<td>CACCCTGTTGCTGTAGCAA</td>
</tr>
<tr>
<td>EGFR</td>
<td>AGACGCAGATAGTCGCCAAAG</td>
<td>TCCATCAGGGCACGGTAGAAG</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>CCAATGTTTCGAAATACAGGA</td>
<td>CACCCTGTTGCTGTAGCAA</td>
</tr>
<tr>
<td>Akt</td>
<td>AGACGCAGATAGTCGCCAAAG</td>
<td>TCCATCAGGGCACGGTAGAAG</td>
</tr>
<tr>
<td>RAF1</td>
<td>AGACGCAGATAGTCGCCAAAG</td>
<td>TCCATCAGGGCACGGTAGAAG</td>
</tr>
<tr>
<td>KRAS</td>
<td>GTGACGAAATATGGCAAAATAG</td>
<td>TCCATCAGGGCACGGTAGAAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCAACCGGCTACGCTTTA</td>
<td>GCCACCCCCACATACATAATCAA</td>
</tr>
</tbody>
</table>

Transferred to an Immobilon-polyvinylidene difluoride membrane (Millipore, Billerica, MA, United States). The membranes were blocked and incubated with the following primary antibodies: FOXQ1 (Abcam, Cambridge, MA, United States), HB-EGF (Abcam, Cambridge, MA, United States), phosphor-PI3K (Cell Signaling Technology, Cold Spring Harbor, NY, United States), PI3K (Proteintech, Wuhan, China), Akt (Proteintech, Wuhan, China), phosphor-Akt (Proteintech, Wuhan, China), phosphor-MAPK (Cell Signaling Technology, Cold Spring Harbor, NY, United States), MAPK (Proteintech, Wuhan, China), EGFR (Proteintech, Wuhan, China), KRAS (Proteintech, Wuhan, China), RAF (Proteintech, Wuhan, China), E-cadherin, N-cadherin, vimentin and β-actin (Proteintech, Wuhan, China). Blots were then incubated with the appropriate peroxidase-conjugated secondary antibody as follows: HRP-Rb-anti-goat (Cell Signaling Technology, Cold Spring Harbor, NY, United States) or HRP-goat-anti-mouse (Proteintech, Wuhan, China), respectively. The proteins were detected using an ECL system (Millipore, Braunschweig, Germany) and visualized with a ChemiDoc XRS system (Bio-Rad, Hercules, CA, United States).

**RNA isolation and qRT–PCR**

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer’s protocol. Total RNA (1 μg) was reverse transcribed into cDNA using PrimeScript RT reagent (Takara, Japan), and qRT–PCR was performed using a LightCycler 480 (Roche, United States) with SYBR Premix Ex Taq (Takara, Japan). Each sample was analyzed in triplicate, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference. Quantitative results were calculated using the 2^(-∆∆CT) method. Primers used for qRT–PCR were designed and synthesized by Takara (Dalian, China) (Table 1).

**Epidermal growth factor/platelet-derived growth factor pathway cDNA array assay**

The human epidermal growth factor (EGF)/platelet-derived growth factor (PDGF) Signaling RT² Profiler™ PCR Array (SABiosciences), which profiles the expression of 84 genes related to EGF/PDGF-mediated signal transduction, five housekeeping genes and three controls, was used to analyze the effect of FOXQ1 on EGF/PDGF signaling-related gene expression (Table 2). Total RNA was extracted with TRIzol reagent according to the manufacturer’s manual. DNase treatment was performed by amplification grade I DNase I (Sigma–Aldrich, St. Louis, MO, United States) according to the manufacturer’s instructions. Each total RNA preparation (5 μg) was digested with 1 μL of DNase I (1 unit/μL) and 1 μL of 10 reaction buffer in a volume of 10 μL. After incubation and addition of Stop Solution, DNase I was denatured by incubation at 70 °C for 10 min. The RNA samples were kept on ice for another 5 min and then converted into cDNA with the RT² PCR Array First Strand Kit (SuperArray) according to the manufacturer’s protocol. cDNA (20 ng) was combined with RT² SYBR Green/Fluorescein PCR master mix (SuperArray), and equal amounts of this mixture (25 μL) were added to each well of the RT² Profiler PCR plate containing the predispensed gene-specific primer sets. PCR cycles were performed according to the manufacturer’s instructions. The relative level of mRNA expression for each gene in each sample was first normalized to the expression of GAPDH in that sample and then normalized to the level of mRNA expression in the DLD1-shControl.

**ELISA**

Exogenous recombinant HB-EGF protein at a final concentration of 50 ng/mL was added to the cell culture medium when the cell density reached 80%, and the culture medium was changed after incubation for 24 h [22]. The supernatant was collected after the cells were cultured for another 24 h. The protein concentrations of soluble HB-EGF, ADAM9, ADAM10, ADAM12 and MMP-7 in the cell culture medium were determined by ELISA detection kits against human HB-EGF, ADAM9, ADAM10, ADAM12 and MMP-7, respectively (R&D Systems, Minneapolis, United States).
Table 2 List of up- and down-regulated genes between DLD1-shFOXQ1 and DLD1-shControl with a fold-change ≥ 2.0 or ≤ -2.0 by using epidermal growth factor/platelet-derived growth factor SuperArray

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene information</th>
<th>Fold change (shFOXQ1/shControl)</th>
<th>P value</th>
<th>FOXQ1 binding sites (TSS: -2k_+1k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAT</td>
<td>Plasminogen activator, tissue</td>
<td>-8.01</td>
<td>0.0375</td>
<td>3</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Collagen, type I, alpha 1</td>
<td>-5.72</td>
<td>0.0138</td>
<td>1</td>
</tr>
<tr>
<td>ELK1</td>
<td>ELK1, member of ETS oncogene family</td>
<td>-5.45</td>
<td>0.0228</td>
<td>1</td>
</tr>
<tr>
<td>BCAR1</td>
<td>Breast cancer anti-estrogen resistance 1</td>
<td>-3.97</td>
<td>0.0479</td>
<td>2</td>
</tr>
<tr>
<td>PIK3R2</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 2</td>
<td>-3.63</td>
<td>0.0197</td>
<td>3</td>
</tr>
<tr>
<td>NCK2</td>
<td>NCK adaptor protein 2</td>
<td>-3.51</td>
<td>0.0237</td>
<td>6</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene v-jun</td>
<td>-3.43</td>
<td>0.0077</td>
<td>12</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>-3.36</td>
<td>0.0166</td>
<td>11</td>
</tr>
<tr>
<td>LTA</td>
<td>Lymphotoxin alpha (TNF superfamily, member 1)</td>
<td>-3.18</td>
<td>0.0796</td>
<td>3</td>
</tr>
<tr>
<td>PDGFB</td>
<td>Platelet-derived growth factor beta</td>
<td>-2.91</td>
<td>0.1896</td>
<td>5</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3 (acute-phase response factor)</td>
<td>-2.49</td>
<td>0.0038</td>
<td>6</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF-like growth factor</td>
<td>-2.19</td>
<td>0.0278</td>
<td>3</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
<td>-2.10</td>
<td>0.0005</td>
<td>6</td>
</tr>
<tr>
<td>FASLG</td>
<td>Fas ligand (TNF superfamily, member 6)</td>
<td>-2.08</td>
<td>0.2026</td>
<td>17</td>
</tr>
<tr>
<td>HRAS</td>
<td>V-Ha-ras Harvey rat sarcoma viral oncogene homolog</td>
<td>-2.06</td>
<td>0.0058</td>
<td>2</td>
</tr>
<tr>
<td>MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>-2.02</td>
<td>0.0243</td>
<td>5</td>
</tr>
<tr>
<td>AKT3</td>
<td>V-akt murine thymoma viral oncogene homolog 3</td>
<td>-2.02</td>
<td>0.1440</td>
<td>11</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
<td>-2.01</td>
<td>0.0470</td>
<td>5</td>
</tr>
</tbody>
</table>

**Cell proliferation**

DLD1-shFOXQ1 and SW480-shFOXQ1 cells (2,000 cells/well in a 96-well plate) were incubated with medium containing 10% FBS at 37 °C for 24, 48, 72 and 96 h. At the end of incubation, 10 μL of Cell Counting Kit-8 (CCK-8) solution (Beyotime Biotech, Shanghai, China) was added to each well with 100 μL of medium and incubated for another 4 h at 37 °C, and the OD at 450 nm was measured by a microplate reader (BioTek, Winooski, VT, United States). The effect of siRNA FOXQ1 on CRC cell viability was assessed as the percent of cell viability compared to vehicle-treated control cells, which were arbitrarily assigned as 100% viability.

**Cell migration and wound-healing assay**

Cell migration was analyzed using Transwell inserts with 8.0 μm membrane pores (BD, San Jose, CA, United States) according to the manufacturer’s protocol. Migration was additionally evaluated with the wound-healing assay. Briefly, DLD1-shFOXQ1/SW480-shFOXQ1 and DLD1-shControl/SW480-shControl cells were seeded in 6-well plates at a density that enabled a confluency of 80% to be attained 24 h after plating. A 10 μL filter tip was used to gently scratch the cell monolayer across the center of the well. Cells were then gently washed twice with PBS to remove the dislodged cells, and fresh medium was added. The first images of the scratch area were then acquired. Cells were cultured in serum-free medium for another 48 h, and a second set of images was then acquired to determine the extent of wound closure.

**CRC tissue microarray**

Tissue microarrays containing a total of 90 pairs of colorectal tumor tissues and matched adjacent normal tissues, together with pathological staging data in accordance with TNM classification of the American Joint Committee on Cancer (2010) and follow-up survival time after surgery, were obtained from Shanghai Biochip Co. Ltd., Shanghai, China (HCol-Ade180Sur-06). FOXQ1 (ab51340) and HB-EGF (ab192545) antibodies were purchased from Abcam (Cambridge, MA, United States). Tissue microarray analysis was performed using a standard immunohistochemistry (IHC) protocol. The median value of the immunoreactivity score (IRS) was selected as the cutoff for high and low protein expression levels based on a measure of heterogeneity according to the log-rank test with respect to disease-specific survival (DSS) as described previously. Cutoff values for the scoring system were assigned as follows:
### Table 3 Correlation between heparin binding epidermal growth factor expression and clinicopathological characteristics of colorectal cancers in cohort of human colorectal cancer tissues

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>Cohort tumor HB-EGF expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n = 26)</td>
<td>Positive (n = 39)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>65.72 (10.37)</td>
<td>69.43 (7.98)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Size of tumor (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 10</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>21</td>
<td>32</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Present</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>III-IV</td>
<td>14</td>
<td>15</td>
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<tr>
<td>TNM stage</td>
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<td></td>
</tr>
<tr>
<td>I-II</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>III-IV</td>
<td>15</td>
<td>31</td>
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<td>AJCC clinical stage according to 7th issue</td>
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<td></td>
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<tr>
<td>1 and 2A</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>3 and 3B</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>


### Table 4 Correlation analysis of forkhead Box q1 and heparin binding epidermal growth factor expression in cohort (n = 65) colorectal cancer tissues

<table>
<thead>
<tr>
<th>FOXQ1, negative (n = 31)</th>
<th>FOXQ1, positive (n = 34)</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB-EGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 26)</td>
<td>20</td>
<td>6</td>
<td>4.116</td>
</tr>
<tr>
<td>Positive (n = 39)</td>
<td>11</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

FOXQ1: Forkhead Box q1; HB-EGF: Heparin binding epidermal growth factor.

High expression of FOXQ1 and HB-EGF were defined as an IRS of ≥ 4 (4, 6, 8, 9 and 12); and low expression was defined as an IRS of < 4 (0, 1, 2 and 3)\[23\]. Immunostained sections were scanned using a microscope (Axiovert 200, Carl Zeiss, Gttingen, Germany). Data for 25 patients were excluded because the dots were off the chips during the experiment. In total, data for 65 patients with CRC were included in the final analysis (Tables 3 and 4).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 8 and SPSS v.19. An unpaired two-tailed Student’s t-test was performed for two-group comparisons, and one-way analysis of variance (ANOVA) was performed for multiple group comparisons. Survival curves were calculated using the Kaplan–Meier algorithm and log-rank test. P < 0.05 was considered to indicate a statistically significant difference.

### RESULTS

**Expression and prognosis of FOXQ1 and HB-EGF in CRC and normal colorectal tissues**

To determine the expression levels of FOXQ1 and HB-EGF in CRC, we investigated the expression and prognosis of FOXQ1 and HB-EGF in CRC in the Gene Expression Profiling Interactive Analysis (GEPIA) online database (Figure 1). The results showed that FOXQ1 was upregulated in CRC compared to normal samples according to GEPIA (Figure 1A). Increased expression of FOXQ1 was also associated with worse overall survival (Figure 1C). The expression of HB-EGF in CRC was not significantly different from that in normal colorectal tissues (Figure 1B), and its expression had no significant effect...
Zhang JJ et al. FOXQ1 promotes CRC invasion and metastasis

WJG https://www.wjgnet.com 1787 May 7, 2022 Volume 28 Issue 17

Figure 1 Expression and prognosis of forkhead Box q1 and heparin binding epidermal growth factor in colorectal cancer and normal colorectal tissues. A and B: Forkhead Box q1 (FOXQ1) (left) and heparin binding epidermal growth factor (HB-EGF) (right) expression was analyzed in human colorectal cancer (CRC) tissues and normal tissues using the Gene Expression Profiling Interactive Analysis online database. *P < 0.05; C and D: Overall survival rate of FOXQ1 (left) and HB-EGF (right) in CRC patients. FOXQ1: Forkhead Box q1; HB-EGF: Heparin binding epidermal growth factor; GEPIA: Gene Expression Profiling Interactive Analysis.

on the overall survival of patients with CRC (Figure 1D).

Construction of FOXQ1 knockdown CRC cell lines
To elucidate the functional roles of FOXQ1 in CRC, we generated CRC lines with stable FOXQ1 knockdown. Among the CRC lines we tested, DLD1 and SW480 cells had relatively high endogenous FOXQ1 expression as described previously [21]. Because the high expression of FOXQ1 in these two cell lines has also been confirmed in several other independent studies [3,4,8,9,24], we selected these two cell lines for knockdown studies. qRT–PCR assays verified significant FOXQ1 knockdown in DLD1-shFOXQ1 and SW480-shFOXQ1 cells. FOXQ1 mRNA expression in DLD1-shFOXQ1 and SW480-shFOXQ1 cells was significantly reduced compared to that in the control DLD1-shControl and SW480-shControl cells (Figure 2A). Western blot analysis also verified that the expression of FOXQ1 was significantly downregulated (Figure 2B).

Knockdown of FOXQ1 suppresses the expression of HB-EGF and blocks the EGF/PDGF signaling pathway in vitro
A panel of PCR arrays consisting of 84 representative genes related to the EGF/PDGF signaling pathways was used to detect the transcriptional signatures of DLD1-shFOXQ1 and DLD1-shControl cells. Differentially expressed genes with statistical significance were identified by volcano plot filtering. The results showed that 18 genes had expression changes with a fold-change ≥ 2.0 (P < 0.05) (Figure 2C). Among these 18 genes, 12 genes were associated with EGFR signaling pathways, including HB-EGF (FC = -2.19), and there were several novel genes of the three main EGF/PDGF downstream signaling
Zhang JJ et al. FOXQ1 promotes CRC invasion and metastasis

Figure 2 Forkhead Box q1 knockdown induces heparin binding epidermal growth factor suppression and epidermal growth factor/platelet-derived growth factor signaling pathway blockade in vitro according to transcriptional analysis of the generated forkhead Box q1 knockdown cell line. A: Quantitative real-time polymerase chain reaction was performed to evaluate mRNA expression in the knockdown of forkhead Box q1 in DLD1 cells (DLD1-shFOXQ1) and SW480-shFOXQ1 cells (two-tailed, unpaired Student’s t test). Bars represent the mean ± SE of three independent experiments; B: Western blotting was performed to evaluate FOXQ1 protein expression in DLD1-shFOXQ1 and SW480-shFOXQ1 cells; C: Volcano plot illustrating differentially expressed genes using the epidermal growth factor (EGF)/platelet-derived growth factor (PDGF) array to compare DLD1-shFOXQ1 and DLD1-shControl cells. The vertical blue points in the plot represent 84 genes related to the EGF/PDGF signaling pathways. The vertical green line indicates a fold-change in gene expression of 1. The vertical pink lines indicate the threshold for fold-change (≥ 2.0 and ≤ -2.0). The horizontal blue line indicates the threshold for the P value of the t test. *P < 0.05; **P < 0.01; ***P < 0.001. DLD1-shFOXQ1: The knockdown of forkhead Box q1 in DLD1 cells; SW480-shFOXQ1: The knockdown of forkhead Box q1 in SW480 cells.

Effect of FOXQ1 expression on HB-EGF expression and extracellular release ability in CRC cells

Western blot analysis confirmed that knockdown of FOXQ1 in CRC cells resulted in a significant decrease in the expression of HB-EGF in DLD1 cells (Figure 3A), but no significant changes in HB-EGF were observed in SW480 cells (Figure 3A). Flow cytometry analysis confirmed that knockdown of FOXQ1 expression in CRC cells did not affect the expression level of proHB-EGF (Figure 3B). ELISA results indicated that knockdown of FOXQ1 significantly reduced the shedding of soluble HB-EGF (sHB-EGF) in DLD1 cells but did not affect the shedding of HB-EGF in SW480 cells (Figure 3C). Among the four proteins that affect the release of the extracellular domain of proHB-EGF, three (ADAM9, ADAM12 and ADAM7) were decreased significantly in both DLD1-shFOXQ1 and SW480-shFOXQ1 cells, while ADAM10 was not changed. The feedback regulation of ADAM9, ADAM12 and MMP7 secretion by HB-EGF from CRC cells was studied by adding exogenous recombinant human HB-EGF (rhHB-EGF). The results showed that rhHB-EGF reversed the decline in the expression of ADAM9, ADAM12 and MMP7 in DLD-shFOXQ1 cells. However, only the decreased expression of ADAM9 was reversed by rhHB-EGF in SW480-shFOXQ1 cells (Figure 3D).

FOXQ1 regulates EGFR and downstream signaling pathways by regulating HB-EGF

To verify the role of FOXQ1 in activating the HB-EGF/EGFR signaling pathway, we performed qRT–PCR analysis in DLD1-shFOXQ1 and SW480-shFOXQ1 cells. The results confirmed that FOXQ1 knockdown resulted in a significant decrease in the mRNA expression of HB-EGF, EGFR and downstream genes (AKT, RAF and KRAS) in DLD1-shFOXQ1 and SW480-shFOXQ1 cells (Figure 4A and B). In addition, the Western blot analysis showed that knockdown of FOXQ1 resulted in a significant decrease in HB-EGF and EGFR expression as well as decreased AKT and MAPK pathways (MAPK/ERK1/2, PI3K/AKT and JAK/STAT3). These novel genes included RAS, PI3K and STAT3 as well as several intracellular transcription factors activated by these signaling pathways, such as c-JUN and c-FOS (Table 2).
Figure 3: Effect of forkhead Box q1 expression on heparin binding epidermal growth factor expression and extracellular release ability in colorectal cancer cells.

A: Quantitative analysis of western blots was performed to detect the expression of heparin binding epidermal growth factor (HB-EGF) with forkhead Box q1 (FOXQ1) inhibition; B: Flow cytometry was performed to detect the effect of changes in FOXQ1 expression on the expression of proHB-EGF in DLD1 and SW480 cells; C: ELISA verified the effect of FOXQ1 knockdown on sHB-EGF expression in DLD1 and SW480 cells; D: ELISA confirmed the effect of FOXQ1 on the expression of proHB-EGF extracellular release proteins (ADAM9, ADAM10, ADAM12 and MMP-7) in DLD1 and SW480 cells. *p < 0.05; **p < 0.01; ***p < 0.001. FOXQ1: Forkhead Box q1; HB-EGF: Heparin binding epidermal growth factor; ProHB-EGF: Membrane-bound HB-EGF; sHB-EGF: Soluble HB-EGF; ADAM9: A disintegrin and a metalloprotease 9; ADAM10: A disintegrin and a metalloprotease 10; ADAM12: A disintegrin and a metalloprotease 12; MMP7: Matrix metallopeptidase 7.
Figure 4 Forkhead Box q1 regulates epidermal growth factor receptor and downstream signaling pathways by regulating heparin binding epidermal growth factor. A: Quantitative real-time polymerase chain reaction (qRT–PCR) was performed to analyze the levels of forkhead Box q1 (FOXQ1), heparin binding epidermal growth factor (HB-EGF), epidermal growth factor receptor (EGFR), Akt, kRas and Raf in DLD1-shFOXQ1 and DLD1-shControl cells; B: qRT–PCR was performed to analyze the levels of FOXQ1, HB-EGF, EGFR, AKT, RAF and KRAS in SW480-shFOXQ1 and SW480-shControl cells; C: Western blot analyses were performed to analyze the protein levels of FOXQ1, HB-EGF, EGFR, RAF and KRAS as well as the phosphorylation levels of PI3K, Akt, and MAPK in
phosphorylation in DLD1-shFOXQ1 cells compared to DLD1-shControl cells. In SW480-shFOXQ1 cells, EGFR and HB-EGF expression was significantly decreased, and AKT and PI3K phosphorylation was inhibited compared to that in SW480-shControl cells (Figure 4C). Furthermore, Western blot analysis confirmed that the decreased expression of important downstream genes was rescued by rhHB-EGF protein in either DLD1-shFOXQ or SW480-shFOXQ1 cells (Figure 4D).

**rhHB-EGF reverses the FOXQ1 knockdown-induced suppression of CRC cell proliferation and migration in vitro**

CCK-8 results confirmed that FOXQ1 knockdown significantly inhibited the proliferation of DLD1-shFOXQ1 and SW480-shFOXQ1 cells, and the inhibitory effect was partially reversed by exogenous rhHB-EGF (Figure 5A). The results of the Transwell migration assay confirmed that FOXQ1 knockdown also reduced the migration of DLD1 and SW480 cells, and the inhibitory effect was also reversed to a large extent by rhHB-EGF (Figure 5B and C). Scratch experiment results confirmed that FOXQ1 knockdown reduced the wound-healing ability of DLD1 and SW480 cells, which was also reversed to a large extent by rhHB-EGF (Figure 5D and E). We next analyzed the protein expression during cell invasion and metastasis (E-cadherin, N-cadherin, Vimentin and Snail), Western blot analysis indicated that FOXQ1 knockdown reduced the expression in DLD1-shFOXQ1 and SW480-shFOXQ1 cells (Figure 5F).

**Prognostic value of the combination of FOXQ1 and HB-EGF**

To verify the clinical relevance of our findings, we evaluated the expression of FOXQ1 and HB-EGF in human CRC tissue biopsies (cohort, n = 65) (Tables 3 and 4). IHC analysis showed that FOXQ1 was significantly upregulated in CRC tissues compared to adjacent nontumorous tissues and that HB-EGF was moderately upregulated (Figure 6A). Although the overexpression of HB-EGF had no significant correlation with any clinicopathological characteristics of CRCs (Table 3), further analysis verified the positive correlation between FOXQ1 and HB-EGF (Table 4). In a cohort of 65 CRC patients, Kaplan–Meier survival analysis results showed that CRC patients with positive expression of FOXQ1 had shorter overall survival than those with negative expression of FOXQ1. Furthermore, Kaplan–Meier survival analysis of CRC patients with positive coexpression of FOXQ1 and HB-EGF had the shortest overall survival times compared to the corresponding single-negative or double-negative groups in the cohort of 65 CRC patients (Figure 6B).

**DISCUSSION**

Studies have indicated that FOXQ1 is an oncogene in multiple tumors, including CRC [24], breast cancer [25], lung cancer [26], gastric cancer [27], liver cancer [28], pancreatic cancer [29], ovarian cancer [30], and neuroglioma [31]. In CRC, FOXQ1 promotes tumor invasion and metastasis through the Wnt signaling pathway, and it affects the prognosis of patients [24]. EGFR is overexpressed in a variety of cancers, including CRC. EGFR overexpression and activation have a positive effect on the cell growth and metastasis of a variety of solid tumors, including CRC [17]. Metastasis is a multistep process in which tumor cells spread from the primary site to distant sites and form secondary tumors [32], and is the leading cause of death in CRC patients. Many downstream targets of HB-EGF and EGFR include MAPK, p-MAPK, RAS, RAF, PI3K, p-PI3K, AKT, p-AKT, and AKT protein kinases, leading to many processes related to tumor progression, including cell growth [33], epithelial-mesenchymal transition (EMT) [34], metastasis [35], and angiogenesis [36]. FOXQ1 can regulate FAK, PI3K, AKT, and many other key proteins in the PI3K/AKT signaling pathway and promotes the phosphorylation of the above proteins to maintain the activation of PI3K/AKT signaling [37]. FOXQ1 can also combine with VEGFR2 and VE-cadherin to promote angiogenesis and endothelial cell migration and rearrangement [24].

In our previous studies, we found that the mRNA and protein expression levels of FOXQ1 gradually increase with the pathological development of colorectal adenoma to colorectal adenocarcinoma, and the increased expression of FOXQ is not only involved in the process of colorectal adenoma carcinogenesis, but is also closely related to invasion and metastasis of CRC [38]. The differential expression of genes that are involved in the process of colorectal adenoma carcinogenesis screened by the gene chip has been analyzed by signal pathway analysis, suggesting that the abnormally high expression of FOXQ1 is closely related to the activation of the EGFR signaling pathway [39]. Studies have shown that the abnormal activation of the EGFR pathway plays an important role in malignant growth, invasion,
Zhang JJ et al. FOXQ1 promotes CRC invasion and metastasis
Zhang JJ et al. FOXQ1 promotes CRC invasion and metastasis

Figure 5 Recombinant human heparin binding epidermal growth factor reverses the forkhead Box q1-induced suppression of colorectal cancer cell proliferation and migration in vitro. A: A cell counting Kit-8 (CCK-8) assay was performed to detect the effects of recombinant human heparin binding epidermal growth factor (rhHB-EGF) on the proliferation of forkhead Box q1 (FOXQ1)-deficient DLD1 and SW480 cells; B: The Transwell migration assay was performed to evaluate the migration ability after knocking out FOXQ1 and after adding rhHB-EGF; C: A statistical analysis of the results of the Transwell migration assay was performed. *P < 0.01; D: After 0 h and 24 h, the scratch experiment evaluated the migration ability of DLD1 and SW480 cells with downregulated FOXQ1 and rhHB-EGF; E: Statistical analysis of the above scratch experiment results. *P < 0.05; *P < 0.01; *P < 0.001; F: Quantitative analysis of Western blot to detect the expression of E-cadherin, N-cadherin, vimentin and Snail protein levels in DLD-shFOXQ1 and SW480-shFOXQ1 cells compared to DLD1-shControl and SW480-shControl cells. β-actin was used as the loading control. CCK-8: Cell Counting Kit-8.

and metastasis of colorectal tumors. When sHB-EGF binds to EGFR, the tyrosine kinase activity of EGFR is activated, mainly by activating the MAPK/ERK1/2, PI3K/AKT, and JAK/STAT3 signaling pathways, leading to the proliferation, invasion, metastasis, and apoptosis of tumor cells[40]. The results of this study also proved that knockout of FOXQ1 caused a decrease in gene expression in these three signaling pathways.

Combined with the analysis in Figure 3B-D, these results suggested that FOXQ1 may regulate the EGFR pathway by promoting the separation of ProHB-EGF into sHB-EGF. AMAD7, AMAD9, and AMAD12 are also factors that affect the separation of ProHB-EGF[41]. In this study, decreased expression levels of AMAD7, AMAD9, and AMAD12 were also observed in DLD1-shFOXQ1 and SW480-shFOXQ1 cells, but this indirect regulation should be further verified in other CRC cell lines.

