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Insights into the biology and treatment strategies of pancreatic neuroendocrine tumors

Jessica L. Buicko[1,#], **Brendan M. Finnerty**[1,#], **Tiantian Zhang**[2], **Bu Jung Kim**[2], **Thomas J. Fahey III**[1], **Yi-Chieh Nancy Du**[2]

[1]Department of Surgery, Weill Cornell Medicine, New York, NY 10065, USA

[2]Department of Pathology & Laboratory Medicine, Weill Cornell Medicine, New York, NY 10065, USA

Abstract

Pancreatic neuroendocrine tumors (PNETs) are the second most common primary pancreatic neoplasms after pancreatic ductal adenocarcinoma. PNETs present with widely various clinical manifestation and unfavorable survival rate. The recent advances in next generation sequencing have significantly increased our understanding of the molecular landscape of PNETs and help guide the development of targeted therapies. This review intends to outline a holistic picture of the tumors by discussing current understanding of clinical presentations, up-to-date treatment strategies, novel mouse models, and molecular biology of PNETs. Furthermore, we will provide insight into the future development of more effective targeted therapies that are necessary to manage PNETs.

Keywords

Pancreatic neuroendocrine tumors (PNETs); therapies; mouse models; molecular characterization

Introduction and epidemiology

The incidence and prevalence of neuroendocrine tumors (NETs) have continued to rise in the United States (1). Pancreatic neuroendocrine tumors (PNETs) are rare tumors that account for approximately two percent of all pancreatic neoplasms (2). The estimated incidence of PNETs is 1 to 1.5 per 100,000 with a prevalence of 35 per 100,000 in the United States (3). According to the Surveillance, Epidemiology, and End Results (SEER) database, peak incidence occurs in the eighth decade of life and survival rate is unfavorable for the patients (1,4).

Correspondence to: Yi-Chieh Nancy Du, PhD. Department of Pathology & Laboratory Medicine, Weill Cornell Medicine, New York, NY 10065, USA. nad2012@med.cornell.edu; Thomas J. Fahey III, MD, FACS. Division of Endocrine and Minimally Invasive Surgery, Department of Surgery, Weill Cornell Medicine, New York, NY 10065, USA. tjfahey@med.cornell.edu. .

[#]These authors contributed equally to this work.

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PNETs are biologically and clinically different from pancreatic ductal adenocarcinoma. Due to the heterogeneous molecular and clinical presentations of PNETs, screening test is not readily available yet and patients often present with metastatic disease upon initial evaluation.

Classification

The most accepted consensus classification system comes from the World Health Organization (WHO), 2017 (5). PNETs are classified based on histological features and graded by the proliferation rate of the neoplastic cells, as determined by the mitotic count and/or the Ki67 labeling index (Table 1).

Subtypes of PNET

In general, PNETs are categorized as either functional or nonfunctional based on whether or not they are associated with a hormonal syndrome due to the secretion of a biologically active substance (3,7). It is estimated that 70–80% of PNETs are nonfunctional (8). Functional PNETs include: insulinomas, gastrinomas, VIPomas, glucagonomas, and somatostatinomas (Table 2). Less frequently, they may secrete peptide-hormones including growth hormone releasing hormone (GHRH), adrenocorticotropic hormone (ACTH), parathyroid hormone-related protein (PTHrP), and calcitonin. Both functional and nonfunctional PNETs often secrete other peptides such as chromogranin A, neuron-specific enolase, pancreatic polypeptide, ghrelin, and human chorionic gonadotropin among others (7).

Inherited PNET syndromes

PNETs are usually sporadic but can also occur as part of inherited syndromes. The most frequently encountered syndromes are multiple-endocrine neoplasia type 1 (MEN1), von Hippel-Lindau (VHL), von Recklinghausen disease neurofibromatosis 1 (NF1), and tuberous sclerosis (Table 3). Each of these syndromes is autosomal dominant. Among the 80–100% of MEN1 patients who develop a PNET, the majority develop nonfunctional tumors, while 54% of the patients develop gastrinomas (mostly duodenal) and 18% develop insulinomas (10).

Clinical features of specific PNETs

Functional PNETs

Insulinoma—Insulinomas are the most common functional PNET. The majority are sporadic, but approximately 4% of patients with insulinomas have MEN1 syndrome (10). Insulinomas are twice as common in women. The sporadic form is usually unifocal while those with inherited syndromes may present as multifocal (3). Insulinomas commonly present with sympathoadrenal or neuroglycopenic symptoms associated with hypoglycemia, and relief of the symptoms with glucose intake. Classical picture of these symptoms, known as Whipple’s triad, must be documented before pursuing further diagnostic evaluation and management.

Upon presentation of Whipple's triad, the role of insulin must be evaluated as the potential cause of hypoglycemia. The diagnosis of an insulinoma can usually be established by measuring glucose, insulin, C-peptide, proinsulin, beta-hydroxybutyrate, and sulfonylurea levels during symptomatic episodes, typically a fasting or post-prandial state. Individuals will have elevated insulin, proinsulin, and C-peptide, along with low-blood glucose and have relief of symptoms after oral glucose intake. Beta-hydroxybutyrate will be low due to the anti-ketogenic effect of the hyperinsulinemic state. In addition, the absence of urinary or plasma sulfonylureas can confirm the diagnosis. Insulin autoantibodies can be measured at any time point to rule out insulin autoimmune hypoglycemia.

