Chapter 2
Inherited and Somatic Genetics of Pancreatic Neuroendocrine Tumors

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2.1 Introduction

Pancreatic neuroendocrine tumors (PNETs) are tumors derived from endocrine cells of the pancreas found in the islets of Langerhans. PNETs are rare tumors with an incidence in the United States of 3.65 per 100,000 [1]. On autopsy studies, up to 10 % of individuals have PNETs, suggesting many tumors remain undiagnosed [2]. PNETs can occur at any age, with a peak incidence in the fourth to sixth decades of life, and are believed to follow a classic model of tumor progression. The tumors are broadly classified into functional (15 %) and nonfunctional (85 %) PNETs, based on whether they can retain the ability to release one or more hormones such as insulin, gastrin, or glucagon. Nonfunctional PNETs have a worse prognosis, likely due to relative delay in diagnosis, as they are usually discovered at later stages and often are more poorly differentiated [3, 4]. The most common functional tumor, the insulinoma, often is diagnosed while still small and localized because of the severity of symptoms associated with insulin hypersecretion, and thus, the five-year survival rate is quite high at 85–95 %. Seventy percent of patients with the more common nonfunctional tumors present with unresectable disease, often with liver metastases, and the five-year survival rate is only 30–40 % with a median survival of 24 months [1, 5–7]. However, in centers dedicated to neuroendocrine tumor treatment, the five-year survival rate for metastatic disease can be as high as 60 % [8]. For this reason, consensus guidelines from the North American Neuroendocrine Tumor Society
(NANETS) recommend that all patients with metastatic PNETs be treated at specialized centers [9].

The poor prognosis for patients with metastatic or regional disease underscores the urgent need for more effective therapies. As PNETs are relatively rare tumors, the impetus to study their tumor biology has been limited. However, recent technology has allowed for broad-based genetic studies, which have identified novel biomarkers and increased our understanding of tumorigenesis. A thorough understanding of the molecular biology and tumor genetics of PNETs may lead to discovery of novel targets for therapeutic intervention. The goal of this chapter is to summarize the current understanding of inherited and somatic genetics in PNETs.

2.2 Inherited Syndromes Associated with PNETs

Approximately 10–15 % of PNETs are associated with inherited cancer susceptibility syndromes including Multiple Endocrine Neoplasia type 1 (MEN1), von Hippel–Lindau syndrome (vHL), and more rarely Neurofibromatosis type 1 (NF1) and Tuberous Sclerosis Complex (TSC) (Table 2.1).

2.3 MEN1 Syndrome

Multiple Endocrine Neoplasia type 1 (MEN1) is an autosomal dominant cancer susceptibility syndrome, which has an incidence of approximately 0.25 % [10]. In most cases, the syndrome is caused by an inherited germline mutation in the MEN1 gene. MEN1 is defined by the presence of parathyroid adenomas or hyperplasia, gastroenteropancreatic tumors (GEPNETs), and anterior pituitary adenomas [10]. Hypercalcemia from primary hyperparathyroidism is often the presenting feature of MEN1, and it occurs in almost 100 % of cases by age 50 and often affects all four parathyroid glands. MEN1-associated primary hyperparathyroidism develops at an earlier age than sporadic primary hyperparathyroidism (20–25 years old vs. 55 years old, respectively) [10]. Patients who do not have primary hyperparathyroidism by age 50 have not been found to carry mutations in MEN1. Pituitary adenomas occur in up to 60 % of patients with MEN1. Lactotroph adenomas are the most common anterior pituitary tumor observed; somatotroph and somatomammotroph adenomas occur in 5 % of associated pituitary adenomas [11, 12]. Approximately 10 % of MEN1 patients have bronchial or thymic carcinoids and 20–40 % have adrenal cortical tumors. Other non-cancer features include facial angiofibromas, collagenomas, lipomas, and leiomyomas. Of note, both angiofibromas and collagenomas also are observed in TSC. However, patients with MEN1 have fewer angiofibromas, which tend to be non-erythematous and located on the nose rather than nasolabial folds. PNETs are a common feature of MEN1, present in 40–80 % of patients [10, 13]. Mutations in MEN1 are the most common inherited mutations
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<td>Menin</td>
<td>Regulates cellular proliferation Role in genomic stability Role in epigenetic regulation</td>
<td>Multiple Endocrine Neoplasia type 1 Hyperparathyroidism GEPNETs Pituitary adenomas Bronchial carcinoids Adrenal adenomas Angiofibromas</td>
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<td>VHL</td>
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<td>von Hippel–Lindau protein</td>
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<td>von Hippel–Lindau Disease Hemangioblastomas of the CNS Endolyphatic sac tumors Epididymal cystadenomas Pheochromocytomas Renal cell carcinomas Pancreatic cysts PNETs</td>
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<td>NF1</td>
<td>17q11.2</td>
<td>Neurofibromin</td>
<td>Acts as a GTPase to inactivate Ras to regulate the MAPK pathway</td>
<td>Neurofibromatosis type 1 Cutaneous neurofibromas Plexiform neurofibromas Café-au-lait spots Lisch nodules (benign iris hamartoma) Inguinal or axillary freckling Long bone dysplasia Optic gliomas Rare PNETs</td>
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<td>TSC1</td>
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leading to increased susceptibility to PNETs. PNETs in MEN1 patients are typically diagnosed at an earlier age (30–50 years old) than in patients with sporadic PNETs, which may represent a screening bias. PNETs associated with MEN1 are often multiple and small (defined as less than 0.5 cm) and most are nonfunctional. Gastrinomas and insulinomas are the most common functional PNETs (40 % and 10 %, respectively) found in MEN1 patients; glucagonomas, VIPomas and somatostatinomas are rare [11, 14]. MEN1 patients with PNETs tend to have a better prognosis than patients with PNETs without MEN1, likely reflecting a screening bias and earlier diagnosis in patients with MEN1. Approximately 10 % of all PNETs and 25 % of gastrinomas (Zollinger–Ellison syndrome) occur in patients with MEN1.

