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REVIEW AND PERSPECTIVE

Molecular profiles of gastroenteropancreatic endocrine tumors

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Abstract Neuroendocrine tumors of the gastroenteropancreatic system are defined by their endocrine phenotype and share many histopathological and clinical features. However, the fact that the hormone production of tumors depends on their site of origin, that the tumors differ in their biology, and that the association with familial syndromes is nonrandom suggests heterogeneity. It is therefore conceivable that the gastroenteropancreatic neuroendocrine tumors also differ in their molecular profile. This review summarizes and discusses the available data in this field.

Keywords Gastrointestinal · Pancreatic · Endocrine tumor · Genetic · Expression array · Comparative genomic hybridization · LOH · Mutation analysis

Introduction

Well-differentiated gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are defined by their capacity to express neuroendocrine markers and to produce and/or secrete peptide hormones, biogenic amines, and tachykinins.

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Pancreatic endocrine tumors (PETs) may arise in association with a multiple endocrine neoplasia type 1 (MEN1), von Hippel–Lindau (VHL), or rarely neurofibromatosis type 1 (NF1) or tuberous sclerosis (TSC) syndrome, and the genes responsible for these familial diseases have been described. However, about 90% of PETs and the vast majority of gastrointestinal endocrine tumors are sporadic, and still little is known about the molecular basis of GEP-NETs.

Activation of oncogenes is rare in sporadic PETs [21, 23, 29]. Activating mutations of β-catenin were described in 37% of gastrointestinal NETs [14] but were not detected in exon 3 in a series of 15 PETs (own unpublished data). Mutations of *TP53*, *K-ras*, *p16*, *DPC4*, or *DCC*, which are found frequently in gastrointestinal adenocarcinomas, were not identified in GEP-NETs [22, 28–30, 33, 41]. CGH and loss of heterozygosity (LOH) data point toward the involvement of other chromosomal regions. In this article, we review the genetic alterations that have been identified in GEP-NETs, using ribonucleic acid expression arrays, comparative genomic hybridization (CGH), LOH, and gene mutation analysis.

Pancreatic endocrine tumors

Genome-wide analysis: RNA expression arrays

With the development of high-throughput techniques, analysis of the expression of thousands of genes in a given tumor has become possible. The use of RNA expression arrays can further our understanding of tumor classification. It can also provide signatures (gene lists classifying tumors into distinct groups) that may have prognostic implications. While this technique has led to new (sub)classifications in other tumors (lymphoma, breast cancer), only limited

results have been published for GEP-NETs. For these tumors, class comparison studies rather than class prediction or class discovery studies have been performed. Class comparison studies analyze the gene expression in groups of specimens defined by other methods such as histopathological, immunohistochemical, or clinical features. This analysis identifies genes that are differentially expressed between the different tumor types.

As biological behavior is difficult to predict in PETs, the largest published RNA expression array study on PETs compared metastasized with nonmetastasized PETs [10]. By comparing 12 localized to 12 metastasized PETs, the signature of 51 genes that are downregulated in malignant PETs and 72 that are upregulated in malignant PETs emerged. These genes were involved in pathways related to (1) angiogenesis and remodeling, (2) signal transduction through tyrosine kinases, (3) calcium-dependant cell signaling, and (4) response to therapeutic drugs.

