Chapter 14
Molecular Pathology and Diagnostics of Pancreatic Endocrine Neoplasms

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Contents

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Abstract Pancreatic Endocrine Neoplasms (PENs) are a group of rare tumors thought to arise from the endocrine cells of the pancreas. These tumors may be functional (hormone producing), or non functional. They have a wide range of presenting symptoms. Recent research efforts have shown the complex biology of these tumors, and have started to uncover the molecular alterations responsible for the genesis of these neoplasms. In this chapter we give an overview of the molecular tests available to detect such alterations, and of their diagnostic and prognostic significance.

Keywords Pancreatic endocrine neoplasms • Pancreatic endocrine tumors/carcinomas • Classifications of PENS • Molecular genetic • Islets of Langerhans

Abbreviations

AP1	Activator protein-1
bFGF	Basic fibroblast growth factor
CLP-PET	Clinically localized pancreatic endocrine tumors
CPGs	Candidate progression genes
EFR1	Estrogen receptor gene
FHIT	Fragile histidine triad
hMLH1	Human MutL homologue
IGF	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor binding protein 3
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
MAG	Metastasis-associated gene
MECC	Monohormonal endocrine cell clusters
MEN1	Multiple endocrine neoplasia type 1
MP	Metastatic primary
mTOR	Mammalian target of rapamycin
NF1	Neuofibromatosis type 1

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NGF	Nerve growth factor
PECAs	Pancreatic endocrine carcinomas
PENS	Pancreatic endocrine neoplasm
PETs	Pancreatic endocrine tumors
PRAD-1	Parathyroid adenoma-related protein
SDHD	Succinate dehydrogenase subunit D
SST	Somatostatin
SSTRs	G-protein-coupled transmembrane receptors
TGF	Transforming growth factor
TSC1, TSC2	Tuberous sclerosis genes 1 & 2
VHL	von Hippel-Lindau genes

14.1 Introduction

The endocrine pancreas is composed of groups of endocrine cells known as islets of Langerhans, which are scattered throughout the organ and in the vicinity of pancreatic ducts [1–3]. The pancreatic islet cell population consists of alpha (α) cells, beta (β) cells, pancreatic polypeptide (PP) cells and delta (δ) cells [4]. Pancreatic endocrine neoplasms (PENs) are characterized by diverse clinical presentation and outcome and complex biology. PENs comprise about 2 % of all pancreatic neoplasms [5]. Every year, about 2,500 new PENs are diagnosed [6, 7]. The incidence of PENs is <1 per 100,000 person-years, although autopsy incidence ranges from 0.8 % to 10 % as they often remain clinically silent and undiagnosed [8]. PENs are associated with a better prognosis than exocrine pancreatic cancers, with an overall 5-year patient survival rate of 35–60 % [9]. There has been no strong association between environmental risk and the development of PENs. A recent study found no association with first-hand tobacco exposure or alcohol use [8].

14.2 Functional and Pathologic Classification of PENs

PENs are categorized as functional or nonfunctional. Functional tumors secrete polypeptide hormones such as insulin, gastrin, glucagon, somatostatin, vasoactive intestinal peptide, adrenal corticotrophic hormone, parathyroid hormone-related peptide, growth hormone, calcitonin, melanocyte-stimulating hormone, vasopressin, and norephinephrine, and elicit a clinically recognizable, hormone-related syndrome. Nonfunctional tumors secrete biologically inactive peptides and do not produce hormone-associated symptoms [10]. Approximately half of all PENs are nonfunctional [7].

Pathologic classification of PENs based on tumor size, proliferative activity (Ki-67 index), angioinvasion, invasion of adjacent organs, the presence or absence of distant metastases, hormone activity and clinical syndromes has been shown

to be of some use in predicting the clinical behavior of these neoplasms [7, 11]. However, a better understanding of the molecular diagnostic aspects of these unpredictable neoplasms will contribute to improved diagnostic and therapeutic strategies and better patient outcome.

Current molecular techniques have shown that the biology of PENs is complex, without a particular biologic pathway driving these clinically challenging neoplasms. These tumors appear to develop in stages with multiple sequential mutations that contribute to ultimate progression to malignancy [12]. Several factors interact in complex ways to influence development, differentiation, secretion and the interaction of PENs with the tumor microenvironment [13]. Major events involved in pancreatic endocrine neoplasm initiation, progression and distant metastasis include basic fibroblast growth factor (bFGF), fragile histidine triad (FHIT), multiple endocrine neoplasia type 1 (MEN1), neurofibromatosis type 1 (NF1, neurofibromin), nerve growth factor (NGF), parathyroid adenoma–related protein (PRAD-1), transforming growth factor (TGF), tuberous sclerosis genes 1 and 2 (TSC1, TSC2), vascular endothelial growth factor (VEGF) and von Hippel-Lindau (VHL) genes.