In this study, FOXQ1 was determined to be upregulated in CRC cell lines. The results showed that FOXQ1 knockdown inhibited the proliferation, migration and repair capabilities of CRC, which was consistent with our previous results[21,42]. When rhHB-EGF protein was added, the proliferation ability of the cells was not completely restored, but the migration and repair ability of CRC cells was partially restored. These results indicated that the effect of FOXQ1 in promoting the proliferation of CRC cells is not directly mediated by HB-EGF; however, the regulation of the invasion and metastasis of CRC cells by FOXQ1 is partly mediated by HB-EGF. HB-EGF is related to the abnormal proliferation of skin and mucosal cells, and high expression of HB-EGF is closely related to the occurrence and development of a variety of tumors; the expression of the HB-EGF gene is significantly increased in various human cancers and cancer-derived cell lines, indicating that HB-EGF plays an important role in tumor invasion and metastasis[43-46].

Studies have shown that FOXQ1 is related to the poor prognosis of CRC[47]. In this study, we conducted pathological and survival analyses on 65 CRC patients. These findings suggested that the expression of FOXQ1 and its coregulatory protein HB-EGF may have a prognostic correlation with CRC. Thus, FOXQ1 may serve as a therapeutic target for CRC treatment by blocking the HB-EGF/EGFR pathway. Our research suggests that FOXQ1 activates the EGFR signaling pathway by regulating the expression of HB-EGF, thereby affecting the invasion and metastasis of CRC. Regarding the limitations
of this study, it is necessary to construct a FOXQ1 high-expressing cell line and combine it with the dual luciferase reporter gene system to further verify whether FOXQ1 is directly involved in the transcriptional regulation of HB-EGF. Next, we will further explore the regulation of FOXQ1 on EGFR and its downstream signaling pathways through in vivo and in vitro studies. We will conduct a more comprehensive study on the role of HB-EGF in the invasion and metastasis of CRC to provide more possibilities for the treatment of CRC.

**CONCLUSION**

In conclusion, we have demonstrated that decreased FOXQ1 expression was also associated with a lower ability to invade and metastasize in CRC. FOXQ1 promotes the invasion and metastasis of CRC by activating the HB-EGF/EGFR pathway. These data indicated that FOXQ1 and HB-EGF may be potential biomarkers to improve the accuracy of CRC diagnosis and treatment.

**ARTICLE HIGHLIGHTS**

**Research background**

Invasion and metastasis play important roles in tumorigenesis, resulting in the death of most colorectal cancer (CRC) patients. Forkhead Box q1 (FOXQ1) is a well-established oncogene in multiple tumors,
including CRC. However, the role and mechanism of how FOXQ1 promotes tumorigenesis in CRC by activating the heparin-binding epidermal growth factor (HB-EGF)/epidermal growth factor receptor (EGFR) pathway remain largely unknown.

**Research motivation**
Our study aims to elucidate the critical role of FOXQ1 and HB-EGF/EGFR pathways in CRC, and to provide theoretical support for the clinical application of targeted FOXQ1 in the treatment of CRC.

**Research objectives**
To determine the role of FOXQ1-induced invasion and metastasis, which are related to activating the HB-EGF/EGFR pathway, and to explore the mechanism by which FOXQ1 promotes tumorigenesis by activating the HB-EGF/EGFR pathway in CRC.

**Research methods**
We analyzed the correlation between FOXQ1 and the HB-EGF/EGFR pathway by constructing FOXQ1 knockdown cells, tissue microarray, cell function experiments, quantitative real-time polymerase chain reaction (qRT–PCR), flow cytometry, ELISA, western blot, and the Gene Expression Profiling Interactive Analysis (GEPIA) website.

**Research results**
GEPIA showed that the expression of FOXQ1 in CRC tissues was relatively high and was related to a lower overall survival rate. PCR array results showed that FOXQ1 is related to the HB-EGF and EGFR pathways. Knockdown of FOXQ1 suppressed the expression of HB-EGF and led to a decrease in EGFR and its downstream genes AKT, RAF, KRAS expression levels. After knockdown of FOXQ1 in CRC cell lines, cell proliferation, migration, and invasion were attenuated. Adding HB-EGF restored the migration and invasion ability of CRC, but the cell proliferation ability was not restored. Kaplan–Meier survival analysis results showed that the combination of FOXQ1 and HB-EGF may serve to predict CRC survival.

**Research conclusions**
FOXQ1 promotes the invasion and metastasis of CRC by activating the HB-EGF/EGFR pathway.

**Research perspectives**
In this study, our results indicated that FOXQ1 and HB-EGF may be potential biomarkers to improve the accuracy of CRC diagnosis and treatment.

**FOOTNOTES**

**Author contributions:** Zhang JJ and Cao CX contributed equally to this work; Tang H and Guo Q designed the project; Cao CX and Zhang JJ analyzed data and wrote the manuscript; Zhang JJ and Wan LL performed qRT-PCR, Elisa, and Western blotting; Zhang W and Wang JL carried out migration assay; Liu ZJ and Tang H analyzed colorectal tissue microarray and survival data; all authors participated in critical revision of the manuscript and approved the final manuscript.

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**Conflict-of-interest statement:** The authors declare no competing interests.

**Data sharing statement:** No additional data are available.

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Zhang JJ et al. FOXQ1 promotes CRC invasion and metastasis

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Basic Study

Sirtuin1 attenuates acute liver failure by reducing reactive oxygen species via hypoxia inducible factor 1α

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Abstract

BACKGROUND
The occurrence and development of acute liver failure (ALF) is closely related to a series of inflammatory reactions, such as the production of reactive oxygen species (ROS). Hypoxia inducible factor 1α (HIF-1α) is a key factor that regulates oxygen homeostasis and redox, and the stability of HIF-1α is related to the ROS level regulated by Sir2u1 (Sirt) family. The activation of Sirt1 will lead to a powerful antioxidant defense system and therapeutic effects in liver disease. However, little is known about the relationship between HIF-1α and Sirt1 in the process of ALF and the molecular mechanism.

AIM
To investigate whether HIF-1α may be a target of Sirt1 deacetylation and what the effects on ALF are.

METHODS
Mice were administrated lipopolysaccharide (LPS)/D-gal and exposed to hypoxic conditions as animal model, and resveratrol was used as an activator of Sirt1. The cellular model was established with L02 cells stimulated by LPS. N-acetyl-L-cysteine was used to remove ROS, and the expression of Sirt1 was inhibited by nicotinamide. Western blotting was used to detect Sirt1 and HIF-1α activity and related protein expression. The possible signaling pathways involved were analyzed by immunofluorescent staining, co-immunoprecipitation, dihydroethidium staining, and Western blotting.

RESULTS
Compared with mice stimulated with LPS alone, the expression of Sirt1 decreased, the level of HIF-1α acetylation increased in hypoxic mice, and the levels of carbonic anhydrase 9 and Bcl-2-adenovirus E1B interacting protein 3 increased significantly, which was regulated by HIF-1α, indicating an increase of HIF-1α activity. Under hypoxia, the down-regulation of Sirt1 activated and...
Acute liver failure (ALF) refers to a large number of necrosis of liver cells or severe liver damage caused by various reasons[1]. ALF is often accompanied by coagulation dysfunction and progressive multiple organ failure due to liver metabolism disorders and decreased immune function[2]. The occurrence and development of ALF is closely related to a series of inflammatory reactions, such as the release of inflammatory cytokines and the production of reactive oxygen species (ROS)[3].

Hypoxia-inducible factor (HIF)-1 consists of an oxygen-regulated subunit HIF-1α and a constitutive expression subunit HIF-1β. The activity and stability of the alpha subunit of HIF are regulated by its post-translational modifications such as acetylation[4]. Under hypoxic conditions, HIF-1α acts as a primary transcription factor to regulate hypoxia-related anti-inflammatory responses[5]. HIF-1α is a key factor that regulates oxygen homeostasis and redox and promotes effective adaptation to hypoxia[6]. During the development of liver diseases such as liver cancer, hypoxia is a common finding. Hypoxia promotes the stabilization of HIF-1α. HIF signal in innate immune cells and liver cancer cells is beneficial to the recruitment and maintenance of primordial tumorigenic immune cells and promotes immune evasion[7].

The monitoring of HIF-1α activity by members of the Sirtuin (Sirt) family has been a topic of interest in recent years[8-10]. According to reports, HIF-1α has been confirmed to be related to Sirt1, Sirt2, and Sirt3 in the Sirt family, and the stability of HIF-1α is related to the ROS level regulated by Sirt3 and the oxygen level regulated by Sirt6[11-14]. Sirt2 causes protein hydroxylation and ubiquitination by increasing the binding of HIF-1α to propylamine hydroxylase[8]. However, the regulation mechanism of Sirt1 on HIF-1α activity has always been a controversial topic.

Sirt1 in the sirtuin family is a nicotinamide adenine dinucleotide-dependent protein lysine deacetylase with diverse physiological functions such as anti-inflammation, neuronal signaling, DNA repair, and stress response. Sirt1 has been shown to be an important target for the treatment of various diseases[15,16], and its activation will lead to a powerful antioxidant defense system and therapeutic effects in liver ischemia reperfusion[17]. Studies have shown that Sirt1 regulates HIF-1α through the formation of physical complexes between proteins, and Sirt1 may have a negative regulatory effect on HIF-1α[18]. Sirt1 has also been reported to regulate HIF-1α actively by stabilizing the protein[19]. Whether Sirt1 is used as a negative regulator or a positive regulator of HIF-1α depends on the experimental conditions or experimental models remains to be further studied.

In this study, we examined the regulation of Sirt1 on HIF-1α activity in ALF and explored its possible molecular mechanisms.
MATERIALS AND METHODS

**Mice**
Male C57BL/6j wild-type mice aged 5-6 wk were purchased from Wuhan Biomedical Research Institute of Wuhan University. All mice were raised in the specific pathogen free animal facility of Renmin Hospital of Wuhan University with conditions of light-controlled, room temperature 25 °C, and humidity 55 ± 5%, and they were free to eat and drink. All animal operations were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, China (Approval No. WDRY2021-K016).

**Animal models**
The mice were randomly divided into six groups with 6 mice in each group: Saline control group; Hypoxia group; Lipopolysaccharide (LPS) group; Resveratrol group; and LPS + Hypoxia + Resveratrol group. Hypoxia group and Hypoxia + LPS group were cultured in COY Vinyl Anaerobic Chambers (COY, Grass Lake, MI, United States). To avoid pulmonary and cerebral edema caused by a rapid drop in oxygenation, the fraction of inspired oxygen (FIO$_2$ (1%/d) was gradually decreased from 21% normoxia (room-air oxygen) to 8% oxygen (severe hypoxia) over the course of 2 wk, followed by continual exposure to 8% oxygen for an additional 2 wk. On the 14th d after being exposed to 8% oxygen, Resveratrol (10 mg/kg; Sigma–Aldrich, St. Louis, MI, United States) was given intragastrically in Resveratrol group and LPS + Hypoxia + Resveratrol group while LPS (100 μg/kg; Sigma–Aldrich) was administrated by intraperitoneal injection combined with D-Gal (400 mg/kg) in LPS group and LPS + Hypoxia + Resveratrol group[21]. Twenty-four hours after LPS administration, animals were quickly euthanized with inhaled CO$_2$, followed by the collection of blood samples and liver tissues[21].

**Cell culture**
Human embryonic liver cell line L02 was purchased from China Center for Type Culture Collection (Wuhan, China). N-acetyl-L-cysteine (NAC) (Beyotime, Shanghai, China) (5 mmol/L)[22], nicotinamide (NAM) (Beyotime) (5 mmol/L)[23], GW6471 (Sigma–Aldrich) (3 μM)[24] or Compound C (Sigma–Aldrich) (10 μM)[25], which were dissolved in dimethyl sulfoxide (Sigma-Aldrich), were used to pretreat L02 cells for 1 h, followed by LPS (5 μg/mL)[26] treatment. Hypoxic conditions (1% O$_2$) were obtained using humidified variable aerobic workstation InVivo2 400 (Ruskinn, Pencoed, United Kingdom)[27]. For transient transfection, cells were transfected with 2 μg plasmid of pECE-flag-Sirt1 (Addgene, Cambridge, MA, United States) and pECE empty vector (Addgene).

**Biochemical analyses**
Blood samples were collected after mice were anesthetized. The level of malondialdehyde (Cat. No. GM1134), superoxide dismutase (Cat. No. GM1133), and glutathione peroxidase (Cat. No. GM1135) were determined with commercial kits (Servicebio, Wuhan, China), respectively, according to the manufacturer’s instructions.

**Histopathological examination**
The liver tissues were sliced completely and stained with hematoxylin-eosin. The pathological changes of liver tissue were observed and evaluated by light microscope (Olympus, Tokyo, Japan). The degree of liver damage in the ALF models were assessed by the liver histology score.

**Immunofluorescent staining**
Liver tissue sections were intact, and L02 cell suspensions were fixed on glass slides. Sections were fixed with 4% paraformaldehyde for 30 min, and 50-100 μL membrane rupture working solution and 3 % hydrogen peroxide solution were added in sequence according to the manufacturer’s instructions. Primary antibody against acetyl-lysine or HIF-1α (Santa Cruz Biotechnologies, Dallas, TX, United States) diluted 1:100 with 5% bovine serum albumin was added on the slides and tissue sections, and the slides were incubated overnight at 4 °C in a wet box. Then, slides were incubated with secondary antibody (1:50 dilution, Beyotime), and they were imaged using a fluorescent microscope (Olympus).

**Immunoprecipitation**
Approximately 1 mg of total protein was incubated with anti-Sirt1 antibody (Servicebio) or anti-HIF-1α antibody (Servicebio) overnight at 4 °C followed by precipitation with 20 μl of protein A/G-Plus-Agarose (Servicebio) for 4 h at 4 °C. The precipitated complex was immunoblotted with anti-Sirt1, anti-HIF-1α, or anti-acetyl-lysine.

**Detection of ROS production**
L02 cell suspensions were fixed on glass slides. Cell culture fluid (2 mL) was added and the culture was continued for about 6 h. Dihydroethidium (1 mL) (Cat. No. GDP1018), which was dissolved in dimethyl
sulfoxide at a ratio of 1:1000, was added to each well, and the samples were incubated in the dark. An approximate amount of DAPI solution was added to the wells and stained. Then, a drop of anti-fluorescence quenching medium was added into the hole; the slides were imaged under a fluorescent microscope.

**Western blotting**

Proteins were extracted from cells and tissues as directed by the radioimmunoprecipitation assay kit (Sigma-Aldrich). An appropriate amount of concentrated sodium dodecyl sulfate polyacrylamide gel electrophoresis protein loading buffer was added to the collected protein samples, and then 5-10 μL of the sample was loaded in the sodium dodecyl sulfate polyacrylamide gel electrophoresis gel sample holes. Low voltage constant pressure electrophoresis for the upper gel and high voltage constant voltage electrophoresis were applied, when bromophenol blue entered the lower gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes. The following primary antibodies were used: Sirt1 (Cat. No. 9475, Cell Signaling Technology, Danvers, MA, United States), peroxisome proliferator-activated receptor alpha (PPARα, Cat. No. 23398R, Bioss), AMPK (Cat. No. 32047, Abcam, Cambridge, United Kingdom), p-AMPK (Cat. No. 131357, Abcam), Bnip3 (Cat. No. 109414, Abcam), and glyceraldehyde-3-phosphate dehydrogenase (Cat. No. 8245, Abcam). Image Lab statistical software (Bio-Rad, Hercules, CA, United States) was used to evaluate band intensities on Western blots.

**Statistical analyses**

Statistical analysis was performed using GraphPad Prism software version 8.0 (San Diego, CA, United States). The Y axis was labeled as fold of control mean. Data were expressed as the means ± standard deviations. Differences among multiple groups were evaluated using conventional Student’s t test or analysis of variance. Statistical significance was considered at P < 0.05.

**RESULTS**

**Hypoxia aggravated ALF and increased the expression and acetylation of HIF-1α**

The liver structure of each group was shown by histopathological examination. Compared with the control group, large-scale hepatocyte necrosis in the LPS and Hypoxia groups and the number of infiltrating inflammatory cells were significantly increased, while the inflammatory response was significantly more severe in the LPS + Hypoxia group (Figure 1A). Next, we tested the expression of some key proteins in ALF. As shown in Figure 1B, compared with the control group, the expression of Sirt1 in the LPS group was significantly reduced, and hypoxia aggravated this effect. The expression of Bcl-2 adenovirus E1B-interacting protein 3 (Bnip3) in the LPS + hypoxia group was significantly increased, as was carbonic anhydrase 9 (CA9), both of which are regulated by HIF-1α, suggesting that hypoxia significantly increased the activity of HIF-1α in the LPS group. Of note, the expression of HIF-1α in the LPS + Hypoxia group was significantly increased in the form of acetylation. LPS significantly increased HIF-1α acetylation induced by hypoxia (Figure 1C).

**Hypoxia reduced the expression of Sirt1, causing the activation and acetylation of HIF-1α**

To detect changes in the expression of Sirt1 in L02 cells during hypoxia, we measured the expression levels of Sirt1, HIF-1α, and Bnip3 using Western blotting. Compared to the control group, hypoxia reduced Sirt1 expression and upregulated HIF-1α and Bnip3 expression in a time-dependent manner (Figure 2A-D). Through immunofluorescence experiments, we found that as the duration of hypoxia increased, the expression of HIF-1α increased significantly in the form of acetylation (Figure 2E). We then analyzed the interaction between Sirt1 and HIF-1α. After hypoxia induced endogenous HIF-1α, Sirt1-HIF-1α binding was observed (Figure 2F). We next examined whether Sirt1 deacetylates HIF-1α. Immunoblotting with anti-acetyl-lysine in HIF-1α immunoprecipitates was used to detect the lysine acetylation level of HIF-1α. As shown in Figure 2G, Sirt1 overexpression significantly decreased HIF-1α acetylation, suggesting that Sirt1 regulated lysyl acetylation of HIF-1α. These results suggested that hypoxia-induced enhancement of HIF-1α activity and lysine acetylation were related to the down-regulation of Sirt1.

**The inhibition of Sirt1 induced activation of HIF-1α and subsequently increased the production of ROS induced by hypoxia**

Next, we explored the possible molecular mechanisms of the interaction between Sirt1 and HIF-1α. As shown in the Figure 3A, LPS increased the expression of HIF-1α, and the expression of Sirt1 was further reduced after HIF-1α was increased by hypoxia in L02 cells. At the same time, the use of a specific Sirt1 inhibitor NAM to inhibit Sirt1 further aggravated this effect. Sirt1 appear to interact with HIF-1α in L02 cells. Studies have found that excessive production of ROS is considered harmful and related to hypoxia [28]. Oxidative stress has been shown to promote inflammation during ALF[29]. How oxidative stress is
Cao P et al. Sirt1 attenuates ALF via HIF-1α

Figure 1 Hypoxia aggravated acute liver failure and increased the expression of hypoxia inducible factor-1α and its acetylation. A: The representative images of hematoxylin and eosin staining of liver in each group; B: Western blotting was performed to measure the levels of Sirtuin1 (Sirt1), Bcl-2 adenovirus E1B-interacting protein 3 (Bnip3) and carbonic anhydrase 9 (CA9) in liver tissues; C: The representative images of immunofluorescence staining for Acetyl-lysine and hypoxia inducible factor (HIF)-1α. Data shown are means ± standard deviation of three separate experiments. *P < 0.05 vs Control group; †P < 0.05 vs LPS-treatment group; one-way analysis of variance combined with Bonferroni's post hoc test; the error bars indicate the standard deviations. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

involved in inflammation during ALF remains unclear. Therefore, we examined the antioxidant effect of Sirt1 during hypoxia. DHE staining showed that the level of ROS stimulated by LPS was significantly increased by hypoxia, and this effect was enhanced when NAM was used to inhibit the Sirt1 signaling
Figure 2 Hypoxia decreased Sirtuin1 expression leading to the acetylation and activation of hypoxia inducible factor-1α. A-D: Western blotting was performed to measure the levels of Sirtuin1 (Sirt1), Bcl-2 adenovirus E1B-interacting protein 3 (Bnip3), and hypoxia inducible factor (HIF)-1α in L02 cells; E: The representative images of immunofluorescence staining for Acetyl-lysine and HIF-1α; F and G: Equal amounts of protein were subjected to immunoprecipitation with Sirt1 antibody or HIF-1α antibody followed by immunoblotting with antibody against Sirt1, HIF-1α, or acetyl-lysine and effect of Sirt1 overexpression (O/E) was shown. Data shown are means ± standard deviations (SDs) of three separate experiments. *P < 0.05 vs Control group; one-way analysis of variance combined with Bonferroni’s post hoc test; the error bars indicate the SDs.
Figure 3 The inhibition of Sirtuin1 induced activation of hypoxia inducible factor-1α and subsequently increased hypoxia-induced
reactive oxygen species production. A: Western blotting was performed to measure the levels of Sirtuin1 (Sirt1) and hypoxia inducible factor (HIF)-1α in L02 cells; B: Reactive oxygen species (ROS) productions were detected by dihydroethidium (DHE) staining. Representative images of the DHE staining in different groups; C: ROS productions were evaluated by quantification of mean fluorescence intensity in DHE staining; D: Western blotting was performed to measure the levels of Sirt1 and HIF-1α in L02 cells; E and F: ROS productions were detected by DHE staining. Data shown are means ± standard deviations (SDs) of three separate experiments. *P < 0.05 vs Control group; **P < 0.05 vs Lipopolysaccharide (LPS)-treated group; ***P < 0.05 vs LPS + Hypoxia-treated group; one-way analysis of variance with Bonferroni’s post hoc test; the error bars indicate the SDs.

The inhibition of Sirt1/PPARα signaling pathway increased hypoxia-induced ROS production in vitro

Some studies have shown that liver PPARα expression is lower in patients with hepatitis C and advanced nonalcoholic fatty liver disease, perhaps due to the inhibitory effect of multiple cytokines[30]. This also shows that increasing PPARα may help reduce liver inflammation. In our study, as shown in Figure 4A, in the L02 cells stimulated by LPS, PPARα expression was decreased and aggravated after hypoxia intervention, and its effect was further aggravated when NAM was used to inhibit the Sirt1 signaling pathway, suggesting that hypoxia-induced PPARα inhibition was closely related to Sirt1. In addition, Sirt1 expression was further reduced by the PPARα inhibitor GW6471, while HIF-1α was opposite (Figure 4B) and the levels of ROS were also improved (Figure 4C), suggesting that the inhibition of Sirt1/PPARα signaling pathway might increase hypoxia-induced ROS production in L02 cells.

The inhibition of Sirt1/AMPK signaling pathway increased hypoxia-induced ROS production in vitro

AMPK acts as a regulator of cellular energy metabolism and redox homeostasis. More and more evidence shows that AMPK plays a protective role by regulating the redox system[31]. Next, we further studied whether Sirt1 can regulate AMPK and its role in cell hypoxia in L02 cells. As shown in Figure 5A, the phosphorylation level of AMPK in L02 cells induced by LPS after hypoxia treatment was significantly reduced, while NAM pretreatment aggravated this effect, indicating AMPK could be modulated by hypoxia via Sirt1. In addition, AMPK inhibitor Compound C further reduced the expression of Sirt1, the expression of HIF-1α was further increased (Figure 5B), and the levels of ROS were also improved (Figure 5C). Therefore, these results suggested that Sirt1/AMPK signaling pathway might be involved in modulating ROS in LPS-stimulated L02 cells during hypoxia.

The activation of Sirt1 induced the inactivation and deacetylation of HIF-1α and subsequently rescued the progressive aggravation of ALF induced by hypoxia in vivo

Finally, to determine further whether Sirt1 attenuated the progressive aggravation of ALF induced by hypoxia through the Sirt1/AMPK or the Sirt1/PPARα pathway, LPS-stimulated mice were exposed to hypoxia with or without resveratrol treatment, which is a Sirt1 activator. Compared with the LPS group, activation of Sirt1 by resveratrol alleviated the more severe liver tissue damage in the LPS + Hypoxia group (Figure 6A and B). LPS + Hypoxia group mice showed lower activity of superoxide dismutase and glutathione peroxidase, while malondialdehyde levels were increased, indicating that hypoxia led to decreased antioxidant activity. However, resveratrol treatment could significantly improve the activity (Figure 6C). As shown in Figure 6D, resveratrol dramatically alleviated hypoxia-induced reduction levels of PPARα protein and the phosphorylation of AMPK in LPS-stimulated mice, suggesting that Sirt1 was a key regulator on the activation of PPARα and the phosphorylation of AMPK during hypoxia in ALF. Finally, we demonstrated with animals whether Sirt1 has a regulatory effect on hypoxia-induced HIF-1α lysine acetylation and HIF-1α activity. As shown in Figure 6E, with the intervention of resveratrol, the expression of HIF-1α and the level of acetylation decreased significantly. These findings indicate that the activation of Sirt1 induced HIF-1α inactivation and deacetylation, thereby alleviating the progressive aggravation of ALF induced by hypoxia.

DISCUSSION

Recently, more and more studies have confirmed the effect of Sirt1 in liver disease. Sirt1 has been confirmed to have a protective effect in a variety of disease models, including liver fibrosis[32], drug-induced liver injury[33], non-alcoholic fatty liver disease[34], and fatty liver[35]. As well known, HIF-1α is a transcription factor that can promote the adaptive response of cells to hypoxia. Some reports have mentioned the connection between Sirt1 and HIF protein, but there are still many controversies about the results. According to reports, in hypoxic Hep3B or HEK293 cells, Sirt1 targeted HIF-2α and increased the transcriptional activity of HIF-2α but not HIF-1α[36]. On the contrary, another group of studies
Figure 4 The inhibition of Sirtuin1/peroxisome proliferator-activated receptor alpha signaling pathway increased hypoxia-induced reactive oxygen species production. A: Western blotting was performed to measure the levels of peroxisome proliferator-activated receptor alpha (PPARα) in L02 cells; B: The levels of Sirtuin1 (Sirt1) and hypoxia inducible factor (HIF)-1α in L02 cells; C: Reactive oxygen species productions were detected by dihydroethidium (DHE) staining and evaluated by quantification of mean fluorescence intensity in DHE staining. Data shown are means ± standard deviations (SDs) of three separate experiments. \( \ast P < 0.05 \) vs Control group; \( \ast \ast P < 0.05 \) vs Lipopolysaccharide (LPS)-treated group; \( \ast \ast \ast P < 0.05 \) vs LPS + Hypoxia-treated group; one-way analysis of variance with Bonferroni’s post hoc test; the error bars indicate the SDs.

showed that Sirt1 interacted with HIF-1α, causing HIF-1α deacetylation to promote its activity in Hep3B and Huh7 cells[19]. Therefore, the regulation of Sirt1 on the activity of HIF-1α and its expression seems to be cell-type-specific, which is currently unclear. It has not been reported that the beneficial effect of Sirt1 activation is related to its HIF-1α deacetylation against ALF.

In our research, we found that the activity of HIF-1α increased after acetylation and promoted hepatocyte apoptosis in ALF models and hypoxia models in vitro. In addition, we demonstrated that the expression of Sirt1 in L02 cells decreased in a time-dependent manner due to hypoxia, which was closely related to the activation and acetylation of HIF-1α. During hypoxia, with the decrease of the level of nicotinamide adenine dinucleotide, the activity of Sirt1 decreased and HIF-1α transcription activity further increased[18,19]. Therefore, the insufficient expression of Sirt1 in the liver or the acetylation of HIF-1α might be the key mediators of ALF.