A different approach was taken in the study by Maitra et al. [31]. In this study, transcripts from eight well-differentiated PETs were compared with transcripts of three enriched pancreatic islet cell samples with 80–90% purity. Their aim was to identify potential therapeutic targets and novel tumor markers in PETs. None of the patients had an associated endocrine hypersecretion syndrome; therefore, all eight tumors were nonfunctioning tumors. One of the tumors revealed somatostatin expression. Two tumors had lymph node metastases and measured 2 cm or less in diameter. The authors described 66 transcripts with threefold overexpression in PETs and 119 underexpressed transcripts. The underexpressed genes included cell cycle checkpoint proteins (p21, MICT) and genes involving deoxyribonucleic acid (DNA) damage repair and genomic stability (*O*-methylguanine DNA-methyltransferase and GADD 45). The number of differentially expressed genes was rather low in this study. One reason might be the applied high threshold of "threefold change" and the other, the selection of tumor types. The group of nonfunctioning tumors might be a potpourri of PETs expressing hormones at low levels. Furthermore, the study included many large tumors that may have accumulated extensive genetic changes, at least as expected from CGH studies. While the aim of the study to detect therapeutic markers is not yet accomplished, these gene lists will provide useful candidate genes for further detailed analyses.

In a second study, this group extended their analysis to the differential expression of genes in well-differentiated metastatic and nonmetastatic PETs [18]. The expression profiles of seven metastatic PETs were compared with the profiles of five nonmetastatic PETs. Sixty-five transcripts were overexpressed, and 57 transcripts were underexpressed in metastatic PETs compared to nonmetastatic ones. In this attempt to find a progression model from nonmetastatic to metastatic PETs, the gain of function of genes involved in growth regulation, cholesterol homeostasis, osmotic regulation, and hypoxia-inducible factors were described. Underexpression of genes involved in cell cycle regulation, developmental regulation, and DNA damage repair was described. Theses two studies showed a continuum of upregulation of IGFBP3 from normal islets to nonmetastasizing PETs and to metastasizing PETs. Malignant tumors showed an overall pathway activation of the insulin-like growth factor-signaling cascade, but the underlying mechanisms still have to be elucidated.

The rate of differentially expressed genes described was dramatically higher in the study by Carpuso et al. [7]. Comparing RNA extracts of 13 nonfunctioning PET (NF-PET) samples (eight primaries and five metastases) to extracts of four isolated islet preparations, they found 990 differentially expressed individual genes. Only 20 of the reported 667 upregulated genes were also described in the aforementioned similar studies (fibronectin [31], elongation of very long fatty acid-like 2 [6], coagulation factor V [18], and mitogen activated protein kinase 3 [17]). The authors described a striking similarity between primary malignant PETs and their metastases concerning their genetic profile: Metastases clustered with their corresponding primary tumors in most instances. This is evidence that the metastatic potential is acquired in an early stage of tumor transformation because cells of the primary tumor already carry the metastatic signatures.

Shalev et al. [42] compared isolated islets with and without glucose challenge. Fourteen genes were upregulated by glucose in all three independent experiments, and only six genes were downregulated. The authors were able to show that upregulation of transforming growth factor β (TGFβ) signaling was induced by glucose. As TGFβ is also known to be involved in differentiation, this raises the intriguing possibility that there might be crosstalk between glucose metabolism, development, and the immune system in human pancreatic islets.

The research on PETs using expression array techniques has only recently started, and it is anticipated that significant results will be published in the years to come. Future studies comprising larger numbers of well-characterized tumors are needed, and class prediction studies rather than simple class confirmation studies will allow the identification of subsets of endocrine tumors that are caused by different genetic changes and that might therefore respond to targeted therapies.

Genome-wide analysis: CGH studies

Four conventional CGH studies included a total of 102 PETs. These studies indicate that chromosomal losses occur slightly more frequently than gains, whereas amplifications