If the diagnosis is equivocal, a 72-hour fast should be performed, typically as an in-patient. A patient is strictly limited to non-caloric liquid intake while plasma glucose, insulin, C-peptide, and beta-hydroxybutyrate are drawn every 6 hours until hypoglycemia is documented (<60 mg/dL), and then every 1–2 hours thereafter— insulin, C-peptide, and proinsulin should be measured. The fast ends in the following circumstances: when glucose <45 mg/dL and the patient have signs or symptoms of hypoglycemia; when glucose <55 mg/dL without signs or symptoms provided Whipple's triad has been documented prior; or when 72 hours has elapsed. All lab values should be redrawn at the end of the fast, including a sulfonylurea panel, and 1 mg glucagon should be injected with subsequent glucose measurements (11).

Insulinomas are usually small tumors, less than two centimeters in size, and are equally distributed among the pancreatic head, body, and tail. In light of their small size, pre-operative localization can be difficult. Computed tomography (CT) scanning can detect the majority of tumors. Magnetic resonance imaging (MRI) is often second line imaging. In addition, endoscopic ultrasound (EUS) with or without fine needle aspiration biopsy can aid in detection of these tumors. Intraoperative ultrasonography is another useful adjunct. When non-invasive modalities fail to localize an insulinoma, intra-arterial calcium stimulation with hepatic venous sampling for insulin can also be used for localization.

Gastrinoma—Gastrinomas are the second most common functional PNET. They are sporadic and are located predominantly within the duodenum followed by the pancreas. The mean age at presentation is 50 years and they are found more often in males (3). The majority of gastrinomas are malignant. Gastrinomas are associated with MEN1 in approximately 25% of the cases and the remaining 75% arise sporadically (9,12). Conversely, gastrinomas occur in approximately one-half of patients with MEN1 and are the most common functional NET in this condition (13).

Gastrinomas secrete gastrin, which leads to gastric acid hypersecretion and the clinical syndrome known as Zollinger-Ellison Syndrome (ZES). Patients commonly present symptoms of heartburn, weight loss, and nausea; astute recognition is crucial, as the average time to diagnose ZES is over five years after evaluation of initial symptoms. As a result, patients with gastrinomas often develop intractable gastrointestinal ulcers, and up to ten percent are present with sequelae of severe peptic ulcer disease prior to ZES diagnosis, such as bleeding, perforation, or gastric outlet obstruction (14). Therefore, ZES should be suspected in patients with severe peptic ulcer disease, including those with ulcers in unusual

locations associated with hypergastrinemia, such as in the duodenal bulb. The distribution of symptoms is similar between sporadic and MENl-associated gastrinoma; however, MEN1 patients present less commonly with abdominal pain (66%), and instead may display signs of concomitant hyperparathyroidism and/or pituitary adenoma (15).

The diagnosis is made by measuring fasting gastrin levels (FSG), which are often ten times higher than the upper limit of normal. The diagnosis can be more difficult if patients are on proton pump inhibitors (PPIs) or histamine-2 receptor antagonists since the medications can falsely elevate gastrin levels (16). Thus, these medications should be stopped prior to testing if possible. Intra-gastric pH testing, either via trans-nasal pH probe monitoring or intraluminal gastric fluid analysis using pH indicator paper, can aid in the diagnosis of ZES. In equivocal cases where FSG is less than ten times the upper limit of normal, secretin stimulation testing is used to help establish a diagnosis. Furthermore, evaluation for MEN1 should be considered, including calcium, parathyroid hormone, and prolactin levels, with or without MEN1 genetic testing.

Imaging modalities for localization of gastrinomas are similar to insulinomas, which include CT, MRI, and EUS. Somatostatin receptor scintigraphy (SRS), known as octreotide scanning, is another modality that can aid in the localization of gastrinomas (3). Several prospective studies demonstrated an improved sensitivity in detecting gastrinomas using [111In-DTPA-D-Phe1]- octreotide (primary tumors 58–78%, metastases 92–100%) as compared to other imaging modalities (17,18). More recently, gallium-labeled somatostatin analogues (usually ^{68}Ga) have been used to accurately identify the location of gastrinoma and other PNETs. In gastrinoma, only one small series evaluated the efficacy of ^{68}Ga-DOTANOC as compared to contrast-enhanced CT; ^{68}Ga-DOTANOC identified tumors in 36% and 93% of negative and equivocal CT scan results, respectively (19).

VIPoma—VIPomas are usually associated with Verner-Morrison syndrome, also known as WDHA syndrome. Symptoms include watery diarrhea, hypokalemia, and achlorhydria. They are usually found as single tumors in the pancreatic tail and are often metastatic at presentation (10). The diagnosis of VIPoma is made by an elevated serum VIP level in a patient with large-volume diarrhea.

Glucagonoma—Glucagonomas are frequently larger tumors (greater than 6 centimeters) that occur between the ages of 40–50 (2,7). The majority are malignant. Patients frequently present with weight loss, glucose intolerance, and a characteristic rash known as necrolytic migratory erythema. The tumors are usually found in the pancreatic body and tail.

Diagnosis is made by measuring increased plasma levels of glucagon, usually over ten times greater than the normal level. Since they are usually large tumors, CT scan is usually sufficient for imaging.