MEN1 demonstrates variable expressivity. Members of the same family, who carry the same mutation, can have diverse clinical manifestations. Identifying individuals with MEN1 mutations is not only important for the proband’s medical management but also for testing family members. Genetic testing is an important medical management tool, and screening and surveillance for the clinical manifestations of disease can be initiated once a mutation is identified. Additionally, having mutational information will allow for preconception genetic counseling and testing, such as preimplantation genetic diagnosis. Finally, identifying individuals in the kindred who do not carry the familial mutation (true negatives) is critical as well, since they do not need lifelong screening for tumors.

### 2.4 MEN1 Gene and Mutations

MEN1 is located on chromosome 11q13 and is comprised of ten exons spanning over 7 kb of genomic DNA [15]. The coding region spans 1,830 bp and encodes the protein menin, which is 610 amino acids. Tumors have one germline mutation in MEN1 with a second hit, often being loss of heterozygosity (LOH), at the other allele. Mutations occur throughout the gene [16, 17]. More than 1,336 different MEN1 mutations have been reported, both germline (N=1,133) and somatic (N=203) [17]. The 1,133 germline mutations reported in 2008 were found throughout the entire coding region and splice sites. Of the 459 unique mutations, 23 % were nonsense, 41 % frameshift deletions or insertions, 6 % in-frame deletions or insertions, 9 % splice-site mutations, 20 % missense mutations, and 1 % whole or partial gene deletions [17]. Approximately 5–10 % of patients, who meet the clinical diagnostic criteria for MEN1, do not have an identifiable mutation in the coding region of MEN1 [15–18]. These patients may have mutations in the regulatory regions, such as the promoter, which are not routinely evaluated with clinical genetic testing.

Despite the absence of hotspot mutations in the MEN1 gene, mutations at nine sites account for 20 % of all germline mutations [17]. Five of these nine mutations are deletions or insertions, one is a novel splice-site acceptor, and three are nonsense mutations. It is hypothesized that the MEN1 deletion/insertion mutations are caused by replication slippage at areas of repetitive sequence in the gene. There are 24 known polymorphisms in the MEN1 gene, including two nonsynonymous amino acid changes, which must be differentiated from mutations in clinical genetic testing [17].
2.5 Somatic MEN1 Mutations

*MEN1* is the most common somatically mutated gene in sporadic PNETs, often accompanied by loss of heterozygosity at the second hit. Whole-exome sequencing of ten sporadic PNETs and subsequent targeted sequencing of an additional 58 sporadic PNETs in a validation set found that 44% had somatic mutations in *MEN1* [19]. These data are consistent with prior work showing that somatic mutations in *MEN1* were found in 30% of sporadic PNETs, 7% of insulinomas, 36% of gastrinomas, 67% of glucagonomas, and 44% of VIPomas [20, 21]. As with the known germline mutations, the somatic mutations are scattered throughout the coding sequencing, and 18% are nonsense mutations, 40% are frameshift deletions or insertions, 6% are in-frame deletions or insertions, 7% are splice-site mutations, and 29% are missense mutations [17]. Losses of heterozygosity of segments of chromosome 11 (over the *MEN1* locus) have been seen in 38.6% of nonfunctioning PNETs and 15–20% of gastrinomas and insulinomas [22, 23]. Most *MEN1* mutations in sporadic PNETs appear to be associated with regions of the gene involved in nuclear localization or protein–protein interactions, and these tumors tend to have abnormally low to absent nuclear staining of menin [21].

