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Frequent Methylation-Associated Silencing of the *Tissue Inhibitor of Metalloproteinase-3* Gene in Pancreatic Endocrine Tumors

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Molecular mechanisms contributing to the tumorigenesis of pancreatic endocrine tumors (PETs) are still not well understood. Allelic deletions at chromosome 22q12.3 were detected in about 30–60% of PETs, suggesting that inactivation of one or more tumor suppressor genes on this chromosomal arm is important for their pathogenesis. Because the putative tumor suppressor gene *tissue inhibitor of metalloproteinase-3* (*TIMP-3*) has been located at 22q12.3, we undertook a genetic analysis of *TIMP-3* to determine its role in the tumorigenesis of PETs. Single-strand conformational polymorphism analysis, methylation-specific PCR, RNA expression analysis, and immunohistochemistry of *TIMP-3* were performed in 21 sporadic PETs. Thirteen of 21 PETs (62%) revealed *TIMP-3* alterations, including promoter hypermethylation and homozygous deletion. The predominant *TIMP-3* alteration was promoter hypermethylation, identified in 8 of 18 (44%) PETs. It was tumor-specific and corresponded to loss or strong reduction of TIMP-3 protein expression. Notably, 11 of 14 (79%) PETs with metastases had *TIMP-3* alterations, compared with only 1 of 7 (14%) PETs without metastases ($P < 0.02$). These data suggest a possibly important role of *TIMP-3* in the tumorigenesis of human PETs, especially in the development of metastases, which has to be further evaluated in large-scale studies. (*J Clin Endocrinol Metab* 88: 1367–1373, 2003)

PANCREATIC ENDOCRINE TUMORS (PETs) represent a distinct group of rare tumors that are frequently associated with unique clinical syndromes that result from an excess of endogenously produced hormones, such as insulin or gastrin (1). Due to the rare occurrence of PETs, our knowledge of genetic changes associated with the initiation and progression of these neoplasms is still limited, although significant knowledge has been gained in the last 5 yr. Important genetic findings identified in human PETs are summarized in Table 1 (2–20). In particular, we and others detected significant loss of heterozygosity (LOH) at chromosome 22q12.3 in about 30% of insulinomas (19) and 35–59% of gastrinomas and nonfunctioning neuroendocrine carcinomas (17, 18, 20). These data indicated the possible presence of one or more tumor suppressor genes at this chromosomal region that might be involved in the tumorigenesis of PETs. One putative tumor suppressor gene at chromosome 22q12.3 is the *tissue inhibitor of metalloproteinase-3* (*TIMP-3*) gene. TIMPs are a family of secreted molecules that inhibit the proteolytic activity of the matrix metalloproteinases (MMPs; Ref. 21). Tumors frequently show an increase in MMP expression and/or decrease of TIMP expression leading to an imbalance in proteolytic activity during tumor progression. TIMP-3 is a secreted 24-kDa protein that, unlike other TIMP family members, binds to the extracellular matrix. TIMP-3 antagonizes the activity of MMPs by binding covalently to the active site of the enzymes (22). It is thought that reduced expression of TIMP-3 contributes to primary tumor growth, angiogenesis, apoptosis, tumor invasion, and metastasis by allowing increased activity of MMPs in the extracellular matrix (23–25). Recent studies on methylation-associated silencing of *TIMP-3* suggested a tumor suppressor role in kidney, brain, breast, and colon cancers (26). In the present study, the involvement of *TIMP-3* in the tumorigenesis of 21 human PETs was evaluated. We demonstrate for the first time frequent alterations of *TIMP-3* in these neoplasms, primarily targeted by tumor-specific methylation-associated silencing leading to lack of protein expression.

Materials and Methods

Patients and tissue samples

Twenty-one fresh-frozen sporadic PETs were obtained from the tumor bank of the Department of Surgery, Philipps-University Marburg, including nine nonfunctioning neuroendocrine pancreatic carcinomas (NNPCs), five insulinomas, five gastrinomas, one vipoma, and one renin-producing PET (27). The diagnosis of insulinoma required a symptomatic hypoglycemia (<40 mg/dl) with concomitant endogenous hyperinsulinemia (>0.12 nmol/liter) during a supervised fast and a positive immunohistochemistry of the tumor for insulin. 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 more than 200 pg/ml, and a positive immunohistochemistry for gastrin in the tumor. The diagnosis of vipoma was confirmed by secretory diarrhea (>6 liter/d) and a fasting vasoactive intestinal polypeptide concentration greater than 130 pg/ml. Tumors were considered as nonfunctioning if clinically no symptoms of hormonal excess were present and plasma hormone levels were within normal limits. Malignancy was determined on the basis of the strict criteria of infiltrating overgrowth, lymph node, or distant metastases. Clinical follow-up was obtained through the patient's personal physi-