Somatostatinoma—Somatostatinomas are very rare and encompass only 1% of all PNETs. They are usually malignant and over 75% have metastasized by the time of presentation (3). Somatostatinomas are large, solitary, and intrapancreatic in location (10).

They commonly present with diabetes, gallstones, and steatorrhea. Diagnosis is confirmed by an elevated somatostatin level.

Other rare functional PNETs—GHRHomas secrete GHRH, which can result in acromegaly. These tumors can be found in the pancreas, lung, or other abdominal locations (10). Diagnosis is made by measuring elevated plasma growth hormone levels. ACTHomas are associated with ectopic Cushing syndrome and are often associated with liver metastases. PNETs secreting PTH-rP classically present with hypercalcemia.

Nonfunctional PNETs (NF-PNETs)

As previously mentioned, about 70–80% of PNETs are nonfunctional. They are often diagnosed during radiographic screening for unrelated issues or nonspecific abdominal complaints. NF-PNETs do not have a specific clinical syndrome but between 60–100% of NF-PNETs secrete a number of peptides that can help with diagnosis, including: chromogranin A, neuron-specific enolase, pancreatic polypeptide, ghrelin, neurotensin, motilin, or human gonadotrophin (20).

NF-PNETs are usually slow-growing, indolent tumors. The majority are asymptomatic and are thought by some to be over-diagnosed—especially when small—prompting controversy as to whether these tumors need to be treated. Nonetheless, incidentally discovered NF-PNETs are being diagnosed more frequently. Crippa *et al.* studied 355 patients with NF-PNETs over an 18-year period [1990–2009] and found that the frequency of incidentally found tumors increased from 9% [1992–1996] to 40% [2007–2009] (21). A case series from Massachusetts General Hospital reported that over 80% of NF-PNETs were incidentally found (139 *vs.* 30 patients) (22). Decisions regarding treatment of NF- PNETs are multifactorial and often based on size and grade, combined with consideration of patient comorbidities and risks (20).

Treatment of PNETs

Surgical management

The treatment for functional PNETs should be surgical resection whenever possible. Surgery improves longterm survival and helps prevent metastases. Many PNETs, including insulinomas—especially if smaller than two centimeters—can be treated by surgical enucleation. Enucleation removes the area of the tumor and tumor capsule and spares otherwise normal pancreatic parenchyma (23). Tumors that are larger, deeper in the pancreatic parenchyma, higher grade, or suspicious for malignancy should undergo anatomic resection. In general, resection of functional PNETs should be offered as long as the patient has an acceptable surgical risk. Sporadic gastrinomas have high incidence of lymph node metastasis and surgical resection should include a systematic lymph node dissection (23). The role of surgery in patients with MEN1 and ZES syndrome is controversial. These tumors are often small, microscopic, and multifocal. Some studies have shown that these patients have excellent prognoses even without surgery if there are no tumors greater than 2 cm (10,24). MEN1 is associated with decreased rates of the immediate post-surgical disease-free status (50% *vs.* 15%), 5-year disease-free survival (40% *vs.* 4%), and 10-year disease-

free survival (34% *vs.* 0%); however, importantly, there are no differences in overall survival between sporadic and MEN1 associated gastrinoma (25,26). The most important predictor of worse survival in gastrinomas is liver metastasis, which is associated with tumors greater than 2 cm (26–29).

For NF-PNETs, surgery is the treatment of choice for tumors greater than 2 cm. Because more NF-PNETs are being found incidentally, the optimal management of tumors smaller than 2 cm is currently under debate. The majority of lesions of this size are benign or intermediaterisk, and it has been reported that only 6% of NF-PNETs under 2 cm are malignant (30). The most recent ENETS guidelines suggest a conservative approach for NF-PNETs under 2 cm as studies showed no significant changes during follow-up for the majority of these patients (31).

Medical management

Medical management of PNETs involves controlling the symptoms of hormonal hypersecretion. For insulinomas, frequent small meals help manage hypoglycemia. In addition, diazoxide and long-acting somatostatin analogues, such as octreotide and lanreotide, can control symptoms (10). Many patients with gastrinomas are on either histamine H2-receptor antagonists or proton pump inhibitors to help control acid hypersecretion. Longacting somatostatin analogues are also helpful in patients with VIPomas, glucagonomas, somatostatinomas, and GHRHomas (10). Adverse effects of these medications include diarrhea, nausea, and gallstones.

Management of advanced PNETs

Cytoreductive surgery—In stark contrast to exocrine pancreatic cancers, patients with endocrine pancreatic neoplasms benefit from aggressive resection in the setting of hepatic metastases. If untreated, patients with hepatic metastases from NETs have a 5-year survival between 20% and 40% (32). Tumor debulking can help patients who are symptomatic from the hormonal activity from high tumor burden (3). In a Mayo Clinic series, the authors concluded that palliative surgical resection is safe, can provide excellent palliation of symptoms, and may prolong survival if all or more than 90% of gross metastatic disease can be removed (33,34). The first line treatment for PNET liver metastases is surgical resection since other options including chemoembolization, hormonal therapy, and chemotherapy fail to completely eradicate the tumors (35). Nonetheless, undetectable hepatic micrometases by radiologic or gross examination may still remain even after aggressive resection. These micrometases are associated with worse outcomes and higher recurrence rates (36).