2.6 Menin Protein

The *MEN1* gene product, menin, is ubiquitously expressed but functions in a tissue-specific manner, sometimes with opposing functions. Menin is primarily located in the nucleus of nondividing cells and is found in the cytoplasm of dividing cells [24]. Many protein partners have been reported to interact with menin, suggesting a role in various cellular pathways, including the regulation of gene transcription, DNA replication and repair, and signal transduction. Given the wide range of proposed functions and associated protein partners for menin, the discussion below will focus on those pathways and associations most related to neuroendocrine tumorigenesis (Fig. 2.1).

In cell culture, menin represses telomerase activity via interaction with human telomerase reverse transcriptase (hTERT), thereby preventing uncontrolled continued cellular proliferation [25]. Consistent with these data, menin depletion results in immortalization of human fibroblasts [25]. Menin also binds directly to AKT1 to inhibit the PI3K-Akt-mTOR signaling pathway, thereby suppressing proliferation and anti-apoptotic signals [26]. Menin interacts with NF-κB family members to repress the NF-κB-mediated transcriptional activation which is linked to apoptosis and delayed cellular growth [27]. Clearly, menin appears to play a role in controlling cellular proliferation.

Menin also is involved in multiple cell signaling pathways. Menin interacts with the Smad family of proteins to inhibit the transforming growth factor-β (TGF-β) and bone morphogenetic protein-2 (BMP-2) signaling pathways [28–30]. Menin also may be involved in the Wnt signaling pathway through interactions with the transcription factor β-catenin. Interestingly, Wnt signaling stimulates pancreatic islet
β-cell proliferation. Over-expression of menin decreases nuclear β-catenin in part by directly binding and excluding it from the nucleus [31]. In contrast to that, menin also appears to be needed to interact with β-catenin for Wnt signaling in rodent islet tumor cells [32]. Although certainly interesting given the role of Wnt signaling in β-cell proliferation, the exact role of menin in the Wnt signaling pathway still remains to be elucidated.

In addition to roles in proliferation, genomic stability, and cell cycle regulation, menin also plays a role in epigenetic regulation of gene expression through histone methylation and acetylation. Menin is part of the mixed lineage leukemia (MLL) histone methyltransferase complex. Menin binds to MLL and mediates the H3K4 methyltransferase activity promoting histone H3 lysine 4 trimethylation which is linked to transcriptional activation [33–35]. As part of the MLL complex, menin is involved in the regulation of the homeobox genes and increasing expression of cyclin-dependent kinase inhibitors, p27 (CDKN1B) and p18 (CDKN2C) [33, 36–38]. In addition, menin may mediate the repression of genes targeted by JunD through recruitment of the histone deacetylase (HDAC) complex to suppress transcriptional activity [39, 40]. Finally, menin has been shown to interact with suppressor of variegation 3-9 homolog family protein (SUV39H1) to mediate H3K4 methylation and silence transcriptional activity of target genes [41].
Menin has been shown to be involved in neuroendocrine cell development and function. Menin regulates proliferation in normal pancreatic islet cells [33, 42, 43]. Men1−/− mice are embryonic lethal [44]. Men1+/− mice develop pancreatic islet cell hyperplasia and multiple endocrine tumors with a prolonged latency [45]. Conditional Men1 gene knockout in pancreatic β-cells results in the development of insulinomas with full penetrance, and none of the tumors become poorly differentiated [46, 47]. These data suggest that Men1 mutations are drivers for PNET formation; however, additional mutations are needed to convert PNETs into high-grade tumors. Recently, menin ablation in mouse pancreatic islet cells was shown to enhance Hedgehog signaling, a pro-proliferative and oncogenic pathway [48]. These studies demonstrate that menin directly interacts with protein arginine methyltransferase 5 (PRMT5), a negative regulator of gene transcription. Menin recruits PRMT5 to the promoter of the Gas1 gene, a crucial factor for binding of Sonic Hedgehog (Shh) ligand to its receptor. This binding increases the repressive histone 4 arginine dimethylation (H4R3m2s) mark at the Gas1 promoter thereby suppressing expression of Gas1 [48]. Menin mutant mice have reduced binding to PRMT5 and therefore fail to provide the repressive H4R3m2s mark at Gas1 promoter, resulting in elevated gene expression and increased Hedgehog signaling. In mice, pharmacological inhibition of Hedgehog signaling reduces proliferation of insulinoma cells [48]. This novel finding suggests that menin-PRMT5 interaction epigenetically suppresses Hedgehog signaling, making this pathway a potential target for treatment of MEN1 mutated tumors.