14.3 Hereditary Syndromes Associated with PENs

The multiple endocrine neoplasia syndromes associated with PENs include multiple endocrine neoplasia type 1 (MEN1 gene), von Hippel-Lindau disease (vHL gene), neurofibromatosis (NF-1 gene), and tuberous sclerosis (TSC1 and TSC2 genes).

14.3.1 Multiple Endocrine Neoplasia Syndrome, Type 1

Multiple endocrine neoplasia syndrome, type 1 (MEN-1) is an autosomal dominant familial syndrome first described by Moldawer et al. [14] and Wermer [15]. Clinically, it is characterized by parathyroid adenomas, pancreatic endocrine neoplasms, and pituitary hyperplasia or neoplasia [16]. Other rarer neoplasms include bronchial and thymic carcinoids, adrenocortical tumors and cutaneous lipomas and collagenomas [17]. While hyperparathyroidism is the most common endocrine manifestation of MEN-1, PENs are the second most common endocrine manifestation and occur in about 60 % or more of MEN-1 patients. The majority of PENs in MEN1 are non-functional. However, most common functional entero-pancreatic endocrine tumors in MEN-1 patients are gastrinomas [18, 19], which often occur in the wall of the duodenum and in peri-pancreatic lymph nodes, and also insulinomas. Clustering of subvariants of MEN-1 such as insulinomas [20, 21] and aggressive gastrinomas [22] within small MEN-1 families suggest specific MEN-1 mutations may correlate with specific clinical variants. PENs in MEN-1 syndrome are invariably multifocal and may be widely dispersed in the pancreas and duodenum [18].

Consequently, the role of surgical management becomes particularly challenging and controversial [23, 24]. PENs in MEN-1 may also present as multiple clinically silent entero-pancreatic macroadenomas, which may be found at surgery or at autopsy in almost 100 % of MEN-1 patients older than 40 years [20, 25, 74]. Approximately 80 % of MEN1 cases are familial, whereas 20 % appear to be associated with new mutations based on negative familial history [26].

The MEN1 gene is a tumor suppressor gene [25, 27] mapped to a specific region on the long arm of chromosome 11 (11q13). The gene encodes a nuclear protein called menin, which interacts with several other proteins including junD, a member of the activator protein-1 (AP1) transcription factor family, SMAD1, SMAD3, SMAD5, PEM, NM23, nuclear factor kB and runx2 [28–35]. Mutations in MEN1 gene are the principal genetic abnormality in MEN1 syndrome, seen in most PENs in MEN1 patients and in about one third of sporadic PENs, as an early event in the neoplastic process.

The tumorigenesis in MEN-1 patients is thought to be a two-step inactivation of the MEN1 gene, in which both copies of the MEN1 gene must be inactivated in order for tumorigenesis to occur. A "two-hit" hypothesis has been proposed whereby germline inactivation of one allele is followed by somatic inactivation of the second allele in a predisposed cell, leading to clonal proliferation [36]. Virtually all first hits at the MEN1 gene are small mutations involving one to several bases [37, 38]. Most MEN1 gene mutations occur in the locus of exon 2 [39]. However, hundreds of unique germline and somatic mutations, broadly distributed across the MEN1 open reading frame, have been found [16, 38, 40]. Most of the first-hit mutations predict premature truncation of the menin protein, while other mutations predict missense mutations or replacement of one to three amino acids, all of which result in inactivation or absence of menin protein. The second step in MEN-1 tumorigenesis always occurs in somatic tissue, usually as a postnatal event. The second-hit mutations are usually large chromosomal or subchromosomal rearrangements with a resultant deletion that includes the remaining normal MEN1 gene.

14.3.2 Loss of Heterozygosity at 11q13

Loss of heterozygosity (LOH) is used mainly to show loss of the normal copy of the MEN1 gene. In MEN-1 patients, LOH at 11q13 was found in almost 100 % of gastrinomas and other pancreatic endocrine tumors, as well as non-pancreatic endocrine neoplasia [41, 42]. Some sporadic endocrine tumors of the type found in MEN-1 show frequent LOH at 11q13. An underlying mutation at 11q13 has been traced to the MEN1 gene in about 50 % of sporadic MEN-1 like tumors with 11q13 LOH. Somatic mutations of the MEN1 gene occur in about 20 % of sporadic solitary PENs [26, 30, 43]. Therefore, MEN1 gene mutations are among the most common mutations in sporadic PENs. Among functional PENs, MEN1 gene mutations occur in about 25 % gastrinomas, 10–20 % insulinomas and 50 % VIPomas [44–47].

