CLINICAL STUDY

Chromosome 22q in pancreatic endocrine tumors: identification of a homozygous deletion and potential prognostic associations of allelic deletions

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Abstract

Objective: A variety of human tumors frequently show allelic deletions of chromosome 22q, suggesting that inactivation of one or more tumor suppressor genes in this region is important for their tumorigenesis.

Methods: In this study, 23 patients with pancreatic endocrine tumors (PETs), including gastrinomas, VIPomas and non-functioning islet cell carcinomas, were analyzed for loss of heterozygosity (LOH) on chromosome 22q with 12 microsatellite and 7 sequence tagged site markers.

Results: LOH on chromosome 22q was identified in 22 of 23 (96%) PETs. Markers in the chromosomal region 22q12.1 revealed LOH rates up to 85%. Notably, one tumor revealed a homozygous deletion in a second region at 22q12.3. LOH at this locus occurred more frequently in tumors with distant metastases (10 of 11) compared with tumors without distant metastases (3 of 12; $P = 0.0057$) and, overall, allelic loss of 22q is positively correlated with distant metastases ($r = 0.78$; $P < 0.0001$).

Conclusions: These findings are suggestive for novel tumor suppressor gene loci at chromosome 22q that might contribute to the pathogenesis of PETs, especially to the development of distant metastases.

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Introduction

Pancreatic endocrine tumors (PETs) take their direction of differentiation from the pancreatic islet cells and are identified in as many as 1.5% of unselected autopsy cases (1). Functioning PETs, such as gastrinomas, insulinomas and vasoactive intestinal polypeptide producing tumors (VIPomas), are characterized by the extensive production of single polypeptide hormones that lead to specific clinical syndromes. While some PETs occur in well-described familial syndromes, such as multiple endocrine neoplasia type 1 (MEN1) and von Hippel-Lindau (VHL) syndromes, the vast proportion occur sporadically. Although the histological characteristics of these tumors are very similar, their malignant potential varies greatly. Non-functioning PETs, for example, are malignant in up to 92% of cases compared with 50–60% of gastrinomas and 10% of insulinomas (1, 2). Usually PETs have a more indolent behavior than do their highly malignant exocrine counterparts, but they can present with widely metastatic disease. In the absence of local invasiveness or distant metastases, there are no indisputable clinical or histopathological methods to classify PETs as malignant. Consequently, lack of cellular atypia, lymph node or blood vessel invasion are not evidence of a benign

phenotype (3). Thus, prognosis cannot reliably be determined when only a primary endocrine pancreatic lesion is present (1, 4). In certain tumor types, molecular genetic markers have demonstrated the ability to determine malignancy and prognosis in addition to conventional histology. One key example is chromosome 1p loss in neuroblastomas (5). Our understanding of the molecular pathogenesis of PETs, however, is still limited. Loss of heterozygosity (LOH) on chromosome arm 3p has been found in both familial and non-familial PETs with a suggested relation to malignancy (4, 6). Mutations of the *DPC4/Smad4* tumor suppressor gene might indicate PETs with a more aggressive phenotype reflected by an early occurrence of metastases (7). It has also been suggested that deletion of chromosome 1 (8) and 11q13 (4) predicts prognosis in PETs. We performed a fine deletion mapping of chromosome 22q in human PETs and identified potential novel tumor suppressor loci that seem to correlate directly with prognosis.

Materials and methods

Patients and tissue samples

Twenty-three fresh-frozen PETs, including twelve non-functioning neuroendocrine pancreatic carcinomas

(NNPC), eight gastrinomas, two VIPomas and one renin-producing PET, were obtained from the tumor bank of the Department of Surgery, Philipps-University of Marburg. The diagnosis of Zollinger–Ellison syndrome was established by clinical symptoms, an elevated fasting serum gastrin level (>125 pg/ml) in the presence of acid in the stomach, a positive secretin stimulation test defined as increase to a serum gastrin concentration of >200 pg/ml, and positive immunohistochemistry for gastrin in the tumor. The diagnosis of VIPoma was confirmed by secretory diarrhea (>6 liters/day) and a fasting vasoactive intestinal polypeptide VIP concentration >130 pg/ml. Characteristics of the renin-producing PET have been described previously (9). Tumors were considered as non-functioning if clinically no symptoms of hormonal excess were present and plasma hormone levels were within normal limits. Malignancy was determined based on the strict criteria of infiltrating growth, lymph node or distant metastases. Twenty-one tumors were malignant and two gastrinomas appeared to be benign (Table 1). Clinical follow-up was obtained through the patient's personal physician or at outpatient attendance. Survival was calculated from the time of surgical resection to either death or most recent contact. Informed consent was obtained from all patients. All investigations and all patients' material in this study were assessed under a research protocol approved by

the Philipps-University of Marburg Ethic Committee. The tumor samples used for DNA isolation had a neoplastic cellularity between 85% and 100% after cryostat microdissection, whereas constitutional normal DNA was derived from blood lymphocytes. Genomic DNA from fresh-frozen tissue and whole blood samples was isolated using the QIAamp DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