2.7 von Hippel–Lindau Disease

von Hippel–Lindau disease (vHL) is an autosomal dominant hereditary cancer syndrome with an incidence in the United States of 1 in 32,000 and penetrance over 90% by age 65 [49]. vHL is caused by germline mutations in the VHL gene and associate with several benign and malignant tumor types including hemangioblastomas of the central nervous system (brain, spinal cord, and retina), renal cysts and clear cell renal cell carcinoma (RCC), endolymphatic sac tumors, epididymal cystadenomas, pheochromocytomas (PCC), and pancreatic cysts and PNETs. PNETs occur in 9–17% of patients with vHL [50–52]. vHL-associated PNETs display differential expression of genes related to angiogenesis and hypoxia-inducible factor signaling compared to sporadic PNETs [53]. Meta-analysis of 1,442 patients with vHL found that of 420 patients who were assessed for pancreatic lesions, 60% had pancreatic masses, 47% of which were simple cysts [51]. PNETs were found in 15% of patients and only 2% of those were malignant and so vHL-associated PNETs are associated with very different prognosis (improved) than sporadic PNETs [51]. vHL-associated PNETs are nonfunctional. Notably, patients with vHL often have multiple pancreatic cysts and masses. In patients with vHL, surgical removal of PNETs is recommended for pancreatic lesions over 3 cm, as this size cutoff is associated with more aggressive disease. Further indicators of malignant potential are quickly growing tumors and those associated with inherited mutations in the third exon of VHL [50].
2.8  VHL Gene and Mutations

The VHL gene is located on chromosome 3p25-26 and contains three exons which span 639 bp and encodes for two VHL proteins, one full-length 213 amino acids, and a smaller protein that lacks the first 53 amino acids. VHL is a tumor suppressor gene and most vHL-associated tumors show LOH of the wild-type allele as the second hit. Over 1,000 mutations in the VHL gene have been reported to date, which range across the gene and include missense, nonsense, and insertion/deletion mutations [54]. Genotype-phenotype correlations with VHL mutations have been well documented. Patients with type 1 vHL disease have a lower risk of developing PCC and a higher risk of RCC and other manifestations of vHL; they tend to have truncating mutations or exonic deletions. Patients with type 2 vHL disease tend to have missense mutations in the VHL gene, which are associated with much greater penetrance of PCC [55, 56]. Type 2 disease is further stratified into type 2A, which has a lower risk of clear cell RCC; type 2b, which is associated with a high risk of all manifestations of vHL; and type 2C, which is associated only with PCC. PNETs are associated with mutations throughout the VHL gene, but the development of metastatic disease appears to be higher in association with mutations in exon 3 [50].

In contrast to MEN1, somatic VHL point mutations in sporadic PNETs are rarely observed [20, 57]. Rather, up to 25% of sporadic PNETs have been shown to have inactivation of VHL through promoter hypermethylation or gene deletion [57]. The presence of VHL methylation or deletions in sporadic PNETs has been suggested to be associated with worsened outcome [57].

2.9  VHL Protein

The VHL protein forms a complex with elongin B, elongin C, RBx 1 and Cul2 which has ubiquitin ligase E3 activity [58]. The major function of the VHL protein is to regulate the hypoxia-inducible transcription factors (HIF1α and HIF2α). Under normoxic conditions, VHL binds to the hydroxyproline residue on the HIFs targeting them for ubiquitination and proteosomal degradation [59]. Under hypoxic conditions, or if there is a mutation in VHL, this interaction cannot take place, resulting in the loss of ubiquitination of HIFα and thus allowing it to complex with a ubiquitous nuclear transporter HIF1β, also known as ARNT [58, 60]. In hypoxia situations, there is massive upregulation of over 100 genes now known to be induced because of activation by the transcription factors HIF1α and HIF2α [61, 62]. The activation of these target pathways serves to enhance tumorigenesis and includes genes involved in angiogenesis, glucose metabolism, cell survival, and cell migration/invasion properties [61, 62]. Although the consensus binding site for these transcription factors is the same, the factors themselves have overlapping, but not identical sets of target genes.

The VHL protein also has HIF-independent functions relevant to tumor development. VHL appears to be required for extracellular matrix assembly including binding to fibronectin and hydroxyl collagen IV-α2 [63, 64] and regulating some integrin
functions for cellular adhesions [65]. In addition, VHL has been shown to directly bind p53, and the phosphorylation of VHL by checkpoint kinase 2 (Chk2) promotes transactivation of p53, resulting in apoptosis [66, 67]. VHL also promotes the inhibitory phosphorylation of NF-\(\kappa\)B agonist CARD9, which leads to a decrease in NF-\(\kappa\)B activity [68].

### 2.10 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1), also called von Recklinghausen’s disease, is an autosomal dominant disorder caused by inactivating mutations in the tumor suppressor gene, \(NF1\). NF1 occurs in 1 in 3,000 individuals worldwide. The diagnosis is made based on clinical criteria. Patients must have at least two of the following features: six or more café-au-lait macules (at least 0.5 cm in prepubertal patients and 1.5 cm in postpubertal patients), two or more cutaneous neurofibromas or a single plexiform neurofibroma, inguinal or axillary freckling, two or more Lisch nodules (benign iris hamartomas), optic nerve glioma, dysplasia of the long bones, and a first-degree relative with NF1 [69]. Several cancers have been associated with NF1 at a higher frequency than the general population, including malignant peripheral nerve sheath tumors, gastrointestinal stromal tumors, chronic myeloid leukemia, and PNETs [70–72].