14.3.3 Events Following Inactivation of MEN1 Gene

After MEN1 gene inactivation, other unknown genes or undetected mutations in MEN1 gene may contribute to MEN-1 tumor development. Studies suggest that the tumorigenic pathway of MEN-1 overlaps and interacts with other homeostatic cell pathways [48–51]. Multifocal microadenoma is an early monoclonal or oligoclonal islet cell lesion in MEN-1, which may represent a hyperplastic precursor stage to subsequent neoplastic development [52]. Monohormonal endocrine cell clusters (MECCs) develop most frequently within normal pancreatic islets but also in exocrine pancreatic ducts and hyperplastic islets through 11q13 LOH. MECCs progress to microadenomas (MA) with disruption of the normal islet structure and transformation into neoplastic process. Evidence in support of microadenomas in MEN1 patients includes hyperplastic foci of gastrin cells seen by light microscopy in the duodenum of gastrinoma specimens from MEN-1 but not from sporadic gastrinomas [53]. Furthermore, in the heterozygous knockout of the MEN1 gene in mice, giant hyperplastic islets precede the development of insulinoma, suggesting that subtle islet hyperplasia may be an unrecognized precursor lesion in MEN-1 of humans despite the presence of one normal MEN1 allele. One could speculate that hyperplasia is an expression of MEN1 heterozygosity [54, 55].Further studies are needed to link these findings with an as yet undiscovered genetic basis of tumor development.

14.3.4 Von Hippel-Lindau Syndrome

Von Hippel-Lindau syndrome (VHL) is an autosomal dominant disorder characterized by hemangioblastomas of the central nervous system, renal cell carcinomas, retinal angiomas, visceral cysts, pheochromocytomas and PENs, which occur in10–20 % of VHL patients [56–59]. The VHL gene, mapped to chromosome 3p25.3 [60], is a tumor suppressor gene that has an inhibitory effect on transcription elongation and facilitates the proteasome-mediated degradation of the hypoxia-inducible factor 1 (HIF-1) protein [16, 61]. The alpha subunit of HIF-1 is highly sensitive to tissue oxygen levels. In the presence of normal oxygen levels, it is bound by the VHL protein complex and covalently linked to ubiquitin in order to be targeted for degradation. In the absence of the VHL protein, HIF-1 alpha levels increase, leading to overproduction of hypoxia-associated cytokines, including erythropoietin, vascular endothelial growth factor (VEGF), and platelet-derived growth factor [5, 62–67]. These cytokines have been implicated in tumor growth. However, the precise mechanism of tumorigenesis is unknown. Other factors contributing to tumor pathogenesis may include matrix metalloproteinases such as MMP1 [68, 69]. VEGF inhibition, with resultant inhibition of angiogenesis, is currently under investigation as a potential therapeutic strategy for PENs [70, 71].

The majority of patients present with a germline mutation of the gene from the affected parent and a normal copy of the gene from the unaffected parent. Tumor develops when both alleles are inactivated, usually as the result of a deletion [5]. In one study 12.3 % of 155 patients with VHL went on to develop PENs [72]. Majority of these neoplasms tend to be nonfunctional, and are frequently composed of cells with foamy, clear cytoplasm, similar to clear cells typical of renal cell carcinomas in VHL [41, 73]. LOH of the VHL gene was found in 100 % of PENs (6 of 6 tumors) analyzed by PCR-single strand conformational polymorphism and fluorescent in situ hybridization. All the tumors in this study were nonfunctional [41]. These findings support a role for VHL gene mutation in the formation of VHL-associated PENs. In patients with sporadic PENs, no mutations were found in the VHL gene, although allelic loss on chromosome 3p was found in 33 % of 43 patients with sporadic PENs [74].

14.3.5 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF-1) is a neurocutaneous syndrome characterized by neurofibromas, Lisch nodules on the iris, dermal café-au-lait spots, as well as a variety of endocrine neoplasms, including somatostatin-producing neuroendocrine tumors of the duodenal wall, pheochromocytomas, hyperparathyroidism, hypothalamic or optic nerve tumors [16] and rarely somatostatinomas of the pancreas [5, 75, 76]. NF-1 is caused by a mutation of the NF-1 gene, a tumor suppressor gene located on chromosome 17q11 that code for the protein neurofibromin. Mutations cause the premature truncation of neurofibromin. The precise role of the NF-1 gene in the development of PENs still remains to be elucidated.

14.3.6 Tuberous Sclerosis

Tuberous sclerosis is a rare autosomal dominant syndrome associated with the development of hamartomas and benign tumors in multiple organs, including skin, brain, and kidney. Two gene mutations have been described: TSC1 on 9q34 encoding hamartin [77] and TSC2 on 16p13.3 encoding tuberin, with identification of the tuberous sclerosis gene on chromosome 16 [78]. Together, these proteins function as a tumor suppressor gene complex and control the activity of mammalian target of rapamycin (mTOR) [16]. A complex of hamartin and tuberin is thought to regulate cell-cycle progression, possibly through upregulation of the mTOR cell-signaling pathway [79, 80]. mTOR is an intracellular protein that is key in the control of cell growth, protein synthesis, and autophagy [71] and is involved in the regulation of β-catenin stability and activity [5]. One to five percent of patients with tuberous sclerosis can develop PENs that demonstrate LOH on 16p13.3 or lack of tuberin immunoreactivity [5, 81–83]. Based on these findings, the RADIANT 1 (RAD In

Advanced Neuroendocrine Tumors) trial, a phase II study, focused on the evaluation of everolimus, an mTOR inhibitor, in patients with advanced PENs who have failed cytotoxic chemotherapy [71].