Abbreviations: LOH, Loss of heterozygosity; MMP, matrix metalloproteinases; MSP, methylation-specific PCR; NNPC, nonfunctioning neuroendocrine pancreatic carcinoma; PET, pancreatic endocrine tumor; SSCP, single-strand conformational polymorphism; *TIMP-3*, *tissue inhibitor of metalloproteinase-3*.

TABLE 1. Summary of genetic alterations in sporadic PETs

Gene/region	Genetic alteration	Type of PETs	PETs with alteration (%)	Ref. no.
MEN1	Somatic mutation	Ins, Gas, NPT	30	2, 3
K-ras	Somatic mutation	Ins, Gas, NPT, VIP	Rarely	4–6
p53	Somatic mutation	Ins, Gas, NPT, VIP	Rarely	6, 7
p^{16INK4}*(CDKN2a)*	Hypermethylation, homozygous deletion	Gas, NPT, Ins	52–90	8, 9
		Ins	17	10
DPC4 (*Smad4*)	Somatic mutation, homozygous deletion	NFT, Ins, Gas, VIP	0–55	6, 11
PTEN	Somatic mutation	NPT, Ins, Gas	3	12
Chromosome 1	LOH	Ins, Gas, NPT	34	13
Chromosome 3p	LOH	Ins, Gas, NPT	33–62	14–16
Chromosome 6q	LOH	Ins, Gas, NPT	60	17
Chromosome 10q	LOH	Malignant PET	53	12
		Benign PET	0	
Chromosome 11q	LOH	Ins, Gas, NPT	55–63	16–18
Chromosome 16p	LOH	Ins, Gas, NPT	36	18
Chromosome 22q	LOH	Ins, Gas, NPT	30–59	17–20

Ref., Reference; Ins, insulinoma; Gas, gastrinoma; NPT, nonfunctioning endocrine pancreatic tumor; VIP, vipoma.

cian 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 patient material in this study were assessed under a research protocol approved by the Philipps-University of Marburg Ethic Committee. Tumor samples used for DNA and RNA isolation had a neoplastic cellularity between 85% and 100% after cryostat microdissection, whereas constitutional normal DNA was derived from peripheral blood lymphocytes or normal tissue specimens if available. 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, whereas total RNA was extracted with the TRIzol RNA kit as advised by the manufacturer (Life Technologies, Inc., Karlsruhe, Germany).

Single-strand conformational polymorphism (SSCP) and DNA sequence analysis of TIMP-3

The oligonucleotides for SSCP analysis of exons 1 to 5 of the *TIMP-3* gene have been chosen from the published National Center for Biotechnology Information sequence (accession no. AH003680) as follows—Ex1For: 5′-gcacggcaactttggaga-3′; Ex1Rev: 5′-ggcaccaggagcgcttac-3′; Ex2For: 5′-ctgatgtggttcaggccttc-3′; Ex2Rev: 5′-cactttgcctcccttagcag-3′; Ex3For: 5′-acccagcagtggattaggac-3′; Ex3Rev: 5′-caaaccctggcctagaagc-3′; Ex4For: 5′-gctgcaggaattctctctgg-3′; Ex4Rev: 5′-ccagtgaccccagagcttac-3′; Ex5For: 5′-gagtccatcaactgctgcctg-3′; and Ex5Rev: 5′-gaagggagggaagtgaggtg-3′. PCR amplification and SSCP analysis were performed as described previously (19).