Intra-arterial therapies (IAT)—Liver-directed surgery for PNET liver metastases has a high rate of recurrence with estimates that over 90% of patients will recur by 5 years (32). Some studies suggest that various types of IAT are best suited for PNET liver metastases since these tumors commonly derive the majority of their blood supply from the hepatic artery, unlike other types of hepatic metastases. Different types of IAT for these tumors include: trans-arterial chemoembolization (TACE), transarterial bland embolization (TAE), chemoembolization with drug-eluting beads, and radioembolization using Y90 spheres (3). Most studies of IAT are retrospective in nature. An Australian study by Bester and

colleagues evaluated survival and safety of using Y90 spheres in patients with refractory liver metastases. They found improved symptom control and survival with Y90 treatment (37). A large study by Mayo *et al.* sought to identify which patients were best managed with surgery versus IAT. They concluded that patients with significantly high burdens of intrahepatic disease were likely best managed with locoregional IAT rather than surgical debulking (38).

Other treatment options—Radiofrequency ablation (RFA) and cryotherapy are also available modalities of treatment performed either percutaneously or via a laparoscopic approach. Some studies have shown the benefit in local and hormonal symptom control but long-term outcome studies in these patients are lacking (3).

In addition, most PNETs overexpress somatostatin receptors. This has led to newer investigations into using targeted radiolabeled cytotoxic agents to treat unresectable, malignant PNETs. Peptide receptor radionuclide therapy (PRRT) is a new promising systemic therapy. Studies have shown PRRT has an improved progression-free survival rate at 20 months (65% *vs.* 11%), objective response rate (18% *vs.* 3%), and overall survival (interim analysis) as compared to long-acting octreotide in inoperable somatostatin-receptor positive metastatic midgut NETs (39). A small retrospective study from Israel examined eleven patients with metastatic gastrinomas and found that PRRT induced symptomatic improvement in all patients, in addition to decreasing serum gastrin levels (40). Surveillance showed that 1 patient (9%) had a complete response, and 5 patients (45%) had a partial response and tumor stabilization, demonstrating that PRRT is a promising systemic therapy in these patients (40).

Chemotherapy combinations such as streptozocin, doxorubicin, and fluorouracil have shown some antitumor activity in metastatic PNETs. Kouvaraki *et al.,* retrospectively studied 84 patients with metastatic PNET and found that 39% of patients had either complete or partial response to treatment with fluorouracil, doxorubicin, and streptozocin (41). A newer temozolomide based regimen (+/− capecitabine) has shown objective response rates widely ranging from 15–70% (41–43).

Recent clinical trials have investigated targeted therapies directed against the mammalian target of rapamycin (mTOR) pathway and angiogenic growth factors. Yao *et al.* published the third trial study (RADIANT-3), examining whether an mTOR inhibitor, everolimus, would prolong progression-free survival among patients with advanced PNETs (44). They found that patients with advanced inoperable PNETs had a median progression-free survival of 11.0 months compared to 4.6 months in the placebo group ($P<0.001$) (44). A multinational, randomized, double-blind, placebo-controlled phase III trial of a multitargeted tyrosine kinase inhibitor, sunitinib, in patients with advanced, well-differentiated PNETs showed promising results with improved progression-free survival (11.4 *vs.* 5.5 months), overall survival, and objective response rate compared with placebo in this patient cohort (45).

In summary, surgical resection for oncologic cure or hormonal symptom control should be considered for the majority of PNETs, even in the setting of metastatic diseases. Recurrent

and metastatic diseases should also be considered for adjuvant therapies such as peptide receptor radionuclide therapy, mTOR inhibitors, tyrosine kinase inhibitors, chemotherapy, and somatostatin analogs, while liver-directed interventional therapies are reserved for unresectable liver metastases.

Mouse modeling for progression and metastasis of PNETs

In this section, we summarize different mouse PNET models, including genetically engineered mouse models *(Table 4)* and xenografts, which have been used to gain important insights into the biology and treatment of PNETs.

Genetically engineered mouse models

The RIP1-Tag2 (RIP-Tag) transgenic mice—The *RIP-Tag* mouse model, developed by Douglas Hanahan, was one of the first genetically engineered transgenic mouse lines expressing oncogenes (46). In this model, the rat insulin promoter (RIP) drives the expression of SV40 T antigen (Tag), providing the driving force for tumor initiation by inhibiting two tumor suppressors, p53 (54) and Rb (55). Mice developed insulinomas with 100% penetrance through well-defined stages that are similar to human tumorigenesis, including hyperplasia, angiogenesis, adenoma, and invasive carcinoma. Although expression of Tag began in the developing pancreas at embryonic day 9 and was sustained in all ~400 pancreatic islets throughout the life of the mice (56), islets were histologically “normal” until ~5 weeks of age. Then, hyperplastic islets emerged stochastically from relatively quiescent β cells. From this hyperplastic stage, angiogenic islets emerged, wherein the growth of new blood vessels took place in ~10 % of the total islets. Islet tumors then arose from the angiogenic islets, either with well-defined margins or locally invasive carcinoma. At 12–16 weeks of age, 2–4% of the islets became adenomas and less than 1% of the total islets developed into invasive carcinoma. The majority of tumors produce insulin and cause mice to die from hypoglycemia, typically before the formation of distant metastasis. The highly predictable tumor progression and relatively short tumor latency have made the *RIP-Tag* mouse model extremely useful for the discovery for genes contributing to various steps of tumorigenesis. Importantly, preclinical trials in *RIP-Tag* mice have predicted that both sunitinib and everolimus would be effective in treating human PNETs (57–59).

