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STOMACH AND DUODENUM (J PISEGNA AND J BENHAMMOU, SECTION EDITORS)

Pathophysiology of Gastric NETs: Role of Gastrin and Menin

Sinju Sundaresan[1] · **Anthony J. Kang**[1] · **Juanita L. Merchant**[1,2]

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Abstract
Purpose of review Neuroendocrine tumors (NETs) were initially identified as a separate entity in the early 1900s as a unique malignancy that secretes bioactive amines. GI-NETs are the most frequent type and represent a unique subset of NETs, because at least 75% of these tumors represent gastrin stimulation of the enterochromaffin-like cell located in the body of the stomach.

The purpose of this review is to understand the specific role of gastrin in the generation of Gastric NETs (G-NETs).
Recent findings We review here the origin of enterochromaffin cells gut and the role of hypergastrinemia in gastric enteroendocrine tumorigenesis. We describe generation of the first genetically engineered mouse model of gastrin-driven G-NETs that mimics the human phenotype. The common mechanism observed in both the hypergastrinemic mouse model and human carcinoids is translocation of the cyclin-dependent inhibitor $p27^{kip}$ to the cytoplasm and its subsequent degradation by the proteasome.
Summary Therapies that block degradation of $p27^{kip}$, the CCKBR2 gastrin receptor, or gastrin peptide are likely to facilitate treatment.

Keywords MEN1 · ECL cells · Somatostatin · Proton pump inhibitors · $p27^{Kip}$

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✉ Juanita L. Merchant
Merchanj@med.umich.edu

[1] Division of Gastroenterology, Department of Internal Medicine, University of Michigan, BSRB, 2051, 109 Zina Pitcher PL, Ann Arbor, MI 48109-2200, USA

[2] Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

Introduction

The term "carcinoid" was initially coined in 1907 by the German pathologist Oberndorfer to describe slow growing tumors that did not exhibit a full-blown malignant phenotype [1, 2]. The term carcinoid implied "carcinoma-like" and was described as "a nest of cells" surrounded by a thick stroma. In 1925, the Estonian pathologist Harry Kull noticed that scattered cells in the gastrointestinal tract shared a similar characteristic with adrenal chromaffin cells, which arise from the neural crest. This characteristic was the ability to form a black precipitate with chromium and silver salts (argentafffin-staining). Subsequently, it was discovered that the cells comprising carcinoid tumors not only showed an affinity for silver but also displayed endocrine characteristics such as the ability to secrete bioactive amines, specifically 5-hydroxytryptamine (serotonin) and histamine [3••]. In 1966, Anthony Pearse reclassified the foregut, midgut, and hindgut derived carcinoids that were initially characterized by their embryological origins as amine precursor uptake and decarboxylation (APUD) tumors, a designation which recognizes their common chemical characteristic of bioamine synthesis [4, 5]. In 1995, in an effort to avoid the "carcinoid tumor" designation, Capella and European NET pathologists coined the term neuroendocrine tumors (NETs), which the World Health Organization revised again in 2010 to avoid the term "carcinoid" and add the term neuroendocrine carcinoma (NEC) [6, 7].

NETs originate primarily from endocrine tissues such as the pituitary, parathyroid, islet pancreas, and scattered endocrine cells in the gastrointestinal tract as well as the lung and ovaries. Within the luminal gastrointestinal (GI) tract, the small intestine is the most frequent location for NETs. As slow growing tumors, they are potentially curable with surgery if discovered early, when they often are asymptomatic. When they cause symptoms, they secrete vasoactive amines such

as histamine and serotonin that induce flushing, smooth muscle contractions, diarrhea, dyspnea, bronchospasm, palpitations, and right heart failure, collectively called the carcinoid *syndrome*. However, carcinoid syndrome primarily represents the secretion of bioamines from Type 3 gastric NETs (G-NETs) and mid-gut NETs that metastasize to the liver once they achieve a size greater than 2.0 cm [8•].