Next, we carefully evaluated Sirt1’s regulatory effect on HIF-1α activity in ALF and explored its possible molecular mechanisms. ROS are by-products of normal metabolism in living cells, but
Cao P et al. Sirt1 attenuates ALF via HIF-1α

**Figure 5** The inhibition of Sirtuin1/AMP-activated protein kinase signaling pathway increased hypoxia-induced reactive oxygen species production. A: Western blotting was performed to measure the levels of AMP-activated protein kinase (AMPK) and p-AMPK in L02 cells; B: The levels of Sirtuin1 (Sirt1) and hypoxia inducible factor (HIF)-1α in L02 cells; C: Reactive oxygen species (ROS) productions were detected by dihydroethidium (DHE) staining and evaluated by quantification of mean fluorescence intensity in DHE staining. Data shown are means ± standard deviations (SDs) of three separate experiments. *P < 0.05 vs Control group; **P < 0.05 vs Lipopolysaccharide (LPS)-treated group; ***P < 0.05 vs LPS + Hypoxia-treated group; one-way analysis of variance combined with Bonferroni’s post hoc test; the error bars indicate the SDs.

Excessive ROS accumulation can damage organelles, leading to increased oxidative stress[37,38]. ALF produces excessive amounts of ROS due to insufficient detoxification of toxic substances in the liver [39]. Sirt1 has been reported to play an important role in anti-inflammatory and antioxidant processes [40]. Here, we demonstrated that HIF-1α was over-activated in hypoxia due to increased level of ROS in the absence of Sirt1, and the effect was inhibited by the antioxidant NAC, indicating that ROS was involved in this activation.

In particular, PPARα is reported to be a potent inhibitor of NF-κB signaling pathway and inflammation[41]. The positive effect of Sirt1 on the inflammatory pathway may be related to PPARα[42], and the interference of PPAR transcriptional activity may disturb estrogen/androgen receptor expression and impair steroidogenesis and ROS metabolism[43]. In addition, PPARα contributes to the protection of redox homeostasis[44]. Previous studies have confirmed that Sirt1 can regulate AMPK, which is an important energy sensor[45]. AMPK acts as a regulator of cellular energy metabolism and redox homeostasis. More and more evidence shows that AMPK plays a cardiovascular protective role by...
Cao P et al. Sirt1 attenuates ALF via HIF-1α

(A) Representative images of liver histological scores under different conditions: Con, LPS, Hypoxia, LPS+Hypoxia, Resveratrol, LPS+Hypoxia+Resveratrol.

(B) Bar chart showing liver histological scores for different conditions.

(C) Graph showing fold of control mean for MDA content, SOD activity, and GSH-Px activity under different conditions: Con, LPS, Hypoxia, LPS+Hypoxia, Resveratrol, LPS+Hypoxia+Resveratrol.

(D) Western blot analysis of Sirt1, HIF-1α, PPARα, p-AMPK, AMPK, and GAPDH under different conditions: Con, LPS, Hypoxia, LPS+Hypoxia, Resveratrol, LPS+Hypoxia+Resveratrol.

(E) Immunofluorescence images of Acetyl-lysine, HIF-1α, DAPI, and Merge under different conditions: Con, LPS, Hypoxia, LPS+Hypoxia.
Cao P et al. Sirt1 attenuates ALF via HIF-1α

Figure 6 The activation of SirT1 induced the deacetylation and inactivation of hypoxia inducible factor-1α, and subsequently rescued the progressive aggravation of acute liver failure induced by hypoxia. A: Mice were pretreated with resveratrol or exposed to hypoxia and then stimulated with lipopolysaccharide (LPS). The representative images of hematoxylin and eosin staining of liver in each group; B: The liver histological score of liver in each group; C: The levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) of mice in each group; D: Western blotting was performed to measure the levels of SirT1 (SirT1), hypoxia inducible factor (HIF)-1α, peroxisome proliferator-activated receptor alpha (PPARα) and p-AMP-activated protein kinase (AMPK) in liver tissues and the protein expression were quantified; E: The representative images of immunofluorescence staining for Acetyl-lysine and HIF-1α. Data shown are means ± standard deviations (SDs) of three separate experiments. *P < 0.05 vs Control group; †P < 0.05 vs LPS-treated group; ‡P < 0.05 vs LPS + Hypoxia-treated group; one-way analysis of variance combined with Bonferroni's post hoc test; the error bars indicate the SDs.

regulating the redox system[46]. In diabetes, the activation of AMPK increases the expression of mitochondrial antioxidant enzymes and leads to a decrease in the production of mitochondrial ROS in endothelial cell[47].

Our experiments revealed that the inhibition of PPARα and the phosphorylation of AMPK induced by hypoxia were closely related to SirT1, and the inhibition of SirT1/PPARα or SirT1/AMPK signaling pathway might increase hypoxia-induced ROS production in L02 cells. In order to determine whether the activation of SirT1 induced inactivation of HIF-1α, the progressive aggravation of ALF induced by hypoxia in vivo was rescued in mice treated with resveratrol. As expected, the activation of SirT1 significantly alleviated the degree of liver damage in ALF and enhanced antioxidant activity. Resveratrol dramatically alleviated the hypoxia-induced reduction level of PPARα protein and the phosphorylation of AMPK in LPS-stimulated mice. In addition, the activation of SirT1 induced the deacetylation of HIF-1α compared to LPS-stimulated mice exposed to hypoxia; the expression of HIF-1α and the level of acetylation decreased significantly.

One limitation of our study is that we did not use HIF-1α overexpressing mice in vivo to test whether the increase of SirT1 activity can rescue ALF. We need to conduct further experiments to solve this problem.

CONCLUSION

In summary, we have demonstrated that SirT1 reduced oxidative stress in ALF by regulating the activity and acetylation of HIF-1α, achieved by normalizing the SirT1/PPARα and SirT1/AMPK pathway. Our research showed that the deacetylation and inactivation of HIF-1α induced by the activation of SirT1 might have therapeutic benefits in reducing liver damage during ALF.

ARTICLE HIGHLIGHTS

Research background

Acute liver failure (ALF) is a life-threatening disease that can rapidly develop into multiple organ failure. The mortality rate is high. If effective treatment measures are not taken, various complications will occur, including cerebral edema, sepsis, renal failure, gastrointestinal bleeding, and respiratory failure. Hypoxia inducible factor 1α (HIF-1α) is a transcription factor that regulates oxygen homeostasis. In ALF, HIF-1α contributes to early liver cell necrosis. SirT1 (SirT1) plays a key role in health by deacetylating target proteins in many tissues, including the liver. The activation of SirT1 will result in a powerful antioxidant defense system. However, the role of SirT1 in ALF and the relationship between SirT1 and HIF-1α remain unclear and require further investigation.
**Research motivation**
The results of this study might provide a basis for the application of Sirt1 in the treatment of ALF and further understanding of the mechanism of Sirt1 and HIF-1α in the process of ALF.

**Research objectives**
This study detected the changes in the expression of Sirt1 and HIF-1α in liver tissues and hepatocytes under hypoxia during the ALF process as well as the differences in the expression levels of key enzymes. In addition, this study further explored the relationship and mechanism of Sirt1 signaling pathway and HIF-1α expression.

**Research methods**
Western blotting was used to detect the expression levels of Sirt1 and HIF-1α related proteins in mouse liver tissues, and immunofluorescence staining was used to observe the acetylation level of HIF-1α. Detection of HIF-1α and reactive oxygen species (ROS) levels and the correlation analysis between Sirt1 and HIF-1α were performed. Finally, Sirt1 was activated to observe the influence of the Sirt1 signaling pathway and HIF-1α on ALF, and changes in the expression levels of related markers were detected.

**Research results**
The expression of Sirt1 decreased and the level of HIF-1α acetylation increased in hypoxic mice, and the levels of carbonic anhydrase 9 and Bcl-2-adenovirus E1B interacting protein 3 increased significantly, which was regulated by HIF-1α, indicating an increase of HIF-1α activity. Under hypoxia, the down-regulation of Sirt1 activated and acetylated HIF-1α in L02 cells. The inhibition of Sirt1 significantly aggravated this effect and the massive production of ROS. The regulation of ROS was partly through peroxisome proliferator-activated receptor alpha or AMP-activated protein kinase (AMPK). The activation of Sirt1 effectively relieved ALF aggravated by hypoxia, the production of ROS, and cell apoptosis. It also induced the deacetylation of HIF-1α and inhibited the activity of HIF-1α.

**Research conclusions**
The inhibition of peroxisome proliferator-activated receptor alpha and the phosphorylation of AMPK induced by hypoxia were closely related to Sirt1, and the inhibition of Sirt1/PPARα or Sirt1/AMPK signaling pathway might increase hypoxia-induced ROS production. The activation of Sirt1 reduced oxidative stress in ALF by regulating the activity and acetylation of HIF-1α.

**Research perspectives**
The results of this study showed that the deacetylation and inactivation of HIF-1α induced by the activation of Sirt1 might have therapeutic benefits in reducing liver damage during ALF. This study preliminarily clarified the role of Sirt1 and HIF-1α in ALF, so as to deepen the understanding of the mechanism of ALF, and provided guidance for the selection of ALF treatment targets. The results of this study indicate that Sirt1 activator may have a certain prospective application as a therapeutic drug for ALF.

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**FOOTNOTES**

**Author contributions:** Cao P and Gong ZJ designed the study; Cao P, Chen Q and Shi CX performed most of the experiments and wrote the article; Wang LW analyzed the data; All authors approved the final version of the article.

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Hwang Y, Kim JC, Tae G. Significantly enhanced recovery of acute liver failure by liver targeted delivery of stem cells via
Cao P et al. Sirt1 attenuates ALF via HIF-1α


Basic Study

Peroxisome proliferator-activated receptor-alpha activation and dipeptidyl peptidase-4 inhibition target dysbiosis to treat fatty liver in obese mice

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Abstract

BACKGROUND
Obesity and comorbidities onset encompass gut dysbiosis, altered intestinal permeability, and endotoxemia. Treatments that target gut dysbiosis can cope with obesity and nonalcoholic fatty liver disease (NAFLD) management. Peroxisome proliferator-activated receptor (PPAR)-alpha activation and dipeptidyl-peptidase-4 (DPP-4) inhibition alleviate NAFLD, but the mechanism may involve gut microbiota modulation and merits further investigation.

AIM
To address the effects of PPAR-alpha activation and DPP-4 inhibition (isolated or combined) upon the gut-liver axis, emphasizing inflammatory pathways in NAFLD management in high-fat-fed C57BL/6J mice.

METHODS
Male C57BL/6J mice were fed a control diet (C, 10% of energy as lipids) or a high-fat diet (HFD, 50% of energy as lipids) for 12 wk, when treatments started, forming the groups: C, HF, HFA (HFD + PPAR-alpha agonist WY14643, 2.5 mg/kg body mass), HFL (HFD + DPP-4 inhibitor linagliptin, 15 mg/kg body mass), and HFC (HFD + the combination of WY14643 and linagliptin).
RESULTS
The HFD was obesogenic compared to the C diet. All treatments elicited significant body mass loss, and the HFC group showed similar body mass to the C group. All treatments tackled oral glucose intolerance and raised plasma glucagon-like peptide-1 concentrations. These metabolic benefits restored Bacteroidetes/Firmicutes ratio, resulting in increased goblet cells per area of the large intestine and reduced lipopolysaccharides concentrations in treated groups. At the gene level, treated groups showed higher intestinal Mucin 2, Occludin, and Zo-1 expression than the HFD group. The reduced endotoxemia suppressed inflammasome and macrophage gene expression in the liver of treated animals. These observations complied with the mitigation of liver steatosis and reduced hepatic triacylglycerol, reassuring the role of the proposed treatments on NAFLD mitigation.

CONCLUSION
PPAR alpha activation and DPP-4 inhibition (isolated or combined) tackled NAFLD in diet-induced obese mice by restoration of gut-liver axis. The reestablishment of the intestinal barrier and the rescued phylogenetic gut bacteria distribution mitigated liver steatosis through anti-inflammatory signals. These results can cope with NAFLD management by providing pre-clinical evidence that drugs used to treat obesity comorbidities can help to alleviate this silent and harmful liver disease.

Key Words: Nonalcoholic fatty liver disease; High-fat diet; Peroxisome proliferator-activated receptor-alpha; Dipeptidyl-peptidase-4-inhibitor; Dysbiosis; Inflammation

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Core Tip: Chronic high-fat diet (HF) intake alters the phylogenetic microbiota composition, sending harmful proinflammatory signals to the liver that elicit nonalcoholic fatty liver disease in mice. Here, we treated HF diet-induced obese mice with a peroxisome proliferator-activated receptor-alpha agonist (WY14643), a dipeptidyl-peptidase-4 inhibitor (linagliptin), or their combination, focusing on gut–liver axis modulation. The treatments rescued gut dysbiosis and endotoxemia due to increased tight junction gene expression, mucin production, and numerical density of goblet cells in the intestinal crypts. Treated mice benefited from downregulated Tlr4, Cd206, and Nlrp3, alleviating fatty liver through anti-inflammatory signals such as increased IL-10 and IL-13.

INTRODUCTION
The prevalence of obesity worldwide has tripled during recent decades, necessitating large budgets for public health systems due to associated comorbidities[1,2]. Excessive dietary saturated fat intake triggers insulin resistance, white adipocyte hypertrophy, low-grade inflammation (metainflammation), brown adipose tissue dysfunction (whitening), fatty liver, and, as more recently described, alteration in the composition of the gut microbiota (dysbiosis)[3,4].

The digestive tract is populated by several microorganisms, predominantly including bacteria from the Firmicutes and Bacteroidetes phyla, and this population is influenced by the quality of the diet[5]. Excessive saturated fat in the diet increases the proportion of gram-negative bacteria with lipopolysaccharides (LPS) in the composition of their outer wall[6]. LPS is an endotoxin that compromises the integrity of the intestinal mucosa through alterations in the structural proteins of tight junctions (TJs), resulting in increased intestinal permeability and the migration of LPS to other tissues[5,7].

The gut–liver axis comprises the anatomical communication between these two organs via the portal vein[8]. A recent study by our group has demonstrated phylogenetic changes in the gut microbiota of mice after seventeen weeks of high-fructose diet feeding, with a significant increase in liver steatosis and inflammation, indicating the progression of nonalcoholic fatty liver disease (NAFLD) (fatty liver disease associated with metabolic dysfunction) to more harmful forms of liver diseases[9]. Given this scenario, the identification of metabolic pathways that rescue gut dysbiosis and mitigate the liver changes arising...
from a dietary excess of saturated fat is pertinent, considering the high prevalence of obesity, its deleterious health effects, and the fact that there is, thus far, no treatment directed exclusively toward NAFLD[10].

Peroxisome proliferator-activated receptors (PPARs) are transcription factors involved in several metabolic pathways. The pharmacological activation of the PPAR-alpha isoform promotes reduced body mass, increased insulin sensitivity, the formation of beige adipocytes[11], and a significant reduction in NAFLD by increasing mitochondrial beta-oxidation[9]. Recently, PPAR-alpha deletion promoted intestinal dysbiosis and inflammation in mice[12]. The dipeptidyl-peptidase-4 (DPP-4) inhibitor linagliptin extends the glucagon-like peptide-1 (GLP1) time of action through beneficial brown and white adipocyte remodeling, browning induction, and M2 macrophage polarization, in addition to enhancing liver vascularization, suppressing de novo lipogenesis, and alleviating endoplasmic reticulum stress[13,14].

This study aimed to address the effects of PPAR-alpha activation and DPP-4 inhibition (isolated or combined) on the gut–liver axis, emphasizing inflammatory pathways in NAFLD management in high-fat-fed C57BL/6J mice.

MATERIALS AND METHODS

Animals and diet
Adult male C57BL/6J mice were group-housed (n = 5 per cage) and maintained under controlled temperature (21 ± 2 °C) and humidity (60 ± 10%) with free access to water and food in a ventilated rack containing cages for the mice (NexGen mouse 500, Allentown, PA, United States). The environment comprised a 12/12 h light–dark period and air renewal cycles (15 min/h). The procedures followed the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by our local Ethics Committee (Institute of Biology, CEUA number 041/2018).

Experimental protocol
Fifty adult male C57BL/6J mice (3 month old) from the Central Biotery of the Federal Minas Gerais University were used in this study. Initially, the animals were randomly assigned to two nutritionally different groups: (1) Control group (C) – animals that received a control diet (14% of energy as protein, 10% as fat, and 76% as carbohydrates; total energy 15 KJ/g, n = 10); and (2) High-fat group (HF) – animals that received a high-fat diet (14% of energy as protein, 50% as fat and 36% as carbohydrates; total energy 21 KJ/g, n = 40).

After twelve weeks, the C group and ten animals from the HF group continued the same food scheme for an additional five weeks, whereas the remaining animals from the HF group were randomly subdivided according to the treatment into the following groups: (1) HFA received the PPAR-alpha agonist (WY14643, Sigma–Aldrich, 3.5 mg/kg body mass) incorporated into the HF diet (n = 10) for five weeks; (2) HFL received the DPP-4 inhibitor (linagliptin, Boehringer Ingelheim, 15 mg/kg body mass) incorporated into the HF diet (n = 10) for five weeks; and (3) HFC received the combination of a PPAR-alpha agonist with a DPP-4 inhibitor (at the same doses used in the groups receiving monotherapy) incorporated into the HF diet (n = 10) for five weeks.

The entire experimental protocol lasted for 17 wk (12 wk of obesity induction + 5 wk of treatment). The doses of WY-14643 and linagliptin were based on previous experiments conducted by our group[4,9]. PragSoluções (Jau, São Paulo, Brazil) produced the experimental diets according to the recommendations of the American Institute of Nutrition (AIN 93M)[15]. All groups were treated following the order in which the groups were described.

Food/energy intake and body mass
Food intake was measured daily by subtracting the remainder of the diet verified on the following day from the amount of diet offered on the previous day. Energy intake comprised the product of the food consumption and the energy contained in 1 g of each diet (in kJ). Animal body masses were assessed on a digital scale once a week (BL-3200H, precision 0.01 g).

Metabolic analysis
One week before sacrifice, the animals were subjected to the oral glucose tolerance test (OGTT). Under a 6-h fast (time 0) and after 15, 30, 60, and 120 minutes of the orogastric gavage of a glucose solution (2 g/kg body mass), blood samples were obtained from the caudal vein. A manual glucometer (Accu-Chek, Roche, São Paulo, SP, Brazil) was used to measure the blood glucose levels at different times. The area under the curve (AUC) indicated the oral glucose tolerance (GraphPad Prism, version 8.3 for Windows, GraphPad Software, La Jolla, CA, United States).
**Sacrifice and ELISA**

Mice were fasted for 6 h. Under intraperitoneal anesthesia with ketamine (240 mg/kg) and xylazine (30 mg/kg), blood samples obtained by cardiac puncture were separated by centrifugation (712 × g) to obtain plasma samples to perform biochemical analyses. The liver, large intestine (cecum), and small intestine (jejunum and ileum) were carefully dissected, weighed, and analyzed following the protocols for different techniques.

An ELISA was performed to measure plasma GLP1 (multispecies GLP1 ELISA Kit Cat. #EZGLP1T-36K, Millipore, Missouri, United States), and LPS (multispecies LPS ELISA Kit Cat. #SEB526Ge-96T, Cloud-C1one Corp., Katy, United States). A semiautomatic spectrophotometer and a commercial kit (K117, Bioclin, Quibasa, Belo Horizonte, MG, Brazil) were used to measure hepatic triacylglycerol (TAG) as previously described[16].

**Histology**

Liver and cecum fragments fixed in Millonig-buffered formalin (pH 7.2-7.4) were subsequently dehydrated, diaphanized, embedded in Paraplast Plus (Sigma–Aldrich, St. Louis, MO, United States) and sectioned (5 μm thick) with a microtome. Slides stained with hematoxylin and eosin (liver) or Alcian Blue (Sigma Chemical Company–pH 2.5) plus Periodic Acid–Schiff (PAS, Intestine–Sigma Chemical Company) were photographed using a Leica DMRBE microscope (Wetzlar, Germany) and an Infinity Lumenera digital camera (Ottawa, ON, Canada). The images were analyzed in a blinded manner with STEPanizer ([www.stepanizer.com](http://www.stepanizer.com)) as described below:

**Hepatic stereology:** Five animals per group and ten images per animal were analyzed. The volume density of liver steatosis [Vv (liver, st)] was estimated by the point-counting technique, following the formula: Vv (liver, st) = Pp (liver, st)/PT (Pp is the number of points that reached fat droplets, and PT is the total test points). The images were analyzed with STEPanizer using a 36-point test system[17].

**Gut stereology:** The number of goblet cells per area [Qc (goblet)] was estimated using STEPanizer. All goblet cells within the test area were counted, except those touching the forbidden lines. The result was divided by the test area measured in mm².

**16S rDNA polymerase chain reaction amplification**

The feces found in the mouse cecum were used to extract microbial DNA using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer’s instructions. The DNA quantity, purity, and concentration were determined using Qubit (Life Technologies, Carlsbad, California, United States) and horizontal electrophoresis (1% agarose gel). Real-time quantitative polymerase chain reaction (PCR) assays were used for the relative quantification of specific phyla of microorganisms (Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria) in fecal microbiota from the mouse guts by detecting 16S rRNA genes. For relative quantification, the abundances of different phyla were normalized by the total bacterial amount in the samples[18]. The indicators used are described in Table 1.

**Real-time reverse transcriptase–PCR**

Total RNA was extracted from 50 mg of the liver and 70 mg of the small intestine (jejunum and ileum) using TRIzol reagent (Invitrogen, CA, United States). Afterward, the addition of 200 μL of chloroform was followed by centrifugation (1200 g for 10 min at 4 °C for liver samples and 12000 g for 15 min at 4 °C for intestine samples). The RNA extract portion was separated, and 500 μL of isopropanol was added and allowed to stand for 10 min (liver samples) or 15 min (intestinal samples) to precipitate the RNA. Then, the samples were centrifuged (1200 g for 10 min at 4 °C for liver samples and 12000 g for 10 min at 4 °C for intestine samples). The isopropanol was removed, and the formed pellet was resuspended in 500 μL of 75% ethanol for liver samples or 70% ethanol (ice-cold) for intestine samples and then centrifuged (1200 g for 5 min at 4 °C for liver samples and 10000 g for 5 min at 4 °C for intestine samples).

The ethanol was removed, and the pellet was resuspended in 20 μL (liver)/50 μL (intestine) of deionized water (Milli-Q). The samples were subjected to a dry bath (50 °C for 5 min) and quantified by using Nanovue equipment (GE Life Sciences). For RNA transcription into complementary DNA (cDNA), 1.0 μg RNA was treated with DNase I (Invitrogen, CA, United States). First-strand cDNA synthesis was performed using Oligo (dT) primers for reverse transcriptase mRNA and Superscript III (both from Invitrogen). qPCR was performed using a CFX96 recycler (Bio–Rad, Hercules, CA, United States) and SYBR Green mix (Invitrogen, Carlsbad, CA, United States). Beta-actin was used to correct the expression of the target genes in liver samples, and Gapdh was used for intestine samples. The primer sequences used are shown in Table 2 (liver) and Table 3 (intestine). All gene symbols are italicized (the first letter capitalized), and protein symbols are italicized in uppercase[19].

**Data analysis**

Sample size calculation considered that, in metabolic and molecular biology analyses, if a factor
increases or decreases in five replicates, the probability of occurrence is $P = (1/2)^5 = 0.05$. Therefore, a minimum of 5 replicates was adopted for the analyses\([20]\). The data are shown as the mean and SD. During the first 12 wk, statistical analysis comprised Student’s t test and Welch’s correction. At the treatment phase, data were analyzed using Brown–Forsythe and Welch one-way ANOVA with the Dunnett T3 post hoc test\([21]\). A $P < 0.05$ was considered significant (GraphPad Prism version 8.3 for Windows, GraphPad Software, La Jolla, CA, United States).

### RESULTS

**Treatment reduced body mass without altering energy intake**

The animals in the C and HF groups had equal body mass (BM) at baseline. All animals tolerated the diets and treatments well. The protocol was maintained as previously stated. In the 13th week, the HF group was overweight compared to the C group (+30%), and this characteristic persisted until the end of the experiment. Figure 1A depicts these results. Although food intake did not differ between the groups (Figure 1B), the energy intake in the HF-fed groups was higher than that in the C group (Figure 1C).

**All proposed treatments rescued glucose tolerance and increased GLP1 concentrations**

Figure 2A shows the OGTT curve, in which the HF group showed a significant increase in fasting glucose (T0) compared to the C group. This difference remained until the end of the test (T120). The C group and the treated groups exhibited rescued baseline blood glucose levels at the other evaluation times (T30, T60, and T120). On the other hand, the HF group did not reach baseline glucose levels,
Silva-Veiga FM et al. Dysbiosis and NAFLD treatment in HF-fed mice

Figure 1 Body mass and food behavior. A: Body mass evolution; B: Food intake; C: Energy intake. Brown-Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± SD, n = 6). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.0001. C: Control diet; HF: High-fat diet; HFA: High-fat diet plus PPAR-alpha agonist (WY14643); HFL: High-fat diet plus DPP-4 inhibitor (linagliptin); HFC: High-fat diet plus the combination of WY14643 with linagliptin.

indicating a delay after glucose overload and implying oral glucose intolerance, as confirmed by the higher AUC for OGTT in the HF group than in the C group (+23%, Figure 2B). In contrast, all treated groups showed lower AUCs than the HF group, indicating oral glucose intolerance alleviation.

Plasma GLP1 concentrations diminished in the HF group compared to the C group (Figure 2C). As expected, treatment with linagliptin enhanced GLP1 concentrations in the HFL group (+13%), which also occurred in the HFC group and after the single treatment with the PPAR-alpha agonist in the HFA group compared to the HF group (Figure 2C).

Treatments recovered the microbiota composition and reversed endotoxemia in HF-fed mice

The amplified 16S rRNA genes of cecal gut bacteria were measured at the end of the experiment to evaluate the microbiota composition. The HF group showed a Firmicutes phylum increase coupled with decreases in the Proteobacteria and Bacteroidetes phyla compared to the C group, as shown in Figure 3A. However, all treatments reversed these phylogenetic alterations in the treated groups. The treatments restored the amount of Bacteroidetes to resemble that in the C group and caused a significant decrease in Proteobacteria, which can play a decisive role in the beneficial effects observed due to the proposed treatments. Changes in the microbiota composition in the HF group triggered increased LPS concentrations (+10%, Figure 3B), while the HFL and HFC groups showed significantly reduced plasma LPS concentrations (-11% for HFL vs HF and -12% for HFC vs HF).

The hepatic mRNA expression of both Lbp (Figure 3C) and Tlr4 (Figure 3D) genes increased in the HF group compared to the C group, while the treated groups showed significantly reduced expression.

DPP-4 inhibitor and PPAR-alpha agonist improved the intestinal barrier structure and protection

The HF group showed an 80% decrease in intestinal Mucin2 gene expression compared with the C group. Conversely, the HFA, HFL, and HFC groups showed significant increases in Mucin2 expression (+439% for HFA vs HF, +345% for HFL vs HF, and +670% for HFC vs HF; Figure 4A).
In agreement with the previous result, the HF group also had reduced intestinal Zo-1 (-38%, Figure 4B) and Occludin (-78%, Figure 4C) expression compared to the C group. On the other hand, the HFA group showed a 238% increase in Occludin expression, whereas the HFL and HFC groups had >500% increases in the expression of this gene (Figure 4C). Regarding Zo-1 gene expression, all treated groups had a significant increase (+166% for HFA vs HF, +397% for HFL vs HF, and +102% for HFC vs HF; Figure 4B).