LOH analysis

Eleven highly polymorphic microsatellite markers D22S345, D22S1174, D22S351, D22S1167, D22S1144, D22S929, D22S280, D22S283, D22S423, D22S1140, D22S1169, and one expressed sequence tag (EST) marker A006E25 have been chosen for deletion mapping. Their location as shown in Fig. 1 and genomic sequences were confirmed by the published National Center for Biotechnology Information or Sanger Centre chromosome 22q sequences. PCR amplification was performed with fluorescence-labeled oligonucleotides on a MWG Primus 25 PCR cyclor (MWG Biotech, Ebersberg, Germany) with a standard protocol. PCR product analysis was performed on a 310 Genetic Analyzer (ABI Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. LOH was defined as a reduction in intensity of 50% or

Table 1 Clinical and tumor characteristics of patients with PETs analyzed by 22q allelotyping.

Patient	Age at surgery (years)	Tumor type	Signs of malignancy†	Primary tumor size (mm)	Follow-up (months)	LOH/informative markers on 22q	LOH on 22q12.3‡
20/96	50	Vipoma	Liver metastases	20 × 20	71; AWD	6/9	No
53/96	61	Non-functioning	IG	35 × 35	66; NED	1/11	No
74/96	56	Gastrinoma	Liver metastases	25 × 25	2; DOD	10/11	Yes
91a	64	Non-functioning	LN metastases	35 × 35	54; NED	5/9	No
106a	45	Non-functioning	LN metastases	70 × 80 × 50	73; AWD	1/11	No
108a	62	Non-functioning	Liver metastases	30 × 16	21; DOD	7/9	Yes
122a	44	Reninoma	Liver metastases	100 × 50 × 30	12; DOD	9/10	Yes
141/98	28	Gastrinoma	LN metastases	20 × 20	59; NED	1/11	No
155/98	63	Non-functioning	LN metastases	100 × 100	42; NED	8/12	Yes
160a	64	Non-functioning	LN metastases	100 × 65 × 60	29; NED	6/11	Yes
162/98	49	Non-functioning	Liver metastases	200 × 200	106; AWD	7/11	Yes
164/98	58	Gastrinoma	LN metastases	5 × 6	97; NED	7/12	No
166/98	43	Non-functioning	Liver metastases	26 × 26	192; AWD	10/11	Yes
167/98	50	Gastrinoma	None	20 × 20	127; NED	7/12	No
171/98	53	Non-functioning	LN metastases	40 × 40 × 25	84; NED	6/11	Yes
172/98	50	Non-functioning	Liver metastases	31 × 31	76; DOD	7/10	Yes
177/98	57	Gastrinoma	None	9 × 30 × 5	99; NED	0/10	No
179/98	53	Non-functioning	Liver metastases	40 × 40	138; AWD	11/11	Yes
183/98	43	Gastrinoma	Liver metastases	8 × 8	48; AWD	8/9	Yes
F2II18*	40	Gastrinoma	LN metastases	7 × 7	127; NED	1/9	No
F5II1*	31	Vipoma	Liver metastases	350 × 350	155; AWD	6/11	Yes
F7III4*	34	Non-functioning	Lung metastases	20 × 15	114; NED	10/11	Yes
F8IV23*	32	Gastrinoma	LN metastases	20 × 20	80; AWD	4/10	No

LN, lymph node; IG, infiltrating overgrowth; NED, no evidence of disease, partially after up to three re-operations; AWD, alive with disease; DOD, dead of disease.

* Familial, MEN1-associated.

† At diagnosis or during follow-up.

‡ Determined by markers D22S280 and D22S283.

