

## **MOLECULAR EVENTS IN GASTROINTESTINAL AND PANCREATIC NEUROENDOCRINE TUMORS**

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### **BACKGROUND**

GI and pancreatic NETs originate from the cells of the diffuse endocrine system derived from the embryonic neural crest, neuroectoderm and endoderm. NETs can be functional (release hormones) or non-functional (1-3).

It is important to recognize that molecular studies of GI and pancreatic NETs to date have been affected by several factors:

- 1) Heterogeneity of tumors;
- 2) Difficulty in predicting tumor behavior and prognosis;
- 3) Rarity of tumors and relatively small study sample size;
- 4) Use of different GI-NET classifications by investigators for tumor selection;
- 5) Differences in genetic composition of GI-NETs vs. epithelial tumors;
- 6) Tendency to study known epithelial genes/oncogenes first.

### **GI-NET Classifications Used in Molecular Studies (1, 4-6)**

**GI CARCINOIDS** are traditionally considered serotonin (5-hydroxytryptamine)-secreting and argentaffin positive:

**Foregut:** stomach, first part of duodenum and pancreas

**Midgut:** small intestine (second portion of duodenum, jejunum, ileum) and large intestine (appendix, ascending colon)

**Hindgut:** transverse colon, descending colon and rectum

**PANCREATIC AND DUODENAL NETS** (functional) are commonly classified according to the predominant hormone secreted: gastrinoma, insulinoma, VIPoma, glucagonoma, somatostatinoma. Clinically silent NETs are called non-functional (2).

## **WHO CLASSIFICATION OF ENDOCRINE TUMORS (4)**

Well-differentiated endocrine tumors (benign or uncertain behavior)

Well-differentiated endocrine carcinomas (low-grade malignant behavior)

Poorly differentiated endocrine carcinomas (high-grade malignant behavior)

## **MOLECULAR GENETICS OF GI- AND PANCREATIC NETs**

The hope is that the finding of specific genetic alterations that are characteristic of GI-PNETs will lead to improved diagnosis and characterization of these tumors and will enable a modification of current morphologic classifications.

Because the enteroendocrine cells are epithelial cells derived from the same stem cell as the other cell lineages, the investigation of the same mechanisms of neoplastic progression leading to adenocarcinoma were applied to many studies of gut endocrine tumors. However, the results of such studies led to conclusions that GI-PNETs do not share the same mechanism of development and neoplastic progression with GI epithelial tumors (2, 3).

## **Epithelial Carcinoma Genes Are Not Involved in GI-PNET Tumorigenesis (2, 3)**

Despite *APC* (adenomatous polyposis coli; familial adenomatous polyposis) gene's ubiquitous expression and crucial role in cellular homeostasis and neoplastic progression in colon and small bowel adenocarcinomas, it does not seem to have an impact on neoplastic progression in enteroendocrine cells. *DCC* (deleted in colon cancer gene) gene is not involved. DNA mismatch repair (*MSH2* and *MLH1*) genes, (Lynch syndrome) are not involved.

## **MOLECULAR EVENTS IN TUMORS OF FOREGUT**

### **PANCREAS**

Pancreatic NETs occur in three hereditary syndromes such as Multiple Endocrine Neoplasia type 1 (MEN1), von Hippel-Lindau disease (VHL) and von Recklinghausen's disease (Neurofibromatosis 1) (2). The syndromes are caused by defects in three known tumor suppressor genes, respectively: *MEN1* on chromosome 11q13 (610-amino acid protein, *MENIN*); *VHL* on chromosome 3p25.5 (213-amino acid protein, *VHL*) and the gene on chromosome 17q11.2 (2485 -amino acid protein, *neurofibromin*). Pancreatic tumors are multiple but their type, location and incidence are different in each syndrome (2). MEN1 pancreatic NETs are located in the pancreas and duodenum with the incidence of 80-100% (non-functioning pancreatic tumors>gastrinomas>insulinomas). Pancreatic NETs in VHL disease occur only in the pancreas, are non-functioning and are seen in 12-17% of patients. Pancreatic somatostatinomas occur in 6% of patients with Neurofibromatosis 1.