The high intake of saturated fat altered the histochemical pattern of the intestinal mucosal cells, as revealed by the reaction with Alcian Blue and PAS. Figure 4D shows decreased mucus in the HF group, while the treatments elicited an increase in mucus production in the apical region of the crypts, followed by an increased presence of goblet cells (mucus-producing cells). Gut stereology confirmed these observations with the results of Qa (goblet), which showed a reduction in the HF group compared to the C group (-44%), while the HFA (+68%), HFL (+47%), and HFC (+56%) groups showed an increase in the number of goblet cells per tissue area (Figure 4E).

DPP-4 inhibitor and PPAR-alpha activation mitigated liver steatosis

HF-fed mice exhibited noticeable microvesicular liver steatosis, while mice in all treated groups showed liver steatosis mitigation, with the liver parenchyma resembling that in the C group (Figure 5A).

In accordance with these histological findings, Vv (liver, st) in the HF group was higher than that in the C group (+38%, Figure 5B). All treated groups showed drastic reductions in liver steatosis (-72% for HFA vs HF, -50% for HFL vs HF, and -77% for HFC vs HF).

Consistent with the stereological findings, hepatic TAG levels increased in the HF group (+38%). Conversely, the treated groups showed lower hepatic TAG concentrations than that in the HF group (-11% for HFA, -16% for HFL, and -13% for HFC; Figure 5C).
Figure 3 Gut-liver axis. A: Phylogenetic microbiota composition; B: Plasma lipopolysaccharide concentrations; C: Hepatic Lbp gene expression; D: Hepatic Tlr4 gene expression. Brown-Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± SD, n = 6). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. C: Control diet; HF: High-fat diet; HFA: High-fat diet plus PPAR-alpha agonist (WY14643); HFL: High-fat diet plus DPP-4 inhibitor (linagliptin); HFC: High-fat diet plus the combination of WY14643 with linagliptin; LPS: Lipopolysaccharide.

DPP-4 inhibition and PPAR-alpha activation attenuated macrophage activation and reduced liver inflammation in HF-fed mice

The HF group showed higher expression of Cd206, which is a specific marker of macrophages, than the C group (+103%, Figure 6A). In contrast, the treated groups showed reduced Cd206 expression (-67% for HFA vs HF, -65% for HFL vs HF, and -74% for HFC vs HF).

In addition to the Cd206 results, the HF diet significantly reduced IL-10 expression compared to the C group (-54%), whereas the combined treatment yielded an 80% decrease in HFC IL-10 expression compared to HF (Figure 6B). Nlrp3 showed similar results to those of the Cd206 gene, with higher expression in the HF group than in the C group (+128%) and reduced expression in the HFA (-72%), HFL (-88%), and HFC (-85%) groups compared to the HF group (Figure 6C).

Regarding the IL-13 cytokine, the HF group exhibited a significant increase compared to the C group (+290%). Only the HFA and HFC treatments were able to reduce the expression of this cytokine in comparison with the HF group (-39% and -67%) (Figure 6D).

DISCUSSION

Excessive saturated fat intake causes overweightness, oral glucose intolerance, gut dysbiosis, and morphological and functional intestinal barrier alterations. Hence, HF animals had endotoxemia with proinflammatory signals directed toward the liver, causing substantial NAFLD. Single treatment with the PPAR-alpha agonist or DPP-4 inhibitor and the combined treatment yielded beneficial results, rescuing body mass, oral glucose tolerance, gut goblet cell numerical density per area, TJ gene expression, phylogenetic microbiota distribution, and LPS concentrations. Thus, treated animals showed liver steatosis and inflammation mitigation due to gut dysbiosis and endotoxemia control.

The chronic ingestion of a diet with a high content of saturated fats increases body mass and impairs glucose and lipid metabolism[22]. Accordingly, HF-fed mice were overweight and showed oral glucose intolerance, along with difficulty in rescuing glycemic levels during the OGTT compared to the C group. These metabolic alterations were paralleled by gut dysbiosis in the HF group, confirming that dietary patterns interfere with the gut-liver axis, favoring NAFLD pathogenesis and progression.

The integrity of the intestinal barrier structure and function relates to the gut microbiota composition. The gut microbiota comprises a great diversity of symbiotic bacteria, whereas the intestinal barrier relies on junctional proteins that make this epithelium less permeable to pathogens and toxins. In this way,
both mechanisms prevent metabolic dysregulation and contribute to maintaining gut homeostasis[23-25].

Dysbiosis caused by the chronic consumption of fats is usually characterized by an increase in *Firmicutes* relative to *Bacteroidetes*, as shown by the HF group. These two phyla constitute more than 90% of the phyllogenetic category currently known and characterized in the intestine of experimental models [26]. Conversely, all treated groups showed a reduction in the quantitative percentage of *Firmicutes*, especially the groups treated with the DPP-4 inhibitor and the combined therapy. The rescuing of gut dysbiosis in the treated groups resulted in marked improvements in TJ gene expression. An HF diet also impairs the junctional components present in the intestinal epithelium, making it more permeable and consequently more susceptible to the translocation of microorganisms and toxins into systemic circulation. The increase in intestinal permeability is known as a leaky gut [25, 27].

The leaky gut was rescued through enhanced expression of TJ genes in all treated groups. MUCIN2 knockout mice exhibit alterations in TJ structural proteins, in addition to mitochondrial damage and inflammation, which agrees with a leaky gut[28]. The treatments significantly augmented Mucin2, which is the major goblet cell gene responsible for mucin secretion, in addition to the gene expression of the TJs Occludin and Zo-1[29]. PPAR-alpha has recently been described as essential for lipid droplet formation and crypt expansion in the intestine during chronic HF diet intake[30]. Sitagliptin, as a DPP-4 inhibitor, exerted protective effects on the intestinal barrier (high occludin and Zo-1 levels) by GLP-2 induction in experimental colitis[31]. Herein, the combination of these drugs resulted in the highest intestinal expression of Mucin2 and Occludin, indicating that adequate function of goblet cells and well-preserved TJs may underlie the mechanisms involved in the beneficial results obtained in the HFC group.

HF diet intake also impairs the epithelium lining the intestinal mucosa. The goblet cells found in the intestinal villi and crypts are mucin producers that play a crucial role in protecting and lubricating the intestinal mucosa. The number of goblet cells indirectly reflects the ability to secrete mucus[25]. Herein, the HF diet caused a reduced number of goblet cells per area of the gut crypt, while all treatments
Figure 5 Liver histology, stereology, and biochemistry. A: Hematoxylin-eosin liver sections; B: Volume density (Vv) (liver steatosis); C: Hepatic triacylglycerol. Liver sections show widespread hepatic steatosis after chronic HF diet intake and expressive reduction in all treated groups (scale bar = 50 μm). Both stereology (Vv steatosis) and biochemical analyses (hepatic triacylglycerol) confirm these observations. Brown-Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± SD, n = 6 for biochemistry and n = 5 for stereology). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. C: Control diet; HF: High-fat diet; HFA: High-fat diet plus PPAR-alpha agonist (WY14643); HFL: High-fat diet plus DPP-4 inhibitor (linagliptin); HFC: High-fat diet plus the combination of WY14643 with linagliptin; Vv: Volume density.

Figure 6 Liver gene expression. A: C206; B: IL-10; C: Nlrp3; D: IL-13. Brown-Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± SD, n = 5 or 6). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. C: Control diet; HF: High-fat diet; HFA: High-fat diet plus PPAR-alpha agonist (WY14643); HFL: High-fat diet plus DPP-4 inhibitor (linagliptin); HFC: High-fat diet plus the combination of WY14643 with linagliptin.

reversed this alteration with the normalization of the numerical density of goblet cells and Mucin2 gene expression. Hence, the treatments supported intestinal barrier integrity and reduced the translocation of endotoxins, such as LPS, into systemic circulation, rescuing the mice from a condition called metabolic endotoxemia[32].
The high translocation of endotoxins derived from the gut microbiota induces Toll-like receptor (TLR) activation. LPS is the most common pathogen-associated molecular pattern (PAMP), and its binding to TLR4 is catalyzed by lipopolysaccharide-binding protein (LBP), which is mainly expressed in the liver and adipose tissue[32].

LPS stems from the destruction of the bacterial cell wall. An increase in LPS in systemic circulation triggers the release of proinflammatory cytokines and an inadequate amplification of the immune response, causing tissue damage[26]. An increase in plasma LPS levels was observed in the HF group, characterizing the involvement of the epithelium with a consequent increase in intestinal permeability, making the epithelium more permeable to the entry of microorganisms. Additionally, we showed that hepatic Lbp and Tlr4 gene expression increased in the HF group, inducing the translocation of cytokines associated with inflammation and changing hepatic lipid metabolism, reconfiguring this metabolism to become a potent inducer of fatty liver, and contributing to the pathogenesis of NAFLD.

In contrast, the treatments normalized Lbp and Tlr4 expression, with both emerging as targets for NAFLD treatment. The DPP-4 inhibitor alogliptin suppressed TLR4 via ERK activation, resulting in decreased matrix metalloproteinases and proinflammatory cytokines in U937 histiocytes[33]. Sitagliptin was previously shown to attenuate NAFLD by suppressing inflammation and insulin resistance due to TLR4/NF-kB pathway downregulation in diabetic rats[34]. PPAR-alpha activation by WY-14643 mitigated liver steatosis in high-fructose-fed mice by reducing LPS concentrations, improving the intestinal barrier ultrastructure, upregulating hepatic beta-oxidation, and suppressing lipogenesis in mice[9].

A recent study showed that systemic LBP blockade or decreased LBP levels in the liver normalized glucose homeostasis, mainly by reducing fasting glucose levels, without changing adiposity or liver steatosis[35]. In this study, the group treated with the PPAR-alpha agonist as monotherapy did not show reduced LPS concentrations. However, the treatment reduced Lbp expression, producing anti-inflammatory and anti-steatotic effects similar to those of the other treatments.

In this context, macrophages play roles in acute and chronic inflammatory liver diseases. Macrophages have receptors, such as CD163 and CD206, which participate in the phagocytosis of harmful substances. Due to its high affinity for macrophages, CD206 is a potential biomarker of hepatic macrophage (Kupffer cells) activation, which indicates inflammation and fibrosis in chronic liver diseases[36]. In response to LPS and other stimuli, the metabolic profile of macrophages and dendritic cells stimulates the glycolytic pathway, resulting in the metabolic accumulation of citrate and succinate, which, in turn, regulate the gene expression of cytokines such as interleukin 10 (IL-10)[37]. Recent evidence shows that IL-10 may play a dual role in some contexts by stimulating the immune response rather than suppressing it. However, the cytokine IL-10 has emerged as an anti-inflammatory mediator determining the protection of its host in response to pathogens[38].

Our results showed that Cd206 macrophages were activated in the HF group, reinforcing the idea that the chronic consumption of this type of diet activates inflammatory pathways that contribute to the development of liver disease. In contrast, the groups treated with the PPAR-alpha agonist and DPP-4 inhibitor showed reduced expression of Cd206 macrophages, conferring a protective effect against the activation of inflammatory pathways. Recent evidence showed that highly fibrous livers had a higher density of Cd206 macrophages[36]. IL-10 gene expression was reduced in the HF group. However, only the combined treatment increased IL-10 expression, implying that the isolated treatments might have acted through another anti-inflammatory pathway. IL-13 overexpression in HF diet-fed mice is a pathway related to fatty liver and insulin resistance onset. Combined treatment and PPAR-alpha activation alone markedly reduced hepatic IL-13 expression, which combined with reduced macrophage activation and glycemic homeostasis to alleviate fatty liver[39].

Concerning inflammasomes, NLRP3 is present mainly in immune and inflammatory cells, such as macrophages, monocytes, dendritic cells, and neutrophils, after activation by inflammatory stimuli. NLRP3 is activated by numerous PAMPs, such as hyperglycemia, fatty acids, bacterial toxins, and bacterial and viral nucleic acids[40]. Studies in macrophages and animal models have shown that oxidized low-density lipoproteins and cholesterol crystals trigger NLRP3 activation. In macrophage and type 2 diabetes animal models, glucose and free fatty acids trigger inflammasome activation, damaging glucose metabolism and favoring insulin resistance[41]. Thus, NLRP3 may contribute to the appearance and progression of several diseases related to metabolic syndrome.

In this study, we showed that treatment with the PPAR-alpha agonist and DPP-4 inhibitor produced a potent anti-inflammatory effect in the liver, as they demonstrated reduced gene expression of Nlrp3 and Cd206. The blockade of NLRP3 has recently improved NAFLD and mitigated liver fibrosis in two models of steatohepatitis[42], highlighting the proposed treatments as viable tools to control fatty liver. Two DPP-4 inhibitors were previously shown to suppress NLRP3 in human macrophages by downregulating the TLR4-IL-1beta pathway[43]. Sitagliptin alleviated liver injury caused by thioacetamide in mice by decreasing NLRP3 and exerting anti-apoptotic effects[44]. Regarding PPAR-alpha activation, oleoylthanolamide protected against LPS-induced liver injury in mice by suppressing NLRP3[45]. However, the present study is the first to report the effects of both drugs and their combination on the gut-liver axis in HF-fed mice. Figure 7 summarizes our main findings.
Silva-Veiga FM et al. Dysbiosis and NAFLD treatment in HF-fed mice

Figure 7 Main findings of the study. All treatments alleviated fatty liver by countering gut dysbiosis and rescuing the intestinal barrier integrity. The reduced lipopolysaccharide (LPS) influx to the liver caused reduced inflammasome and CD206 macrophage activation. Peroxisome proliferator-activated receptor-alpha activation and DPP-4 inhibition can be regarded as viable tools to treat leaky gut and prevent LPS-driven hepatic steatosis. PPAR: Peroxisome proliferator-activated receptor; DPP-4: Dipeptidyl peptidase-4; LPS: Lipopolysaccharide; LBP: Lipopolysaccharide-binding protein; TLR4: Toll-Like receptor 4; ZO-1: Zonula occludens 1; NLRP3: NLR family pyrin domain containing 3; CD206: Cluster of differentiation 206; IL: Interleukin. Created with Biorender: www.biorender.com.

Some limitations of the present study comprise the absence of female mice evaluations to determine possible sexual dimorphism, the lack of plasma glucose concentration measurements, and the inability to determine LBP concentrations, although its gene expression was evaluated. Future research should also include the evaluation of genera and families in the Proteobacteria phylum, as it seems to be involved in the harmful evolution of NAFLD.

CONCLUSION

In conclusion, HF-fed mice showed impairment of the intestinal barrier and alteration in the phylogenetic diversity of the gut microbiota, leading to dysbiosis, and contributing to the influx of LPS into the liver. An impaired gut-liver axis favors liver damage, making the liver more susceptible to NAFLD through proinflammatory signals (Tlr4 and Nlrp3 upregulation). Treatment with the PPAR-alpha agonist and the DPP-4 inhibitor modulated the gut microbiota and rescued intestinal barrier gene expression and goblet cell numerical density, reducing endotoxemia and liver steatosis through anti-inflammatory signaling. Given the beneficial effects of the treatments found, these treatments have become possible therapeutic strategies for the NAFLD spectrum of diseases.

ARTICLE HIGHLIGHTS

Research background

Gut microbiota can be modified by the dietary composition and play a role in fatty liver pathogenesis through endotoxemia. Peroxisome proliferator-activated receptor (PPAR)-alpha activation has previously rescued the gut-liver axis with anti-steatotic effects in high-fructose-fed mice, whereas a high-dose dipeptidyl peptidase-4 (DPP-4) inhibitor (linagliptin) inhibited hepatic lipogenesis in high-fat-fed mice. The combination of these drugs could restore the gut-liver axis in obese mice.
Silva-Veiga FM et al. Dysbiosis and NAFLD treatment in HF-fed mice

Research motivation
Nonalcoholic fatty liver disease (NAFLD) is highly prevalent among obese individuals and can evolve into harmful liver diseases. Currently, there is no treatment directly prescribed to counter NAFLD. Herein, we propose a drug combination (PPAR-alpha agonist plus DPP-4 inhibitor) that can alleviate fatty liver by modulating the gut–liver axis with anti-inflammatory properties.

Research objectives
To evaluate the effects of monotherapy with a PPAR-alpha agonist (WY14643) or a DPP-4 inhibitor (linagliptin) and their combined treatment on the gut–liver axis, highlighting the intestinal barrier, endotoxemia, and inflammatory pathways in the livers of high-fat-fed mice. These preclinical insights can help establish new strategies for the treatment of NAFLD.

Research methods
Mice were fed a control diet (C, 10% of energy as lipids) or a high-fat diet (HF, 50% of energy as lipids) for 12 wk. Then, the HF group was randomly divided into four groups: HF, HF-A (treated with the PPAR-alpha agonist), HFL (treated with the DPP-4 inhibitor), and HFC (treated with the combination of both drugs). The treatments lasted for five weeks. The gut–liver axis was assessed by histological, biochemical, stereological, and molecular techniques.

Research results
The HF diet yielded overweightness and oral glucose intolerance, altered gut microbiota composition, and decreased the numerical density of goblet cells and tight junction gene expression, with increased plasma lipopolysaccharide (LPS) concentrations and increased fatty liver. The combined treatment rescued all of these metabolic alterations by restoring the gut microbiota and intestinal barrier markers, resulting in decreased LPS concentrations and fatty liver through anti-inflammatory signals. Further studies may focus on the Proteobacteria phylum, whose alteration can trigger harmful signaling to the liver.

Research conclusions
The combination of PPAR-alpha activation with DPP-4 inhibition yielded marked anti-steatotic effects by modulating the gut–liver axis, with reduced endotoxemia and amelioration of the intestinal barrier histology and gene expression. In turn, the livers of obese mice treated with the drug combination benefited from downregulation of the Tlr4 and Nlrp3 pathways and exhibited a hepatic parenchyma similar to that in the C group. Of note, we used stereology to estimate the numerical density of goblet cells in cecum slides stained with Alcian Blue and Periodic Acid-Schiff. This technique can be used to assess the effects of different interventions on the intestinal barrier and leaky gut in further studies.

Research perspectives
This study brings novelty to the treatment of NAFLD, which is a challenge to the scientific community. Our results confirm the importance of the gut–liver axis in the pathogenesis of fatty liver and propose a combined treatment that targets the gut microbiota composition, endotoxemia, and intestinal barrier alterations to alleviate fatty liver through local anti-inflammatory effects in HF-fed mice.

ACKNOWLEDGEMENTS
The authors would like to thank Aline Penna for her technical assistance.

FOOTNOTES
Author contributions: Silva-Veiga FM, Martins FF, Daleprane JB, and Souza-Mello V designed and coordinated the study; Silva-Veiga FM, Miranda CS, Vasques-Monteiro IML, Souza-Tavares H, Martins FF, Daleprane JB, and Souza-Mello V performed the experiments, acquired and analyzed data; Silva-Veiga FM, Miranda CS, Vasques-Monteiro IML, Martins FF, Daleprane JB, and Souza-Mello V interpreted the data; Silva-Veiga FM and Souza-Mello V wrote the manuscript; all authors approved the final version of the article.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at souzamello.uerj@gmail.com.

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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Silva-Veiga FM et al. Dysbiosis and NAFLD treatment in HF-fed mice


Retrospective Cohort Study

Epidemiological characteristics of Asian children with inflammatory bowel disease at diagnosis: Insights from an Asian-Pacific multi-centre registry network

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Abstract

BACKGROUND

There remains a dearth of Asian epidemiological literature for paediatric inflam-
matory bowel disease (PIBD).

**AIM**
To describe the presenting features of PIBD from 7 Asia-Pacific pediatric gastroenterology centers via a central standardised electronic data platform.

**METHODS**
Clinical, endoscopic and radiologic data at diagnosis from the registry were extracted between 1st January 1995 to 31st December 2019. Disease phenotypic characteristics were classified as per the Paris classification system.

**RESULTS**
There was a distinct rise in new PIBD cases: Nearly half (48.6%) of the cohort was diagnosed in the most recent 5 years (2015-2019). The ratio of Crohn’s disease (CD):Ulcerative colitis (UC):IBD-Unclassified was 55.9%:38.3%:5.8%. The mean age was 9.07 years with a high proportion of very early onset IBD (VEO-IBD) (29.3%) and EO-IBD (52.7%). An over-representation of the Indian/South Asian ethnic group was observed which accounted for 37.0% of the overall Singapore/Malaysia subcohort (6.8%-9.0% Indians in census). Indian/South Asian CD patients were also most likely to present with symptomatic perianal disease ($P = 0.003$). CD patients presented with significantly more constitutional symptoms (fever, anorexia, malaise/fatigue and muscle-wasting) than UC and higher inflammatory indices (higher C-reactive protein and lower albumin levels).

**CONCLUSION**
We observed a high incidence of VEO-IBD and an over-representation of the Indian ethnicity. South Asian CD patients were more likely to have symptomatic perianal disease.

**Key Words:** Asia; Inflammatory bowel disease; Paediatrics; Crohn’s disease; Ulcerative colitis; Registry

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**Core Tip:** We describe the presenting features of paediatric inflammatory bowel disease (IBD) in 7 paediatric gastroenterology centers across six Asia-Pacific regions via a centrally-hosted electronic data capture platform. Clinical, endoscopic and radiologic data of 311 paediatric patients diagnosed with IBD between 1995 and 2019 were extracted. The ratio of Crohn’s (CD):Ulcerative colitis:IBD-Unclassified was 55.9%:38.3%:5.8%. The mean age was 9.07 years with a high proportion of very early onset IBD (VEO-IBD) and EO-IBD. An over-representation of the Indian/South Asian ethnic group was observed in the multiethnic subpopulations of Singapore and Malaysia. Patients of Indian/South Asian ethnicity were also most likely to present with symptomatic perianal CD.


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**INTRODUCTION**
The epidemiology of inflammatory bowel disease (IBD) is a dynamic and rapidly evolving landscape. Recent publications over the past decade document the rise in IBD incidence in traditionally low-prevalence regions (Asia, Africa, South America) while the incidence is somewhat stabilising in high-prevalence western nations[1]. In particular, there is an increased impetus to focus on paediatric IBD (PIBD) epidemiology as the rising incidence of childhood-onset disease appears to be driving this overall trend in certain regions[2]. Very-early onset IBD (VEO-IBD), generally defined as disease onset prior to the age of 6 years, was once considered rare but now represents the fastest growing age bracket of IBD incidence in certain countries[3]. Yet in spite of these global trends, there remains a dearth of epidemiological literature for PIBD in Asia, in particular the regions of Central, South and Southeast Asia. This begets the question whether these countries are truly ‘low prevalence’ in the absence of national IBD registries such as those established in Japan[4] and South Korea[5]. Apart from studies
published out of these East Asian cohorts, current literature is mostly limited to single centre publications[6-8] from countries in the Asia-Pacific region. Epidemiological data from these publications would only represent ‘the tip of the iceberg’ of the true burden of IBD and may not necessarily encapsulate recent regional epidemiological trends in disease incidence and behaviour.

There are numerous challenges in establishing a robust disease registry within Asia, especially with chronic diseases of emerging importance such as PIBD. For one, there is significant heterogeneity in disease awareness, diagnostic evaluation processes and therapeutic strategies across the vast Asian continent. This inevitably results in variations in case definitions with consequent epidemiological data inconsistencies. The aim of the present study was to describe the presenting features of PIBD managed in 7 paediatric gastroenterology centers across six Asia-Pacific countries and regions via a centrally-hosted electronic data capture platform using international standardised definitions.

**MATERIALS AND METHODS**

**Establishing a multi-centre Asian PIBD registry network**

The concept and goal of establishing a standardised disease data capture platform across Asian paediatric gastroenterology centres were first discussed amongst paediatric gastroenterologists practising across 7 tertiary centres in 6 Asian countries (Malaysia, Philippines, Singapore, Sri Lanka, Taiwan R.O.C., Thailand) in 2017. A consensus was established amongst the network committee members with regards to the standardisation of disease data collected both retrospectively and prospectively. Anonymised clinical data was to be collected on patient demographics, diagnostic evaluation and disease characteristics based on clinical examination, laboratory, endoscopy and radiology investigations. The diagnosis and classification of IBD into one of three disease subtypes [Crohn’s disease (CD), Ulcerative colitis (UC) and IBD-unclassified (IBD-U)] was determined by each participating centre based on the revised Porto criteria[9] as agreed within the network. The Paris Classification[10] (paediatric modification of the Montreal classification for IBD) system was then utilised to describe disease behaviour and phenotype. Electronic case report forms were thereafter loaded on a secure REDCAP data-capture platform centrally hosted by the Singapore Clinical Research Institute.

**Ethics approval**

Ethics approval for the storage of anonymised clinical data on a multi-centre central data registry hosted by the Singapore Clinical Research Institute was granted by the National Healthcare Group (NHG) Domain Specific Review Board (Approval letter for study code NUH/2019-00060 dated 23rd January 2020), followed by a separate ethics approval for data extraction and analysis (Approval letter for study code 2019/00751 dated 20th October 2021 to 19th October 2022).

**Data extraction and analysis**

For the purposes of analysis of clinical features at diagnosis, data from the registry was extracted for patients diagnosed with IBD between 1st January 1995 to 31st December 2019. The patient’s inclusion criteria were: (1) Gastroenterologist-confirmed diagnosis of IBD at the participating site; (2) Age below 18 years at the point of diagnosis; and (3) Resident (citizens, permanent residents or long-term visitors) of the country of the participating site. Medical tourists (non-resident patients visiting the country solely for the purpose for medical assessment/treatment) were excluded. We stratified the cohort (1995-2019) into five equal time-intervals, each of 5-year duration, for the intent of analysing change in IBD incidence and evolution of diagnostic practices with time.

VEO-IBD was defined by an age of onset less than 6 years and EO-IBD, by an age of onset less than 10 years. Symptomatic perianal disease was defined as perianal pain or discharge due to a fistula or abscess/collection. A ‘perianal manifestation’ of CD, symptomatic or otherwise, was defined broadly as large inflamed perianal tags, poorly healing perianal fissures ascribed to CD or perianal fistula/abscess or collection evident clinically or via imaging. South Asians were defined as people of Indian, Bangladeshi, Pakistani, Sri Lankan (Sinhalese/Tamil) or Maldivian origin. Anthropometric Z-scores for children of age 0-5 years were measured based on the World Health Organization (WHO) Child Growth Standards, while those for children aged 5-19 years were measured on the WHO Reference 2007. Linear growth failure was defined as a height Z-score of -2 and lower.

Disease severity was defined based on the patients’ respective Paediatric Crohn’s Disease Activity Index (PCDAI) (Inactive: ≤ 10, mild: 11-30, moderate-severe: > 30)[11] and Paediatric Ulcerative Colitis Activity Index (Inactive: < 10, mild: 10-34, moderate: 35-64, severe ≥ 65)[12] scores.

The date of IBD diagnosis was taken as the date of diagnostic endoscopy. For the purposes of this publication discussing solely the presenting clinical features at diagnosis, we only included clinical data occurring within 3-mo prior or after the date of diagnosis and before any therapeutic intervention.