Figure 1 Deletion map of chromosome 22q in PETs. Homozygous deletion (shaded star); retention of heterozygosity (open squares); LOH (shaded squares); tumors with distant metastases (solid circles). NI, not informative; cen, centomere; tel, telomere. LOH rates are given in percentage of informative markers. The boxed area indicates the markers D22S280 and D22S283 on 22q12.3 where LOH is significantly correlated with the presence of distant metastases ($r = 0.78$; $P < 0.0001$).

more in either of the two alleles as compared with those in constitutional normal DNA, considering the calculated difference between the relative intensity in tumor and corresponding alleles by semiquantitative analysis on a 310 Genetic Analyzer (Fig. 2). Data were analyzed using Genescan version 2.1.1 software (ABI Applied Biosystems).

Qualitative fine deletion mapping

Since one NNPC (166/98) showed a reproducible homozygous deletion on chromosomal band 22q12.3 corresponding to marker D22S280, we undertook a finer deletion mapping with seven sequence tagged site markers from contiguous clones Z75744 (G05050), Z72521 (G03684), Z72520 (Z72520-STS1) and Z73495 (G60706) centromeric of D22S280, and telomeric contig clones Z73979 (G49417), Z98256 (G49339) and AL031592 (G60943). Deletion mapping was performed in all tumors that showed LOH at this locus and all tumors without LOH after standard PCR protocols. PCR products were visualized by ethidium-bromide staining

after electrophoresis on 6% polyacrylamide mini gels. Homozygous deletion was determined as absence of a marker specific signal, verified by primer site mutation analysis (Fig. 3).

Statistics

Descriptive statistics are expressed as the mean and s.d. value system. The χ^2 -test with Yates' correction was performed for nominal data. Because of unknown distribution, non-parametric correlation between distant metastases and rate of LOH was analyzed using the Spearman rank correlation. Survival analysis was performed using the Kaplan–Meier method with the log rank test. P values < 0.05 were considered as statistically significant. Data were analyzed using SPSS software (version 10).

Results

We determined the frequency of LOH in 23 primary PETs for LOH at 12 loci on chromosome 22q. Tumor

Figure 2 Examples of LOH on chromosome (A) 22q12.1 in NNPC 53/96 with marker D22S1167 and (B) on 22q12.3 in NNPC 171/98 with marker D22S280. Arrows indicate allelic loss. T, tumor DNA; N, 'normal' constitutional DNA; numbers indicate internal DNA length standard given in bp.

Figure 3 Qualitative fine deletion mapping with markers D22S280, G49417, G49339 and G60943. The tumor DNA of NNPC 166/98 revealed a homozygous deletion in markers D22S280, G49417 and G49339 as shown by the absence of a marker specific signal. T, tumor DNA; N, corresponding 'normal' constitutional DNA; WT, control DNA from wild-type peripheral blood lymphocytes; H₂O indicates negative control; DNA length standard, 50 bp ladder. Distances between markers are given in kb.

phenotypes included twelve NNPCs, eight gastrinomas, two VIPomas and one renin-producing PET. Nineteen cases were sporadic and four patients had MEN1 (Table 1). There were 12 males and 11 females with a mean age of 49 (range 28–64) years at the time of surgery. The tumor size ranged from 5 mm to 350 mm in diameter (Table 1). All patients were explored, staged and underwent resection of the tumor and/or detectable metastases. At the time of surgery, six patients had localized disease, defined by the absence of any extra-pancreatic spread of the tumor. Nine patients had tumors with lymph node metastases. Three patients presented with synchronous liver metastases and one patient with a synchronous single lung metastasis. During mean follow-up of 81 (range 2–192) months seven patients developed liver metastases. At the conclusion of the study, eleven patients had no evidence of disease, eight patients were alive with disease and four patients were deceased due to diffuse liver metastases.

The 19 markers used in this study are localized on chromosomal bands 22q11.22 to 22q13.32 and are distributed over a genomic region of about 25 Mb according to the published sequence data of chromosome 22q (10). LOH was identified in 22 of 23 (96%) PETs (Fig. 1). All 11 tumors with distant metastases revealed LOH in more than 60% of the markers (Fig. 1). Highest LOH rates were detected on 22q12.1 at markers D22S1167 and D22S1144 with 85% (17/20) and 76% (16/21) of informative tumors respectively (Fig. 2). In our material, the deletions were large in all but three cases, contributing to different markers on chromosomal bands 22q11.23 and 22q12.1 (Fig. 2). Therefore a smallest common region of overlap for LOH is hard to define. Notably, NNPC 166/98 showed a homozygous deletion on chromosomal band 22q12.3 corresponding to marker D22S280 that revealed an LOH rate of 59% (Figs. 1 and 3). After exclusion of a primer site mutation, a fine deletion mapping with seven STS markers spanning approximately 300 kb around D22S280 was performed in all tumors at this locus. None of the other tumors revealed any homozygous deletion, whereas the homozygous deletion of tumor 166/98 spanned about 70 kb centered between markers D22S280 and STS marker G49339 (Fig. 3).