are uncommon [45–47, 58]. Genetic alterations seem to accumulate during tumor progression: The total number of genomic changes per tumor appears to be associated with both the tumor volume and the stage of the disease [46] Thus, large and/or malignant tumors and especially metastases harbor a larger number of genetic alterations than small and clinically benign neoplasms [46, 58] These findings point toward a tumor suppressor pathway and chromosomal instability as important mechanisms associated with malignancy in PET. These data have recently been confirmed for insulinomas in a large array CGH analysis studying 30 insulinomas. At least 20 chromosomal alterations and six telomeric losses were the best predictors of malignancy [26]. The alterations described are not randomly distributed on chromosomes but are particularly common in distinct chromosomal regions. Gains are common on 4pq (17% of the tumors), 5q (25%), 7pq (41%), 9q (28%), 12q (23%), 14q (32%), 17pq (31%), and 20q (27%), whereas genomic losses frequently occur on 1p (21%), 3p (19%), 6q (28%), 10pq (14%), 11q (30%), Y (31%), and X (31%) [45–47, 58]. Furthermore, genomic alterations in some chromosomal regions appear to be associated with the stage of the disease and, additionally, with particular tumor types. For example, losses of chromosome 1 and 11q as well as gains of 9q appear to be early events in the development of PETs because they are already present in many small tumors (<2 cm) [46]. Some defects, e.g., losses of 3p, 6pq, and 10pq and gains of 5q, 12q, 18q, and 20q, were shown to be associated with malignant behavior [2, 3, 46], whereas gains of chromosomes 4 and 7 in the presence of losses of 21q prevailed in metastases [58].

Insulinomas exhibit a lower number of genomic alterations than other PETs [45–47, 58]. Insulinomas often have chromosomal aberrations consisting of a gain of 9q32 and loss of 22q13.1, which may occur in the same tumor and appear to be early genetic events in these tumors [25]. Losses of 3p and 6q associated with malignancy are particularly rare in insulinomas [2, 3]. Malignant insulinomas, in contrast, harbor a large number of chromosomal alterations similar to that seen in other types of malignant PET [26].

In pancreatic gastrinomas, only limited chromosomal imbalances are encountered: Losses at 3p and 18q21 occur in approximately 33 and 22% of cases, respectively [46, 47, 58]. It is interesting to know that 18q losses are also common in gastrointestinal NETs, indicating that NETs and pancreatic gastrinomas might be related. NF-PETs in general harbor higher numbers of chromosomal gains and losses than functioning tumors. These genetic aberrations occur in chromosomal loci frequently involved in malignant tumors [58]. Gains of chromosome 4 and losses of 6q appear to be early events because they are already detectable in 40 and 50% of tumors with a diameter of less than 2 cm [46].

Loss of heterozygosity and mutation analysis

Analyses of LOH using polymerase chain reaction microsatellite markers identify the same chromosomal regions as those described by CGH. The rate of alterations detected by microsatellite analysis is, however, approximately twice that of the allelic losses detected by CGH. In the regions 3p23, 6q22, 9p, 11q13, 18q21, and 22q12.1, the differences are even more pronounced, indicating that small deletions that are not detectable by CGH may be involved [2, 3, 16, 19, 20, 32, 33, 37, 41, 59]. These regions displaying losses might harbor tumor suppressor genes involved in the initiation and progression of PETs. However, only a few candidate genes have been thoroughly investigated, and many genes remain to be tested.

The most important candidate gene is the *MEN1* gene. We know that almost all PETs in MEN1 patients, including microadenomas show allelic loss of the wild-type allele [19, 34, 51]. However, somatic mutations of the *MEN1* gene located on 11q13 are detectable in only 21% (33 of 155) of sporadic PETs [11, 16, 32, 43, 53, 59]. When PETs are stratified according to the hormonal syndrome they cause, the rate of *MEN1* mutations varies considerably between the subgroups. Thus, *MEN1* mutations are only detected in 7.7% (5 of 65) of insulinomas and 8% (2 of 26) of NF-PETs. In contrast, gastrinomas, glucagonomas, and VIPomas show a higher mutation rate of 37, 67, and 44%, respectively [11, 15, 16, 32, 43, 53, 56, 59].