Although gastric carcinoids (GC) are relatively rare and represent about 7% of all carcinoid tumors, their incidence is increasing due to more frequent endoscopies, endoscopic ultrasound, and physician awareness [9]. Surprisingly, the NET location varies by race with the lung being the most common site in Caucasians and the rectum in Asians, Native Americans, and African Americans [10]. There are three types of GCs. The most common GC is Type 1 (approximately 70–80% of total), which typically arises from enterochromaffin-like (ECL) cells in the gastric corpus as a multifocal lesion in response to hypergastrinemia associated with autoimmune and chronic atrophic gastritis from *Helicobacter pylori* infection [11]. Partial or complete atrophy of the parietal cell leads to hypo- or achlorhydria, which through a feedback mechanism results in gastrin (G) cell hyperplasia and hypersecretion. Type 2 GC develops from ECL cells in response to constitutively high gastrin levels from a gastrin-secreting tumor (gastrinoma). Therefore, due to their association with the pancreatic or duodenal gastrinomas, Type 2 GCs, which comprise approximately 5–8% of gastric NETs, are associated with the Zollinger-Ellison syndrome (ZES). Sporadic gastrinomas primarily arise in the pancreas, while gastrinomas due to mutations in the multiple endocrine neoplasia Type 1 locus (MEN-1) tend to develop within the duodenal submucosa [12••]. About 20–25% of patients with ZES and multiple endocrine neoplasia 1 (*MEN1*) mutations develops Type 2 lesions, and about 10–20% of these NETs metastasize. Both Type 1 and Type 2 GCs are multifocal and primarily arise in the corpus where scattered ECL cells are most abundant. Type 3 GCs are sporadic, usually solitary and arise in either the corpus or antrum due to the absence of hypergastrinemia, since they are unrelated to ECL cell hyperplasia. Unlike Types 1 and 2, Type 3 GC exhibits male predominance and is locally aggressive. Type 3 also displays a high rate of metastasis at >50% [11]. A fourth type of GC has been proposed in which there is parietal cell hyperplasia and achlorhydria [13, 14••]. The oxyntic glands exhibit large cysts lined by ciliated epithelium similar to bronchioles. Although hyperplastic, the parietal cells are vacuolated and swollen suggesting a secretory abnormality. Large cysts lined by the abnormal parietal cells are filled with glassy-appearing colloid material. Hyperplastic and dysplastic nests of ECL cells are scattered throughout the corpus, and there is no evidence of autoimmune gastritis, ZES, or MEN1 mutations [14••] (Table 1).

Pathophysiology

Types 1 and 2 GCs are gastrin-dependent tumors. Normally, food stimulates the vagus nerve to initiate the cephalic phase of gastric acid secretion that includes the release of gastrin from antral G cells [15]. During the gastric phase, food in the stomach stimulates antral G cells to secrete gastrin and augments vagal stimulation. Secreted gastrin binds to the cholecystokinin B receptor (CCKBR), which is normally expressed on ECL cells [16, 17]. Moreover, gastrin binding to its receptor stimulates CCKBR gene expression. Therefore, hypergastrinemia amplifies CCKBR signal transduction by increasing the abundance of the receptor. The CCKBR has about a 1000-fold higher affinity for gastrin than for cholecystokinin, which is why this receptor is considered the gastrin-specific receptor. Ligand-receptor binding triggers histamine release from ECL cells that subsequently binds to histamine H2 receptors on parietal cells to stimulate acid. In addition to activation of the ECL cell, gastrin also stimulates gastric epithelial cell proliferation [18]. With autoimmune atrophic gastritis, the parietal cells are attacked by the immune system due to the generation of parietal cell antibodies resulting in hypo- or achlorhydria [19]. Lack of acid triggers G cell hyperplasia leading to hypergastrinemia. Excess gastrin chronically stimulates ECL cells, which subsequently becomes hyperplastic and in some cases becomes transformed into Type 1 gastric carcinoids. However, only a subset of patients with atrophic or autoimmune gastritis develops carcinoid tumors, suggesting that additional factors are required for tumor development. A combination of genetic mutations, bacterial infections, and crosstalk with underlying mesenchyme has been proposed to explain the occurrence of these tumors. As such, infection with *H. pylori* has been implicated as a cause of the atrophic gastritis that can lead to hypergastrinemia and carcinoid tumor development [19–22]. Loss of heterozygosity at the MEN-1 gene locus (11q13, menin) in the stomach occurs in almost all Type 2 GCs, in 17–73% of Type 1 and in 25–50% of Type 3 [23].