We further analyzed whether chromosome 22q allelic loss correlates with clinical features of PETs. Remarkably, there was a strong positive correlation between the presence or development of distant metastases (liver, lung) and the rate of LOH ($r = 0.78$; 95% CI: 0.54–0.91; $P < 0.0001$), whereas no association between 22q loss and patient sex, age, tumor type or tumor size was identified. It was also of note that ten of eleven informative tumors with distant metastases showed LOH on chromosome band 22q12.3 compared with only three of twelve tumors without distant metastases ($P = 0.0057$; Table 1). After a mean follow-up of

81 months, four of thirteen patients with tumors exhibiting LOH at 22q12.3 died due to diffuse liver metastases compared with none of the patients without LOH at this locus. Survival analysis revealed strong positive correlation (log rank test $P = 0.032$; $df = 1$) between the presence of distant metastases and premature death, whereas a correlation between the presence of LOH at 22q12.3 and premature death was not quite achieved (log rank test $P = 0.073$; $df = 1$).

Discussion

LOH studies are a powerful tool to assess the status of particular gene loci in the development and progression of human neoplasms (11). The goal of this study was to evaluate whether chromosome 22q contains a tumor suppressor gene region associated with the development or progression of PETs. Utilizing a large number of microsatellite markers we could show, for the first time, that 22 of 23 (96%) reveal LOH on chromosome 22q with marker D22S1167 showing an LOH rate of 85% (17 of 20 informative tumors) at locus 22q12.1. This is in contrast to three previous reports that observed LOH at the long arm of chromosome 22 in only 0–38% of PETs (12–15). There are several possible explanations for this discrepancy of overall LOH rates. First, three of the studies (12–14) used at most three markers that were in the main different from ours, whereas we used 12 markers enhancing the chance for detecting LOH. The study of Rigaud *et al.* (15) analysed seven markers on 22q in 16 non-functioning PETs, including markers D22S280, D22S283 and D22S423, and found LOH rates of 35% 42% and 35% respectively. These LOH rates are statistically not different from the LOH rates of 59%, 48% and 36% detected in the present study, given the small number of PETs analyzed and the relatively high number of non-informative cases in the study of Rigaud *et al.* (15). Second, the differences may relate to different tumor populations and to different amounts of contaminating normal DNA in the tumor samples. Third, intermediate levels of allele retention could theoretically also be due to a variable degree of intratumoral gene amplification of one chromosomal copy. Fourth, intratumoral genetic heterogeneity is a known feature of PETs (14, 16), that can result in complete loss of chromosomal material or varying levels of allelic retention.

The present examination of a large number of microsatellite markers encompasses more than 25 Mb and about 82% of the coding regions of chromosome 22q. PETs with distant metastases revealed loss of larger portions of 22q, whereas PETs without distant metastases often showed smaller regional deletions. In previous comparative genomic hybridization (CGH) and karyotyping studies of PETs, only very few genomic losses on chromosome 22q, comprising either the entire

chromosomal arm or large portions of 22q, were identified (17–21). This discrepancy can be explained by the limited resolution (10–20 Mb) of the CGH and karyotyping methods so that smaller deletions (<10 Mb) usually cannot be detected (22). However, in concordance with these CGH studies, our data show that PETs from patients with advanced metastatic disease had higher numbers of chromosomal alterations than tumors from patients with localized tumors. These data underscore the hypothesis that the progression of PETs towards an aggressive malignant phenotype goes along with an accumulation of genetic changes, as has been suggested by previous studies (21, 22). Furthermore, it can be speculated that genes guarding chromosomal stability by their involvement in mitotic replication and segregation of chromosomes might be important players in PET progression.