14.4 Molecular Genetic Analyses in Sporadic PENs

The majorities of PENs are sporadic and lack any association with germline mutations. The genetic aberrations implicated in sporadic PENs are poorly understood. Oncogenes and tumor suppressor genes that are mutated in common human malignancies (p53, APC, Rb, K-ras) do not appear to be associated with PENs [84–86]. Both within genetic syndromes characterized by PENs and in sporadic PENs, genomic studies have identified specific molecular patterns that characterize individual PENs. In the case of MEN1 associated insulinoma, these molecular abnormalities include gain of function in dlk1, PCNA, TERT, ASK and NFkB [93, 257, 262, 283], and loss of function of menin, p27, p18, JunD and FANCD2 [87–89]. Sporadic insulinomas are characterized by gain in function of cyclD/CDK4, Akt1, Abl, TGFa/EGFR and Bcl2 [193, 194, 263, 263, 264, 265, 266, 277, 280] and loss of function of several proteins, including PTEN, p16, p15 and RKIP [168, 190, 260, 261, 270, 271]. Several of these genes and markers have also been studied in other PENs with variable findings.

14.5 Comparative Genomic Hybridization Studies in PENs

Several studies have used comparative genomic hybridization (CGH) to identify chromosomal aberrations in PENs. One of the most consistent chromosomal alteration is loss of 11q, which harbors the MEN1 gene [90]. Zikusoka et al. [81] compared six studies using comparative genomic hybridization to detect gains and losses in various chromosomes in PENs. The most frequent gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. Chromosomal aberrations associated most frequently with malignant behavior and metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q [91–93]. Male patients with malignant insulinomas have been reported to show loss of Y chromosome and gain of Xp [75, 91]. Similarly, PENs in females have been shown to exhibit loss of X chromosome [75]. Furthermore, loss of sex chromosome has been associated with metastatic disease, local invasion and poor patient survival [75]. Other chromosomal aberrations identified in PENs include gains of chromosomes 19, 5, 14p, 12q, 17, 20q, 15, 18, 9q, and 17p, as well as losses of chromosomes 1p, 6, 11q, 3q, 11p, and Xq [91, 92, 94–97]. Overall, nonfunctional PENs contained more genetic aberrations than functional PENs [91], metastases had a higher average number of chromosomal aberrations than matched primaries [94] and 11q losses and 7q gains were reported in multiple studies, supporting their role in the development of PENs.

14.6 Loss of Heterozygosity (LOH) Studies in PENs

LOH analysis is a powerful molecular tool used to identify tumor suppressor gene loci that are involved in the formation and progression of neoplasms, including PENs. An LOH frequency greater than 35 % at a specific chromosomal locus exceeds the rates of random genomic instability and strongly suggests a relevant tumor suppressor gene at that locus [98, 99]. A technical advantage with LOH is that it can detect even smaller chromosomal deletions that could be missed by CGH. Several chromosomal aberrations have been identified in sporadic PENs. Using LOH, most common abnormalities are found in chromosomes 1, 3 and 6. LOH at chromosome 1 was found in 34 % of PETs and was found to be more common in tumors with hepatic metastasis [100, 101]. Chen et al. [102] identified chromosome 1 LOH on 1q31–32 and 1q21–23 in almost half of the gastrinomas studied and found an association with aggressive growth, liver metastasis and post-surgical recurrence of liver metastasis, suggesting a worse prognosis for patients with chromosome 1 aberrations. Overall, 56 % of 273 metatsatic PENs analyzed in several different studies were found to show various chromosomal aberrations, especially in chromosomes 1, 3, 6, X and Y, as opposed to 39 % of 264 non-metastatic PENs [75, 91, 98, 100, 102–109]. These data point toward a potential association between chromosomal aberrations and progression of PENs.

Chromosome 3 is the location of the vHL gene (3p25.3) [60], which has been associated with vHL syndrome-associated PENs. Recent studies have shown LOH at loci proximal to vHL locus [103] and LOH at 3p14.2–3p21 loci occur more often in malignant insulinomas than in benign insulinomas. Barghorn et al. [104] found highly statistically significant increased frequency of LOH at 3p25.3–p23 in malignant as compared to benign PENs, and in metastasizing as compared to non-metastasizing PENs. Additionally, a strong correlation was found between the loss of alleles on chromosome 3p and clinically metastatic PENs. These findings suggest a tumor suppressor gene at 3p25.3–p23 that may be associated with sporadic PEN development and that losses of larger centromeric regions are associated with metastatic progression. In another study, LOH at 3q was found in half of sporadic PENs with hepatic metastases, while PENs without hepatic metastasis showed no LOH at this location [105]. Microsatellite markers demonstrate the smallest common deleted region at 3q27-qter, the region of p51 (a member of the p53 tumor suppressor family) [110]. These findings are suggestive of a late event in the molecular pathogenesis of PENs, consistent with advanced stage of tumor development [81].