In contrast to the low rate of 21% *MEN1* mutations, 68% of all PETs exhibit losses at 11q13 and/or of more distal parts of the long arm of chromosome 11, indicating that there might be a haploinsufficiency of the *MEN1* gene or that the other allele might be inactivated by epigenetic mechanisms. CpG island methylation in the *MEN1* promoter, however, was not identified [8]. Another explanation for this high rate of LOH is that further, yet unknown tumor suppressor genes in this region might be involved [11, 12, 16, 32, 43, 53, 59]. An important role of the *SDHD* gene (one of the candidate genes on 11q23) in the pathogenesis of PETs and other endocrine tumors has recently been excluded [35].

Another familial syndrome associated with PETs is VHL. Intriguingly, somatic *VHL* gene mutations have been detected in only 1 of 22 sporadic PETs, despite the high LOH rate on 3p25, indicating that the gene is less frequently involved than expected [9, 32]. NF1 and TSC patients may also develop PETs. However, the role of the *NF1* and *TSC1/2* genes in sporadic tumors has not been examined so far.

Several other tumor suppressor genes or oncogenes have been examined in PETs. These genes include *p16*, *PTEN*, *k-RAS*, and *p53*, which are only occasionally mutated and therefore seem to be of minor importance [5, 26, 33, 36, 41, 54]. *RET*, *hZAC*, *BRAF*, and *SMAD3* have been excluded as candidate genes [28, 38, 44, 49]. Furthermore, we recently were unable to detect any somatic mutation of the *HRPT2* gene on 1q25 in 25 examined PETs (unpublished observations).

The role of *DPC4/Smad4* in sporadic PETs also seems to be of minor importance. The high mutation rates described by one group [4] could not be confirmed by subsequent studies on a larger number of tumors by several other groups [30, 33, 37]. The classical tumor suppressor genes *PTEN*, *p53*, and *DPC4* are only rarely mutated, mostly in malignant tumors, and alterations of these genes therefore most likely represent late events in PET progression.

Gastrointestinal endocrine tumors

Genome-wide analysis

Knowledge about the genetic background of sporadic gastrointestinal NETs is even sparser than that of PETs. No results of RNA expression array analyses have been reported so far. Only three studies using CGH [50, 57] or 131 microsatellite LOH markers [30] examined genome-wide allelic imbalances in gastrointestinal NETs. The average number (2.9) of genomic changes was lower in gastrointestinal NETs than in PETs, and there was no clear correlation between the number of aberrations and tumor stage [57]. Furthermore, the number of different chromosomes involved was low, genetic alterations apparently being concentrated on chromosome 18. The study by Zhao et al. [57] described losses of the entire chromosome 18 or of its long arm in 38% of gastrointestinal NETs, whereas others reported these losses to occur in 50 [50] or even in 88% of tumors [30]. The high percentage reported by Lollgen et al. [30] is based on microsatellite LOH analysis, in which small deletions are detected with a higher sensitivity than obtained by CGH. The loss of chromosome 18 in gastrointestinal NETs is strong evidence that important candidate tumor suppressor genes are located on this chromosome. Most of these genes are most likely located on the long arm of chromosome 18.

In the three aforementioned studies [30, 50, 57], significant genetic differences between bronchial NETs, gastrointestinal NETs, and PETs were described, suggesting a different genetic background for these tumor groups. For example, losses of chromosome 18 are rare in bronchial tumors and PETs, whereas losses of chromosome 11 are common [57]. Furthermore, losses of 9p, which are detectable in 50% of gastrointestinal NETs, are rare in PETs, which in contrast more frequently exhibit gains of 17q and losses of 11q.37.

LOH and mutation analysis

The candidate genes *DPC4*, *DCC*, and *Smad2* were investigated because of the frequent allelic loss on 18q (88% of gastrointestinal NETs) [30] but were found to be unaltered in a small series of tumors. In the study by Lollgen et al. [30], all eight examined gastrointestinal NETs contained a wild-type C-terminal *DPC4* sequence encoded by exon 8–11 and conserved DPC4 protein expression as demonstrated by immunohistochemistry [30]. In our study on 21 gastrointestinal NETs, *DPC4* and *DCC* mutations were not detected using single-strand conformation polymorphism analysis (unpublished observations). Therefore, other possible tumor suppressor genes located on chromosome 18 remain to be investigated.