Numerous case reports of PNETs associated with NF1 are found in the literature; up to 10% of NF1 patients are described as having PNETs. The most frequent NF1-associated PNET is the somatostatinoma. NF1-associated somatostatinomas more often are found in the duodenum rather than the pancreas [73]. Up to 48% of duodenal somatostatinomas have been reported to be associated with NF1 [71, 73]. In one of the largest case series including 26 NF1 patients with somatostatinomas, the patients were more often female than male and ranged in age from 21 to 70 years old [74]. Interestingly, NF1-associated somatostatinomas infrequently present with symptoms of somatostatin syndrome because the tumors are less likely to hypersecrete hormones compared to sporadic duodenal somatostatinomas or pancreatic somatostatinomas [73]. Instead, these tumors tend to present with obstructive symptoms such as jaundice, weight loss, and abdominal pain. In other aspects, NF1-associated duodenal tumors are similar to sporadic tumors with both types having frequent psammoma bodies on pathologic examination and less frequent metastases compared to pancreatic somatostatinomas [73].

### 2.11 \(NF1\) Gene and Protein

The \(NF1\) gene is large, spanning 360 kb and over 60 exons located on chromosome 17q11.2. There are no hot spots for mutations and no genotype/phenotype correlations. No specific mutations are associated with PNET development. Up to 50% of NF1 patients arise from a de novo mutation, and interestingly, there is variable
penetrance and expressivity of the disease even in patients with the same mutation [70]. The protein neurofibromin is composed of 2,818 amino acids, and the most well-characterized function of this large protein is as a GTPase which inactivates Ras to inhibit the MAPK signaling pathway. When NF1 is mutated, there is constitutive activation of Ras and hence the downstream MAPK, PI3K, and mTOR pathways, leading to uncontrolled cellular growth and differentiation [75–77]. NF1 has been found to be mutated in a multiplicity of tumor types, ranging from glioblastoma multiforme to melanoma, lung, ovarian, and bladder cancers among others based on data from the Cancer Genome Atlas.

### 2.12 Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC) is another autosomal dominant disease with prevalence of 1 in 6–10 thousand individuals [78]. The clinical manifestations include abnormalities of the brain (cortical tubers, subependymal nodules, seizure disorders, developmental delay), skin (facial angiofibromas, ungual and periungual fibromas, hypomelanotic macules), kidney (renal angiomyolipomas, cysts), lungs (lymphangiomomatosis), and eyes (hamartomas) [78]. Two-thirds of patients have no family history of TSC and are thought to be due to de novo mutations, particularly in TSC2. Eighty to 85% of patients meeting clinical criteria for TSC are found to have mutations in one of two genes, TSC1 and TSC2 [79, 80]. No other genes are thought to be associated with TSC; rather it is thought that the mutations may not be detectable in some cases due to issues such as somatic mosaicism or being outside the region interrogated by clinical testing (e.g., promoter region). TSC1 is located on chromosome 9q34 and spans 55 kb of DNA encoding 23 exons. TSC2 is located on chromosome 16p13 and spans 40 kb encoding 41 exons. The gene products are hamartin and tuberin, respectively, which share no homology. These two proteins dimerize to control cellular proliferation through the PI3 kinase/mTOR pathway [81]. Truncating mutations span both genes without particular hot spots, although mutations in TSC2 are more common in both familial and sporadic cases [82]. Missense mutations in TSC2 tend to cluster in the GTPase-activating protein-binding domain and are rare in TSC1. Large genomic deletions also are more frequent in TSC2 than in TSC1 [83]. PNETs in TSC are extremely rare with only a handful of cases reported in the literature with most having mutations in TSC2 when tested [84]. The tumors tend to be well differentiated and can be secretory.

### 2.13 Somatic Genetic Mutations in PNETs

Candidate gene approaches to identify somatic mutations in PNETs have been performed in small studies with variable results. Activating mutations in exon 3 of β-catenin were described in 37% of gastrointestinal NETs [85] but not in PNETs [86].
PTEN, KRAS, TP53, and CDKN2A are rarely mutated in PNETs [87–92]. One small study suggested that loss of PTEN expression by immunohistochemistry correlated with advanced tumor stage [93]. Another small study showed that although no point mutations were found in CDKN2A, the gene was homozygously deleted in 42% and methylated in 58% of gastrinomas and nonfunctioning PNETs [94]. Several small studies found no mutations in RET, BRAF, and SMAD3 [95–98]. One small study did find DPC4/SMAD4 mutations in a high percentage of sporadic PNETs [99], but this was not confirmed in subsequent larger studies [89, 100].