LOH detected chromosomal loss at 6q in 6 % of sporadic PENs overall and in 100 % of insulinomas, suggesting a chromosomal aberration specific to insulinomas [91]. Additional findings were smallest regions of allelic deletions at 6q22 (50 %) and 6q23–24 (41.2–56.3 %). Also, more chromosome 6 aberrations were detected by FISH in metastatic relative to benign PENs [106]. Thus, chromosome 6 alterations may play a specific role in the molecular pathogenesis and progression of insulinomas. LOH at chromosomal arm 9p, the home of p16, is a frequent finding in PENs. However, Moore et al. [111] found a p16 mutation in only one insulinoma

out of 41 PENs, none of which showed methylation. Current studies seem to indicate that p16 inactivation by promoter methylation may be restricted to functional gastrinomas. Rare homozygous p16 gene deletions have also been reported in PENs [107]. Using CGH, Speel et al. [93] found 9q gain to be the most common gain in insulinomas (50 %).

The tumor suppressor gene, PTEN, is located at chromosome 10q23 [112] performed a mutation analysis of the entire coding region of PTEN in 33 PENs and found only one tumor with a somatic mutation in exon 6. However, 10q23 region LOH was detected in more than half of malignant and in none of 7 benign PENs. All samples with LOH at 10q23 were malignant PENs. This suggests that allelic loss of this region could be associated with malignant behavior. In non-neoplastic pancreatic islet cells, immunohistochemical expression of PTEN protein is localized to the nucleus. PTEN expression was lost in the single malignant PEN with two structural hits; however, all of the PENs with LOH remained PTEN-immunoreactive but PTEN was localized predominately in the cytoplasm and cell membrane in 23 of 24 (96 %) PENs. No increase in malignant behavior is associated with this shifting of PTEN from the nucleus but is associated with the neoplastic state in general. Based on the above findings [112] it was proposed that inappropriate compartmentalization of PTEN (cytoplasmic as opposed to nuclear localization of PTEN) could be an initiating event in the genesis of PENs, whereas physical loss of 10q leads to progressive malignancy.

Chromosome 11q13, discussed previously, is associated with *MEN1* and the development of most MEN1-associated PENs as well as some sporadic PENs. Chromosome 11p13–15 was studied in a comparative genomic hybridization investigation of 25 PENs from 23 patients. 11p13–15 loss was found in 24 % of cases, likely representing uncharacterized tumor suppressor genes in this region [94]. Chromosome 11q23 harbors the tumor suppressor gene *succinate dehydrogenase subunit D (SDHD)* [113]. A number of studies have shown significant allelic loss of 11q extending to 11q23, or distal to 11q13, and have thus postulated that a previously unrecognized tumor suppressor in this region plays a role in PEN development [47, 91, 108]. Chromosome 12p12 is the location of the K-ras gene, which is commonly mutated in pancreatic ductal adenocarcinomas but is found only rarely in PENs [111], supporting the idea that exocrine and endocrine pancreatic neoplasms involve different genetic pathways. Chromosome 17p13 is home to TP53, which plays a significant role in the tumorigenesis of pancreatic ductal carcinomas, but not PENs. A study by Moore et al. [114] supported previous suggestions that the presence of a tumor suppressor gene other than TP53 on chromosomal arm 17p is involved in the molecular pathogenesis of nonfunctional PENs.

Chromosome 18q21 mutations may play a role in the molecular pathogenesis of nonfunctional PENs, whereas select functional tumors lack this change [115]. 18q21 is the location of the *DPC4/Smad4 gene*, a cell cycle regulator [116]. However, in another series of PENs, these chromosomal aberrations were not detected in any of the 19 nonfunctional PENs analyzed [117]. Chromosome 22 was studied in gastrinomas, insulinomas, VIPomas, and nonfunctional PENs, and LOH was found

on chromosome 22q in 22 of 23 tumors [109]. Another study found LOH in 57 % of insulinomas at 22q12.1–q12.2 [118]. This site is the location of the hSNF5/INI1 gene, implicated in medulloblastoma and other pediatric central nervous system tumors [119]. Further studies could not find an alteration in this gene suggesting it is not the cause of tumor development [81, 118]. X chromosome losses were seen in patients with functional and nonfunctional PENs and were associated with shorter patient survival [8] and clinically aggressive behavior [75, 98, 120]. Y chromosome losses were found frequently in PENs from males (36 %) and were associated with metastasis, local invasion, and high proliferation rates [75].