Methylation-specific PCR (MSP) assay

Genomic DNA was extracted as mentioned above, and 1 μg of DNA was bisulfite-modified as described by Herman *et al.* (28). The sequence difference of bisulfite-treated DNA was detected by PCR using primers specific for either the methylated or unmethylated DNA. Oligonucleotide sequences and PCR conditions were chosen according to Ueki *et al.* (29). MSP primer sequences that specifically recognized unmethylated *TIMP-3* sequence were 5′-TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT-3′ (upstream) and 5′-CCCCCCAAAAACCCCACCTCA-3′ (downstream); and the methylated *TIMP-3* sequence 5′-CGTTTCGTTATTTTTTGTTTTCGGTTTTC-3′ (upstream) and 5′-CCGAAAACCCCGCCTCG-3′ (downstream). MSP was performed on 2 μl bisulfite-treated DNA under the following conditions: 96 C for 3 min; 40 cycles of 96 C for 30 sec, 59 C for 30 sec, and 72 C for 30 sec; and a final extension of 3 min at 72 C. DNA from peripheral blood lymphocytes treated with *Sss* I methylase (New England Biolabs, Inc., Beverly, MA) was used as a positive control for methylated alleles. Ten microliters of each PCR product were loaded onto 6% polyacrylamide mini gels, ethidium bromide stained, and visualized under UV transillumination.

Immunohistochemistry

Immunohistochemical stainings for TIMP-3 were performed by a standard avidin-biotinylated immunohistochemistry technique using a commercial rabbit antihuman TIMP-3 polyclonal antibody (Chemicon International, Inc., Temecula, CA) and following a standard protocol (26). Each experiment contained normal pancreas and normal kidney as positive controls. Staining results of tumor sections were interpreted by an experienced pathologist (A.R.) without knowledge of clinical and molecular data. The TIMP-3 results were scored as follows: −, negative; +, less than 10% of cells positive; ++, 10–50% of tumor cells positive; +++, more than 50% of tumor cells positive.

RT-PCR of TIMP-3

Enough material for total RNA preparation was available for only 12 PETs (4 insulinomas, 4 gastrinomas, 3 NNPCs, and 1 renin-producing PET). RT-PCR analysis was performed with the One-step RT-PCR kit as described by the manufacturer's protocol (Life Technologies, Inc.) with some modifications (29). The oligonucleotides for *TIMP-3* RT-PCR, coding for a 648-bp amplicon, were chosen as follows: 5′-GGAATTCATGACCCCTTGGCTCGGG-3′ (upstream) and 5′-ggaattcagggtctggcgctcaggg-3′ (downstream). Integrity of RNA was verified by β-actin amplification with the following oligonucleotides: (upstream) 5′-GATGATGATATCGCCGCGCTCGTCGTC-3′ and (downstream) 5′-GTGCCTCAGGGCAGCGGAACCGCTCA-3′ (778-bp fragment; Ref. 30). The products were visualized on 6% polyacrylamide mini gels, followed by ethidium bromide staining.

LOH analysis on 22q12.3

The LOH data on chromosome 22q12.3 has been published for most of the tumors previously (20). In brief, microsatellite markers D22S280 and D22S283 have been chosen for determination of LOH at chromosome 22q12.3. PCR amplification was performed with fluorescence-labeled oligonucleotides on a MWG Primus 25 PCR cycler (MWG Biotech, Ebersberg, Germany) with a standard protocol. Semiquantitative PCR product analysis was performed on a 310 Genetic Analyzer (PE Applied Biosystems, Foster City CA) as described by the manufacturer. Only microsatellites showing two distinct alleles in normal DNA were considered as informative. LOH was defined as a reduction in intensity of one allele in the tumor sample with respect to the matched allele from corresponding normal tissue. LOH was scored when the relative intensities of the tumor alleles compared with the corresponding normal tissue alleles differed by at least a factor of two, symbolizing a reduction of at least 50% or more in either of the two tumor alleles. Data were analyzed using Genescan version 2.1.1 software (PE Applied Biosystems).

Statistics

Descriptive statistics were expressed as mean and SD value system. The Fisher's exact test was performed for nominal data. *P* values less than 0.05 were considered as statistically significant. Data were analyzed using SPSS software (version 10; SPSS, Inc., Chicago, IL).

Results

We screened the putative tumor suppressor gene *TIMP-3* for alterations in 21 sporadic PETs, including 9 NNPCs, 5 insulinomas, 5 gastrinomas, 1 vipoma, and 1 renin-producing PET (27). Thirteen males and 8 females with a median age of 52 (range, 28–66) yr at the time of surgery were included in this study. The tumor size ranged from 5 mm to 350 mm in diameter. Sixteen tumors were malignant, whereas three insulinomas and two gastrinomas appeared to be benign. At the time of surgery, 10 patients had localized disease, defined by the absence of metastases. Eight patients had tumors with lymph node metastases, and three patients presented with synchronous liver metastasis. During median follow-up of 69 (range, 2–339) months, five patients developed liver metastases. At conclusion of the study, 12 patients had no evidence of disease, 4 patients were alive with disease, and 5 patients were deceased due to diffuse liver metastases.