Animal Models

Few animal models develop gastric carcinoids, which has presented challenges to studying these tumors and testing potential therapeutic strategies. The African rodent *Praomys (Mastomys) natalensis* develops gastric carcinoids spontaneously at about 2 years and was the first example of an animal

Table 1 Four Types of Gastric NETs

Characteristics	Type 1 G-NET	Type 2 G-NET	Type 3 G-NET	Type 4 G-NET
% of G-NETs	70–80%	5–10%	10–15%	<1%
Gender	Women > men	Women = men	Women < men	Unknown
Tumor #	Multiple	Multiple	Single	Multiple
Tumor size	<10 mm	<10 mm	>10 mm	>10 mm
ECL cell hyperplasia	Yes	Yes	No	Yes
Gastric pH	High	Low	Normal	High
Gastrin	High	High	Normal	High
Histology	Parietal cell atrophy	Parietal cell hyperplasia	Normal corpus	Corpus cysts, colloid filled, vacuolated, hyperplastic, parietal cells
Carcinoid location	Body	Body	Body or antrum	Body , Hyperplastic and dysplastic nests of ECL cells
Ki-67 mitotic index	<2%	<20%	>20%	Not determined
Prognosis	Good	Fair	Poor	
Associated disorders	Pernicious anemia, thyroiditis, primary biliary cirrohosis	MEN1, Zollinger-Ellison Syndrome, NF1	Sporadic	Unknown, no evidence of autoimmune gastritis, ZES or MEN1 mutations
H. pylori	Yes	No	No	No

model with this tumor [24, 25]. Apparently, these animals express a mutant CCKBR rendering the receptor constitutively active increasing its sensitivity to hypergastrinemia [26]. Accordingly, suppression of gastric acid by histamine 2 receptor (H2R) blockade and the associated hypergastrinemia accelerates GC development in these animals to about 4 months instead of 24 months [27]. Although numerous genetic changes or mutations have been identified as potential triggers, there is no direct evidence that *Men1* mutations are sufficient to induce ECL cell transformation [23, 28]. For example, while deletion of the *Men1* locus is sufficient to induce insulinomas in the pancreas and adenomas in the pituitary, no tumors develop in the luminal GI tract [29–32].

Although deleting the *Men1* locus in the GI epithelium using Villin-Cre generates hypergastrinemia, G cell hyperplasia, and epithelial dysplasia, no ECL tumors developed [33], suggesting that more than one mutational "hit" to the genome is required. We previously showed that somatostatin stimulates menin expression in vitro and that both are known inhibitors of gastrin gene expression and secretion. Recently, we reported that conditional deletion of the *Men1* locus on a somatostatin (Sst) null background (*Villin-Cre: Men1*$^{FL/FL}$*; Sst*$^{-/-}$) generated carcinoid hyperplasia and tumors in the corpus that infiltrated the submucosa and smooth muscle layer representing the first genetically engineered mouse model for gastric carcinoid tumors [34••]. Treatment of these mice with the acid suppressing proton pump inhibitor (PPI), omeprazole accelerated the occurrence of these tumors, to within 6 months of beginning acid suppression with omeprazole [34••]. Gastrin-mediated signaling with increased CCKBR expression led to nuclear export of the cyclin kinase inhibitor, $p27^{Kip1}$, correlating with ECL hyperplasia and eventually tumor formation. Importantly, human gastric carcinoids that were also examined showed elevated CCKBR expression and low $p27^{Kip1}$ expression in the cytoplasm that was determined by immunohistochemistry. Moreover, we recently found that *Helicobacter* infection of this genetically engineered mouse model induced ECL hyperplasia and carcinoid tumors, consistent with the anecdotal observations reported in human subjects (Fig. 1). The appearance with Helicobacter infection required 12 months in contrast to 6 months with omeprazole treatment [34••]. One might predict that the combination of chronic Helicobacter infection and acid suppression with PPI will increase the likelihood of carcinoid development. Thus, collectively, this mouse model provides insights into potential therapeutic strategies such as avoiding PPIs in patients with chronic atrophic gastritis, considering CCKBR antagonists to treat patients with types 1 and 2 gastric carcinoids and aggressively eradicating *Helicobacter* infection.