14.7 Cell Cycle Regulars in Sporadic PENs

Regulation of the cell cycle keeps cell death (apoptosis) in balance with cell growth (proliferation). Loss of cell cycle regulation is one of the hallmarks of neoplasia. Understanding the regulatory mechanisms of the cell cycle are complex, as multiple, often repetitive pathways may be involved. A number of studies have shown that common cell cycle regulators are involved in the molecular pathogenesis of PENs.

P27KIP1 is a cell kinase inhibitor that opposes cell cycle progression and is located on chromosome 12p12–p13.1 [121]. A study by Guo et al. [122] found overexpression of P27KIP in sporadic PENs. An elevation of P27KIP1 expression was found to be inversely related to Ki-67 in a study of 109 gastroenteropancreatic NETs, suggesting that P27KIP1 may inhibit proliferation in these tumors [123].

Loss of p16INK4/p14ARF, a tumor suppressor gene located on 9p21 [124] l, leads to tumorigenesis as a result of deregulation of p53 and cyclin-dependent kinase/retinoblastoma pathways [125]. Reports found inactivating p16INK4 gene alterations (such as homozygous deletion and methylation at the 5′CpG islands of promoter regions) in 92 % of gastrinomas and nonfunctional PENs. Loss of expression of genes in the 9p21 region was found in 57 % of nonfunctional PENs, 30 % of insulinomas, and 22 % of gastrinomas. This study also found CpG promoter methylation of the p16 gene [126].

Cyclin D1, on chromosome 11q13 [127, 128], plays an important role in cell cycle regulation. Nuclear expression of Cyclin D1 was found to be increased in almost half of the PENs [129]. Sporadic PENs were specifically studied and Cyclin D1 overexpression was found in 65 % (20 out of 31) of the PENs studied compared to normal pancreatic tissue [130]. Pathways associated with Cyclin D1, specifically the P38/mitogen-activated protein kinase and Akt/PKB pathways, were activated in PENs, whereas down-regulation of the extracellular signal-regulated kinase pathway was also found with overexpression of Cyclin D1 [131–133].

Other cell cycle regulators have been studied with controversial results, including the retinoblastoma tumor suppressor gene initially found to be deleted in insulinomas [134, 135] but without further confirmation [74, 136, 137] and, as mentioned previously DPC4/Smad4 on chromosome 18q21 [116].

Cell cycle regulators studied and found not to be important contributors to PEN development to date include P53 located on chromosome 17p13.1 [114, 138–140], β-catenin located on chromosome 3p21 [141, 142], phospholipase CB3 located on chromosome 11q13 [143], and retinoic acid receptor β located on chromosome 3p24 [144, 145].

14.8 Other Molecular Genetic Abnormalities in Sporadic PENs

PI3K-Akt/PKB pathway participates in the mediation of β-cell mass up-regulation [146–148]. This pathway activates downstream messengers and transcription factors such as PDX-1, Ngn-3, Isl-1, NeuroD/Beta2, and Nkx2.2, known to act during pancreatic embryogenesis. Glucose and insulin-like growth factor (IGF) induce activation of the PI3K-Akt/PKB pathway and promote in vitro proliferation of insulinoma cells [147]. A persistent stimulus that promotes proliferation is seen in other tumors, including the persistence of achlorhydria inducing gastrinomas. Mouse studies found that up-regulation of the PI3K-Akt/PKB pathway is not sufficient for neoplastic transformation [146].

K-RAS2, on chromosome 12p12.1 [149], is an important oncogene that transduces cell growth signals, mutations of which lead to growth factor-independent stimulation of cell proliferation [150]. K-RAS2 mutations were not detected in PENs in a study by Yashiro et al. [151]. Another study found strong K-RAS2 immunoreactivity and mutations in 4 of 6 insulinomas studied [152].

Somatostatin (SST) and G-protein-coupled transmembrane receptors (SSTRs) seem to play a role as regulators of islet cell proliferation [1]. Loss of SST/SSTR signaling may contribute to the genesis of PENs. In support of this theory, MEN-1 studies showed decreased expression of SST and islet amyloid polypeptide in MEN syndrome type 1 [153], providing a link between loss of menin, suppression of SST and islet amyloid polypeptide expression and oncogenesis.

The dominant effect of the protooncogene c-Myc is apoptosis of the islet cells. However, increased expression of c-Myc has been demonstrated in glucose-induced hyperplasia of pancreatic islets. Thus, the effects of this gene depend on the environmental effects and the influences of other genes and proteins. Pelengaris and Khan [154] proposed a model of accumulating mutations leading to progression from hyperplasia to neoplasia. c-Myc is thought to be an early event in hyperplastic islets. c-Myc expression was found to be increased in hyperplastic islets and benign and malignant insulinomas.