<b>Pancreatic and GI NETs in Hereditary Syndromes</b>		
Syndrome	Gene location (product)	NET Frequency
Multiple Endocrine Neoplasia type 1	11q13 (610-amino acid protein, <i>MENIN</i> )	80-100% pancreas+duodenum (NF>gastrinoma>insulinoma Gastric carcinoids)
Von Hippel-Lindau disease	3p25.5 (213-amino acid protein, <i>VHL</i> )	12-17% pancreas all non-functioning
Von Recklinghausen's disease	17q11.2 (2485 -amino acid protein, <i>neurofibromin</i> )	6% pancreatic somatostatinoma

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MEN1 patients have multiple endocrine tumors of the parathyroid glands, pituitary, pancreas, duodenum as well as gastric, lung and thymic carcinoids (7). Like in

hereditary tumors in patients with MEN1, *MEN1* gene alteration is an important initiating event in about 1/3 of sporadic non-functioning pancreatic NETs, insulinomas, and gastrinomas and is documented in tumors regardless of metastases (2, 8-9). Genetic analysis of the counterpart sporadic pancreatic NETs demonstrated somatic *MEN1* gene mutations accompanied by loss of the wild-type allele in 10-27% of insulinomas and 39-45% of gastrinomas (8, 10). The mutation rate in non-functioning NETs in different reports is 15-26% (10-11). Because the rate of 11q13 LOH in sporadic pancreatic NETs is on average 46%, and LOH is not always accompanied by somatic mutation, other mechanisms of *MEN1* gene inactivation or other genes may play a role in sporadic tumor development.

Thus LOH analysis for more telomeric markers on 11q showed that allelic loss consistently and continuously spanned to 11qter (12). These findings support the hypothesis that additional onco/suppressor gene(s) may reside at 11q distal to the *MEN1* gene and may play a role in the pathogenesis of pancreatic NETs (13-15).

VHL disease patients develop CNS and retinal hemangiomas, renal cysts and carcinomas, pancreatic and epididymal cystadenomas and pheochromocytomas. Pancreatic non-functioning NETs are seen in 12-17% of VHL patients (16-17). Loss of heterozygosity at 3p25.5 gene locus is documented in only 30% of sporadic pancreatic NETs and is usually not accompanied by somatic *VHL* gene mutation (18-20). These data indicate that *VHL* gene is not a factor in sporadic pancreatic NET development and another gene telomeric to the *VHL* 3p locus may be involved.

Variations in the genetic makeup of functioning versus non-functioning pancreatic NETs have been demonstrated in small tumors (<2 cm in diameter) by CGH (21). 9q gains with a common region of involvement at 9q34 were observed in 46.4% of functioning tumors, of which in 50% of insulinomas. However, diffuse genetic instability with multiple chromosomal aberrations per tumor was observed, making it difficult to evaluate the significance of specific findings.

In sporadic gastrinomas, either homozygous deletion or hypermethylation at the 5' region of the *p16/MTS1* or *p16<sup>INK4a</sup>* tumor suppressor gene on chromosome 9p21 was demonstrated (22-23). Such an abnormality was not observed in 17 insulinomas (24) and in 41 pancreatic NETs of different types (25), despite the presence of 9p LOH in 30% of cases. CGH and high-resolution allelotype analysis confirmed the involvement of 9p defects in 20–30% of cases investigated (26-28). Interestingly, *p16/MTS1* gene mutation was observed in only one out of a total of 68 tumors investigated in different reports. Overall, these findings indicate that other potential tumor suppressor genes on chromosome 9p are involved in the genesis of pancreatic NETs and suggest that *p16/MTS1* or *p16<sup>INK4a</sup>* defect is restricted to gastrin-producing tumors.

No defect of the retinoblastoma *Rb* gene on chromosome 13q was observed in any type of pancreatic NET investigated to date (29-30).

High LOH rates for markers on chromosome 22q (93%) were observed in both benign and malignant insulinomas (31). An overexpression of cyclin D1 was demonstrated by both immunohistochemistry and Northern analysis in 43% of PET, although there was no correlation with any specific tumor phenotype (32). Although possibly important in initiation of tumorigenesis in both benign and malignant pancreatic NETs these changes were not accompanied by gene mutations and, therefore, require further investigation.

The promoter region CpG island methylation of 12 genes potentially involved in endocrine tumor development such as *p14*, *p16*, the estrogen receptor (*ER*), retinoic acid receptor-beta 2 (*RAR-β*), *O<sup>6</sup>*-methyl-guanine-methyltransferase (*O<sup>6</sup>-MGMT*), *MEN1* and cyclooxygenase 2 (*COX2*), recently proved to affect especially the *ER* gene in 9 out of 11 pancreatic NETs (2 gastrinomas and 1 insulinoma) (33). A recent similar investigation for 11 candidate tumor suppressor genes in 48 well-differentiated tumors (of which three were functional) proved a high frequency of methylation for the Ras-associated domain gene family 1A (*RASSF1A*) (75% of

cases), *p16* (40%), *O<sup>6</sup>-MGMT* (40%), *RAR-β* (25%) and *hHMLH1* (23%) (34). In addition, a recent investigation of pancreatic cancer showed *RASSF1A* promoter methylation in 10 out of 12 pancreatic NETs with a similar incidence in both malignant (*n*=6) and benign lesions (35). Since *RASSF1A* gene mutation is very rarely observed in human cancer (36) these findings strongly support the methylation mechanism for multiple gene inactivation in PETs and suggest that the *ras* pathway is involved via *RASSF1A* methylation.