Gastrin Dependence in ECL Hyperplasia

Normal enteroendocrine cells of the human GI tract are nonproliferating and terminally differentiated. However, under the influence of hypergastrinemia, ECL cells proliferate and become hyperplastic. Binding of gastrin to CCKBR is crucial in regulation of ECL cell proliferation, as demonstrated by targeted CCKBR gene disruption in mice [35]. It was known

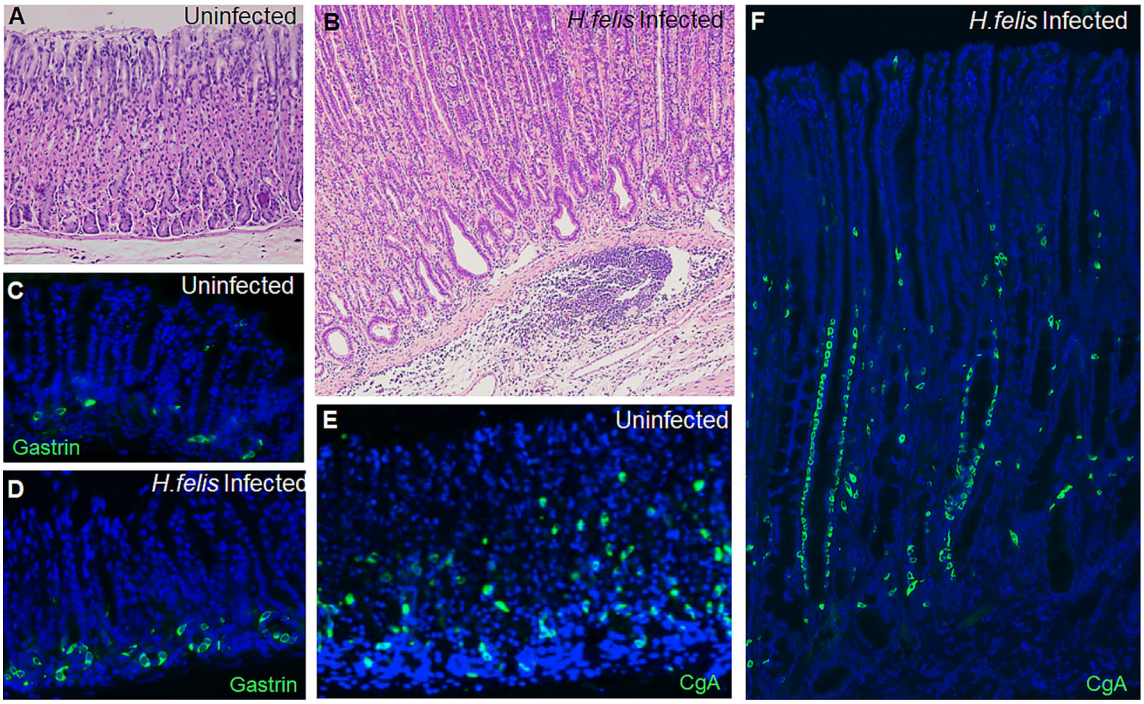

Fig. 1 *H. felis* infection induces G cell and ECL cell hyperplasia in *VC:Men1*$^{fl/fl}$; *Sst*$^{-/-}$ mice. (**a**, **b**) H&E staining of corpus from uninfected *VC:Men1*$^{fl/fl}$; *Sst*$^{-/-}$ mice (**a**) and *VC:Men1*$^{fl/fl}$; *Sst*$^{-/-}$ mice infected with *H. felis* for 6 months (**b**). (**c**, **d**) Immunofluorescent (IF) staining of gastrin in antrums from uninfected (**c**) and *H. felis*-infected *VC:Men1*$^{fl/fl}$; *Sst*$^{-/-}$ mice (**d**). (**e**, **f**) IF staining of chromogranin A (CgA) in corpi from uninfected (**e**) and *H. felis*-infected *VC:Men1*$^{fl/fl}$; *Sst*$^{-/-}$ mice (**f**)

that gastrin binding to CCKBR activates the gene encoding histidine decarboxylase (HDC), the rate-limiting enzyme for histamine synthesis triggering histamine production. Additionally, genes encoding vesicular monoamine transporter molecule 2 (VMAT2), responsible for the storage of histamine in secretory vesicles and chromogranin A (CgA) secreted with other products into ECL vesicles, are also upregulated [36, 37]. However, the signaling and molecular events leading up to ECL hyperplasia were not understood. We showed that binding of gastrin to CCKBR induces phosphorylation and subsequent export of p27^{Kip1} from the nucleus. Loss of p27^{Kip1} expression has been extensively studied in the pathology of endocrine tumors and has been reported to be involved in malignant transformation [34••]. Its export from the nucleus de-represses cell cycle progression, leading to uncontrolled proliferation and hyperplasia. It has been observed that hypergastrinemia induced by acid suppression from omeprazole or loxtidine an irreversible H2 receptor antagonist can lead to ECL cell tumors in rodents [28, 38•, 39]. Indeed, reports are emerging that fundic gland polyps and ECL hyperplasia do arise with prolonged PPI use, but the clinical impact remains uncertain [40–42]. In addition, there are also reports that chronic PPI and Helicobacter infection synergize to induce NETs [43, 44], consistent with observations in the mouse model. Although the contribution of various growth factors including bFGF (basic fibroblast growth factor) and TGFα (transforming growth factor alpha) cannot be overlooked [45, 46], the importance of genetic factors was demonstrated by the high incidence of ECL cell tumors in patients with ZES associated with mutations in the tumor suppressor gene MEN1.