RIP-Tag2 oncogene expression in transgenic mice leads to islet cell hyperplasia and neoplasia. Tag oncoprotein inactivates tumor suppressor proteins p53 and pRb. Decreased apoptosis is also seen due to overexpression of antiapoptotic protein Bcl-XL and Bcl-2. These antiapoptotic proteins counteract the effects of proapoptotic c-Myc [154, 155].

HER-2 neu, found on chromosome 11q21 [156], is a well-known oncogene that is overexpressed in some cases of breast carcinoma and is associated with increased

malignant behavior, proliferation, and metastasis [157]. HER-2 neu has been studied in gastrinomas, where its overexpression was found in a minority of these tumors, and was associated with liver metastasis [158].

The CpG island methylation of the estrogen receptor gene (ESR1), located on chromosome 6q24 [159], has also been described in breast carcinoma and has significant therapeutic implications, indicating tamoxifen resistance [160]. Estrogen receptor gene methylation was found in 64 % of PENs in one study [144].

14.9 Molecular Biologic Aspects of PENs

Human MutL homologue 1 (hMLH1), found on chromosome 3p21.3 [161], is a mismatch repair gene. One study found hMLH1 to be hypermethylated in 23 % of PENs with evidence of microsatellite instability [162]. Promoter hypermethylation (gene silencing) was associated with an improved 5-year survival (100 % vs. 56 %).

Telomerase, on chromosome 5p15.33 [163], is an enzyme that maintains the chromosomal telomere. Telomere degradation is a normal part of the cell cycle, but aberrations of telomerase can lead to tumorigenesis [81]. Telomerase activity may predict an unfavorable outcome in PENs [8, 164].

Thrombomodulin, an endothelial anticoagulant, when overexpressed, reduces cellular proliferation and promotes cellular adhesion in vitro, while expression of thrombomodulin in vivo is inversely correlated with metastatic spread [165, 166].

E-cadherin functions to promote cell-cell adhesion. Loss of E-cadherin is associated with invasion and metastasis in many malignancies. Chetty et al. [13] found aberrant E-cadherin expression in more than 50 % of PENs, which strongly correlated with lymph node and liver metastasis. In addition, nuclear E-cadherin was seen in 18/57 cases when stained with antibodies detecting the cytoplasmic fragment of E-cadherin. This is a previously undescribed staining pattern in PENs.

Cell signaling pathways influence tumor growth and hormonal activity. Neuroendocrine cells can express the insulin-like growth factor (IGF) and its receptor (IGFR) [167]. Cell line studies indicate that IGF-1 can act in autocrine and paracrine fashion to inhibit apoptosis and stimulate secretion of chromogranins, possibly by activating the P13K-AKT pathway. VEGF is also expressed by neuroendocrine tumors, and elevated levels of VEGF have been associated with tumor progression [168, 169].

14.10 Gene Expression Profiling Studies in PENs

Pancreatic endocrine tumors/carcinomas (PETs/PECAs) are clinically challenging neoplasms. Based on the presence or absence of metastases and/or gross invasion of adjacent organs, they are classified as pancreatic endocrine carcinomas (PECAs) or pancreatic endocrine tumors (PETs) [WHO-2004] [10]. In the presence of negative metastatic work-up at the time of first tissue diagnosis, it is difficult to predict

which of these tumors will become metastatic ('malignant') or remain localized to the pancreas ('benign'). Standard histologic criteria, including grade, tumor size and Ki-67 index, often fail to predict malignant behavior [170]. Patients who develop metastases are rarely cured and 5-year survival rates diminish significantly. Therefore, discovery of molecular markers of prognosis is vitally important in order to quantify the risk of metastatic recurrence in patients with resected primary tumors.

The broad heterogeneity that characterizes neuroendocrine tumors has always posed problems regarding their correct classification [171]. In recent years, several refined histopathologic classification, grading and staging schemes have been proposed for PETs and, to some extent, validated to categorize these heterogeneous neoplasms into various diagnostic and prognostic subgroups [10, 11, 172–179]. However, due primarily to the diverse biology of these rare neoplasms, there is no single internationally acceptable prognostic scheme that would allow accurate determination of prognosis in a particular case.

In order to identify newer diagnostic, prognostic markers and therapeutic targets, lately there has been an increasing application of high-throughput technologies, including gene expression profiling, to the study of pancreatic endocrine tumors [153, 180–185]. Genes that have been associated with malignant behavior in human PENs include VEGF-C [186], met proto-oncogene, insulin-like growth factor binding protein 3 (IGFBP-3) [187], MEN-1 gene [188], c-N-ras , HER-2/neu [158] and c-myc, TGF-alpha, c-K-ras and p53 [152]. Most of these studies were conducted on small numbers of specimens and have not been fully validated. In a recent study [182], gene expression profiles of primary PECAs and their hepatic metastases were found to be similar, suggesting that most genetic abnormalities accumulate at the level of primary PETs. Therefore, identification of a set of genes and/or their protein products in the primary tumor tissue in clinically localized pancreatic endocrine tumors (CLP-PET), may have significant prognostic and therapeutic implications. Clinical utility of a metastasis-associated gene (MAG) signature as a predictor of future metastases has been shown in breast [189] and prostate [190] cancers and in medulloblastoma [191]. However, molecular markers of progression in primary pancreatic endocrine tumors are largely unknown.