A comprehensive cross-species analysis of mRNA and miRNA transcriptomes of PNETs from this mouse model and human supports for the *RIP-Tag* mouse model as representative of the human PNETs without MEN1 mutations (60).

RIP-Tag; RIP-tva bitransgenic mice—To generate a system in which genetic alterations can be rapidly tested for their roles in tumorigenesis, we have utilized the *RIP-Tag* mouse model and developed a bitransgenic mouse model, *RIP-Tag; RIP-tva,* in which the expression of the tva receptor for subgroup A avian leukosis virus is also driven by RIP (47), in an RIP- Tag background. As such, genetic alternations can be introduced into premalignant lesions of pancreatic β cells by infection with an avian retroviral vector such as RCASBP via intracardiac injection (47,48). This approach mimics sporadic tumor development. This strategy also avoids any potential perturbation of normal tissue formation often observed in conventional transgenic models due to the ectopic expression of the gene

of interest during development. It is much faster to generate vectors carrying genes of interest than to generate transgenic mice. Importantly, genes that promote metastasis can be easily identified, because macroscopic metastases are not typically evident in *RIP-Tag; RIP-tva* mice. Moreover, by deriving cell lines from *RIP-Tag; RIP- tva* tumors, further biochemical and cellular analyses can be performed in vitro.

Using this model, we have gained insights into factors and pathways that contribute to cancer metastasis, some of which are summarized in the next section.

Men1 knockout and conditional knockout mice—MEN1 patients, who are heterozygous for a loss-of- function mutation in one *MEN1* allele, have a high incidence of PNETs (61). The MEN1 gene encodes a 610-amino acid protein, menin, which is ubiquitously expressed and located in the nucleus (62). Menin is a component of a histone methyltransferase complex with diverse functions (63). Mouse menin is 97% identical to the human protein. Homozygous *Menl* knockout mice are embryonic lethal, while heterozygous mutant mice develop features of endocrine tumors similar to those of the human disorder (64,65). Various mouse models of menin loss have been described in detail by Li *et al.* (49). The pancreatic-specific *Men1* knockout mice developed PNETs and, less frequently, pituitary adenomas, and parathyroid adenomas (49). The tumor latency is between 5 to 13 months.

The pINS-c-MycER^{TAM} and pINS-c-MycER^{TAM}; RIP7- Bcl-xL inducible transgenic mice—The transcription factor, c-Myc, can induce cell proliferation and apoptosis (66). In the *pINS-c-MycER^{TAM}* mouse model, an inducible c-Myc protein, c-MycER (human c-Myc fused to the ligand-binding domain of a modified estrogen receptor) is expressed in pancreatic β cells under control of an insulin promoter (50). Activation of c-MycER by daily intraperitoneal administration of the ligand, 4-hydroxytamoxifen (4-OHT) led to pancreatic β cell proliferation within 24 hours. However, apoptosis was evident, after 72 hours of c-MycER activation. Thus, after 6–10 days of continued c-MycER activity, almost complete ablation of P cells ensued. Interestingly, islets can be regenerated after deactivation of c-MycER by stopping 4-OHT administration.

Alternatively, when c-Myc-induced apoptosis was blocked by co-expression of an anti-apoptotic protein, Bcl- xL, in the *pINS-c-MycER^{TAM}; RIP7-Bcl-xL* model, c-MycER then induced rapid and uniform tumor progression in β cells (50). Islets became hyperplastic throughout the pancreas within 7 days of 4-OHT treatment. Angiogenesis arose with similar kinetics in each islet, suggesting c-MycER induced an angiogenic response coincident with its induction of hyperplasia in P cells. Also, within 7 days of c-MycER activation, widespread loss of E-cadherin coincided with loss of P cell-cell contacts. Within 2 weeks of c-MycER activation, 50% of islets were locally invasive. By 6 weeks, much of the exocrine pancreas was replaced by β cell tumors. At 8 weeks, disseminated β cells were detected in blood vessels, pancreatic ducts, and pancreatic lymph nodes. Nonetheless, deactivation of c-MycER led to regression of tumors and reversion to phenotypically normal islets in *pINS-c-MycER^{TAM}; RIP7-Bcl-xL* mice.

Compared to the *RIP-Tag* model of sporadic tumor progression only in a subset of progenitors, c-Myc triggers rapid and uniform tumor progression in all islets as long as apoptosis is suppressed. This result suggests that secondary lesions are not required for the development of invasive tumors induced by c-Myc in this mouse model.

LSL-MYCN; hGFAP-Cre mice—MYCN amplification drives pediatric neuroblastoma (67) and neuroendocrine prostate cancer (68), but its role in PNET progression is unknown. Using hGFAP promoter to drive the expression of Cre recombinase and MYCN in neuroendocrine cells and in the developing nervous system in adulthood, *LSL-MYCN; hGFAP-Cre* mice developed PNETs and, less frequently, pituitary gland malignancies with an incidence of 59% (32/54) and 6 to 11 months of tumor latency (51). These PNETs strongly produced and secreted glucagon. Cell lines, derived from the mouse PNETs, were susceptible to MYCN-directed small-molecule inhibitors *in vitro* and *in vivo* (51).