Activating mutations in the *ras* family of proto-oncogenes, *K-ras*, *H-ras*, *N-ras*, are absent or exceedingly rare in large series of pancreatic NETs investigated (20, 37-39) although it was reported as a frequent event in a single study of malignant insulinomas(40). Overall, the *ras* oncogene seems not play a direct role in the development of most pancreatic NETs, with the possible exception of malignant insulinomas. However, the *ras* pathway may be involved in pancreatic endocrine tumorigenesis via promoter methylation of the *RASSF1A* gene (see above) (33, 35).

Several studies have tried to address the relationship between genetic defects and tumor progression or malignancy. Most relevant data, however, require further confirmation. The deletion of either arm of chromosome 1 was found in 10 out of 17 metastatic pancreatic NETs, with no tumor type prevalence (41). This finding was not confirmed by CGH and genome-wide allelotype studies (26-27). LOH at 3p25, centromeric to the locus for VHL disease, was shown to be associated with malignancy (18). This observation was further supported by CGH data on 44 tumors of different types (26) and by a large study of 99 pancreatic NETs (42). The frequent occurrence of allelic loss at 6q was documented in pancreatic tumors, although its association with malignancy and tumor progression was observed by one group (26, 43) and not confirmed by another (27).

Well differentiated NETs only rarely display *p53* mutations (20, 44-45). LOH of *p53* gene chromosomal markers on 17p13 was reported in 25% of investigated cases and found to be associated with malignancy (38). The absence of relevant *p53* mutation

suggested that an additional tumor suppressor gene might occur on 17p telomeric to *p53*. Poorly differentiated NE carcinomas of any site show high chromosomal instability and frequent *p53* changes (46). It is likely that *p53* alteration is not involved in pancreatic NET initiation but that it is a late progression event in a poorly differentiated endocrine carcinoma of the pancreas

Allelic loss for markers on chromosome X has been frequently demonstrated in malignant compared with benign endocrine tumors (47). The analysis was extended to chromosome Y in male patients, resulting in a significant association with short survival, the presence of metastases, local invasion and a high Ki-67 proliferation rate (48).

Published data suggest that multiple genetic defects may accumulate, resulting in tumor progression and malignancy. LOH for markers of seven different oncosuppressor genes was significantly more frequent in malignant (40%) than in benign (17%) tumors (38). A paired CGH study of primary tumors and their metastases with a control group of non-metastatic tumors displayed more frequent genomic aberrations in metastases than in the corresponding primary tumors when compared with non-metastatic cases (49). In non-functioning pancreatic tumors, a high frequency of chromosomal markers loss (fractional allelic loss) correlates with aneuploid status and a poorer clinical outcome (27). Finally, in an investigation of multiple chromosomal markers, higher percentages of allelic imbalances were reported in pancreatic NETs, suggesting chromosomal instability as basis for malignant progression (50).

## STOMACH AND DUODENUM

NETs of stomach and duodenum display frequent LOH for the *MEN1* locus at 11q13 in both familial and sporadic cases (15, 51-52). LOH at the *MEN1* locus occurred in 75% of gastric (ECL) carcinoids in 23 familial cases and 41% in 46 sporadic cases (10). Four out of five poorly differentiated tumors of the stomach also showed allelic loss of the *MEN1* gene (15, 51, 53). A similar frequency of 11q13 LOH was observed

in both familiar and sporadic duodenal gastrinomas (28% and 25% respectively), whereas mutation for the *MEN1* gene was found only in 22 out of 67 sporadic cases (33%) (10). The findings support the initiating role of the *MEN1* gene in the development of many gastric carcinoids and duodenal gastrinomas.

Other data are scattered through small studies on various GI-NETs and are therefore fragmented. The promoter methylation of several genes, including *p14*, *p16*, *COX2* and *ER*, was frequently observed in two gastric and two duodenal well-differentiated tumours(33). Larger series are needed to confirm such observations.

An investigation of multiple chromosomal markers in nine gastric NETs, six of which were poorly differentiated, demonstrated frequent and diffuse allelic imbalances mostly in aggressive carcinomas (50). This finding was further demonstrated in 19 cases of aggressive gastric endocrine carcinoma, 10 of which were poorly differentiated, by an independent study (47). Both studies showed frequent LOH for markers at the *MEN1* and *p53* gene loci and demonstrated high chromosomal instability. In addition, a loss of *Rb* expression was frequently observed in 6 out of 9 well-differentiated gastric endocrine carcinomas and in 7 out of 10 poorly differentiated endocrine carcinomas (54). Finally, extensive losses of X chromosomal markers were present in four malignant tumors (two of which were poorly differentiated) but virtually absent in 29 benign neoplasms investigated (55).