Patients suffering from MEN1 often develop NETs; most of which are localized in the duodenum and the stomach. Allelic loss of the corresponding chromosomal arm 11q has been detected in these types of endocrine tumors associated with the MEN1 syndrome. LOH of 11q is also consistently found in a subset of sporadic endocrine tumors of various localizations, although the observed LOH frequency varies. Although it is very low in the ileum (14% of tumors), it amounts to 25% in the ascending colon, to 30% in the pancreas and stomach, to approximately 50% in NETs of the descending colon and rectum, and to 60% in the duodenum [13, 15, 16, 24, 30, 56].

Enterochromaffin-like (ECL)-cell gastric NETs associated with chronic atrophic gastritis (type I gastric NETs) arise in association with ECL-cell hyperplasia and are often multiple [40]. LOH of 11q was present in only one of six such tumors examined. In the setting of the MEN1 syndrome, *MEN1* LOH, however, occurred in 15 of 20 type II gastric NETs arising in patients with a Zollinger–Ellison syndrome because of duodenal gastrinomas [13]. Two examined sporadic gastric NETs (type III gastric NETs) exhibited allelic loss of 11q microsatellite markers [16].

With the exception of sporadic gastrinomas, only few gastrointestinal NETs have been examined for *MEN1* gene mutations. The *MEN1* mutation rate in sporadic gastrinomas was high (31%). This ratio was comparable to that found in PETs [15]. This is not surprising, as duodenal gastrinomas and PETs are both classical MEN1-associated tumors. Somatic *MEN1* mutations have also been detected in a small subset of NETs of the ileum (one of four tumors examined) and colon, indicating that these mutations are not restricted to foregut NETs but may also occur rarely in midgut and hindgut tumors [16].

The tumor suppressor genes *p16* and *TP53* have also been investigated in NETs. Like in PETs, no mutations of the *p16* tumor suppressor gene have been identified. However, a 5′ CpG island of the p16 gene promoter was methylated in 52% of gastrinomas [41] and in other types of gastrointestinal NET [8]. Furthermore, methylation of *p16* was significantly more frequent in NETs than in PETs and thus represents an additional molecular difference between the two tumor groups [8]. It is interesting to know that a recent study also described higher rates of promoter methylation of the *APC*, *MEN1*, *HIC1*, and *RASSF1a* genes in gastrointestinal NETs [1]. This is good evidence that CpG island methylator phenotype (CIMP) is frequent in gastrointestinal NETs. Microsatellite instability does not occur in PETs or gastrointestinal NETs, and the *hMLH1* gene is not methylated [1, 27]. The high rate of *RASSF1a* promoter methylation might explain the frequent expression of extracellular signaling-related kinase (ERK) 1/2, an important downstream point of convergence in the ras-RAF-mitogen-activated protein ERK pathway [49].

Accumulation of the *TP53* protein was detected by immunohistochemistry in only one duodenal gastrinoma with an identified 17p loss and thus seems to be a rare event in NETs [50]. However, in 25% of goblet cell carcinoids of the appendix, *TP53* mutations were reported to occur [39]. The most frequently reported mutated gene in gastrointestinal NETs is β-*catenin*. Mutations in exon 3 of this gene protecting the corresponding protein from phosphorylation and degradation have been reported in 38% (27 of 72) of NETs [14].This leads to cytoplasmic and nuclear accumulation of β-*catenin* (demonstrated by immunohistochemistry) in 79% of these tumors, indicating other, yet unknown, mechanisms of accumulation of this protein. Only one of these 72 tumors (1.4%) displayed a mutation of the APC gene. However, the rate of gastrointestinal NETs with nuclear translocation of β-*catenin* was only 30% in a later study, and exon 3 mutations were absent [48]. The reported overexpression of cyclin D1 and c-*myc* [52] may be a downstream effect of the alterations of the Wnt signaling pathway. Other examined oncogenes appear to be of minor importance. Hardly any tumors show amplifications of the *HER2* gene [50], and in contrast to gastrointestinal adenocarcinomas, no *K-Ras* mutations were detected in gastrointestinal NETs [55] (Table 1).