Ren-Cre; $p53^{loxP/loxP}$; $Rb^{loxP/loxP}$ mice—The renin gene is classically associated with expression in the kidney, but also expressed in the pancreas (69). Conditional deletion of p53 and Rb by Cre recombinase under the renin promoter led to the development of glucagonomas and highly penetrant metastases in the liver and pancreatic lymph nodes (52). These mice died by 29 weeks of age.

RIP-rtTA; tet-o-PyMT-IRES-Luciferase inducible transgenic mice—Polyoma middle T antigen (PyMT) is a potent oncoprotein, and it stimulates at least two signaling pathways that are important in human cancers—the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades (70). It has been shown that PyMT can sufficiently induce tumor formation in mammary glands, endothelial cells, exocrine pancreas, and liver (71–73).

Mice with doxycycline-inducible PyMT linked to a luciferase reporter *(tet-o-PyMT-IRES-Luciferase)* were generated. Intriguingly, induction of the potent PyMT oncogene in pancreatic β cells only leads to irreversible expansion of β cells, regardless of the developmental stage at which it is expressed (53). PyMT-induced β-cell hyperplasia is associated with a low proliferation index (as judged by Ki67 staining: ~4%), compared to Tag-induced β-cell hyperplasia (~21%) (53) and c-Myc-induced β-cell hyperplasia (near 100%) (50). The P-cell hyperplastic lesions do not progress to malignant tumors even after one year of PyMT induction and even in an *Ink4a/Arf-null* or *Arf-null* background (53). Upon PyMT de-induction, the proliferation of β cells was greatly reduced. However, no apoptosis was detected and the islets remained enlarged.

Therefore, unlike Tag and c-Myc, PyMT expression is not sufficient for tumor initiation in p cells, indicating that MAPK and PI3K pathway activation is inadequate for initiation of PNETs.

Xenograft mouse models with cell lines

One challenge in treating PNETs is that they are often metastasized by the time of diagnosis, with the liver being the most common site of metastasis. Orthotopic modeling by intrasplenic injection of human PNET cells into mice can be used to study liver metastasis

and to test novel therapeutic strategies (74,75). The limitations of this orthotopic model of liver metastasis are that (I) it starts with introducing single tumor cell suspension into mice, which bypasses early steps of metastasis; and (II) immunodeficient mouse hosts are required for human cell lines, which prohibits the study of contribution of immune cells in metastasis. If mouse pancreatic tumor cell lines are used, syngeneic mouse strains with intact immune system can also be used to establish liver metastasis via intrasplenic injection or tail vein injection (76).

Three human PNET cell lines, BON1, CM, and QGP1, and a few mouse PNET cell lines are available. BON1 was established from a peri-pancreatic lymph node from a 28-year-old male patient with metastatic PNET (77). CM was derived from the peritoneal ascitic fluid of a patient with insulinoma (78). QGP1 was established from a well-circumscribed nodular lesion in the tail of pancreas from a 61-year-old male patient. This patient had vascular invasion and metastases to peri-pancreatic lymph nodes and the liver (79). Both of the primary tumor and QGP1 produce carcinoembryonic antigen (CEA). QGP1 secretes somatostatin, but not insulin and glucagon. QGP1 is deficient for Rb expression (80). To detect the localization and growth of the tumor cells inside mice by *in vivo* luciferase bioluminescence imaging, our group has engineered these PNET cell lines to express a luciferase reporter [(81) and unpublished work].

Molecular characterization of PNETs

Understanding the biology of PNETs can guide the treatment and pave the way for the development of new therapeutic agents. We discuss the recent molecular characterization of PNETs in this section. It is important to bear in mind that PNETs are clinically and genomically heterogeneous.

mTOR pathway

Genes in mTOR pathway such as phosphatase and tensin homolog (PTEN), tuberous sclerosis 2 (TSC2), and Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA) are dysregulated in PNETs. The dysregulation of PTEN in PNETs could come from DNA mutations, reduced RNA expression, and altered protein subcellular localization. Normal islet cells exhibited predominantly nuclear PTEN immunostaining, but 19 of 23 PNETs (82.6%) had a predominantly cytoplasmic PTEN staining (82). Another study showed that expression of PTEN and TSC2 was downregulated in most of the 72 primary PNETs analyzed by microarray studies and their low expression was associated with shorter disease-free and overall survival (83). Moreover, whole-exome sequencing of sporadic PNETs showed that 14% of their cohort of PNETs had mutations in genes in the mTOR pathway, including PTEN, TSC2, and PIK3CA (84).

MEN1, DAXX, and ATRX

A study of the molecular landscape of sporadic PNETs using whole-exome sequencing revealed that 44% of the tumors had somatic inactivating mutations in MEN1, and 43% had mutations in genes encoding either of the two subunits of a transcription/chromatin remodeling complex consisting of death-domain associated protein (DAXX) and alpha

thalassemia/mental retardation syndrome X-linked (ATRX) (84). Mutations in DAXX and ATRX promote alternative lengthening of telomeres and chromosomal instability (85,86).