The *TIMP-3* gene was screened for mutations by SSCP analysis in all 21 PETs. Exons 1 to 5 were successfully amplified in all tumors but NNPC 166/98. This was suggestive for a homozygous deletion of *TIMP-3* in this sample, because the DNA integrity was confirmed in several control experiments, including the successful amplification of *K-ras*, *p53*, and $p16^{INK4}$ genes (data not shown). SSCP analysis revealed no variant bands in any of the 21 PETs. Thus, no sequence alterations in the coding regions of *TIMP-3* have been identified (Table 2).

To elucidate the mechanism of gene silencing by promoter hypermethylation of *TIMP-3*, we performed MSP assays in 19 PETs, whereas for 2 tumors not enough DNA was available. The 5′CpG island of *TIMP-3* was aberrantly methylated in 8 of 18 (44%) tumors (Fig. 1 and Table 2). Six tumors revealed a methylated signal in addition to the signal of the normal unmethylated allele. Two tumors (167/98, 155/98) showed only a methylated signal compared with their unmethylated normal tissues. This implies biallelic methylation in tumor 167/98, revealing no LOH on 22q13.2, and methylation of one allele and deletion of the other methylated or unmethylated allele in tumor 155/98, showing LOH on 22q12.3. *TIMP-3* promoter hypermethylation was tumor specific, because this epigenetic change was restricted to the tumors in all but one matched normal/tumor pair. Only vipoma 20/96 revealed a weak methylated product besides a strong unmethylated MSP product in its corresponding normal pancreatic tissue. Remarkably, none of the five insulinomas was hypermethylated. The sample NNPC 166/98 yielded no PCR products in either the unmethylated or methylated-specific PCR, in accordance with its homozygous deletion of the *TIMP-3* locus.

Expression of TIMP-3 was examined by immunohistochemical staining of normal and tumor tissues in 18 PETs. Immunohistochemistry for TIMP-3 in primary pancreatic tissue revealed strong expression of the protein in normal islet cells. In contrast, 10 of 18 (55%) PETs showed loss or

TABLE 2. Clinical characteristics and results of TIMP-3 analysis in 21 patients with PETs

Patient, tumor type	Signs of malignancy[a]	Follow-up (months)	LOH on 22q12.3[b]	Mutation analysis[c]	Hypermethylation	Expression by IHC[d]	Expression by RT-PCR	TIMP-3 alteration
26/96, Ins	None	75; NED	No	wt	No	na	No	Yes
90, Ins	None	58; NED	Yes	wt	No	+++	Yes	No
158/98, Ins	None	44; NED	No	wt	No	+++	na	No
163/98, Ins	IG	43; NED	Yes	wt	No	+++	Yes	No
SW15122, Ins	LN, L	339; DOD	NI	wt	No	+	No	Yes
74/96, Gas	L	2; DOD	Yes	wt	na	na	No	Yes
141/98, Gas	LN	62; NED	No	wt	Yes	−	Yes	Yes
164/98, Gas	LN	100; NED	No	wt	No	na	No	Yes
167/98, Gas	None	130; NED	No	wt	Yes	−	No	Yes
177/98, Gas	None	102; NED	No	wt	No	+++	na	No
53/96, NNPC	IG	69; NED	No	wt	No	++	na	No
91a, NNPC	LN	57; NED	No	wt	No	+++	na	No
106a, NNPC	LN	76; AWD	No	wt	Yes	+	na	Yes
108a, NNPC	L	21; DOD	Yes	wt	No	++	na	No
155/98, NNPC	LN	45; NED	Yes	wt	Yes	−	No	Yes
160a, NNPC	LN	32; AWD	Yes	wt	Yes	+	na	Yes
166/98, NNPC	L	195; AWD	HD	HD	HD	−	No	Yes
171/98, NNPC	LN	87; NED	Yes	wt	Yes	−	na	Yes
172/98, NNPC	L	76; DOD	Yes	wt	na	++	Yes	No
20/96, VIP	L	74; AWD	No	wt	Yes	+	na	Yes
122, Ren	L	12; DOD	Yes	wt	Yes	−	No	Yes
Total alterations			10/20 (50%)	0/24 (0%)	8/18 (44%)	10/18 (55%)[e]	8/12 (67%)	13/21 (62%)

AWD, Alive with disease; DOD, dead of disease; Gas, gastrinoma; HD, homozygous deletion; IG, infiltrating growth; IHC, immunohistochemistry; Ins, insulinoma; L, liver metastases; LN, lymph node metastases; na, not available; NED, no evidence of disease, partially after up to three reoperations; NI, not informative; Ren, reninoma; VIP, vipoma; wt, wild type.