Taking advantage of information available on the precise position and size of polymorphic markers in this chromosomal area (10), it is possible to estimate the locus of interest as approximately 2.5 Mb (4.3 cM). The deletions in our material were large, in all but three cases extending centromerically to the tumor suppressor gene *hSNF5/INI1* on 22q11.23 and telomerically to the tumor suppressor gene *neurofibromatosis type 2 gene (NF2)* on 22q12.2 in 44% and 75% of positive cases respectively. The tumor suppressor gene *hSNF5/INI1* encodes a widely expressed component of the chromatin-remodeling SWI/SNF multiprotein complex involved in the regulation of gene expression in the cell nucleus (23). Numerous loss-of-function mutations and homozygous deletions have been identified in malignant rhabdoid tumors as well as in medulloblastomas (24). Although *hSNF5/INI1* appears to be an attractive candidate gene, no alterations could recently be identified in insulinomas (25). Somatic mutations in the tumor suppressor gene *NF2*, localized at 22q12.2, have been identified in neurofibromatosis type 2-related tumors, such as sporadic meningiomas and vestibular schwannomas (26). It is suggested that *NF2* may act as a membrane-associated molecular switch that regulates cell–cell and cell–matrix signals transduced by cell surface receptors. Since 75% of the analyzed PETs revealed LOH at the *NF2* locus, this gene warrants future evaluation to determine its role in the development of PETs.

We identified a second region of interest at chromosomal band 22q12.3, since one NNPC (166/98) revealed a homozygous deletion of marker D22S280 in this region that was characterized by an LOH rate of 59%. Fine deletion mapping of tumors exhibiting LOH or without LOH at this locus revealed no additional tumors with homozygous deletions. However, tumor 166/98 showed homozygous deletion of two adjacent markers that allows estimation of the locus of interest as approximately 70 kb. According to the map of chromosome 22q there are two candidate

genes localized in this area, namely *synapsin3 (SYN3)* and *tissue inhibitor of metalloproteinase-3 (TIMP-3)*. *SYN3* belongs to a family of neuron-specific synaptic vesicle-associated phosphoproteins that have been implicated in synaptogenesis and in the modulation of neurotransmitter release, but no association with tumor development has yet been identified (27). The second gene, *TIMP-3*, has been localized within an intron of the *SYN3* gene. *TIMP-3* is a member of the family of secreted inhibitors that blocks the activity of metalloproteinases, therefore regulating matrix composition, cell growth, invasion and migration (28). It has been shown that *TIMP-3* is implicated in tumor progression, including the development of metastases (29). Furthermore, it has also been suggested that *TIMP-3* might function as a tumor suppressor, since methylation-associated silencing and loss of expression was identified in kidney, brain and some lung tumors (30). Thus, *TIMP-3* appears to be an attractive candidate for genetic analysis in PETs to clarify its role in the tumorigenesis of these tumors.

Although the histological characteristics of PETs are very similar, their malignant potential varies greatly. Currently, there is no histopathologic feature other than the presence of gross metastases or infiltrating overgrowth that can reliably distinguish benign from malignant PETs. High expression of chromogranin A, high Ki-67 index or abnormal DNA content have been suggested to indicate malignant tumors (4, 14), but *per se* they are no evidence of a malignant phenotype (3). Previously, LOH on chromosomes 1, 3p and 11q13 have been also proposed as potential predictors of malignancy and prognosis in PETs (4, 6, 8). However, these small-scale studies await confirmation in a larger population of patients with PETs. In our present study, allelic loss on chromosome 22q seems to be strongly correlated with the occurrence of distant metastasis ($r = 0.78$; $P < 0.0001$), especially LOH at chromosomal band 22q12.3 ($P = 0.0057$). As expected, there was a strong positive correlation in survival analysis between presence of distant metastases and premature death (log rank test $P = 0.032$; $df = 1$). The correlation between the presence of LOH on 22q12.3 and premature death did not quite reach significance (log rank test $P = 0.073$). This may be due to the limited number of cases and the fact that survival estimates were censored for the group of tumors without LOH at 22q12.3, since none of the patients died in this group during follow-up. However, the data presented indicate a prognostic distinction between tumors with lymph node involvement alone and those with distant metastases. Our findings correspond well with available clinical studies of gastrinomas showing that sole lymph node involvement has a relatively good prognosis (31). Based on our data, one might hypothesize that loss of a putative tumor suppressor gene on chromosome 22q12.3 is more likely associated with suppression of the metastatic processes

rather than with regulation of other aspects of cellular growth or differentiation.

In summary, the high frequency of LOH on chromosome 22q12.1 and 22q12.3 indicates two loci compatible with harboring tumor suppressor genes that may be associated with the tumorigenesis of PETs. Large-scale controlled studies are warranted to determine whether allelic loss of 22q12.3 may be a useful prognostic marker for PETs.

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