Although it was initially reported that DAXX/ATRX mutations were associated with better prognosis (84), recent studies showed that tumors harboring DAXX or ATRX mutations were associated with a poor prognosis (87,88). The discrepancy between these studies might be attributed to a different composition of the PNET subtypes and treatment.

A recent whole-genome sequencing of 102 sporadic PNETs discovered a larger-than-anticipated germline mutation contribution to PNET development (87). These germline mutations include not only the known mutations in *MEN1* and *VHL,* but also previously unreported mutations in the DNA repair genes *MUTYH, CHEK2,* and *BRCA2.* Altogether, these germline mutations occur in 17% of the patients. In addition to germline mutations, somatic mutations are found in genes involved in four main pathways: chromatin remodeling, DNA damage repair, activation of mTOR signaling, and telomere maintenance. In addition, whole-genome and transcriptome sequencing of PNET liver metastases identified loss of MEN1 and DAXX in 4 of the 5 cases (89).

Cyclin D1, Cdk4, and Retinoblastoma (Rb) pathway

Cyclin D1 has been found to overexpress in PNETs (90,91). Cdk4 staining was detected in 58% of PNETs, and phospho-Rb1 was detected in 68% of PNETs in immunohistochemistry study using a TMA constructed from 92 cases of well-differentiated PNETs (92). Cdk4, cyclin D1, and phospho-Rb1 could be detected in normal islets. The correlation between phospho-Rb1 and Cdk4 protein expression is significant as well as the correlation between phospho-Rb1 and cyclin D1 protein expression (92). Growth of a PNET cell line, QGP1, was inhibited *in vitro* and in a xenograft mouse model by the Cdk4/6 inhibitor, PD 0332991, which reactivated the Rb pathway. A recent sequencing study found Rb1 somatic mutation in 1 of the 11 PNETs (93).

p53 pathway

The tumor suppressor p53 protein levels and activities are regulated by many proteins, among which MDM2, MDM4, and WIP1 are key negative regulators. An array-based CGH analysis on 55 PNETs revealed a high percentage of PNET; contain extra gene copies of MDM2 (22%), MDM4 (30%), and WIP1 (51%), which are correlated with expression of corresponding mRNAs and proteins (94). About 70% of PNETs have one or more of these genetic changes in this study. Another study reported somatic mutation in TP53 in 2 of the 11 PNETs (18%) (93). Therefore, downregulation of p53 function could be important for the initiation and progression of PNETs.

UCHL1

To improve risk stratification of aggressive tumors at initial fine needle aspiration (FNA) biopsy, our group has investigated the utility of novel diagnostic biomarkers, including ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). Loss of UCHL1 has been associated with metastasis in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) via CpG promoter hypermethylation, and was further shown to be an independent risk factor for

metastatic disease at the time of index operation (95). In a cohort of well- and moderately-differentiated PNET (Ki67 <20%) samples obtained by EUS-FNA, weak UCHL1 staining on immunocytochemistry had 80% sensitivity, 65% specificity, 63% positive predictive value, and 81% negative predictive value to identify primary tumors associated with metastatic disease. Combining weak UCHL1 staining and high Ki67 (>3%) increased the test specificity to 95%, and, on multivariable analysis, this combined positive test was an independent predictor of metastatic disease (96). Mechanistically, its re-expression in the UCHL1-deficient cell lines BON1 and QGP1 induces a less aggressive phenotype, in part by inducing cell-cycle arrest through post- translational regulation of phosphorylated CHK2 (97). Using UCHL1 expression as a definitive diagnostic biomarker should be confirmed in a prospective cohort, and targeting its downstream effectors needs to be investigated as a potential therapy.

Bcl-xL

Bcl-xL is overexpressed in a variety of cancers. Bcl-xL has long been known for its function in regulating apoptosis on mitochondria. Any role that Bcl-xL might play in tumor metastasis has been ascribed to its anti-apoptotic function; i.e., Bcl-xL may increase metastasis by lending survival advantage to tumor cells during the course of metastasis. However, specific Bcl-xL mutants defective in anti-apoptotic function still promoted metastasis of PNETs in spontaneous RIP-Tag; RIP-tva mouse model and xenograft mouse models (81). ABT-737, the prototype of Bcl-xL small molecular inhibitors, was designed to block the anti-apoptotic function. However, ABT-737 did not affect the ability of Bcl-xL-mediated cell migration. Moreover, prominent nuclear Bcl-xL were found in 3 of 7 PNET liver metastases (81). Therefore, Bcl-xL's metastatic function is independent of its canonical anti-apoptotic activity and may require a novel nuclear function in PNETs. Therefore, development of therapeutics that blocks the dual functions of Bcl-xL in anti-apoptosis and metastasis is required.

RHAMM

A screen using a library of cancer genes in RIP-Tag; RIP-tva mice identified the first gene that promotes liver-specific metastasis of PNETs. This gene encodes Receptor for hyaluronan-mediated motility isoform B, RHAMM^{B} (76). Liver-specific metastasis was recapitulated in a tail vein assay of metastasis in which mouse PNET cells overexpressing RHAMM^{B}, but not the control cells, initially circulated through the lung capillary beds of the recipient immunodeficient mice (76). RHAMM is not expressed in normal adult pancreas and its expression is restricted in other normal adult human tissues (98). Our studies showed that RHAMM^{B} is overexpressed in human primary PNETs and liver metastases and it is a key driver for liver metastasis of PNET in mouse models (99). Therefore, RHAMM^{B} could be a potential therapeutic target in PNETs.