Massively parallel sequencing studies on PNETs have confirmed some of the previously known somatically mutated genes in PNETs and also have identified novel genes involved in tumorigenesis (Table 2.2). Whole-exome sequencing (WES) of ten advanced PNETs identified 157 somatic mutations in 149 genes with an average mutation rate of 16 mutations per tumor [19]. This low number of somatic mutations reflects the often indolent nature of PNETs compared to other more aggressive carcinomas which often have a median of 44 non-silent somatic mutations per tumor [101]. The most frequently mutated genes in the discovery PNET set were selected to be sequenced in a validation set of 58 additional PNETs. In total, this study confirmed the most commonly somatically mutated gene in well-differentiated PNETs is MEN1 (in 44% of cases) [19]. Fifteen percent of PNETs had a mutation in one of the PI3K/mTOR signaling pathway genes including TSC2 (in 8.8% of cases) and PTEN (in 7.3% of cases) as well as one tumor with a PIK3CA activating mutation. The fact that a significant percentage of PNETs have mutations in genes regulating the mTOR pathway is consistent with the observation that patients with PNETs respond to the mTOR inhibitor everolimus with improved progression-free survival [102]. Perhaps identifying patients with a somatic mutation in the mTOR pathway before treatment could serve as a biomarker to predict response to directed therapy with everolimus in the future.

| Table 2.2 Common somatic mutations in PNETs |
|----------|---------|--------------------------------------------------------------------|
| Gene     | Loci    | Protein                                                                 |
| MEN1     | 11q13.1 | Menin Regulates cellular proliferation |
|          |         | Role in genomic stability                                             |
|          |         | Role in epigenetic regulation                                          |
| DAXX     | 6q21.3  | Death-domain-associated protein Histone H3.3 chaperone                |
| ATRX     | Xq21.1  | Alpha thalasemia/mental retardation syndrome X-linked protein Member of the SWI-SNF family of chromatin remodeling proteins |
| PTEN     | 10q23.3 | Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase Phosphatase which preferentially dephosphorylates phosphoinositide substrates Key modulator of the AKT-mTOR signaling pathway |
| TSC2     | 16p13.3 | Tuberin Dimerizes with hamartin to control cellular proliferation through the PI3K/mTOR pathway |

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WES also identified that the second most commonly identified somatic mutations are in the DAXX/ATRX complex (in 43% of cases) which was not previously known to play a role in the biology of PNETs [19]. DAXX and ATRX are both part of the chromatin remodeling complex (Fig. 2.2). Mutations in either DAXX (death-domain-associated protein, in 25% of cases) or ATRX (alpha thalassemia/mental retardation syndrome X-linked in 18% of cases) were mutually exclusive with each other but sometimes demonstrate overlap with mutations in MEN1 (in 23.5% of cases) [19]. Interestingly, DAXX/ATRX mutations were associated with a statistically significant increase in overall survival, which improved further if there was an additional MEN1 mutation [19]. These data should be interpreted with caution given the small numbers of tumors with MEN1 and DAXX/ATRX mutations in the study. Nevertheless, these data are consistent with a study examining protein expression in well-differentiated PNETs versus poorly differentiated neuroendocrine carcinomas. In this study, DAXX and ATRX expression by immunohistochemistry (IHC) are mutually exclusively lost in 45% of well-differentiated PNETs, similar to the mutation rate and pattern seen by WES, whereas p53 and Rb showed normal expression [103].
Conversely, p53 and Rb protein expression by IHC was altered in poorly differentiated neuroendocrine carcinomas compared with well-differentiated PNETs, whereas expression of ATRX and DAXX was the same in both tumor sets [103]. However, recently a larger study of 149 PNETs had contrasting results with the absence of DAXX/ATRX staining by IHC associated with chromosomal instability and decreased relapse-free survival in patients with PNETs [104]. Given the differing results, additional studies need to be performed to understand the true association of DAXX/ATRX mutations with prognosis in PNETs. Nonetheless, mutations in this complex appear to play a significant role in PNET tumorigenesis, although the mechanism is still being elucidated.