Statistical analysis was done through SPSS Version 27 whereby we compared presenting symptoms, biochemical indices and growth indices between the key phenotypic subgroups (CD and UC); the overall effect of ethnicity on IBD phenotype and behaviour. Continuous variables (laboratory invest-
igation values, anthropometric indices) were compared through the student’s t-test while most of the other outcomes (clinical findings, disease phenotype and behaviour) were classified as categorical variables/dichotomous outcomes and these were compared through the chi-square test. A P value of less than 0.05 was deemed as a significant difference in outcomes, otherwise stated as ‘NS’ (non-significant). Whereby multivariate analysis was required in determining the effect of South Asian ethnicity on the incidence of perianal disease in CD adjusting for inter-ethnic variability in disease phenotype, logistic regression was used to calculate an adjusted odds ratio.

RESULTS

Three hundred and eleven children were recruited from seven participating tertiary centres across six countries within the Asia-Pacific region. There was a distinct rise in the number of new PIBD cases recorded across the network, with nearly half (48.6%, 151/311) of the entire cohort being diagnosed in the most recent 5 years (2015-2019) of recruitment (Figure 1). Table 1 illustrates the overall distribution of CD vs UC vs IBD-U, in which the CD:UC ratio was approximately 1.5:1 while the proportion of IBD-U was 5.8%. While there were more males in the overall cohort (58.2% male), we did not observe any significant gender predilection across the three disease subtypes: Male CD 58.0% vs male UC 57.0% vs male IBD-U 66.7% (P = 0.75).

High incidence of VEO and EO disease

The mean age of disease presentation was 9.07 years for the entire IBD cohort which is within the age definition of EO disease. UC patients presented at a significantly younger age than CD patients (7.73 years vs 10.18 years, P < 0.001), with the mean age of IBD-U patients (7.34 years) similar to that of UC. When stratifying age of onset into VEO disease and EO disease, 29.3% of the entire cohort was classified as VEO-IBD and more than half (52.7%) was early-onset. There was a significantly higher proportion of VEO-IBD and EO-IBD presenting as a UC phenotype as compared to CD (Table 1). Across a period of 20 years, the proportions of VEO-IBD and EO-IBD remained relatively stable, approximately at 30% and 50% of the cohort per time interval respectively (Figure 2).

Over-representation of the Indian/South Asian ethnicity

For a meaningful interpretation of ethnic predilection amongst Asian IBD patients, the multi-racial sub cohorts from Singapore and Malaysia were chosen as the other participating sites were relatively more homogeneous in ethnic group distribution. The three main ethnic groups in these two countries are Chinese, Indian/South Asian and Malay as listed in Table 2[13,14]. A distinct over-representation of the Indian/South Asian ethnic group was observed, with Indians/South Asians accounting for 37.0% of the overall Singapore/Malaysia IBD cohort relative to a minority representation of 6.8%-9.0% in both the countries’ respective population census. There was a significantly higher proportion of UC patients relative to CD amongst the Malay ethnic group (CD:UC ratio 0.7, P = 0.037).

Increased constitutional symptoms, extraintestinal manifestations and higher inflammatory indices in CD

Abdominal pain (P = 0.004) and clinically evident abdominal tenderness (P = 0.003), fever (P < 0.001), loss of appetite (P = 0.001) and malaise/fatigue (P = 0.016) were significantly more common amongst CD patients relative to UC. While there was a non-significant higher proportion of CD patients presenting with weight loss (46.6% CD vs 37.0% UC), clinically evident muscle wasting was significantly more common in CD (14.4% CD vs 5.9% UC, P = 0.022).

Although CD patients had numerically lower mean weight Z-scores [-1.4] vs [-1.0]) and height Z-scores [-0.8] vs [-0.6]) than UC, only the mean body-mass index Z-score of CD patients was significantly lower [-1.5] vs [-0.9], P = 0.013. There were approximately similar rates of linear growth failure across the three disease subtypes: 18.4% CD vs 16.1% UC vs 17.6% IBD-U (Table 3). There was a non-significant higher proportion of patients with joint pains (7.5% CD vs 5% UC) and clinically evident arthritis (6.9% CD vs 2.5% UC) in CD patients. Other extraintestinal manifestations (EIMs) such as oral ulcers (20.7% CD vs 17.6% UC, P = 0.001) and erythema nodosum (4.6% CD vs 0.0% UC, P = 0.018) were more common in CD than UC. Hepatic involvement (hepatomegaly and elevated transaminases) was however more commonly observed in UC than CD, consistent with a significantly higher association of autoimmune liver disease/primary sclerosing cholangitis with UC than CD (17.6% UC vs 11.1% CD, P < 0.001).

CD patients also presented with significantly higher C-reactive protein (CRP) levels (mean 54.6 CD vs 20.1 UC, P < 0.001) and lower albumin levels relative to UC. However, there was no significant difference in mean haemoglobin and proportion of severe anaemia (defined as ≤ 7.0 g/dL: 4.0% CD vs 5.0% UC, P = 0.601) at presentation between CD and UC.

Faecal calprotectin testing

16.1% of the cohort had a faecal calprotectin (50/211) assayed at baseline. There was no significant
Table 1 Demographic features of 311 children with inflammatory bowel disease in 6 Asia-Pacific regions

<table>
<thead>
<tr>
<th>IBD subtypes</th>
<th>ALL IBD, n = 311</th>
<th>CD, n = 174 (55.9%)</th>
<th>UC, n = 119 (38.3%)</th>
<th>IBD-U, n = 18 (5.8%)</th>
<th>P value (CD vs UC)</th>
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<tbody>
<tr>
<td>Country (n, row %)</td>
<td></td>
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<tr>
<td>Malaysia</td>
<td>95 (30.6)</td>
<td>47 (49.5)</td>
<td>9 (9.5)</td>
<td></td>
<td></td>
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<tr>
<td>Philippines</td>
<td>29 (9.4)</td>
<td>1 (3.4)</td>
<td>4 (13.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singapore</td>
<td>108 (34.6)</td>
<td>49 (45.4)</td>
<td>0 (0.0)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>19 (6.1)</td>
<td>7 (36.8)</td>
<td>2 (10.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan R.O.C.</td>
<td>19 (6.1)</td>
<td>0 (0.0)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>41 (13.2)</td>
<td>15 (36.6)</td>
<td>3 (7.3)</td>
<td></td>
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</tr>
<tr>
<td>Mean age at diagnosis, yr (SD)</td>
<td>9.07 (4.56)</td>
<td>10.18 (4.34)</td>
<td>7.73 (4.39)</td>
<td>7.34 (5.1)</td>
<td>&lt; 0.001</td>
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<tr>
<td>Age groups (n, column %)</td>
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</tr>
<tr>
<td>Very early onset &lt; 6 yr</td>
<td>91 (29.3)</td>
<td>30 (17.2)</td>
<td>51 (42.9)</td>
<td>10 (55.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Early onset &lt; 10 yr</td>
<td>164 (52.7)</td>
<td>75 (43.1)</td>
<td>78 (65.5)</td>
<td>11 (61.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex (n, column %)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>181 (58.2)</td>
<td>101 (58.0)</td>
<td>68 (57.1)</td>
<td>12 (66.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>130 (41.8)</td>
<td>73 (42.0)</td>
<td>51 (42.9)</td>
<td>6 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>

CD: Crohn’s disease; IBD-U: Inflammatory bowel disease- Unclassified; UC: Ulcerative colitis.

Table 2 Ethnic distribution of inflammatory bowel disease vs population census in countries with multiracial populations (Singapore and Malaysia)

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Ethnic group (n, column %)</td>
<td></td>
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</tr>
<tr>
<td>Chinese</td>
<td>70/203 (36.5)</td>
<td>74.3</td>
<td>38 (41.3)</td>
<td>31 (34.1)</td>
<td>1 (11.1)</td>
<td>1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Indian/South Asian</td>
<td>71/203 (37.0)</td>
<td>9.0</td>
<td>35 (38.0)</td>
<td>33 (36.3)</td>
<td>3 (33.3)</td>
<td>1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Malay</td>
<td>51/203 (26.6)</td>
<td>13.5</td>
<td>19 (20.7)</td>
<td>27 (29.7)</td>
<td>5 (55.6)</td>
<td>0.7</td>
<td>0.037</td>
</tr>
</tbody>
</table>

CD: Crohn’s disease; IBD-U: Inflammatory bowel disease-Unclassified; UC: Ulcerative colitis.

difference between the mean values in CD and UC (Table 3). Amongst children with CD, faecal calprotectin values were lower in patients with isolated ileal disease (L1) compared to ileocolonic (L3) disease (468.7 in L1 vs 749.2 μg/g in L3, P = 0.05). No discernible difference in mean faecal calprotectin values was noted amongst children with UC; left sided disease (E1/E2: 547.0 μg/g) vs more extensive disease (E3/E4: 555.6 μg/g).

Small bowel imaging and endoscopy
There was a progressive decline in the use of small bowel follow through studies across the cohort. Since 2010, there has been increasing use of either MR enterography or intestinal ultrasound although approximately 60% of the cohort have not had any form of small bowel imaging (Figure 3). Complete endoscopy as per Porto guidelines (upper and lower endoscopy) was performed in 79.1% of the cohort.

Disease behaviour and phenotype of CD patients
The ileocolonic location was the most common disease location in the entire cohort of CD patients, with pure inflammatory disease being most common (90.7%) followed by luminal penetrating disease (5.3%) and strictureing disease (3.3%). Isolated L4 disease (absence of terminal ileal or colonic disease) was relatively uncommon at 4.0% of the CD cohort. Over half of the CD patients had at least moderate
### Table 3 Clinical features in different subtypes of PIBD in the Asia-Pacific region

<table>
<thead>
<tr>
<th>IBD subtypes</th>
<th>CD, n = 174 (55.9%)</th>
<th>UC, n = 119 (38.3%)</th>
<th>IBD-U, n = 18 (5.8%)</th>
<th>P value (CD vs UC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptoms at diagnosis (n, column %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>107 (61.5)</td>
<td>53 (44.5)</td>
<td>7 (38.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>Chronic diarrhoea</td>
<td>104 (59.8)</td>
<td>69 (58.0)</td>
<td>10 (55.6)</td>
<td>0.760</td>
</tr>
<tr>
<td>Bloody diarrhoea</td>
<td>60 (34.5)</td>
<td>89 (74.8)</td>
<td>11 (61.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Weight loss</td>
<td>81 (46.6)</td>
<td>44 (37.0)</td>
<td>9 (50.0)</td>
<td>0.104</td>
</tr>
<tr>
<td>Fever</td>
<td>66 (37.9)</td>
<td>21 (17.6)</td>
<td>2 (11.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>57 (32.8)</td>
<td>19 (16.0)</td>
<td>6 (33.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Malaise/fatigue</td>
<td>28 (16.1)</td>
<td>8 (6.7)</td>
<td>1 (5.6)</td>
<td>0.016</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>36 (20.7)</td>
<td>1 (0.8)</td>
<td>0 (0.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Perianal symptoms (pain/discharge)</td>
<td>23 (13.2)</td>
<td>3 (2.5)</td>
<td>1 (5.6)</td>
<td>0.002</td>
</tr>
<tr>
<td>Joint pains</td>
<td>13 (7.5)</td>
<td>6 (5.0)</td>
<td>0 (0.0)</td>
<td>0.407</td>
</tr>
<tr>
<td><strong>Physical signs at diagnosis (n, column %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pallor</td>
<td>68 (39.1)</td>
<td>32 (26.9)</td>
<td>5 (27.8)</td>
<td>0.031</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>8 (4.6)</td>
<td>12 (10.1)</td>
<td>2 (11.1)</td>
<td>0.067</td>
</tr>
<tr>
<td>Abdominal tenderness</td>
<td>47 (27.0)</td>
<td>15 (12.6)</td>
<td>2 (11.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Asymptomatic perianal tags</td>
<td>25 (14.4)</td>
<td>3 (2.5)</td>
<td>1 (5.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Perianal fissure</td>
<td>20 (11.5)</td>
<td>2 (1.7)</td>
<td>1 (5.6)</td>
<td>0.002</td>
</tr>
<tr>
<td>Muscle wasting</td>
<td>25 (14.4)</td>
<td>7 (5.9)</td>
<td>1 (5.6)</td>
<td>0.022</td>
</tr>
<tr>
<td>Arthritis</td>
<td>12 (6.9)</td>
<td>3 (2.5)</td>
<td>0 (0.0)</td>
<td>0.095</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>8 (4.6)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>Anthropometric parameters (SD)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean weight Z-score</td>
<td>-1.4 (1.7)</td>
<td>-1.0 (1.7)</td>
<td>-0.7 (2.2)</td>
<td>0.110</td>
</tr>
<tr>
<td>Mean height Z-score</td>
<td>-0.8 (1.5)</td>
<td>-0.6 (1.6)</td>
<td>-0.6 (2.4)</td>
<td>0.583</td>
</tr>
<tr>
<td>Growth failure (Height Z-score less than -2) (%)</td>
<td>18.4</td>
<td>16.1</td>
<td>17.6</td>
<td>0.907</td>
</tr>
<tr>
<td>Mean BMI Z-score</td>
<td>-1.5 (1.8)</td>
<td>-0.9 (1.7)</td>
<td>-0.6 (1.8)</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>Laboratory investigations at diagnosis (SD)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean haemoglobin, g/dL</td>
<td>10.6 (1.9)</td>
<td>11.0 (2.5)</td>
<td>10.9 (1.5)</td>
<td>0.221</td>
</tr>
<tr>
<td>Mean ESR, mm/hr</td>
<td>50.8 (30.5)</td>
<td>42.6 (34.4)</td>
<td>34.9 (32.2)</td>
<td>0.063</td>
</tr>
<tr>
<td>Mean C-reactive protein, mg/L</td>
<td>54.6 (55.0)</td>
<td>20.1 (24.3)</td>
<td>7.1 (9.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean albumin, g/dL</td>
<td>33.0 (8.7)</td>
<td>35.4 (8.4)</td>
<td>37.2 (7.1)</td>
<td>0.040</td>
</tr>
<tr>
<td>Mean ALT, U/L</td>
<td>18.5 (20.5)</td>
<td>72.7 (162.0)</td>
<td>20.4 (13.4)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean AST, U/L</td>
<td>27.0 (32.9)</td>
<td>84.2 (170.7)</td>
<td>30.5 (10.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Mean GGT, U/L</td>
<td>38.3 (79.8)</td>
<td>126.7 (160.8)</td>
<td>30.8 (40.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean faecal calprotectin, μg/g</td>
<td>783 (1009.0)</td>
<td>583.4 (353.1)</td>
<td>593.5 (300.5)</td>
<td>0.447</td>
</tr>
</tbody>
</table>

ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; CD: Crohn’s disease; ESR: Erythrocyte sedimentation rate; GGT: Gamma-glutamyl transferase; IBD-U: Inflammatory bowel disease-Unclassified; UC: Ulcerative colitis.

disease severity at presentation (54.3%), with no differences in mean PCDAI scores noted with the South Asian subgroup. Upper gastrointestinal involvement was seen in 49.0% of the entire CD cohort. Symptomatic perianal disease was seen in 13.2% of the CD cohort and up to 30.5% of the CD cohort had some form of perianal manifestation as earlier defined.
South Asian CD patients were strongly associated with perianal symptoms at presentation ($P = 0.003$), the physical finding of asymptomatic perianal tags (15.5% South Asian vs 7.5%, $P = 0.042$), as well as any form of perianal manifestation (symptomatic and asymptomatic, detected on either clinical examination or perianal imaging; $P = 0.003$). Adjusting for inter-ethnic differences in CD incidence, the
South Asian ethnicity remained strongly associated with symptomatic perianal disease at presentation (B co-efficient 1.264, \( P = 0.001 \)). Sri Lanka was the only participating South Asian site in this study, and only 1 out of 19 Sri Lankan IBD patients had presented with symptomatic perianal disease. All the South Asian CD patients in this subgroup analysis (Table 4) were either first- or second-generation immigrants within Singapore or Malaysia.

**Disease behaviour and phenotype of UC patients**

Overall, pan colonic involvement was the most common location in UC (72.6%) and almost half (48.8%) of UC patients had at least moderate disease severity at presentation. South Asian UC patients had slightly less cases of isolated proctitis than the non-South Asian UC sub cohort (6.9% proctitis South Asian vs 7.8% non-South Asian).

**DISCUSSION**

This first publication from the Asian PIBD registry gives valuable insights into the distinct epidemiological patterns of a rapidly emerging chronic disease in the Asia-Pacific region. Existing literature from published adult cohorts documents the distinct rapid rise in overall IBD incidence across the Asia-Pacific region[15] ascribed to increased urbanisation and industrialisation of developing areas with a consequent change in lifestyle and environmental factors. Our pooled data across major paediatric gastroenterology centres in the Asia-Pacific region verifies the rise in PIBD incidence, particularly in the last decade of data collection (2010-2019). This is also consistent with rising trends observed in other Asian paediatric cohorts in Saudi Arabia[16], Bahrain[17], Japan[18], South Korea[19] and China[20]. There still remains a knowledge gap on the epidemiological trends of PIBD in many parts of Asia, particularly Central and South Asian regions, although recently published studies out of Kazakhstan[21], India[22] and Nepal[23] reaffirm IBD as an emerging health issue.

Several other factors have been proposed as reasons for the observed rise in IBD incidence within the Asia-Pacific region, namely increased disease awareness amongst healthcare professionals, improved access to healthcare resources and better diagnostic modalities in rapidly industrialising areas. While such factors may account for part of the initial observed rise in incidence in the earlier decades, they do not sufficiently explain the sustained year-on-year rise in PIBD incidence seen in developed regions of our network[6,24] as well as other highly industrialised regions in Japan and South Korea.

The key strength of a multi-centre registry in this ethnically and culturally diverse region would be to allow epidemiological comparisons amongst multi-racial Asian populations (e.g., Singapore/Malaysia) and ethnically homogeneous populations (e.g., Philippines/Sri Lanka/Thailand). In Singapore and Malaysia where the Chinese and Malay populations form the majority ethnic group respectively and the Indians are an ethnic minority, we affirm earlier findings from adult Southeast Asian cohorts[25,26] that there is an ethnic predisposition to IBD incidence with an over-representation of the Indian/South Asian subgroup (37% IBD vs 6%-9% general population). This is similar to observations in the United Kingdom and Canada where IBD is seen with greater frequency amongst both South Asians born in the United Kingdom[27] and South Asian migrants to the United Kingdom[28], as well as the paediatric South Asian community in British Columbia, Canada[29]. Similarly, in a study of 30812 IBD subjects diagnosed in the United States, Malhotra et al[30] found that residents of Indian ancestry carried the highest risk for all types of IBD, compared to residents of Jewish, East Asian or Hispanic ancestries.

With combined Singaporean and Malaysian data, we were able to discern unique characteristics of each ethnic group. The CD:UC ratio varied distinctly across the three ethnic groups (Chinese 1.2; Indian/South Asian 1.0 and Malay 0.7) and this is likely attributed to inter-ethnic differences in environmental factors, such as diet, diversity in gut microbiome[31] including *Helicobacter pylori* (H. pylori)[32] as well as socio-economic status[33]. This inter-ethnic variation in IBD incidence has been observed between African Americans and white Americans previously[34]; white Americans were reported to have a CD:UC ratio > 1.0 with Hispanic Americans having an inverse ratio[35], paralleling what is observed between Chinese and Malays in our cohort. A similar variegation of CD:UC ratio was seen in a Latin American systematic review by Kotze et al[36], in which only certain states in Brazil reported a CD:UC ratio > 1 whereas most of the other Latin American countries had a CD:UC ratio < 1. The authors concluded similarly that regions within Latin America that experienced higher degrees of economic development and ‘Westernisation’ had a higher proportion of CD cases.

We also observed a strongly significant association between the South Asian ethnicity and symptomatic perianal disease (pain or perianal discharge) at presentation, only in the South Asian populations of Singapore and Malaysia. Interestingly, despite less South Asians having the CD phenotype than Chinese, the South Asian ethnicity was significantly associated with asymptomatic perianal tags. The latter physical finding may be a prelude to developing symptomatic perianal Crohn’s later in the clinical course, as seen in this study by Singer et al[37] showing a higher rate of perianal fistulisation in patients initially presenting with non-penetrating perianal lesions. The observation of increased rates of symptomatic perianal disease was not appreciated in the native South Asian population of Sri Lanka within the limits of small patient numbers recruited in this sub cohort. A
## Table 4 Disease characteristics of Crohn’s disease and ulcerative colitis

<table>
<thead>
<tr>
<th>Disease characteristics</th>
<th>N</th>
<th>SA</th>
<th>P value (SA vs non-SA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Disease severity based on PCDAI score (n, column %)
  Inactive (≤ 10)                                              | 17 (12.3) | 4 (14.3) | NS                     |
  Mild (11-30)                                                 | 46 (33.5) | 9 (32.1) | 0.505 (SA)             |
  Moderate-severe (> 30)                                       | 75 (54.3) | 15 (53.6) | 0.353 (SA)             |
| Disease location (n, column %)
  L1 (distal 1/3 ileum +/- limited caecal disease)             | 28 (18.7) | 4 (12.5) | 0.505 (SA)             |
  L2 (colonic)                                                 | 54 (36.0) | 10 (31.3) | 0.353 (SA)             |
  L3 (ileocolonic)                                             | 62 (41.3) | 16 (50.0) | 0.353 (SA)             |
  Isolated L4 disease                                          | 6 (4.0)  | 2 (6.3)  | 0.030 (SA)             |
  L4a                                                          | 59 (40.7) | 15 (45.4) | 0.973 (SA)             |
  L4b                                                          | 12 (8.3)  | 3 (9.1)  | 0.973 (SA)             |
  No L4 disease                                                | 74 (51.0) | 17 (51.3) | 0.973 (SA)             |
| Disease behaviour (n, %)
  B1 (inflammatory)                                            | 136 (90.7) | 31 (93.9) | 0.858 (SA)             |
  B2 (stricturing)                                             | 5 (3.3)  | 1 (3.0)  | 0.353 (SA)             |
  B3 (penetrating)                                             | 8 (5.3)  | 1 (3.0)  | 0.353 (SA)             |
  B2/B3                                                       | 1 (0.7)  | 0 (0.0)  | 0.353 (SA)             |
| Perianal involvement (n, %)
  Perianal symptoms at presentation                             | 23 (13.2) | 10 (28.6) | 0.003 (SA)             |
  Any form of perianal manifestation (clinical exam or imaging)| 53 (30.5) | 18 (51.4) | 0.003 (SA)             |
| **UC**                                                       |       |          |                        |
| Disease severity based on PUCAI score (n, column %)
  Inactive (< 10)                                              | 7 (8.3)  | 0 (0.0)  | 0.353 (SA)             |
  Mild (10-34)                                                 | 36 (42.9) | 11 (47.8) | 0.353 (SA)             |
  Moderate (35-64)                                             | 28 (33.3) | 9 (39.1)  | 0.353 (SA)             |
  Severe (≥ 65)                                                | 13 (15.5) | 3 (13.0)  | 0.353 (SA)             |
| Disease location (n, column %)
  E1 (proctitis)                                                | 8 (7.5)  | 2 (6.9)  | 0.353 (SA)             |
  E2 (left sided)                                              | 16 (15.1) | 5 (17.2)  | 0.353 (SA)             |
  E3 (extensive)                                               | 5 (4.7)  | 5 (17.2)  | 0.353 (SA)             |
  E4 (pancolitis)                                              | 77 (72.6) | 17 (58.6) | 0.353 (SA)             |
| Disease severity (n, column %)
  S0 (never severe)                                            | 79 (78.2) | 20 (74.1) | 0.542 (SA)             |
  S1 (ever severe)                                             | 22 (21.8) | 7 (25.9)  | 0.542 (SA)             |

1Disease activity scores available only for 138/174 Crohn’s disease patients.
2Disease severity scores available for 84/119 Ulcerative colitis patients.
CD: Crohn’s disease; PCDAI: Pediatric Crohn’s Disease Activity Index; PUCAI: Pediatric Ulcerative Colitis Activity Index; SA: South Asian; UC: Ulcerative colitis.

Previous questionnaire survey conducted in India estimated the incidence of perianal disease at presentation in Indian paediatric CD patients at 18.0%, which is not strikingly higher than paediatric cohorts in Israel (13.3%) and Canada (16.0%) [38,39]. More recent Indian studies, which included...
perianal disease developed both at diagnosis and subsequent follow-up, however, have found even lower rates than other Asian cohorts. Banerjee et al.[40] reported 7.4% perianal involvement amongst CD patients and a multicentre paediatric study in India reported 10.9% of CD patients with perianal disease(fistula or abscess). These findings suggest South Asian CD patients in their native countries do not experience disproportionately high rates of perianal disease.

On the contrary, countries with a high South Asian immigrant population report findings congruent with our cohort’s. A previous paediatric study in San Francisco likewise demonstrated similar findings in our cohort, where South Asians tended to have more perianal fistulising disease at presentation than their Caucasian counterparts[41]. A recent adult cohort study published by Jangi et al.[42] also showed South Asian CD patients in the United States were more likely to experience symptomatic perianal disease than white patients. Current findings from our study and the above-mentioned cohorts may suggest these phenotypic trends may be unique only to South Asians who have migrated from their native countries to relatively more developed and urbanised regions. A possible explanation is that South Asians may carry an intrinsic genetic predisposition for IBD, which results in a distinct disease phenotype in the setting of specific environmental exposures after migration[43]. A review by Foster and Jacobson[44] discusses these hypothetical gene-environmental interactions in South Asian immigrants, postulating the role of dietary changes from a traditionally high carbohydrate/low saturated fat diet to one which is highly processed. Other possible factors discussed include an increased exposure to urban pollutants and a reduced exposure to potentially protective factors such as vitamin D, H. pylori and helminths.

Overall, we do report a moderately high rate of perianal involvement in our CD patients at presentation (13.2%) with symptomatic perianal disease and up to 30.5% with some form of perianal manifestation of CD either clinically detected or via imaging. An earlier Singaporean paediatric cohort reported a 21.6% incidence of perianal disease amongst CD patients at diagnosis[45] with mainland Chinese and South Korean paediatric CD cohorts reporting even higher rates of perianal involvement 42.4% Shanghai[45]. 47.1% [(perianal fistulising disease) only South Korean[46]]. Some variability in the reported rates of perianal involvement across CD cohorts could be accounted by differences in disease definitions as well as the extent of perianal imaging performed, which may detect indolent perianal fistulising disease in otherwise asymptomatic patients. A large IBD cohort of 5223 white Americans, 35 United States-born Asians and 81 Asian immigrants also reported Asians having a significantly higher rate of perianal disease (at presentation and follow-up) than whites (33% vs 18%)[47]. Further prospective follow-up of our cohort is required to ascertain if an even greater proportion of CD patients will evolve to develop perianal disease beyond the point of diagnosis.

Interestingly, we also found a very high proportion of VEO-IBD (29.3%) in our cohort, which appears to be stably high across a 20-year period of analysis. In comparison, data from the EPIMAD registry in France (n = 1412) reported a VEO-IBD rate of 3.0% based on a similar age definition for VEO-IBD[48]. Other Asian cohorts have similarly reported comparatively high rates of VEO-IBD, albeit using varying ages for definition. A single centre study in Beijing, Mainland China (n = 184) reported 41.8% of hospitalised children with IBD were VEO[49]; a cohort in Saudi Arabia reported 15.9% of patients were younger than 4 years at time of onset[16] and a recent Indian multicentre paediatric study had reported 19.1% of patients being VEO[22]. It is not certain if this epidemiologic trend is unique to regions where IBD is of emerging importance, as a Canadian study across 5 provinces reported VEO-IBD as the subgroup with the most rapid increase in incidence relative to the other age groups[2].