MYC family

The myc family of cellular oncogenes contains three members: c-myc, n-myc and l-myc. c-Myc protein was overexpressed in insulinomas by immunostaining (100). MYCN (N-MYC) was found to have a 38-copy gain in one of the 5 PNET liver metastases (89). This is a

nonfunctional metastatic tumor with wild-type MEN1 and DAXX, but lost of APC and TP53.

microRNAs (miRNAs)

miRNAs are small (~19–24 nucleotide in length) RNA molecules that play essential roles in many biological processes (101). A cross-species study of miRNAs identified stage-specific miRNA expression signatures in human PNETs and RIP-Tag mouse model of PNETs (102). Whether any of the miRNAs contribute to the development of PNETs needs to be investigated.

Up to 20% of metastatic GEP-NETs are without a known primary site. Through a study of miRNA expression profiling from archived pancreatic, ileal, appendiceal, and rectal NETs, the expression of 7 miRNAs (miR- 615, miR-92b, miR-125b, miR-192, miR-149, miR-429, miR-487b) can be used to discriminate these 4 types of GEP-NETs (103).

Future directions

From a clinical and translational perspective, developing cost-effective early detection methods, understanding the molecular pathways of various subtypes of PNETs, and developing more effective targeted therapies are necessary to eradicate PNETs. The mechanisms of resistance that allow tumors to thrive during everolimus or sunitinib therapy need to be fully elucidated and targeted to improve the efficacy of these clinically successful therapies. Additionally, improving upon the efficacy of PRRT is critical to achieve cure, or at least a durable partial response in patients with metastatic PNETs. Lastly, improving detection of aggressive tumors by evaluating novel molecular markers such as UCHL1 during initial FNA biopsy is critical for risk stratification and controlling disease burden at the index operation.

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Table 1

2017 WHO classification of PNETs

WHO classification	Grade	Mitotic count (per 10 high-powered field)	Ki67 index
Well-differentiated neuroendocrine tumors (NETs)	Grade I (GI)	<2	<3%
	Grade II (G2)	2–20	3–20%
	Grade III (G3)	>20	>20%
Poorly differentiated neuroendocrine carcinoma (NECs)	Grade III (G3) small cell or large cell	>20	>20%
Mixed neuroendocrine & non-neuroendocrine subtype (MiNEN)			

* adapted from (6).

Table 2

List of the most frequent functional PNETs

Functional PNET	Incidence	Primary location	Main clinical features	Malignancy (%)
Insulinoma	4 per million	Evenly distributed throughout pancreas	Whipple's triad	5–15%
Gastrinoma	0.2–1 per million	Gastrinoma triangle	Gastric acid hypersection, Zollinger-Ellison syndrome	60–90%
VIPoma	0.05–0.5 per million	Distal pancreas (body, tail)	Watery diarrhea, hypokalemia, achlorhydria	70–90%
Glucagonoma	1 per 20 million	Distal pancreas (body, tail)	Diabetes mellitus, necrolytic migratory erythema, glossitis	60–75%
Somatostatinoma	1 per 40 million	Pancreas, ampulla, duodenum	Cholelithiasis, diabetes mellitus,	40–60%

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Table 3

List of inherited PNET syndromes

Syndrome	Prevalence	Gene, location	Protein	Type of PNET	Frequency PNETs
Multiple endocrine neoplasia type I (MEN1)	1–10/100,000	MEN1, 11q13	Menin	Majority nonfunctional	80–100%
Von Hippel-Lindau (VHL)	2–3/100,000	VHL, 3p25	VCB-CUL2	Majority nonfunctional	10–17%
Von recklinghausen disease (Neurofibromatosis NF1)	1/4,000–5,000	NF1, 17q11.2	Neurofibromin	Duodenal somatostatinomas, rare functional PNETs	≤10%
Tuberous sclerosis	1/10,000	TSC1 and TSC2, 9q34	Hamartin and tuberin	Majority nonfunctional	Rare

* adapted from (9).

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Table 4

List of PNET mouse models

Genetic modification	Phenotype	Incidence	Tumor latency	References
RIP-Tag	Insulinoma	100%	12 to 16 weeks	(46)
RIP-Tag; RIP-tva	Insulinoma	100%	12 to 16 weeks	(47,48)
Men1 knockout and conditional knockout	PNETs and, less frequently, pituitary adenomas, and parathyroid adenomas		5 to 13 months	(49)
pINS-c-MycER^{TAM}	complete ablation of β cells	100%	6~10 days	(50)
pINS-c-MycER^{TAM}; RIP7-Bcl-xL	Insulinoma	100%	within 2 weeks	(50)
LSL-MYCN; hGFAP-Cre	PNETs and, less frequently, pituitary gland malignancies	59%	6 to 11 months	(51)
Ren-Cre; p53$^{loxP/loxP}$; Rb$^{loxP/loxP}$	glucagonomas with metastases	100%	~4 months	(52)
RIP-rtTA, tet-o-PyMT-IRES-Luciferase	p-cell hyperplasia when on doxycycline	100%	No tumors	(53)

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