MEN1 and Menin in Carcinoids

The *MEN1* gene locus at 11q13 encodes menin, a known tumor suppressor protein [47]. Upper GI tract NETs exhibit *MEN1* frameshift, missense, or nonsense mutations, which inactivate its function. The mutated *MEN1* locus results in an autosomal dominant phenotype with endocrine tumors of the pituitary and pancreas being the most frequently affected sites [48]. Within the luminal GI tract, gastrinomas comprise the most frequent and malignant *MEN1* tumor type presaging a poor clinical outcome [49, 50]. Pancreatic gastrinomas develop from the islet pancreas [51•, 52]. However, when gastrinomas are part of the *MEN1* syndrome, they exhibit a greater propensity to develop in the submucosa of the duodenum, where the tumors are multifocal and more invasive than their pancreatic counterparts [49], suggesting possible differences in their behavior and cell origin. While whole exome sequencing of non-functional pancreatic NETs has been reported, none were associated with a familial syndrome [53]. In addition, <50% of *MEN1* duodenal gastrinomas exhibit loss of heterozygosity (LOH) [47, 54, 55], suggesting that

mechanisms other than gene inactivation might mediate loss of menin encoded by the WT allele.

Approximately 40–60% of *MEN1* gastrinomas develop within Brunner's glands, which are tubular submucosal structures in the proximal duodenum that produce mucus-rich secretions including bicarbonate [12••, 56]. Apparently, Brunner's glands arise during embryonic development from the base of the intestinal crypt near the gastrointestinal border (pylorus) and thus are considered modified extensions of this intestinal domain [57]. In addition to mucus, Brunner's glands are a major source of growth factor ligands, specifically, EGF ligand family members, trefoil proteins [56, 58], and express EGF receptor family members, which implies autocrine regulation [59, 60]. Amplification of *HER-2/neu* suggests involvement of growth factor signaling in gastrinoma pathogenesis and aggressiveness [61, 62].

Menin protein levels are regulated by nuclear localization signals and post-translational modifications, including phosphorylation, SUMOylation, ubiquitination, and degradation by the proteasome [63–66]. Case studies of duodenal *MEN1* gastrinomas have identified deletions that disrupt the nuclear export (NES) and localization (NLS) signals, although the functional impact of these modifications remains unclear [65, 66]. In addition, it has been shown that missense *MEN1* mutations accelerate menin protein degradation in various endocrine tumors [66–68]. Menin is a promiscuous scaffold protein that forms complexes with a wide variety of proteins including JUND (transcription factor), GFAP (intermediate filament), and MLL (histone methyltransferase), which in turn impact multiple regulatory pathways [69]. Nevertheless, the functional role of these menin-binding partners in MEN1-mediated carcinoid development has not been explored.

Treatments for GI-NETs

Therapy for gastroenteropancreatic-NETs (GEP-NETs) generally consists of somatostatin analogs and radiolabeled somatostatin analogs because of an increase in somatostatin receptor expression on the surface of the tumor. Thus, somatostatin analogs can be used to target the tumors for diagnosis and has been the mainstay for treatment [70–72]. More recently, targeted therapies have evolved such as gastrin receptor antagonist YM022 (netazepide) to treat the gastrin-dependent ECL-based tumors GC Type 1 and GC Type 2 [73, 74]. Telotristat etiprate blocks serotonin production by inhibiting tryptophan hydroxylase 1 and could be used to palliate symptoms from the carcinoid syndrome [71, 75]. For widely metastatic, poorly differentiated and local unresectable disease, platinum-based regimens remain therapy of choice, while the newer target therapies inhibiting specific signaling molecules such as everolimus for mTOR and sunitinib to block tyrosine kinases are currently being tested in clinical trials [71, 76, 77•].

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human subjects performed by any of the authors. All animal protocols were approved by the University of Michigan Animal Care and Use Committee, which maintains an American Association of Assessment and Accreditation of Laboratory Animal Care facility.

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