Aside from the South Asian predisposition and younger age of onset, the clinical presentation and extra-intestinal manifestations of CD and UC were similar to other Asian and Western paediatric and adult cohorts[50]. Abdominal pain and constitutional symptoms (fever, loss of appetite, oral ulcers, malaise) were more common presenting features in CD than UC; bloody diarrhea and associated liver disease/primary sclerosing cholangitis were more common in UC at presentation, the latter being a well-established association. EIMs such as arthritis and erythema nodosum were more common in CD than UC, as also seen in a Mainland Chinese cohort[20]. We also reaffirm the finding of significant heterogeneity of CRP response between CD and UC, as discussed extensively in published literature[51, 52]. Within the limits of a small sample size, we noted that faecal calprotectin values vary with disease location in CD but not in UC. Previous correlation studies have also similarly observed lower values of faecal calprotectin in CD patients with pure ileal disease[53].

While CD patients had numerically lower weight Z-scores and height Z-scores than UC patients, these comparisons were not statistically significant. This is due to a relatively high rate of linear growth failure (16.1%) reported in UC patients in our cohort, which is higher than the typical prevalence of 3%-10% previously described[54]. It is uncertain if this truly reflects a unique UC phenotype or whether some of these UC patients will be re-classified as CD with evolution of disease and/or further diagnostic modalities performed. As approximately 60% of our cohort did not undergo any form of small bowel imaging and 20% did not undergo an upper gastrointestinal endoscopy, we could expect changes in these initial diagnostic labels had more complete evaluation been performed.

There are a few limitations to our study; firstly, as most of the data was collected retrospectively, we were not able to compute patient disease activity indices in all patients because of incomplete clinical, biochemical, imaging or endoscopy records. Secondly, a lack of access and/or consistency in performing small bowel imaging and upper gastrointestinal endoscopy could have resulted in inaccurate disease
classification. However, as almost half of our cohort was diagnosed in the recent 5 years, we are confident that the disease phenotype has been more accurately assessed and documented in these later years.

Another limitation was the predominance of Southeast Asian participating centres in our network, with the lack of East and West Asian representation. However, we were able to mitigate this as there already exists a substantial amount of published literature from established Japanese, South Korean and mainland Chinese cohorts as previously cited. Hence, we were able to compare certain epidemiological features such as age of onset and the proportion of perianal disease as discussed above.

Lastly, the number of patients captured within our network may only represent ‘the tip of an iceberg’. We acknowledge that access to healthcare services may be very limited in rural regions of the participating countries, and there may be an inevitable selection bias in recruiting patients living in the proximity of major Asian city centres (Singapore, Kuala Lumpur, Bangkok, Manila, Colombo, Chinese Taipei) where our participating sites are located. Hence, there could be a number of children with IBD who do not present to centres equipped with adequate disease knowledge and diagnostic capability, and thus remain undiagnosed. At the same time, there could be a number of adolescent IBD patients who are managed by adult gastroenterologists. The general lack of national chronic disease registries for PIBD in the respective countries of this network further adds to the challenges in obtaining complete epidemiologic data. These factors discussed suggest the true burden of PIBD in the Asia-Pacific region is very likely under-estimated.

CONCLUSION

Our study presents epidemiological data from the largest multi-centre Asian-Pacific paediatric cohort to date, and reaffirms the rising trend of PIBD across our registry, particularly in the past decade (2010-2019). We also report a substantially higher incidence of VEO-IBD than European cohorts and this is similarly observed in other Asian cohorts in mainland China and the Middle East. The unique multi-ethnic demographic composition of our cohort allows for distinct phenotypic differences to be seen between ethnicities, chiefly the over-representation of the Indian/South Asian ethnicity and its strong association with symptomatic perianal CD. Prospective follow-up data from this registry would also ascertain if any of these observed epidemiologic trends within this publication have implications on medium to longer term disease outcomes.

This inaugural publication from our registry likely under-estimates the true burden of IBD in Asia, and the way forward to address these identified knowledge gaps, would be to encourage more tertiary centres from diverse parts of Asia to partake in the Asian PIBD registry. This will improve the representativeness of our cohort and enhance the ability to make both intra- and inter-regional comparisons in disease behaviour and phenotypic trends.

ARTICLE HIGHLIGHTS

Research background
There remains a dearth of epidemiological literature on paediatric inflammatory bowel disease (IBD) in most parts of Asia. While there have been several published cohort studies out of East Asia, little is known on the actual disease burden beyond isolated case series and single-centre studies in the rest of the Asia-Pacific region. This would represent the first cohort study from a multi-centre Asian-Pacific PIBD registry first initiated in 2017.

Research motivation
The main issue was a lack of a standardised data platform in Asian-Pacific paediatric gastroenterology centres. A standardised data platform is vital to allow an accurate and validated way of reporting the disease behaviour and phenotype of PIBD across different healthcare systems in the heterogeneous Asia-Pacific region. This facilitates meaningful epidemiological comparisons with other established PIBD cohorts from North America and Western Europe.

Research objectives
This cohort study describes the epidemiological characteristics of all PIBD patients at the point of initial presentation, whom are managed at one of seven paediatric gastroenterology centres in the Asian-Pacific region (Singapore, Sri Lanka, Malaysia, Thailand, Philippines, Taiwan R.O.C). The objectives were to establish if there were unique disease characteristics of PIBD in the Asia-Pacific region in contrast to East Asian and Caucasian cohorts, as well as inter-ethnic disease phenotypic differences in the multi-ethnic populations of Asia-Pacific.
Research methods
Standardised disease data collection forms using existing validated disease activity indices and disease classification systems for PIBD were stored electronically on a centrally-hosted secure REDCAP platform. Participating Asian-Pacific sites were invited to enrol patients with an established diagnosis of IBD from the point of initial presentation.

Research results
Epidemiological data from our registry demonstrates a rapid rise in PIBD incidence, particularly in the last 5 years. The unique disease characteristics of our Asian-Pacific cohort include a large proportion of very-early onset (VEO)-IBD (29.3%) similar to the high proportions of VEO disease reported from mainland China, Saudi Arabia and India. There is also a relative over-representation of the Indian/South Asian ethnicity in the multi-ethnic countries of Singapore and Malaysia. Patients of Indian ethnicity with Crohn’s disease (CD) were also most likely to present with symptomatic perianal disease.

Research conclusions
The rise in PIBD incidence across the Asian-Pacific region is consistent with the observed rise in incidence in many other global cohorts. The high proportion of very early onset IBD in the Asia-Pacific region may represent a shift in epidemiological trends in PIBD, as other established IBD cohorts similarly report the fastest growth in IBD incidence amongst very young children. The higher incidence of symptomatic perianal CD amongst Indian patients in Singapore/Malaysia suggests inter-ethnic genetic differences may cause variability in disease behaviour.

Research perspectives
Future research should be targeted at establishing national disease data registries to fully encapsulate the true burden of IBD in the Asia-Pacific region, and prospective follow-up data from this multi-centre registry is essential to determine if any of the observed epidemiological trends at diagnosis have implications on longer term outcomes.

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FOOTNOTES

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**L-Editor:** Filipodia  
**P-Editor:** Wang JJ

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Retrospective Study

Clinical outcomes of endoscopic papillectomy of ampullary adenoma: A multi-center study

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Abstract

BACKGROUND
Ampullary adenoma is a rare premalignant lesion, but its incidence is increasing. Endoscopic papillectomy has become the first treatment of choice for ampullary adenomas due to its safety and effectiveness, thereby replacing surgical resection. However, recurrence rates and adverse events after endoscopic papillectomy were reported in up to 30% of cases.

AIM
To review the long-term outcomes of endoscopic papillectomy and investigate the factors that affect these outcomes.

METHODS
We retrospectively analyzed the data of patients who underwent endoscopic papillectomy for ampullary adenoma at five tertiary hospitals between 2013 and 2020. We evaluated clinical outcomes and their risk factors. The definitions of outcomes were as follow: (1) curative resection: complete endoscopic resection without recurrence; (2) endoscopic success: treatment of ampullary adenoma with endoscopy without surgical intervention; (3) early recurrence: reconfirmed adenoma at the first endoscopic surveillance; and (4) late recurrence: reconfirmed adenoma after the first endoscopic surveillance.
RESULTS
A total of 106 patients were included for analysis. Of the included patients, 81 (76.4%) underwent curative resection, 99 (93.4%) had endoscopic success, showing that most patients with non-curative resection were successfully managed with endoscopy. Sixteen patients (15.1%) had piecemeal resection, 22 patients (20.8%) had shown positive/uncertain resection margin, 11 patients (16.1%) had an early recurrence, 13 patients (10.4%) had a late recurrence, and 6 patients (5.7%) had a re-recurrence. In multivariate analysis, a positive/uncertain margin [ Odds ratio (OR) = 4.023, \( P = 0.048 \)] and piecemeal resection (OR = 6.610, \( P = 0.005 \)) were significant risk factors for early and late recurrence, respectively. Piecemeal resection was also a significant risk factor for non-curative resection (OR = 5.424, \( P = 0.007 \)). Twenty-six patients experienced adverse events (24.5%).

CONCLUSION
Endoscopic papillectomy is a safe and effective treatment for ampullary adenomas. Careful selection and follow-up of patients is mandatory, particularly in cases with positive/uncertain margin and piecemeal resection.

Key Words: Endoscopic papillectomy; Ampullary adenoma; Clinical outcome; Recurrence; Adverse event

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Core Tip: This is a multi-center study evaluating the clinical outcomes of 106 patients who underwent endoscopic papillectomy for ampullary adenoma. In our results, margin-positive/uncertain pathologic reports and piecemeal resection were significant factors for the curative resection and recurrences. Unexpectedly, many recurrences were observed in margin-negative resection, but in most cases, they were successfully managed with minimally invasive endoscopic therapies. Since there is no definite factor for predicting and preventing recurrence and re-recurrence, regular follow-up with endoscopy should be performed in every patient regardless of resection margin or resection type, especially in patients with margin-positive/uncertain and piecemeal resection.

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INTRODUCTION
Ampullary adenomas (AAs) are rare lesions, with a prevalence of 0.04%-0.12% in autopsy, and account for 0.2%-5% of newly diagnosed intestinal neoplasms[1-3]. As the number of endoscopic surveillance or computed tomography (CT) scans has increased, the number of AAs detected has also increased. Patients with AA are often asymptomatic, and other complaints are related to biliary or pancreatic obstruction, such as jaundice, biliary colic, or pancreatitis. Even in asymptomatic patients, an AA needs to be removed because of its malignant potential[4]. Furthermore, complete excision of AAs is necessary because of poor diagnostic accuracy with false-negative rates of up to 30% and diagnostic discrepancy of pathologic results, reported as 25%-60%, with forceps biopsy[5,6].

Endoscopic papillectomy (EP) was first introduced for the treatment of AA by Suzuki et al[7] in 1983, and both endoscopic and surgical approaches have been considered for the treatment of AA. EP is now considered as the first treatment of choice for benign AA due to the high recurrence, mortality, and morbidity of surgery[8-11]. Nevertheless, there remain concerns regarding EP. The reported EP adverse event rate is over 20% and while most cases are not severe, this cannot be neglected[12]. The recurrence rate after EP is high at 58.3%, and re-recurrence or persistence of AA has often been reported, requiring patients to undergo additional procedures or surgery[13,14]. Despite recent guidelines, there is no consensus on outcome parameters, and there are no established indication for EP or guidelines for EP technique, and no guidelines for the management of recurrence and re-recurrence[12,15,16].

Here, we aimed to evaluate the clinical outcomes of patients who underwent EP and investigate the factors that affect recurrence and adverse events to assist in improving the outcomes of EP and establishing the guidelines for EP.
MATERIALS AND METHODS

Study design
We retrospectively reviewed the medical charts of patients who underwent EP for AA between January 2013 and December 2019 and their follow-up data until December 2020 at five tertiary hospitals: Korea University Anam Hospital, Korea University Guro Hospital, Korea University Ansan Hospital, Hanyang University Seoul Hospital, and Hanyang University Guri Hospital. We excluded patients who underwent EP or surgical ampulectomy prior to enrollment and those who were followed up for less than a year after EP. Patients with non-adenomatous lesions were also excluded.

Patient baseline characteristics including age, sex, body mass index, clinical presentations, and initial pathologic reports of the lesion were recorded. Patients were screened for familial adenomatous polyposis (FAP), and mean follow-up periods were calculated. Parameters for EP techniques were recorded using written reports of EP, endoscopic images, and fluoroscopic images. These parameters included endoscopic ultrasound (EUS), cholangiogram, pancreatogram, submucosal lifting, type of resection (en-bloc/piecemeal), thermal ablation after resection, complete endoscopic resection, bile duct stent insertion (BDS), and pancreatic duct stent insertion (PDS).

EP procedure
EP was performed at five tertiary hospitals with over 500 endoscopic retrograde cholangiography (ERCP) annual cases by seven experts with over five years of ERCP experience. Before EP, EUS was performed at the endoscopist’s discretion. After adequate sedation, the ampulla of Vater was carefully inspected for its size, extent, and signs of malignancy (Figure 1A). Following the inspection, a cholangiogram and pancreatogram were obtained in cases requiring evaluation of a possible intraductal invasion. Then, snare polypectomy was performed (Figure 1B). Mucosal lifting using saline was performed if needed. With a standard polypectomy snare, the adenoma was tightly grasped, and the electrical current was applied until complete resection of the lesion was achieved.

En-bloc resection of AA was first attempted, and a piecemeal resection was performed if en-bloc resection was not possible. The resected specimen was removed and sent for pathologic evaluation (Figure 1C). The specimen was reviewed by a gastrointestinal pathologist and one or more residents in each hospital.

The EP site was observed for possible remnant lesions and immediate adverse events. Where remnant tissue was suspected, removal was performed with repeated biopsy, snaring, or thermal ablation with argon plasma coagulation (APC). In the event of immediate bleeding, epinephrine was sprayed with additional APC if bleeding persisted. In the event of duodenal perforation, endoscopic hemoclips were applied for the primary closure and surgery was subsequently performed. Sphincterotomies, BDS, and PDS were performed if needed (Figure 1D). The procedure was terminated if there was no more residual tissue or in the absence of immediate adverse events. Subsequently, the patient was observed on the ward with physical examination, monitoring of vital signs, laboratory tests, and X-rays for early adverse events. The details of each endoscopic procedure were determined by the endoscopist.

All patients underwent routine follow-up after EP. Within 3 mo of the procedure, patients underwent endoscopic surveillance for assessment of remnant tissue and recurrence, and stent removal (Figure 1E). Biopsy was performed if any remnant lesion was suspected. If the biopsy result showed remnants or early recurrence, additional therapeutic plans were decided by the endoscopist with the patient (Figure 1F). If no abnormal lesion was identified, the patient underwent further surveillance at six-monthly intervals for the first two years and annually thereafter.

Outcome measures
EP results included the resection specimen size, pathologic findings, accuracy of endoscopic biopsy, resection margin, curative resection, early and late recurrence, re-recurrence, endoscopic success, mean hospital stay, and mean adenoma-free period. EP outcomes were obtained from pathologic reports and medical charts.

Curative resection was defined as complete endoscopic resection without recurrence during follow-up. Early recurrence was defined as reconfirmed adenoma following biopsy at the first surveillance endoscopy. Late recurrence was defined as reconfirmed adenoma following biopsy after the first surveillance endoscopy. Re-recurrence was defined as recurrence of adenoma at the follow-up biopsy after the treatment of early or late recurrence. Endoscopic success was defined as treatment of AA with endoscopy, including cases with residual tissue, recurrence or complications, without surgical intervention. Resection margins were categorized into 3 groups, negative, positive, and uncertain, and they were analyzed as positive/uncertain group and negative group[17,18].

Adverse events of EP were categorized into early events (pancreatitis, delayed bleeding, cholangitis, and perforation) occurring within 30 d of the procedure and late events (papillary stenosis and death) occurring after 30 d following the procedure. Endoscopic adverse events and their severity were graded according to the American Society for Gastrointestinal Endoscopy criteria[19]. We set the minimum follow-up duration to one year to avoid underestimation of recurrence and adverse events. Univariate analysis and multivariate analysis were performed to evaluate the risk factors of early and late

Figure 1 Endoscopic papillectomy of ampullary adenoma. A: Careful inspection of the ampulla was required before the procedure; B: Endoscopic papillectomy was performed using a conventional polypectomy snare; C: The resected specimen was retrieved and pinned on a cork with nails for pathological evaluation; D: The resected area was carefully inspected, and an additional procedure including common bile duct stenting (blue stent) or pancreatic duct stenting (green stent) was performed; E: Endoscopic surveillance was mandatory; F: If recurrence was suspected, additional treatment was considered.

recurrence, non-curative resection, and adverse events.

Statistical analysis
Continuous variables were expressed as mean and standard deviation, and Categorical variables were expressed as a number and percentage. Univariate logistic regression analysis was performed to analyze the risk factors for early and late recurrences, non-curative resection, and adverse events. Variables that were significant in the univariate analysis were included in the multivariate logistic regression analysis. A P value < 0.05 was considered significant. The probability of adenoma-free after EP was analyzed using the Kaplan–Meier method. The statistical analyses were performed using the SPSS version 22.0 software (IBM Corp., Armonk, N.Y., USA).

RESULTS
We retrospectively collected the medical records of 119 patients and their follow-up data (Figure 2). We excluded seven patients who failed to meet the follow-up criteria or were lost to follow-up within a year of the procedure, and six patients because of non-adenomatous lesions. After the exclusion criteria were applied, 106 patients were finally included for analysis.

Patient baseline characteristics are shown in Table 1. Seventy-three patients (68.9%) were asymptomatic, and AA was diagnosed incidentally from screening endoscopy or CT scan. The most frequent symptoms associated with AA were jaundice in 16 patients (15.1%) and abdominal discomfort in 13 patients (12.3%). All patients underwent biopsy before the EP procedure, and their pathology reports were as follows: chronic inflammation in 2 patients (1.9%), atypical proliferative epithelium in 3 patients (2.8%), adenoma with low-grade dysplasia in 91 patients (85.8%), and adenoma with high-grade dysplasia in 10 patients (9.4%).

The EP techniques used for the patients are listed in Table 2. EUS was performed in 37 patients (34.9%), and a cholangiogram and pancreatogram were obtained in 70 patients (66.0%) and 87 patients (82.1%), respectively. Four patients (3.8%) underwent submucosal lifting with normal saline. En-bloc resection was performed in 90 patients (84.9%), and piecemeal resection was performed in 16 patients (15.1%). After the resection, thermal ablation was performed in 24 patients (22.6%) because of remnant tissue or immediate bleeding. Complete endoscopic resection was successfully performed in 105
patients (99.1%), and in one patient the lesion could not be completely removed due to underlying fibrosis and a diagnosis of adenocarcinoma was finally made. BDS and PDS were performed in 25 patients (23.6%) and 78 patients (73.6%), respectively.

Table 2 also summarizes the results of the EP. The mean size of the resected specimen was 13.6 ± 5.5 mm, and the final pathologic results were as follows: chronic inflammation in 3 cases (2.8%), low-grade dysplasia in 81 cases (76.4%), high-grade dysplasia in 18 cases (17.0%), and adenocarcinoma in 4 cases (3.8%). Figure 2 shows the diagnostic discrepancies between the initial and final pathologies. Lesions that showed chronic inflammation or atypical proliferative epithelium on initial biopsy were all low-grade dysplasia on final diagnosis. Out of 91 cases of low-grade dysplasia on the initial biopsy, the final pathologic results were chronic inflammation in three cases (2.8%), low-grade dysplasia in 75 cases (82.4%), high-grade dysplasia in 9 cases (9.9%), and adenocarcinoma in four cases (3.8%). Out of 10 cases of high-grade dysplasia on the initial biopsy, the final pathologic results were low-grade dysplasia in one case and high-grade dysplasia in nine cases. Endoscopic biopsy was accurate in 84 patients (79.2%), with underestimation in 18 patients (17.0%) and overestimation in 4 patients (3.8%).

R0 resection was achieved in 84 patients (79.2%), and curative resection was achieved in 81 patients (76.4%). Early recurrence was found in 11 patients (10.4%), late recurrence was found in 13 patients (12.3%), and all recurrences were local lesions. Re-recurrence occurred in six patients (5.7%), and patient characteristics are summarized in Supplementary Table 1. Figure 2 also shows the number of early and late recurrences from final pathologic results, how these cases were managed, how many re-recurrences occurred after the initial management, and final management of re-recurrences.

Initial management of the 11 patients with early recurrence involved endoscopic therapy in 7 cases (two EPs, two biopsies, and three thermal ablations) and surgery in four cases [two transduodenal ampullectomies (TA) and two pylorus-preserving pancreaticoduodenectomies (PPPD)]. Three patients with re-recurrence were managed with thermal ablation (two cases) and TA (one case). The 13 patients with late recurrence were initially managed endoscopically (six biopsies and seven ablations), and three patients with re-recurrence underwent thermal ablation, biopsy, and TA, respectively. Altogether, 99 patients (93.4%) were managed by endoscopy alone, and seven patients (6.6%) underwent additional surgical management: four patients due to a remnant lesion, two patients due to re-recurrence, and one patient due to incomplete resection and perforation (Figure 2). The mean adenoma-free period was 29.6
Table 2 Techniques and outcomes of endoscopic papillectomy

<table>
<thead>
<tr>
<th>Technique</th>
<th>n (%)/mean ± SD (n = 106)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUS</td>
<td>37 (34.9)</td>
</tr>
<tr>
<td>ERCP</td>
<td></td>
</tr>
<tr>
<td>Cholangiogram</td>
<td>70 (66.0)</td>
</tr>
<tr>
<td>Pancreatogram</td>
<td>87 (82.1)</td>
</tr>
<tr>
<td>Submucosal lifting</td>
<td>4 (3.8)</td>
</tr>
<tr>
<td>Type of resection</td>
<td></td>
</tr>
<tr>
<td>En-bloc</td>
<td>90 (84.9)</td>
</tr>
<tr>
<td>Piecemeal</td>
<td>16 (15.1)</td>
</tr>
<tr>
<td>Thermal ablation after resection</td>
<td>24 (22.6)</td>
</tr>
<tr>
<td>Complete endoscopic resection</td>
<td>105 (99.1)</td>
</tr>
<tr>
<td>Stent implantation</td>
<td></td>
</tr>
<tr>
<td>Bile duct</td>
<td>25 (23.6)</td>
</tr>
<tr>
<td>Pancreatic duct</td>
<td>78 (73.6)</td>
</tr>
<tr>
<td>Resection specimen size, mm</td>
<td>13.6 ± 5.5</td>
</tr>
<tr>
<td>≤ 15</td>
<td>77 (72.6)</td>
</tr>
<tr>
<td>&gt; 15</td>
<td>29 (27.4)</td>
</tr>
<tr>
<td>Final pathology</td>
<td></td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Adenoma</td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>81 (76.4)</td>
</tr>
<tr>
<td>High grade</td>
<td>18 (17.0)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>4 (3.8)</td>
</tr>
<tr>
<td>Accuracy of endoscopic biopsy</td>
<td></td>
</tr>
<tr>
<td>Underestimate</td>
<td>18 (17.0)</td>
</tr>
<tr>
<td>Overestimate</td>
<td>4 (3.8)</td>
</tr>
<tr>
<td>Resection margin</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>84 (79.2)</td>
</tr>
<tr>
<td>Positive/Uncertain</td>
<td>22 (20.8)</td>
</tr>
<tr>
<td>Positive</td>
<td>19 (17.9)</td>
</tr>
<tr>
<td>Uncertain</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Curative resection</td>
<td>81 (76.4)</td>
</tr>
<tr>
<td>Early recurrence</td>
<td>11 (10.4)</td>
</tr>
<tr>
<td>Late recurrence</td>
<td>13 (12.3)</td>
</tr>
<tr>
<td>Re-recurrence</td>
<td>6 (5.7)</td>
</tr>
<tr>
<td>Endoscopic success</td>
<td>99 (93.4)</td>
</tr>
<tr>
<td>Mean hospital stay, d</td>
<td>5.7 ± 3.0</td>
</tr>
<tr>
<td>Mean adenoma-free period, mo</td>
<td>29.6 ± 21.3</td>
</tr>
</tbody>
</table>

EUS: Endoscopic ultrasound; ERCP: Endoscopic retrograde cholangiography.

Figure 2 Flowchart of the study. After applying the exclusion criteria, 106 patients were enrolled, showing the correlation between the initial and final pathology. After the procedure, remnant and recurrent lesions were identified in follow-up surveillances. Most of these lesions were successfully managed with endoscopy. The gray-colored box indicates surgical management.

Table 3, Table 4, and Table 5 show the univariate and multivariate analysis of the risk factors for early recurrence, late recurrence and non-curative resection, respectively. Age over 65, EUS, size > 1.5 cm and positive/uncertain resection margin were statistically significant risk factors for early recurrence in univariate analysis, and positive/uncertain resection margin [Odds ratio (OR) = 4.023; 95%CI: 1.088-16.387; \( P = 0.048 \)] was the significant factor for early recurrence in multivariate analysis. Presence of symptom (OR = 4.659; 95%CI: 1.292-16.797; \( P = 0.019 \)) and piecemeal resection (OR = 7.114; 95%CI: 1.993-25.398; \( P = 0.003 \)) were significant risk factors for late recurrence in univariate analysis, and piecemeal resection (OR = 6.610; 95%CI: 1.760-24.820; \( P = 0.005 \)) was the only significant factor for late recurrence in multivariate analysis. Body mass index over 25, presence of symptom, and piecemeal resection were significant risk factors for non-curative resection, and multivariate analysis showed that piecemeal resection (OR = 5.424; 95%CI: 1.582-18.600; \( P = 0.007 \)) was a significant risk factor for non-curative resection.