## **MOLECULAR EVENTS IN TUMOURS OF MIDGUT**

Midgut NETs rarely display *MEN1* gene alterations. A recent study of 16 ileal, 6 appendicular and 3 rectal well differentiated NETs showed an overall LOH rate of 9% for informative microsatellites at the *MEN1* locus on 11q13 (12). This was recently confirmed in five further cases (50). *MEN1* gene mutation was found in only 1 of 12 midgut endocrine tumors studied (56-57). These data do not support a role for the *MEN1* gene in the development of midgut NETs.

Accumulating evidence suggests the role of genes located on chromosome 18 in the induction of well-differentiated midgut NETs. An imbalance of chromosome 18, especially the loss of 18q markers, is the most frequent abnormality detected by different techniques in these tumors and appears to be typical of midgut carcinoids (58- 61). A combined CGH/LOH study of 18 classical midgut EC cell tumors losses at 18q22-qter were seen in 67% of cases (60), whereas a genome-wide LOH screening of 8 tumors showed 18q21 losses to be very frequent (88% of cases) and highlighted specific alterations in these neoplasms (61). These alterations were telomeric to the loci of the genes *SMAD2*, *SMAD4* and *DCC*, largely involved in colorectal cancer.

The promoter methylation of different genes is reported in 6 out of 7 well-differentiated ileal tumors, indicating that this mechanism of gene inactivation plays a role in such neoplasms and suggesting that different genes are involved compared with pancreatic NETs (33). However, larger studies are necessary to confirm such observations.

Seventeen midgut NETs showed a low frequency of LOH for X chromosome markers in malignant tumors (15% of informative markers investigated) and no losses in benign tumors (55). A CGH study of 13 primary and 5 metastatic classical midgut carcinoids showed that losses at 16q21-qter and gains at 4p14-pter were rare or absent in primary tumors and frequent in most metastatic tissues investigated (61). As for tumors of foregut derivatives, *p53* mutations are only rarely seen in NETs of the small intestine (62).

## **MOLECULAR EVENTS IN TUMORS OF HINDGUT**

Only a few data are available for this area. Of 15 NETs of large intestine (7 in the appendix), 7 showed LOH for *MEN1* gene markers, whereas only 1 of 10 studied for mutation had one (52, 63). A mutation of *p53* was observed in 1 out of 9 'carcinoids' of the colorectum and in 0 of 6 cases in the appendix (44). Similar to conventional adenocarcinoma, poorly differentiated (small-cell) endocrine carcinomas of the large

intestine display frequent LOH for *p53*, *DCC* and the adenomatous polyposis coli (*APC*) tumour suppressor loci (64). In the same report, no abnormality of the *DCC* and *APC* gene chromosomal loci was observed in four well-differentiated tumors, 'carcinoids' (2 from foregut, 1 from the midgut and 1 from the hindgut). Such findings suggest that common genetic events lie at the basis of colon cancer and poorly differentiated endocrine carcinoma.

Interestingly, poorly differentiated endocrine carcinomas may be synchronous with conventional adenocarcinomas or develop within adenomas. An investigation of poorly differentiated carcinomas arising within in situ/early invasive adenocarcinomas suggested a potential clonal divergence with different oncogenic pathways (65). A similar conclusion was drawn by LOH analysis for multiple chromosomal markers in a rare case of small-cell carcinoma mixed with adenocarcinoma of the appendix (66). Indeed, the investigation of 9 small-cell carcinomas of the colorectum compared with 12 adenocarcinomas revealed higher chromosomal instability and different genetic abnormalities (54). All studies on colonic endocrine small-cell carcinomas reported the high frequency of *p53* gene abnormality with nuclear protein hyperexpression/accumulation.

## CONCLUSIONS

- 1) The molecular genetic mechanism of tumor development of GI and pancreatic NETs is complex and largely unknown.
- 2) Multiple genes appear to be involved with significant differences for tumors of different embryological derivatives.
- 3) The *MEN1* gene is involved in initiation of 33% of foregut NETs.
- 4) 18q defects are present almost exclusively in mid/hindgut NETs.
- 5) X-chromosome markers are associated with malignant behavior in foregut tumors only.

6) Poorly differentiated NE carcinomas of any site show high chromosomal instability and frequent *p53* alterations.

### **Future Studies**

- What classification to use when selecting tumors for analysis?
- Need molecular genetic analysis of LARGE tumor series of EACH SPECIFIC tumor type .

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