The ATRX gene is located on the X chromosome at Xq21.1, has 36 exons, and encodes a 2,492 amino acid protein. Germline ATRX mutations lead to ATRX syndrome in which patients develop a neurodevelopmental condition with various degrees of gonadal dysgenesis and alpha thalassemia [105]. Similar to other X-linked disorders, female patients with germline ATRX mutations are generally unaffected or only mildly affected as they exhibit skewed X chromosomal inactivation patterns; hence the syndrome is predominantly seen in males [106]. ATRX is a large nuclear protein with a C-terminal ATPase/DNA helicase domain making it part of the SWI-SNF family of chromatin remodeling proteins. The N-terminal domain has a DNA-binding domain which recognizes the methylation status of lysine residues on histone 3 which typically denotes inactive heterochromatin, including telomeric and pericentric regions. ATRX has also been associated with G-quadruplex formations of DNA which prevent DNA and RNA polymerases from functioning [107]. ATRX is thought to play a role in resolving G-quadruplex DNA formations, thereby promoting gene expression. ATRX depletion leads to loss of structural integrity at telomeres which have high concentration of G-quadruplex formation, while treatment with G-quadruplex-stabilizing agents in ATRX-depleted cells causes DNA damage at telomeres, such as chromosomal end-to-end fusions and telomere deletions [108]. ATRX-deficient mice have defective chromosomal cohesion during mitosis, increased sensitivity to agents that induce replicative stress, and increased p53-mediated apoptosis in response to DNA damage [108]. These studies suggest ATRX serves to help maintain genomic integrity.

Interestingly, the germline mutations associated with ATRX syndrome differ from the somatic mutations found in PNET tumors. Fifty percent of germline mutations are in exons 8–10 in the DNA-binding domain and about 30% are in exons 17–31 in the helicase domains [109]. Furthermore, the inherited ATRX mutations tend to be hypomorphic missense mutations rather than protein-truncating mutations which lead to loss of protein through nonsense-mediated decay as seen with the somatic mutations. This finding is not surprising since ATRX appears to be essential for life as mice deficient in ATRX are embryonic lethal [110].

DAXX is located on chromosome 6q21.3, has eight exons, and encodes a 688 amino acid protein. DAXX also is a nuclear protein and functions as a histone H3.3 chaperone. The ATRX-DAXX complex assembles H3.3 into nucleosomes and, therefore, is implicated in chromatin stabilization [111–113]. ATRX recruits DAXX to bring H3.3 to telomeres and pericentric heterochromatin [113]. It is hypothesized
that disruption of this function leads to tumorigenesis by disrupting regulation of telomeres as protein loss of DAXX or ATRX is correlated with alternative lengthening of telomeres (ALT), a telomerase-independent mechanism of telomere lengthening [114]. Alternative lengthening of telomeres through DNA recombination has been shown in 61% of PNETs in one study and 19 of those 25 tumors had mutations in either ATRX or DAXX [114]. Alternative lengthening of telomeres was seen in all DAXX/ATRX mutated tumors in the whole-exome sequencing study of non-MEN1-associated PNETs [19]. In MEN1-associated PNETs, only a small subset had DAXX/ATRX mutations, but all of the mutation-positive tumors had the ALT phenotype [115]. Interestingly, in a study of multiple cancer types including PNETs, all tumors which had ATRX mutations were ALT positive by a telomere FISH assay [116]. ATRX mutations are not restricted to neuroendocrine tumors but are found in other tumor types as well including gliomas. The ATRX/DAXX complex appears to be critical for genomic integrity, and disruption of this process appears to lead to tumorigenesis. Nevertheless, the precise mechanism of ATRX/DAXX dysfunction in pancreatic neuroendocrine tumorigenesis is still being elucidated.

2.14 Expression Profiling

Several studies have examined expression profiling in PNETs and have highlighted specific altered cellular pathways in subsets of tumors. Comparing nonfunctioning well-differentiated PNETs to pancreatic islet cell samples, very few differentially expressed genes were identified [117]. However, when examining differences in the expression profiles of metastatic and non-metastatic PNETs, there was increased expression of genes involved in growth regulation, cholesterol homeostasis, osmotic regulation, and hypoxia-inducible factors and under-expression of genes involved in the cell cycle and DNA damage response in the metastatic subset [118]. Another study of PNETs with and without metastases showed that metastatic tumors had higher expression in genes involved in angiogenesis, signal transduction through tyrosine kinases, and calcium-dependent cell signaling [119]. Malignant tumors also showed activation of insulin-like growth factor-signaling cascade [118, 119]. Interestingly, another study comparing nonfunctioning PNETs (primary tumors and associated metastases) to islet cell preparations found that similar expression patterns between primary tumors and associated metastatic tumor, suggesting malignant potential, may be acquired at an early stage [120]. ANG2 (angiopoietin-2) has been suggested as a potential molecular marker for malignancy as it was over-expressed in a microarray study in 89% of nonfunctional PNETs compared with 22% of normal pancreas samples [121]. Another potential marker for worsened disease-free and overall survival is co-downregulation of PTEN and TSC2 [122]. RUNXIT1 is under-expressed in well-differentiated metastatic primary PNETs relative to non-metastatic primaries and, therefore, could represent a possible biomarker for prediction of metastases [123]. Most of these studies have been done in small sample sets, and further validation is needed to confirm these genes as possible biomarkers for metastases and survival.
2.15 Copy Number Aberrations