Altogether, adverse events occurred in 26 patients as shown in Table 6. Early adverse events were as follows: pancreatitis in 14 patients (13.2%), delayed bleeding in 11 patients (10.4%), cholangitis in six patients (5.7%), and perforation in one patient (0.9%). No late adverse events were reported. In most cases, the severity of adverse events was classified as either mild or moderate, except for one case with perforation. Table 7 shows the univariate and multivariate analysis of risk factors for adverse events, including pancreatitis and delayed bleeding. FAP, pancreatogram, thermal ablation and PDS were significant risk factors for pancreatitis in univariate analysis, and in multivariate analysis, thermal ablation (OR = 4.128; 95%CI: 1.005-17.128; \( P = 0.048 \)) was a positive risk factor, while PDS (OR = 0.205; 95%CI: 0.044-0.945; \( P = 0.042 \)) was a negative risk factor for pancreatitis. Cholangiogram, piecemeal resection, and BDS were significant risk factors for delayed bleeding in univariate analysis, and piecemeal resection (OR = 6.698; 95%CI: 1.159-28.057; \( P = 0.009 \)) was the only significant risk factor in multivariate analysis. No significant risk factor for cholangitis or perforation was identified.
## Table 3 Risk factors of early recurrence

<table>
<thead>
<tr>
<th></th>
<th>Simple logistic regression (Univariate analysis)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>P value</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td>Age, &gt; 65 yr</td>
<td>4.18 (1.042-16.774)</td>
<td>0.044</td>
<td>3.441 (0.807-14.672)</td>
</tr>
<tr>
<td>Sex, Male</td>
<td>1.8 (0.513-6.319)</td>
<td>0.359</td>
<td></td>
</tr>
<tr>
<td>Body mass index &gt; 25 kg/m²</td>
<td>1.286 (0.251-6.587)</td>
<td>0.763</td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>3.022 (0.851-10.738)</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>EUS</td>
<td>3.792 (1.031-13.946)</td>
<td>0.045</td>
<td>1.622 (0.290-9.073)</td>
</tr>
<tr>
<td>Cholangiogram</td>
<td>1.125 (0.307-4.128)</td>
<td>0.859</td>
<td></td>
</tr>
<tr>
<td>Pancreatogram</td>
<td>2.336 (0.281-19.450)</td>
<td>0.432</td>
<td></td>
</tr>
<tr>
<td>Submucosal lifting</td>
<td>3.067 (0.291-32.329)</td>
<td>0.351</td>
<td></td>
</tr>
<tr>
<td>Piecemeal resection</td>
<td>1.286 (0.251-6.587)</td>
<td>0.763</td>
<td></td>
</tr>
<tr>
<td>Thermal ablation</td>
<td>0.313 (0.038-2.578)</td>
<td>0.280</td>
<td></td>
</tr>
<tr>
<td>BDS</td>
<td>1.244 (0.304-5.096)</td>
<td>0.761</td>
<td></td>
</tr>
<tr>
<td>PDS</td>
<td>0.901 (0.221-3.675)</td>
<td>0.885</td>
<td></td>
</tr>
<tr>
<td>Size, &gt; 15 mm</td>
<td>3.757 (1.048-13.461)</td>
<td>0.042</td>
<td>1.811 (0.344-9.521)</td>
</tr>
<tr>
<td>Initial pathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td>0.685 (0.074-6.272)</td>
<td>0.736</td>
<td></td>
</tr>
<tr>
<td>HGD</td>
<td>2.333 (0.167-32.584)</td>
<td>0.529</td>
<td></td>
</tr>
<tr>
<td>Positive/uncertain resection margin</td>
<td>5.925 (1.610-21.801)</td>
<td>0.007</td>
<td>4.023 (1.088-16.387)</td>
</tr>
<tr>
<td>Complication</td>
<td>2.937 (0.815-10.582)</td>
<td>0.100</td>
<td></td>
</tr>
</tbody>
</table>

OR: Odds ratio; EUS: Endoscopic ultrasound; BDS: Bile duct stent insertion; PDS: Pancreatic duct stent insertion; LGD: Low grade dysplasia; HGD: High grade dysplasia.

## DISCUSSION

Of the 106 patients, curative resection was performed in 81 patients (76.4%) with 26 cases of adverse events (24.5%), 11 early recurrences (10.4%), 13 Late recurrences (12.3%), and 6 re-recurrences (5.7%). Our results were consistent with those of previous studies showing curative resection rates of 73.0%-82.7%, adverse events rates of 15.0%-43.6%, early recurrence rates of 2.7%-19.0%, and late recurrence rates of 0-23.9%[14,20-23]. There are large variations in the reported outcomes, particularly among studies involving small numbers of cases because there is no consensus on which parameter best represents the performance of EP. The parameters used in previous studies are inconsistent, and inclusion criteria for EP vary.

Factors used for the evaluation of outcomes in previous studies include visual resection margin, histologic resection margin, recurrence, adverse events, need for surgery, and combinations of these factors. We suggest that curative resection (negative visual resection margin and no recurrence), adverse events, and endoscopic success (negative visual resection margin and no need for surgery) best represent the outcomes of EP. An ideal outcome for EP is the achievement of complete removal of the AA, without adverse events, and without recurrence, which is curative resection with no adverse event. Moreover, even in the event of recurrence, most of these patients can be and are managed endoscopically, representing cases of endoscopic success. We attempted to identify the factors that could predict and improve these outcomes.

In 84 patients (79.2%) the initial and final pathologic results were consistent, which is comparable to previously reported studies[20,23]. The initial biopsy result for the four patients with adenocarcinoma was reported as low-grade dysplasia. Biopsies of AA can occasionally be insufficient because the biopsy is often performed using a forward-viewing endoscope, making a targeted biopsy difficult. Therefore, even if the initial result is benign, it is important to remove the lesion completely with an adequate margin-free area in case of malignancy.

The ampullary lesions found after EP are often described as remnant/residual or recurrence in the literature[14,20,22-24]. Currently, these two categories are clinically distinguished according to the
Table 4 Risk factors of late recurrence

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Simple logistic regression (Univariate analysis)</th>
<th>P value</th>
<th>Multiple logistic regression (Multivariate analysis)</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, &gt; 65 yr</td>
<td>0.564 (0.162-1.961)</td>
<td>0.368</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex, Male</td>
<td>0.865 (0.263-2.847)</td>
<td>0.812</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index &gt; 25 kg/m²</td>
<td>2.095 (0.645-6.810)</td>
<td>0.219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>4.659 (1.292-16.797)</td>
<td>0.019</td>
<td>4.213 (0.091-16.728)</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>EUS</td>
<td>0.521 (0.134-2.023)</td>
<td>0.346</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholangiogram</td>
<td>1.250 (0.377-4.140)</td>
<td>0.715</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatogram</td>
<td>2.880 (0.351-23.609)</td>
<td>0.324</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submucosal lifting</td>
<td>2.500 (0.240-26.004)</td>
<td>0.443</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piecemeal resection</td>
<td>7.114 (1.993-25.398)</td>
<td>0.003</td>
<td>6.610 (1.760-24.820)</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Thermal ablation</td>
<td>2.434 (0.715-8.293)</td>
<td>0.155</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDS</td>
<td>1.524 (0.426-5.449)</td>
<td>0.517</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDS</td>
<td>4.657 (0.576-37.636)</td>
<td>0.149</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size, &gt; 15 mm</td>
<td>0.444 (0.092-2.140)</td>
<td>0.312</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive/uncertain resection margin</td>
<td>0.286 (0.035-2.326)</td>
<td>0.242</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complication</td>
<td>0.913 (0.231-3.606)</td>
<td>0.897</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR: Odds ratio; EUS: Endoscopic ultrasound; BDS: Bile duct stent insertion; PDS: Pancreatic duct stent insertion.

timing of lesion discovery: most studies define remnant/residual as the part of the previous lesion found at the first or any surveillance endoscopy performed 3-6 mo after EP, and recurrence as the lesion found after the first surveillance endoscopy or 6 mo after EP. Both are confirmed histologically. There is a clear difference between these definitions as remnant/residual refers to the remaining part of the pathologic lesion, while recurrence refers to a pathologic lesion that is newly developed after the procedure[25]. However, it is often difficult to separate these cases clinically. Diagnosis of a remnant could be delayed and the lesion may be found after the first surveillance endoscopy for a number of reasons including small size of remnant tissues and tissue burn from the procedure, and the delay in diagnosis leads to underestimation of remnant/residual lesions and overestimation of recurrence cases [18]. Considering a newly identified lesion at the first surveillance endoscopy as a remnant/residual in R0 resection may also be controversial. Therefore, instead of labeling these two groups differently, it is preferable to refer to both lesions as recurrence and distinguish these cases according to the timing of diagnosis. The recent European Society of Gastrointestinal Endoscopy guideline states that up to two thirds of recurrences are early recurrences[15].

These two groups differ in terms of the timing of the diagnosis and clinical implications, and there may also be differences in patient management. Recurrences were managed at the endoscopist’s discretion using various strategies, including endoscopic and surgical management. Except for early recurrence from adenocarcinoma that was managed with PPPD, it was difficult to identify which factors were considered for a particular treatment. However, early recurrences tend to be managed more aggressively than late recurrences, although the numbers were small for comparisons to be statistically significant (3 vs 1 TA and 2 vs 0 additional EP for early vs late recurrences). This tendency may be explained in that in cases of early recurrence, the initial removal of the lesion has been incomplete, so more invasive treatment may be required compared to the previous treatment method. Conversely, late recurrences are newly developed lesions that are typically small or are early lesions.

It is often difficult to establish which area of the adenoma is responsible for the recurrence because recurrences are typically small, but it can be presumed that they occur from the bile duct, pancreatic orifice, base of ampulla, or resection margin. Reported risk factors for recurrence include age, sex, FAP, intraductal involvement, incomplete resection, piecemeal resection, and final pathology, although the results of these studies are rather inconsistent[13,23,25-27]. Here, a positive/uncertain resection margin in the pathologic report was a significant risk factor for early recurrence, and piecemeal resection was a significant risk factor for late recurrence. This is the first study to analyze the risk factors for both early and late recurrence, considering the different definitions and characteristics of recurrence. A positive resection margin could increase the risk of remnants at the resection margin, but this association was not found to be significant in previous studies[25]. This may be because the positive margin following an EP
**Table 5 Risk factors of non-curative resection**

<table>
<thead>
<tr>
<th></th>
<th>Simple logistic regression (Univariate analysis)</th>
<th>Multiple logistic regression (Multivariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age, &gt; 65 yr</td>
<td>1.485 (0.595-3.703)</td>
<td>0.397</td>
</tr>
<tr>
<td>Sex, Male</td>
<td>1.256 (0.503-3.141)</td>
<td>0.625</td>
</tr>
<tr>
<td>Body mass index &gt; 25 kg/m²</td>
<td>3.340 (1.090-10.255)</td>
<td>0.035</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>2.905 (1.133-7.466)</td>
<td>0.026</td>
</tr>
<tr>
<td>EUS</td>
<td>1.455 (0.572-3.699)</td>
<td>0.431</td>
</tr>
<tr>
<td>Cholangiogram</td>
<td>1.222 (0.475-3.148)</td>
<td>0.678</td>
</tr>
<tr>
<td>Pancreatogram</td>
<td>2.877 (0.615-13.458)</td>
<td>0.179</td>
</tr>
<tr>
<td>Submucosal lifting</td>
<td>3.636 (0.484-27.302)</td>
<td>0.209</td>
</tr>
<tr>
<td>Piecemeal resection</td>
<td>4.625 (1.510-14.162)</td>
<td>0.007</td>
</tr>
<tr>
<td>Thermal ablation</td>
<td>1.185 (0.410-3.427)</td>
<td>0.754</td>
</tr>
<tr>
<td>BDS</td>
<td>1.464 (0.526-4.076)</td>
<td>0.466</td>
</tr>
<tr>
<td>PDS</td>
<td>1.949 (0.601-6.322)</td>
<td>0.266</td>
</tr>
<tr>
<td>Size, &gt; 15 mm</td>
<td>1.452 (0.543-3.881)</td>
<td>0.457</td>
</tr>
<tr>
<td>Initial pathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td>0.446 (0.098-2.036)</td>
<td>0.297</td>
</tr>
<tr>
<td>HGD</td>
<td>0.556 (0.065-4.755)</td>
<td>0.592</td>
</tr>
<tr>
<td>Positive/uncertain resection margin</td>
<td>1.839 (0.648-5.220)</td>
<td>0.252</td>
</tr>
<tr>
<td>Complication</td>
<td>1.778 (0.656-4.817)</td>
<td>0.258</td>
</tr>
</tbody>
</table>

OR: Odds ratio; EUS: Endoscopic ultrasound; BDS: Bile duct stent insertion; PDS: Pancreatic duct stent insertion; LGD: Low grade dysplasia; HGD: High grade dysplasia.

**Table 6 Adverse events of endoscopic papillectomy**

<table>
<thead>
<tr>
<th></th>
<th>n (%) (n = 106)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td></td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>14 (13.2)</td>
</tr>
<tr>
<td>Delayed bleeding</td>
<td>11 (10.4)</td>
</tr>
<tr>
<td>Cholangitis</td>
<td>6 (5.7)</td>
</tr>
<tr>
<td>Perforation</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Late</td>
<td></td>
</tr>
<tr>
<td>Papillary stenosis</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Mortality</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>26 (24.5)</td>
</tr>
</tbody>
</table>

Procedure is occasionally unreliable, as the resected lesions are often too small to be properly manipulated, and cautery may mask a positive margin[17]. Also, many previous studies do not clearly state how they analyzed the lesion with uncertain margin. More studies are needed to understand the clinical implications of a positive/uncertain resection margin and develop further management strategies for margin-positive/uncertain lesions. Additionally, to reduce recurrence after EP, it is important to check the peripheral and deep margin of the lesion meticulously before the EP, including intraductal involvement, and secure the resection margin properly during the EP. This is because recurrence could be caused by the poor selection of patients or inability to secure the margin.
Table 7 Univariate and multivariate analysis of risk factors for pancreatitis and bleeding

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Pancreatitis Univariate</th>
<th>Pancreatitis Multivariate</th>
<th>Bleeding Univariate</th>
<th>Bleeding Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>P value</td>
<td>OR (95%CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age, &gt; 65 yr</td>
<td>0.722 (0.225-2.323)</td>
<td>0.585</td>
<td>0.753 (0.206-2.745)</td>
<td>0.667</td>
</tr>
<tr>
<td>Sex, Male</td>
<td>1.486 (0.481-4.590)</td>
<td>0.491</td>
<td>1.800 (0.513-6.319)</td>
<td>0.359</td>
</tr>
<tr>
<td>Body mass index &gt; 25 kg/m²²</td>
<td>0.412 (0.071-3.902)</td>
<td>0.635</td>
<td>0.533 (0.063-4.480)</td>
<td>0.563</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>0.144 (0.018-1.153)</td>
<td>0.068</td>
<td>1.994 (0.562-7.073)</td>
<td>0.285</td>
</tr>
<tr>
<td>EUS</td>
<td>2.067 (0.664-6.428)</td>
<td>0.210</td>
<td>1.641 (0.465-5.788)</td>
<td>0.442</td>
</tr>
<tr>
<td>FAP</td>
<td>15.167 (1.277-180.166)</td>
<td>0.031</td>
<td>9.363 (0.429-204.392)</td>
<td>0.155</td>
</tr>
<tr>
<td>Cholangiogram</td>
<td>1.550 (0.493-4.870)</td>
<td>0.453</td>
<td>3.983 (1.081-14.669)</td>
<td>0.038</td>
</tr>
<tr>
<td>Pancreatogram</td>
<td>0.278 (0.087-0.885)</td>
<td>0.030</td>
<td>0.534 (0.106-2.678)</td>
<td>0.446</td>
</tr>
<tr>
<td>Piecemeal resection</td>
<td>0.929 (0.187-4.606)</td>
<td>0.928</td>
<td>6.364 (1.661-24.375)</td>
<td>0.007</td>
</tr>
<tr>
<td>Thermal ablation</td>
<td>4.412 (1.366-14.250)</td>
<td>0.013</td>
<td>4.128 (1.005-17.128)</td>
<td>0.048</td>
</tr>
<tr>
<td>BDS</td>
<td>0.868 (0.222-3.393)</td>
<td>0.838</td>
<td>4.800 (1.323-17.418)</td>
<td>0.017</td>
</tr>
<tr>
<td>PDS</td>
<td>0.102 (0.030-0.349)</td>
<td>0.000</td>
<td>0.205 (0.044-0.945)</td>
<td>0.042</td>
</tr>
<tr>
<td>Size, &gt; 15 mm</td>
<td>1.072 (0.308-3.731)</td>
<td>0.913</td>
<td>2.465 (0.690-8.812)</td>
<td>0.165</td>
</tr>
<tr>
<td>Initial pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>Ref</td>
<td></td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>LGD</td>
<td>0.975 (0.109-8.693)</td>
<td>0.982</td>
<td>0.293 (0.050-1.697)</td>
<td>0.171</td>
</tr>
<tr>
<td>HGD</td>
<td>2.333 (0.167-32.584)</td>
<td>0.529</td>
<td>0.429 (0.031-5.985)</td>
<td>0.529</td>
</tr>
<tr>
<td>Positive/uncertain resection margin</td>
<td>3.562 (1.086-11.685)</td>
<td>0.036</td>
<td>0.833 (0.167-4.167)</td>
<td>0.824</td>
</tr>
</tbody>
</table>

OR: Odds ratio; EUS: Endoscopic ultrasound; FAP: Familial adenomatous polyposis; BDS: Bile duct stent insertion; PDS: Pancreatic duct stent insertion; LGD: Low grade dysplasia; HGD: High grade dysplasia.

During the procedure, which is sometimes inevitable due to the characteristics of the lesion or the procedure itself. A more aggressive procedure could secure an adequate margin but may cause adverse events such as perforation, so proper selection of patients and careful approaches are mandatory. Similar considerations apply to the higher risk of late recurrence in piecemeal resection. In piecemeal resection, the resection margin may be unreliable, and a thorough evaluation and follow-up for recurrence is required. Piecemeal resection was a significant risk factor for non-curative resection, meaning en-bloc resection is a significant protective factor for curative resection, while a pathologic margin was not significant. A positive/uncertain margin and piecemeal resection are important factors for recurrence prediction, although their negative predictive values were 79.8% and 77.4%, respectively; thus, curative resection cannot be assumed in lesions with a negative margin or en-bloc resection.

Interestingly, our study was the first to compare the effects of factors including hospital setting and endoscopist experience on the outcome of EP (Supplementary Table 2), and these were not significant for remnant, recurrence, or adverse events. These findings suggest that there was no significant difference in EP results between hospitals with a certain volume of ERCP cases and endoscopists with a certain level of experience. Further studies with larger number of patients are needed to support our

Figure 3 Adenoma-free survival after endoscopic papillectomy. The vertical axis of the graph indicates the adenoma-free probability, and the horizontal axis shows time to remnant or recurrence after endoscopic papillectomy. The longest adenoma-free period before recurrence was 27 mo, and the longest adenoma-free period without recurrence was 94 mo.

suggestion.

Of the six patients with re-recurrence, two patients experienced re-recurrence even after a further session of endoscopic treatment and underwent surgery. Patients with persistent AA showed no specific features to guide the early prediction of the lesion characteristics and early transition to more invasive therapy. The finding that EUS was performed in both patients with re-recurrence suggests that EUS may not adequately predict recurrence or persistence. Moreover, it is unclear as to what extent a benign, although premalignant, AA lesion should be treated at recurrence, considering the adverse events associated with the available treatments. High-quality recommendations or guidelines are necessary.

Our results showed that most adverse events caused by EP showed mild- to moderate-grade severity. The role of thermal ablation in bleeding remains controversial and studies have shown that the risk of pancreatitis increased with the size of the lesion and when hemostasis was performed[22,23,28]. Here, thermal ablation was not significantly associated with bleeding or recurrence although it increased the risk of pancreatitis. The role of PDS is still under debate, but results of several studies, including ours, advocate the use of PDS for prophylaxis of pancreatitis[29-31]. Moreover, no pancreatic stenosis was observed, and this could be explained by our relatively high PDS rate at 73.6%. Hence, it is expected that PDS will help prevent pancreatitis and pancreatic stenosis, and we recommend routine pancreatic stenting, if possible. Also, our result showed that piecemeal resection was the only significant risk factor for delayed bleeding. Piecemeal resection was performed for lesions where en-bloc resection was impossible, therefore, the lesions with piecemeal resection tend to be larger[32]. A previous study did not show the correlation between piecemeal resection and bleeding, based on the small number of piecemeal resection cases, but colonic lesions with piecemeal resection show significant bleeding during endoscopic mucosal resection[32,33]. Further research is needed to support the role and adverse events of piecemeal resection in endoscopic papillectomy.

Our study has several limitations. As the indications for EP have not been established, selection bias could not be avoided. Moreover, due to the lack of guidelines on the optimal EP technique, several decisions made during the procedure were at the discretion of the endoscopist. Not all hospitals distinguished margin-positive cases as vertical or lateral involvement, therefore, we simplified the involvement of the margin as positive/uncertain or negative. Finally, the study design was retrospective, and factors regarding the procedure and follow-up could not be controlled.

CONCLUSION

EP is a feasible treatment option for AA with high technical success. However, diagnostic discrepancy, remnant lesions, recurrence, and adverse events cannot be neglected. Unlike gastric or colon adenoma resection, even in cases of complete resection, remnant lesions, recurrence, and re-recurrence were identified, emphasizing the importance of follow-up. For patients with a positive/uncertain resection margin in particular, close follow-up for early recurrence is required, and the possibility of late recurrence should be considered in patients with piecemeal resection. Especially in patients with a positive/uncertain resection margin or piecemeal resection, the possibility of recurrence should be
considered, and closer follow-up for recurrence is required.

ARTICLE HIGHLIGHTS

Research background
The incidence of ampullary adenoma (AA) is increasing, partly from increasing number of imaging studies and from true increase in incidence. Because of its malignant potential, AA has to be removed either surgically or endoscopically.

Research motivation
The role of endoscopic papillectomy (EP) in treatment of AA has been growing due to its relatively low invasiveness, but recurrences and side effects are reported in up to 30% of cases.

Research objectives
Our study aimed to evaluate the clinical outcomes of EP in patients with AA, performed at five tertiary hospitals.

Research methods
We collected the clinical data of patients with AA who underwent EP at five tertiary hospitals between 2013 and 2020 and analyzed the clinical outcomes and adverse events. Clinical outcomes were curative resection, defined as complete endoscopic resection without recurrence, endoscopic success, defined as treatment of ampullary adenoma with endoscopy alone, and recurrence, defined reconfirmed adenoma in endoscopy. Recurrence was divided into early and late, based on an interval of 6 mo.

Research results
Among 106 patients included, curative resection was achieved in 81 patients (76.4%), endoscopic success was achieved in 99 patients (93.4%), early recurrence was identified in 11 patients (16.1%), and late recurrence was identified in 13 patients, and re-recurrence was identified in 6 patients (12.3%). In multivariate analysis, the risk of early and late recurrences was significantly increased in a positive/uncertain margin and piecemeal resection, respectively. The risk of non-curative resection was significantly increased in piecemeal resection. Twenty-six patients experienced adverse events (24.5%): 14 pancreatitis, 11 delayed bleeding, 6 cholangitis, and 1 perforation.

Research conclusions
EP is a relatively safe procedure with high endoscopic success rate. Due to the diagnostic discrepancy, recurrence, re-recurrence and adverse events, careful selection and follow-up of patients are also needed.

Research perspectives
A positive/uncertain margin and piecemeal resection were significant risk factors for poor outcomes; therefore, every effort should be made to ensure adequate free margin and to perform en-bloc resection during EP.

FOOTNOTES

Author contributions: Choi SJ and Lee HS carried out the concept and design, drafting of the article, and critical revision; Choe JW, Lee JM, Hyun JJ, and Yoon JH collected the data; Kim J, Kim HJ, Kim JS, Choi HS carried out data analysis and interpretation; and all authors approved the final version of the article.

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Institutional review board statement: The study protocol was consistent with the guidelines outlined in the Declaration of Helsinki and was approved by the institutional review boards of each participating institution.

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

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

Data sharing statement: No additional unpublished data are available.
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Retrospective Study

Biliary metal stents should be placed near the hilar duct in distal malignant biliary stricture patients

Mitsuru Sugimoto, Tadayuki Takagi, Rei Suzuki, Naoki Konno, Hiroyuki Asama, Yuki Sato, Hiroki Irie, Yoshinori Okubo, Jun Nakamura, Mika Takasumi, Minami Hashimoto, Tsunetaka Kato, Ryoichiro Kobashi, Takumi Yanagita, Takuto Hikichi, Hiromasa Ohira

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Provenance and peer review: Unsolicited article; Externally peer reviewed.
Peer-review model: Single blind
Peer-review report's scientific quality classification
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Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0
P-Reviewer: Kim JH, South Korea; Sun SY, China; Zeng YY, China
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First decision: November 16, 2021
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Accepted: March 25, 2022
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BACKGROUND
Endoscopic biliary drainage using a self-expandable metallic stent (SEMS) has been widely performed to treat distal malignant biliary obstruction (DMBO). However, the optimal position of the stent remains unclear.

AIM
To determine the ideal position for SEMS placement.

METHODS
In total, 135 DMBO patients underwent SEMS (uncovered or covered) placement over a ten-year period. A total of 127 patients with biliary obstruction between the junction of the cystic duct and Vater’s papilla were enrolled. An SEMS was placed through the upper common bile duct 2 cm from the biliary hilar duct in 83 patients (Hilar group) or near the top of the biliary obstruction in 44 patients (Lower group). Technical and functional success, adverse events, and risk factors for SEMS dysfunction were evaluated.

RESULTS
The stent patency period was significantly longer in the Hilar group than in the Lower group (P value < 0.01). In multivariate analysis, the only statistically significant risk factor for SEMS dysfunction was being in the Lower group.
CONCLUSION
A longer patency period was achieved by positioning the SEMS near the biliary hilar duct.

Key Words: Endoscopic biliary drainage; Malignant biliary obstruction; Uncovered self-expandable metallic stent; Covered self-expandable metallic stent; Biliary hilar duct; Patency period

INTRODUCTION
Stricture of the common bile duct (CBD) can occur in several severe diseases (for example, bile duct cancer, pancreatic cancer, or metastasis of other cancers). Since transpapillary biliary stent insertion was first reported by Sohendra and Reynolds-Frederix[1], it has become the first choice for biliary drainage in patients with malignant biliary obstruction. At present, uncovered self-expandable metallic stents (USEMSs) and covered SEMSs (CSEMSs) have been reported to be more effective at preventing recurrent biliary obstruction (RBO) than plastic stents (PSs) in distal malignant biliary obstruction (DMBO) patients[2,3].

Whether a USEMS or CSEMS should be used remains a topic of debate. Three reports have asserted that the patency period of CSEMSs is superior to that of USEMSs[4-6]. However, others have found that the patency period is similar between CSEMSs and USEMSs[7-9]. Although CSEMS insertion has some disadvantages (such as cholecystitis, pancreatitis, and migration), the stent can be removed[7,10-13].

Based on the above findings, SEMS placement may help drain unresectable DMBOs. Determining which stent (USEMS or CSEMS) should be used has gained increasing attention. However, the optimal position of the inserted SEMS has rarely been discussed and remains unclear. Therefore, we aimed to determine the ideal position for SEMS placement.

MATERIALS AND METHODS

Study design and ethics approval
This was a retrospective study. The patients were not required to provide informed consent because this study used anonymized clinical data obtained after each patient had provided written consent and agreed to undergo medical procedures. Additional details of this study are published on the home page of Fukushima Medical University (approval number 2453).

Patients
A total of 135 DMBO patients underwent SEMS placement between January 2011 and February 2021 (Figure 1). These patients did not undergo previous surgery of the upper gastrointestinal tract and were undergoing SEMS placement for the first time. Seven of these patients whose biliary obstruction was located between the junction of the cystic duct and hilar bile duct were excluded from this study. In addition, one patient who underwent double SEMS placement was excluded. Finally, 127 patients whose biliary obstruction was located between the junction of the cystic duct and Vater’s papilla were enrolled. The SEMS was placed through the upper CBD within 2 cm from the junction of the right and left hepatic ducts in 83 patients (Hilar group) (Figure 2A and B). In the other 44 patients (Lower group),
Figure 1 Patient flowchart. DMBO: Distal malignant biliary obstruction; SEMS: Self-expandable metallic stent; CBD: Common bile duct.

Figure 2 Representative cases from each group. A and B: A patient with distal malignant biliary obstruction (DMBO) in the Hilar group who underwent self-expandable metallic stent (SEMS) placement near the biliary hilar duct; C and D: A patient with DMBO in the Lower group who underwent SEMS placement near the top of the biliary obstruction.

the SEMS was placed near the top of the biliary obstruction (Figure 2C and D).

Endoscopic biliary drainage

With the patient in a prone position, a duodenoscope was inserted after the patient was sufficiently sedated with midazolam. When the duodenoscope reached Vater’s papilla, biliary cannulation was initiated. After the range of the DMBO was confirmed by cholangiography, an SEMS was inserted from the upper part of the obstruction to Vater’s papilla. Endoscopic sphincterotomy (EST) was performed for first-time endoscopic biliary drainage with a PS or before SEMS insertion. The position and type of SEMS (USEMS or CSEMS) were randomly determined by each endoscopist. All the procedures were performed by pancreaticobiliary specialists or trainees under the guidance of specialists.