Conclusion and perspective

The available molecular data indicate that the tumorigenesis of well-differentiated GEP-NETs differs from that of their nonendocrine counterparts. This is supported by results of CGH, LOH, and mutation analyses that point to different genetic pathways leading to the formation of GEP-NETs. In addition, subgroups of GEP-NETs appear to have special genetic pathways. PETs (especially NF-PETs, glucagonomas, and VIPomas) show a higher average number of chromosomal aberrations and allelic losses than gastrointestinal NETs. RNA expression analysis describes deregulation of cell cycle checkpoint proteins as well as genes

Table 1 Genetic changes frequently found in GEP-NETs

	Gene(s)	Involvement		
		Frequent	Rare	Absent
Genes characterizing endocrine tumor syndromes	*men1*	PET, D	G, I	
	vhl		PET	
	nf-1			
	tsc-1/2			
	hrpt-2			PET
	SDHx			PET, gasNET
Wnt signaling pathway	*b-catenin*	gasNET		PET
	APC		gasNET	
TGFbeta signaling pathway	*TGFbR2*			PET, gasNET
	Smad4		PET	
	Smad3			PET
Common tumor suppressor genes/oncogenes	*DCC*			PET, gasNET
	p53		PET, gasNET	
	PTEN		PET	
	K-Ras		PET, gasNET	
Mechanisms of tumorigenesis	CIMP pathway	gasNET	PET	
	Chromosomal instability	PET	gasNET	
	MSI		PET, gasNET	

GEP-NET Gastroenteropancreatic neuroendocrine tumor, *PET* pancreatic endocrine tumor, *D* duodenal endocrine tumor, *I* ileal endocrine tumor, *gasNET* gastrointestinal neuroendocrine tumor, *CIMP* CpG island methylator phenotype, *MSI* microsatellite instability

involved in DNA damage repair and genomic stability. It will be an important task to find the underlying cause of chromosomal instability (that is particularly observed in malignant PETs and less pronounced in benign insulinomas and duodenal gastrinomas) because this might identify new therapeutic targets. Furthermore, some chromosomal loci are preferentially altered in NETs: Gastrointestinal NETs frequently show 18q losses, while PETs commonly exhibit 11q losses involving the *MEN1* gene. Because PETs, in particular insulinomas and NF-PETs, only rarely harbor somatic *MEN1* mutations and there is no known tumor suppressor gene on 18q, the 11q and 18q losses may suggest that potential new tumor suppressor genes are located in both chromosomal areas. *MEN1* gene deletions are very rare in ileal and appendiceal NET, as are somatic *MEN1* mutations. Additional differences between PETs and gastrointestinal NETs concern the tumor suppressor gene p16 and β-*catenin* as well as the CIMP pathway (Table 1, bottom). p16 is more frequently methylated and β-*catenin* more often mutated in gastrointestinal NETs than in PETs, and gastrointestinal NETs more often exhibit alterations associated with the CIMP pathway, whereas advanced PETs frequently reveal chromosomal instability. Taken together, these data may explain why GEP-NETs, depending on their site of origin and phenotype, differ in their biological behavior and, in addition, stress the importance of classifying endocrine tumors according to their localization and hormone production.

Current results combined with those obtained by new technologies, such as DNA and RNA expression and tissue arrays, will probably result in a more clear-cut picture of the molecular background of the oncogenesis and progression of GEP-NETs. These findings, in turn, might subsequently lead to precisely tailored diagnostic and therapeutic strategies in the near future.

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