Array-based comparative genomic hybridization (aCGH) studies have shown that PNETs have multiple chromosomal alterations. Not surprisingly, genetic alterations accumulate during tumor progression and increase in concert with tumor volume and stage [124]. In addition, more copy number gains and losses are found in metastatic disease compared to benign tumors [124, 125]. Loss of chromosome 1 and 11q and gains of 9q appear to be early events because these alterations are seen in many small tumors under 2 cm [124]. The MEN1 gene is located on chromosome 11q, so loss of this region early in tumorigenesis is not surprising. Potential genes of interest in the other commonly disrupted chromosomal regions included tumor suppressor genes on chromosome 1 including TP73 and RIZ and oncogenes on chromosome 9q included ABL and VAV2. In one study of 25 PNETs, 68 % of tumors had gain on chromosome 7 with the minimal overlapping region at 7q11.2 which contains potential genes of interest including MET and EGFR [126]. Loss of chromosome 3pq and 6pq and gains of 14q, 17pq and 20q are associated with advanced stage and malignant behavior [124, 127]. In metastases, gains of chromosome 4pq, 5q, 7pq, and 17q and losses of 11pq, 10p, 3p, and 6q are seen often [125]. Insulinomas have fewer alterations than other PNETs and often have gains of chromosome 9q32 [124–127].

2.16 Epigenetics

Gene-specific analysis has identified some genes commonly epigenetically silenced in PNETs. RASSF1A is a tumor suppressor gene located on chromosome 3q21 and frequently methylated in several cancer types. In PNETs, RASSF1A promoter methylation has been reported in as high as 75–83 % of tumors and correlates with larger tumors and the presence of metastatic disease; however, methylation of RASSF1A also has been found in adjacent normal tissue making the role for silencing this gene uncertain [128–131]. CDKN2A is a tumor suppressor gene located on chromosome 9p21 and encodes the p16 protein which regulates the cell cycle. CDKN2A is commonly silenced by promoter methylation in cancers including 10–58 % of PNETs and correlated with the presence of metastatic disease [91, 94, 129, 132, 133]. Methylation status of other genes has conflicting reports in various studies. The MGMT promoter was methylated in 40 % of 48 PNETs in one study [129] but in none of 11 PNETs in another study [133]. Similarly, TIMP3 encoding an extracellular protease inhibitor, known to play a role in metastatic potential in cancers, was methylated in 44 % of functional PNETs [134] but not in insulinomas or nonfunctional PNETs [129, 134].

CpG island methylator phenotype (CIMP) is known to be associated with colorectal adenocarcinomas. In a series of neuroendocrine tumors, CIMP phenotype was found in 50 % of gastrinomas and up to 100 % of VIPomas and glucagonomas, all associated with high Ki67 proliferative index [135]. High promoter methylation in PNETs has been associated with higher-grade tumors, early recurrence, and
worse prognosis with decreased survival [129, 135]. Future studies using massively parallel sequencing of the methylome may help to further elucidate the role of epigenetic methylation in PNET tumorigenesis.

2.17 Small Intestinal NETS

Massively parallel sequencing of small intestinal NETs (SINETs) identified a low number of somatic alterations similar to PNETs [136]. Banck et al. examined 48 ileal and small bowel carcinoid tumors grades 1 and 2 through whole-exome sequencing and found a total of 197 non-synonymous mutations and 14 splice-site mutations. No recurrent mutations were identified. In fact, only one gene was mutated in more than one tumor (ABCC12 mutations in two of 48 tumors). Several known cancer-promoting genes were mutated in single cases including BRAF, FANCD2, FGFR2, MEN1, and VHL [136].

Somatic copy number analysis in the SINETs showed a low rate of copy number changes per tumor (average 21) suggesting relative genomic stability [136]. The pattern of gains and losses was consistent with previous studies using aCGH and SNP arrays [137, 138]. Copy number gains were found in MTOR (6% of cases) and SRC (23% of cases), whereas copy number loss was common in SMAD4 tumor suppressor gene in 46% of cases [136]. Interestingly, SMAD4 is frequently mutated in pancreatic adenocarcinomas although not seen in PNETs [89, 100].

2.18 Summary and Future Directions

Given the poor prognosis associated with metastatic PNETs, it is essential to identify biomarkers for prediction of malignant potential and to identify novel targets for therapeutics to treat metastatic disease. Recently, our understanding of tumorigenesis in PNETs has expanded from studies focusing solely on the role of menin as a tumor suppressor to massively parallel sequencing studies identifying potential new drivers of tumorigenesis in PNETs. However, further work needs to be done to fully understand the mechanism of tumorigenesis behind the newly discovered somatically mutated genes to enable the development of therapeutics.

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