The USEMSs used in this study were as follows: BileRush, 8 mm × 6 cm, 10 mm × 6 or 8 cm (Piolax, Kanagawa, Japan); Bonastent, 10 mm × 8 cm (Standard Sci Tech, Seoul, Korea); HANARO, 10 mm × 7 cm (Boston Scientific, Tokyo, Japan); Niti-S Large cell, 10 mm × 5, 6, 8, or 10 cm (Taewoong Medical, Gyeonggi-do, Korea); WallFlex, 10 mm × 6, 8, or 10 cm (Boston Scientific); X Suit NIR, 10 mm × 8 cm (Olympus Medical, Tokyo, Japan); and Zilver, 10 mm × 6 cm, and Zilver 635, 10 mm × 6, 8, or 10 cm (Cook Medical Japan, Tokyo, Japan). The CSEMSs used in this study were as follows: Bonastent, 10 mm × 7 cm (Standard Sci Tech); HANARO, 10 mm × 5, 6, or 8 cm (Boston Scientific); Niti-S Comvi, partially covered, 10 mm × 6, 7, or 8 cm (Taewoong Medical); WallFlex, fully covered, 10 mm × 6 cm, and partially covered, 10 mm × 6 or 8 cm (Boston Scientific); and X Suit NIR, 10 mm × 4, 6, or 8 cm (Olympus Medical).
**Outcomes of interest**

The primary outcome was the stent patency period. The secondary outcomes were the technical success rate, functional success rate, adverse events (pancreatitis, post-EST bleeding), severity of adverse events, and stent dysfunction rate. These outcomes were defined according to partially revised versions of the reported criteria\[14\]. The stent patency period was determined as the time from first SEMS insertion to SEMS dysfunction. SEMS dysfunction was defined as the recurrence of hepatic dysfunction, jaundice, or dilated bile tract on ultrasonography or computed tomography (CT), which required secondary SEMS placement. Technical success was defined as successful placement of an SEMS that reached from the upper part of the obstruction to Vater’s papilla. Functional success was defined as the return of alanine transaminase (ALT) or total bilirubin (TB) levels to normal values (ALT < 27 U/L, TB < 1.2 mg/dL) or less than half of the pretreatment values. Adverse events and the severity of adverse events were defined according to Cotton’s criteria\[15\]. Posttreatment pancreatitis was also confirmed by contrast-enhanced CT.

In addition, the patient characteristics (age, sex, serum ALT level, serum TB level, cause of stricture, chemotherapy, duodenal stricture, CBD diameter above the stricture, CBD stricture diameter, CBD stricture length), year of procedure (2011-2015, or 2016-2021), stent diameter, type of SEMS used (USEMS or CSEMS), SEMS shortening, and observational period were compared between the Hilar group and the Lower group. The maximum serum ALT and TB values recorded in the previous week up to endoscopic SEMS insertion were used. The cause of stricture was divided into pancreaticobiliary and metastatic. Duodenal stricture was defined as a stricture that was difficult for the upper gastrointestinal scope to pass through. The diameter and length of the CBD stricture were measured by endoscopic retrograde cholangiography. The year of the procedure was compared between the groups because the techniques and devices have advanced over the approximately ten-year study duration. SEMS shortening was determined as more than 1 cm of shortening evident on X-ray or CT imaging after SEMS placement.

**Statistical analyses**

Student’s t test or Welch’s t test was used to compare continuous variables. Fisher’s exact test was used to compare nominal variables. To analyze the SEMS patency period, the log-rank test was used. To analyze the factors that influenced SEMS dysfunction, a Cox proportional hazard model was used. \( P < 0.05 \) was considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Centre, Jichi Medical University, Saitama, Japan).

**RESULTS**

The patient characteristics are shown in Table 1. There was no significant difference in age, sex, serum ALT level, serum TB level, stricture cause, chemotherapy, duodenal stricture, CBD diameter above the stricture, CBD stricture diameter, or CBD stricture length between the Hilar group and the Lower group.

The outcomes of SEMS placement are shown in Table 2. There was no difference in the procedure year, technical success rate, functional success rate, adverse events, or SEMS shortening between the two groups. The rate of CSEMS use and the SEMS diameter were also not significantly different between the two groups. Regarding the type of SEMS used, the covered WallFlex (Boston Scientific) stent was used significantly more frequently in the Hilar group than in the Lower group (28/83 (33.7%) vs 7/44 (15.9%), \( P = 0.038 \)), and the X Suit NIR stent was used significantly more frequently in the Lower group than in the Hilar group (6/44 (13.6%) vs 0/83 (0%), \( P < 0.01 \)). SEMS dysfunction was observed significantly more often in the Lower group than in the Hilar group [18/44 (41%) vs 2/83 (2.4%), \( P < 0.01 \)]. The causes of SEMS dysfunction were as follows: Ingrowth (1) and overgrowth (1) in the Hilar group, and ingrowth (3), overgrowth (2), ingrowth and overgrowth (8), top edge closed by the CBD wall (4), and dislocation (1) in the Lower group. In the cases in which the top edge was closed by the CBD wall, the SEMSs used were the Zilver 635 (Cook Medical), WallFlex (Boston Scientific), Niti-S large cell (Taewoong Medical), and HANARO (Boston Scientific) stents. A representative case in which the top edge of the SEMS was closed by the CBD wall is shown in Figure 3. The observational period was longer in the Lower group than in the Hilar group (9.12 ± 12.07 mo vs 4.16 ± 5.76 mo, \( P = 0.012 \)).

The results of the stent patency comparison are shown in Figure 4 and Supplementary Figure 1. The stent patency period was significantly longer in the Hilar group than in the Lower group (Figure 4A, \( P < 0.01 \)). The stent patency period was not significantly different between the groups when the patients were divided according to the use of a covered WallFlex stent, use of a covered X Suit NIR stent, observational period (Figure 4B-D), age, sex, serum ALT level, serum TB level, metastatic or pancreaticobiliary status, presence or absence of chemotherapy, presence or absence of duodenal stricture, CBD diameter above the stricture, CBD stricture diameter, CBD stricture length, year of procedure, USEMS or CSEMS, presence or absence of SEMS shortening (Supplementary Figure 1).
### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Hilar group (n = 83)</th>
<th>Lower group (n = 44)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>68.4 ± 11.8</td>
<td>71.2 ± 11.2</td>
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</tr>
<tr>
<td>Sex</td>
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<td></td>
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<tr>
<td>Female</td>
<td>33 (39.8)</td>
<td>16 (36.4)</td>
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<tr>
<td>Male</td>
<td>50 (60.2)</td>
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</tr>
<tr>
<td>ALT, U/L</td>
<td>126.0 ± 116.3</td>
<td>174.8 ± 177.1</td>
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<tr>
<td>TB, mg/dL</td>
<td>5.9 ± 7.5</td>
<td>6.8 ± 6.8</td>
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<td>Cause of stricture</td>
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<td>Pancreatobiliary tumor</td>
<td>73 (88.0)</td>
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<td>Pancreas</td>
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<td>31</td>
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</tr>
<tr>
<td>Biliary tract</td>
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<td>9</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
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<td>4 (9.1)</td>
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<td>Lung</td>
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</tr>
<tr>
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<tr>
<td>Uterine</td>
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<tr>
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<td></td>
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<tr>
<td>Colon</td>
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<td></td>
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<tr>
<td>Lymph node metastasis</td>
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<td>Chemotherapy</td>
<td>35 (42.2)</td>
<td>23 (52.3)</td>
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<tr>
<td>Duodenal stricture</td>
<td>15 (18.1)</td>
<td>7 (15.9)</td>
<td>0.81</td>
</tr>
<tr>
<td>CBD diameter above stricture, mm</td>
<td>11.5 ± 4.3</td>
<td>12.2 ± 4.0</td>
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<tr>
<td>CBD stricture diameter, mm</td>
<td>0.61 ± 0.89</td>
<td>0.72 ± 0.8</td>
<td>0.50</td>
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<tr>
<td>CBD stricture length, cm</td>
<td>2.64 ± 1.35</td>
<td>2.34 ± 1.11</td>
<td>0.21</td>
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</tbody>
</table>

Values are presented as the mean ± SD or n (%).
ALT: Alanine transaminase; TB: Total bilirubin; CBD: Common bile duct.

The risk factors for SEMS dysfunction are shown in Table 3. Serum ALT level and lower placement were statistically significant factors in the univariate analysis [ALT: hazard ratio (HR): 1.003, 95% confidence interval (CI): 1.001–1.01, P value < 0.01; Lower group: HR: 11.42, 95%CI: 2.61–49.83, P value < 0.01]. However, the only statistically significant risk factor in the multivariate analysis was lower placement (HR: 9.94, 95%CI: 2.25–44.0, P < 0.01).

### DISCUSSION

In this study, we investigated the ideal position for SEMS insertion in DMBO patients. The results demonstrated that the SEMS patency period was longer when the stent was placed near the hilar duct.

This finding suggests that this position overcomes several causes of SEMS dysfunction. As shown in Table 2, the main causes of SEMS dysfunction were tumor ingrowth and/or overgrowth and a top edge closed by the CBD wall; notably, overgrowth and a top edge closed by the CBD wall were prevented by using a longer SEMS. Longer SEMSs can delay stent dysfunction due to tumor overgrowth. In the four patients in the Lower group, the top edge of the SEMS was closed by the CBD wall, which may be caused by linearization of the SEMS. The axial force on the stent is thought to be related to the linearization and closing of the top edge by the CBD wall. However, in the four patients with SEMS dysfunction caused by closure of the top edge by the CBD wall, the SEMSs were not necessarily affected...
<table>
<thead>
<tr>
<th>Variable</th>
<th>Hilar group (n = 83)</th>
<th>Lower group (n = 44)</th>
<th>P value</th>
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<tr>
<td><strong>Year of procedure</strong></td>
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<tr>
<td>2011-2015</td>
<td>48 (57.8)</td>
<td>31 (70.5)</td>
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<tr>
<td>2016-2021</td>
<td>35 (42.2)</td>
<td>13 (29.5)</td>
<td></td>
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<tr>
<td><strong>Diameter of SEMS</strong></td>
<td></td>
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</tr>
<tr>
<td>8 mm</td>
<td>1 (1.2)</td>
<td>0 (0)</td>
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<tr>
<td>10 mm</td>
<td>82 (98.8)</td>
<td>44 (100)</td>
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<td><strong>USEMS:CSEMS</strong></td>
<td>35:48</td>
<td>20:24</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>USEMS used</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BileRush</td>
<td>2 (2.4)</td>
<td>1 (2.3)</td>
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</tr>
<tr>
<td>Bonastent</td>
<td>1 (1.2)</td>
<td>0 (0)</td>
<td>1</td>
</tr>
<tr>
<td>HANARO</td>
<td>1 (1.2)</td>
<td>0 (0)</td>
<td>1</td>
</tr>
<tr>
<td>Niti-S Large cell</td>
<td>9 (10.8)</td>
<td>5 (11.4)</td>
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<tr>
<td>WallFlex</td>
<td>24 (28.9)</td>
<td>7 (15.9)</td>
<td>0.13</td>
</tr>
<tr>
<td>X Suit NIR</td>
<td>0 (0)</td>
<td>2 (4.5)</td>
<td>0.12</td>
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<tr>
<td>Zilver</td>
<td>0 (0)</td>
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<tr>
<td>Zilver 635</td>
<td>4 (4.8)</td>
<td>6 (13.6)</td>
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<td><strong>CSEMS used</strong></td>
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<td>Bonastent</td>
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<td>Niti-S Comvi</td>
<td>11 (13.3)</td>
<td>7 (15.9)</td>
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<td>WallFlex</td>
<td>26 (33.7)</td>
<td>7 (15.9)</td>
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<td>X Suit NIR</td>
<td>0 (0)</td>
<td>6 (13.6)</td>
<td>&lt; 0.01</td>
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<tr>
<td><strong>Technical success</strong></td>
<td>83 (100)</td>
<td>44 (100)</td>
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<tr>
<td><strong>Functional success</strong></td>
<td>81 (97.6)</td>
<td>41 (93.2)</td>
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<td><strong>Adverse events</strong></td>
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<tr>
<td>Pancreatitis</td>
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<tr>
<td>Mild</td>
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<td>Post-EST bleeding</td>
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<td>Severe</td>
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<tr>
<td><strong>SEMS shortening</strong></td>
<td>1 (1.3)</td>
<td>2 (4.7)</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>SEMS dysfunction</strong></td>
<td>2 (2.4)</td>
<td>18 (41)</td>
<td>&lt; 0.01</td>
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<tr>
<td>Cause of SEMS dysfunction</td>
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<td>Ingrowth</td>
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<tr>
<td>Overgrowth</td>
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<td>2</td>
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<tr>
<td>Ingrowth and overgrowth</td>
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<td>Top edge closed by CBD wall</td>
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<tr>
<td>Dislocation</td>
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<tr>
<td><strong>Observational period, months</strong></td>
<td>4.16 ± 5.76</td>
<td>9.12 ± 12.07</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Values are presented as n, n (%) , or mean ± SD.

1 The presence or absence of SEMS shortening was confirmed in 79 patients in the Hilar group and 43 patients in the Lower group.

SEMS: Self-expandable metallic stent; USEMS: Uncovered SEMS; CSEMS: Covered SEMS; EST: Endoscopic sphincterotomy; CBD: Common bile duct.
Table 3 Risk factors for self-expandable metallic stent dysfunction

<table>
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<tr>
<th></th>
<th>Univariate analysis</th>
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<th>Multivariate analysis</th>
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<td></td>
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<td>95%CI</td>
<td>P value</td>
<td>Hazard ratio</td>
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<tr>
<td>Lower group</td>
<td>11.42</td>
<td>2.61–49.83</td>
<td>&lt; 0.01</td>
<td>9.94</td>
</tr>
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<td>Age</td>
<td>1.04</td>
<td>0.99–1.09</td>
<td>0.07</td>
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<tr>
<td>Sex, male</td>
<td>0.88</td>
<td>0.35–2.2</td>
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<td>ALT</td>
<td>1.003</td>
<td>1.001–1.01</td>
<td>&lt; 0.01</td>
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<td>TB</td>
<td>1.05</td>
<td>0.99–1.12</td>
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<td>Cause of stricture, pancreaticobiliary</td>
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<td>0.09–1.9</td>
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<td>Chemotherapy</td>
<td>0.89</td>
<td>0.34–2.31</td>
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<td>Duodenal stricture</td>
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<tr>
<td>CBD above diameter stricture</td>
<td>1.06</td>
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<td>CBD stricture diameter</td>
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<td>CBD stricture length</td>
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<td>0.69–1.41</td>
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<td>Use of CSEMS</td>
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<td>Use of covered WallFlex stent</td>
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<td>0.12–1.36</td>
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<tr>
<td>Use of covered X Suit NIR usage stent</td>
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<td>0.92–11.14</td>
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<td>SEMS shortening</td>
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<tr>
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<td>0.98</td>
<td>0.92–1.04</td>
<td>0.43</td>
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</table>

SEMS: Self-expandable metallic stent; ALT: Alanine transaminase; TB: Total bilirubin; CBD: Common bile duct; CSEMS: Covered SEMS; CI: Confidence interval.

Figure 3 A patient with closure of the top edge of the self-expandable metallic stent by the common bile duct wall. A: A patient with distal malignant biliary obstruction who underwent uncovered self-expandable metallic stents (USEMS) placement near the top of the biliary obstruction; B: The top edge of the SEMS was closed by the common bile duct wall (arrows). Upper bile tract dilation was observed; C: An additional USEMS was placed near the biliary hilar duct.

by high axial force, except for the WallFlex (Boston Scientific) stent. When a short SEMS is placed near the top edge of the DMBO, the axial force might be enhanced by the biliary stricture. Using longer SEMSs overcomes this problem because the axial force decreases with increasing distance between the top edge of the SEMS and CBD stricture[16]. In fact, a biliary obstruction was relieved by placing a second SEMS near the biliary hilar duct (Figure 3C).

In past reports, time to adequate expansion, degree of CBD stricture[17], duodenal invasion[18], duodenal SEMS[19], and anticancer treatment[18,20] were reported as risk factors for RBO. The factors...
related to SEMS expansion and CBD stricture were not an issue in this study because functional success was achieved in almost all the patients. Anticancer treatment has been reported to cause RBO as follows. Although anticancer treatment reduces the tumor burden, it can dislocate the CSEMS or induce neutropenia and bacterial overgrowth and, ultimately, cholangitis or sludge formation in the bile duct [20]. However, anticancer treatment was not proposed as a risk factor for RBO in a study that involved patients with a USEMS [21]. This study involved both USEMSs and CSEMSs. Therefore, anticancer treatment may not be a risk factor for SEMS dysfunction. Duodenal invasion from tumors reduces peristalsis and causes food impaction in the biliary duct, and a duodenal SEMS prevents the outflow of bile juice [19]. In this study, any RBO requiring additional SEMS placement was defined as SEMS dysfunction so that SEMS occlusion caused by tumors could be properly evaluated and cases of SEMS obstruction by food impaction could be excluded. Therefore, SEMS placement near the biliary hilar duct was revealed as a new factor related to longer SEMS patency.

There were some limitations to this study. First, this was a retrospective observational study performed at a single institution. In the future, it is hoped that a prospective multicenter study will confirm our findings. Second, the type of SEMS was not unified. The axial force or shortening length varied among the SEMSs. Measurement of the axial force was difficult in this study; instead, different kinds of SEMSs were compared. As a result, the type of SEMS did not influence SEMS dysfunction. The WallFlex stent (Boston Scientific), which has a high axial force and a high shortening rate [22], was used significantly more often in the Hilar group. However, remarkable shortening was rarely observed (the presence or absence of shortening was confirmed in 23 patients 24 h after SEMS placement and in 99 patients more than 48 h after SEMS placement). This was likely due to the placement of an SEMS with a longer than established length because the SEMS could not fully expand in the area of the stricture. As described above, the axial force decreases with increasing SEMS length. Because of these factors, the difference in the type of SEMS did not influence the outcomes. Third, SEMS obstruction of sludge or food debris was not considered SEMS dysfunction. In past reports, sludge formation has been proposed...
to be a cause of SEMS dysfunction[4,5,21]. This factor is surely important for comparisons of patency periods between USEMS and CSEMS. If SEMS obstruction of sludge or food debris was considered stent dysfunction, the patency period was also significantly longer in the Hilar group than in the Lower group (Supplementary Figure 2). Therefore, the obstruction of sludge or food debris did not influence the results of this study. As described above, the SEMS obstruction of sludge or food debris was excluded from SEMS dysfunction to properly evaluate the relationship between the positions of the SEMS and tumor in this study.

CONCLUSION

The results of our study revealed that placement of an SEMS near the biliary hilar duct could delay tumor overgrowth and prevent closure of the top edge of the SEMS by the CBD wall. Thus, in DMBO patients, the SEMS should be placed near the biliary hilar duct to achieve a longer patency period.

ARTICLE HIGHLIGHTS

Research background
Endoscopic biliary drainage using a self-expanding metallic stent (SEMS) has a longer patency period than endoscopic biliary drainage using a plastic stent. Therefore, endoscopic SEMS placement is desirable for the treatment of unresectable distant malignant biliary obstruction (DMBO).

Research motivation
The type of SEMS that should be used for DMBO is a point of active discussion. However, the appropriate position for SEMS insertion is unknown.

Research objectives
To clarify the appropriate SEMS insertion point for DMBO.

Research methods
Among 135 DMBO patients who underwent SEMS placement, 127 patients with biliary obstruction between the junction of the cystic duct and Vater’s papilla were enrolled. In 83 patients (Hilar group), an SEMS was placed through the upper common bile duct within 2 cm from the biliary hilar duct. In the other 44 patients (Lower group), an SEMS was placed near the top of the biliary obstruction. The patency period was compared between the Hilar group and Lower group. The risk factors for SEMS dysfunction were also investigated.

Research results
The patency period of SEMS was significantly longer in the Hilar group patients. Multivariate analysis revealed that the Lower group classification was the only significant risk factor for SEMS dysfunction.

Research conclusions
SEMS placement near the biliary hilar duct extends the patency period in DMBO patients.

Research perspectives
SEMS placement near the biliary hilar duct might prevent obstructive jaundice and cholangitis and contribute to improved prognosis in DMBO patients.

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FOOTNOTES

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Could microbiome analysis be a new diagnostic tool in gastric carcinogenesis for high risk, *Helicobacter pylori* negative patients?

Alla Turshudzhyan, Houman Rezaizadeh

**Abstract**

*Helicobacter pylori* (*H. pylori*) has long been believed to be the major colonizer of the stomach, but recent advances in genetic sequencing have allowed for further differentiation of the gastric microbiome and revealed the true complexity of the gastric microbiome. One of the few studies specifically evaluated the microbiome in the *H. pylori* negative patient population. They concluded that various stages of gastric carcinogenesis are associated with distinct bacterial taxa that could service both a predictive and diagnostic purpose. While the study has some limitations, the conclusions they make are intriguing and should prompt a larger prospective study to be done that spans multiple geographic regions.

**Key Words:** Gastric cancer; Gastric carcinogenesis; Microbiome; Dysplasia; Intestinal metaplasia

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**Core Tip:** Gastric tumorigenesis in *Helicobacter pylori* (*H. pylori*) negative patients remained a mystery for many years until genetic sequencing allowed for a closer look at the composition of the gastric microbiome. Primary colonizers of the stomach in *H. pylori* negative patients at various stages of gastric tumorigenesis and were able to conclude that there are distinct bacterial taxa associated with these stages. Their study is comprehensive but needs a larger prospective study to further support this hypothesis, particularly in other geographic areas with varying risk profiles.
TO THE EDITOR

We read with great interest the case control study by Sun et al[1]. These authors performed a genetic analysis of gastric mucosa from 134 Helicobacter pylori (H. pylori) negative patients, which included a variety of gastric pathology: 56 cases of superficial gastritis, 9 cases of atrophic gastritis, 27 cases of intestinal metaplasia, 29 cases of dysplasia, and 13 cases of gastric cancer[1]. Additionally, gastric juice samples from 18 cases of superficial gastritis, intestinal metaplasia, and dysplasia were included and analyzed[1]. Genetic analysis was performed using a 16S rRNA[1]. The goal of the study was to understand whether there is a distinct pattern in the microbiome of various gastric disease types.

The study demonstrated that microbiota of the gastric mucosa varies across different stages of gastric carcinogenesis[1]. Specifically, Sun et al[1] found that as the stages of carcinogenesis progress, there is less microbiota variability within gastric mucosa[1]. Of note, their data established that different stages of gastric carcinogenesis had distinguishable microbiota taxa for both gastric mucosa and gastric juice[1]. For example, intestinal metaplasia and dysplasia had predominantly Ralstonia and Rhodococcus while Streptococcaceae and Lactobacillaceae were more prominent in pre-cancerous lesions and gastric cancer[1]. Sun et al[1] concluded that their results may facilitate prediction of intestinal metaplasia and dysplasia progression to gastric cancer.

It was long believed that due to the highly acidic environment, H. pylori was the predominant colonizer of the stomach. In the recent years, however, genetic sequencing allowed further differentiation of the gastric microbiota[2]. Similar to the study by Sun et al[1], prior studies established that microbial diversity decreased significantly with gastric carcinogenesis[3,4]. There were a few studies, however, that were arguing that the opposite is true. The studies by Castaño-Rodriguez et al[5] and Eun et al[6] suggested that gastric cancer was associated with increased diversity of microbiome[5,6]. These results were supported by the more recent studies. A recently published study by Dai et al[7] analyzed gastric microbiome of 37 patients with gastric cancer using the same 16s rRNA gene sequencing[7]. They concluded that pre-cancerous and cancerous gastric lesions had an increased diversity in microbiome and specifically an abundance of Lactobacillus, Streptococcus, Bacteroides, and Prevotella[7]. While their conclusions on the increased microbial diversity in gastric cancer argues against conclusions set forth by Sun et al[1], they do agree on the distinguishable bacterial taxa associated with gastric cancer that could be used as a predictive marker of neoplastic conversion in pre-malignant lesions. Perhaps these observational disparities could be attributed to geographic, environmental, and patient population differences or even variability of the microbiome throughout various stages of gastric cancer itself.

Sun et al[1] concluded that there are certain bacteria that predispose patients to development of gastric cancer. With this, we wonder, if there are bacteria that would instead be protective against gastric cancer. Goldin and Gorbach[8] were one of the first to establish an association between probiotics and cancer prevention back in 1980[8]. Since then, multiple studies have attempted to investigate probiotics as a possible adjunct to cancer therapy. Lee et al[9] found that Bacillus polyfermenticus was able to reduce gastric adenocarcinoma cell proliferation by more than 90% in vitro[9]. Similarly, Han et al[10] found that Lactococcus lactis was able to reduce gastric adenocarcinoma cell proliferation by more than 80% in vitro[10]. While both studies were done in vitro, they proposed interesting conclusions that should be further investigated for efficacy in vivo. If proved to be successful and safe in vivo, targeted probiotics could be a new exciting adjunctive therapy for patients with gastric cancer.

The study conducted by Sun et al[1] was retrospective. The patients in the study had a known diagnosis of gastric cancer. Subsequently, it is important to consider a theory that the observed bacterial taxa were a result of neoplastic changes rather than bacteria being responsible for cancer development (i.e. reactive changes rather than causal association). This theory can be better investigated by a prospective study in which patients at high risk for developing gastric cancer are followed over time and changes in their microbiome are documented along with histopathological or endoscopic findings.

Sun et al[1] rightfully excluded patients who were on active antibiotic therapy, however, it is unclear how many of them had significant antibiotic exposure prior to the study. The association between antibiotic use and cancer remains unclear, however, there is literature reporting cases of antibiotic use and subsequent development of malignancy. Petrelli et al[11] conducted a systematic review with meta-analysis and concluded that antibiotics were an independent risk factor for cancer development (OR: 1.18, 95%CI: 1.12-1.24, P < 0.001)[11]. This is the reason we believe a thorough antibiotic use history should be collected on patients in studies investigating microbiome and its effects on cancer development.
Gastric cancer is a prominent malignancy affecting many people worldwide but has a notoriously higher incidence rate in Asia[12]. As a result, many of the studies on this topic originate from Asia. Sun et al[1] study, for example, was limited to Peking University Hospitals patient population in China, which may have introduced a geographic confounding variable. This may make their conclusions less applicable to the patients of other geographic areas. The study recruitment period was limited to September 2019 to October 2020. Lastly, gastric juice data was only collected for superficial gastritis, intestinal metaplasia, and dysplasia patients, and not for atrophic gastritis or gastric cancer patients. Despite some of the limitations, the study by Sun et al[1] had a comprehensive analysis and proposed very interesting conclusions that should be further replicated in larger studies.

In summary, the authors should be commended for their work. They investigated the microbiome in a large group of patients at different stages of gastric cancer tumorigenesis in an H. pylori negative patient population, which is generally understudied. Sun et al[1] study set comprehensive exclusion criteria limiting many confounding factors. They have demonstrated a well conducted analysis that showed there are distinct bacterial taxa associated with each of the stages of gastric carcinogenesis that could be of great clinical value and help triage gastric lesions. Going forward, large prospective randomized controlled trials that encompass multiple geographic areas could help solidify the conclusions set forth by Sun et al[1].

**FOOTNOTES**

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