Gene expression profiling using microarray analysis has identified a number of genes typical of PENs [153, 182, 186, 187, 192]. Similarly, histologically normal pancreatic islets were compared with PENs, showing overexpression of 66 genes, especially IGFBP3 (a growth factor), fibronectin (a cell migration/adhesion molecule), and oncogenes like MLLT10/AF10. One hundred and nineteen genes were under-expressed, including p21CIP1 (a cell cycle regulator), JunD (a transcription factor), and NME3 (a metastasis suppressor gene). Another gene expression study compared normal islet cells and three neuroendocrine tumor cell lines; 667 genes were up-regulated, and 323 were down-regulated [182, 192].

Recently, we selected pathologically well-characterized subsets of frozen PEN tissues and adjacent histologically normal pancreatic islets, and used a comprehensive genome-wide expression profiling approach and discovered a novel set of candidate progression genes (CPGs) that were differentially expressed in surgically resected metastatic primary (MP)-PECAs relative to clinically localized primary

(CLP)-PETs [193–195]. Many of these genes, including RUNX1T, palladin, insulin receptor, CD24 antigen and NRCAM had not previously been associated with progression in PENs. We have successfully validated two of our leading candidate progression genes (RUNX1T1, palladin) at the protein level using immunohistochemistry (IHC) on larger independent test sets of MP-PECAs and CLP-PETs [196, 197]. We are now focused to advance our discovery of novel molecular markers (CPGs) to the next step of larger scale clinical validation, using the roadmaps proposed in recent literature with regard to the discovery and validation of gene-based biomarkers. Although gene expression profiling studies have identified newer candidate genes that are providing newer insights into the molecular pathology and biology of PENs, comparison among these published studies continues to be a challenge due to variations in study designs, patient populations investigated, and tumor samples analyzed.

14.11 Summary

A large number of studies have contributed significantly to our current understanding of molecular pathology and diagnosis of pancreatic endocrine neoplasms. PENs exhibit a diverse spectrum of molecular genetic aberrations similar to their broad clinical presentation. Nonfunctional PENs exhibit more molecular aberrations than functional PENs. Also, malignant behavior seems to be associated with increasing genetic aberrations, suggesting specific genes may be associated with metastases in PENs. Genome wide studies have identified several genetic abnormalities in PENs, some of which predict malignant behavior in various subtypes of PENs and adverse clinical outcome while others provide molecular evidence in support of earlier stages of neoplastic transformation of PENs. Comparative genomic hybridization studies found that most frequent chromosomal gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. The chromosome aberrations most frequently associated with metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q. LOH analyses have identified multiple tumor suppressor gene loci that contribute to the pathogenesis of PENs. Genetic syndromes, which include PENs as one of their components, allow for the identification of genes associated with the genesis of PENs, including MEN1, vHL, NF-1, TSC1 and TSC2. Gene expression profiling using microarray analyses has identified a large number of genes differentially expressed in PENs when compared with normal islets, suggesting their involvement in initiation or progression of PENs. These include IGFBP3, fibronectin, oncogene MLLT10/AF10, p21C1P1, JunD, NME3, RUNX1T1, paladin and p21. While these studies are providing critical scientific evidence to refine our understanding of the molecular complexity of these neoplasms, pooling of large data sets and comparison among these molecular studies is challenging because of variations in study designs, patient populations, and tumor samples (fresh-frozen versus archival) and variation in the technical platforms used. A number of interesting lines of investigation are lending credence to various hypotheses regarding the molecular pathogenesis,

progression and therapeutic responsiveness of PENs. Some the recent studies have focused on cathepsins, CD44, NESP-55, hMLH1, telomerase, thrombomodulin, and E-cadherin expression/activity. Advanced molecular testing, is currently making it more feasible to pursue newer lines of genetic studies to unravel an increasing number of chromosomal aberrations associated with PENs. Multiple molecular alterations, involving cell migratory, cell cycle and angiogenic functions have been found to promote PEN development, growth, invasion, and metastases. As a result of these studies, phase III trials of novel therapies targeting cell regulators such as mTOR, VEGF and other targets have been completed. Focused investigation of various mediators/mechanisms implicated in the molecular pathogenesis and progression of PENs will contribute to novel diagnostic, therapeutic and preventive strategies. These efforts need to be integrated with the development and validation of robust assays for prognostic and predictive biomarkers that can be used in the clinic. All of these efforts will ultimately translate into more efficacious, safer and cost-effective therapeutic and monitoring options for patients with PENs.

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