[a] At diagnosis or during follow-up.

[b] LOH as determined by markers D22S280 and D22S283 (partially published in Refs. 19 and 20).

[c] By SSCP analysis.

[d] Staining was graded as follows: −, negative; +, less than 10% positive cells; ++, 10–50% positive cells; +++, more than 50% positive cells.

[e] Loss of TIMP-3 expression or strongly reduced expression (<10% positive cells).

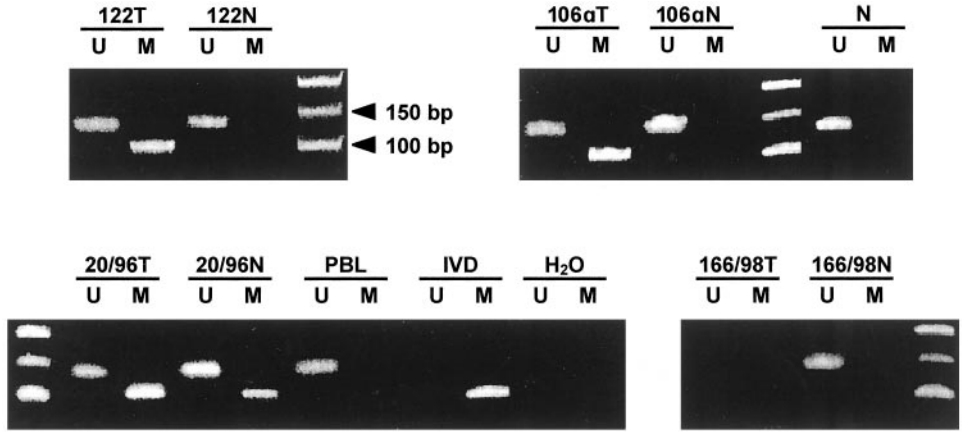

FIG. 1. Representative example of *TIMP-3* MSP assay. Methylation analysis of *TIMP-3* promoter region CpG island in human PETs (T) and corresponding normal tissue (N). Lane U, Amplified product with oligonucleotides recognizing unmethylated *TIMP-3* sequences. Lane M, Amplified product with oligonucleotides recognizing methylated *TIMP-3* sequences. Normal tissue (N) and peripheral blood lymphocytes (PBL) as negative controls are completely unmethylated, whereas the *in vitro* methylated DNA (IVD) was used as a positive control for methylation. H_2O, Water blanks. The used DNA size standard is a 50-bp ladder.

strongly decreased expression (<10% positive cells) of TIMP-3 compared with positive controls (Fig. 2 and Table 2). To determine whether inactivation of the *TIMP-3* gene in PETs is due to transcriptional silencing, we analyzed *TIMP-3* mRNA transcripts in 12 PETs by RT-PCR where adequate RNA material was available. Loss of expression of TIMP-3 was observed in eight tumor specimens (Fig. 3 and Table 2), whereas cDNA integrity was verified in all 12 samples by β-actin expression. Results of immunohistochemistry and RT-PCR were concordant in all but one tumor. Gastrinoma 141/98T was immunonegative for TIMP-3, but revealed a weak *TIMP-3* mRNA transcript (Fig. 3). Given the higher sensitivity of RT-PCR compared with immunostaining, this result is probably due to admixed normal cells, because the tumor 141/98T had only a neoplastic cellularity of 85% after microdissection. Thus, 10 of 16 (63%) malignant PETs and 2 of 5 benign tumors revealed loss or strongly reduced expression of TIMP-3.

In total, 13 of 21 (62%) PETs revealed *TIMP-3* alterations. Loss of expression was due to promoter hypermethylation in eight tumors and homozygous deletion of *TIMP-3* in one tumor, respectively. In three PETs (26/96, SW 15122, 164/98) associated with loss of TIMP-3 expression, mutation, deletion, and promoter hypermethylation could not be identified. In gastrinoma 74/96, the MSP assay could not be performed due to lack of DNA, precluding the evaluation of this mode of *TIMP-3* inactivation in this sample. LOH on chromosome 22q12.3 was identified in 10 of 20 (50%) informative PETs. Six of 10 tumors with allelic loss revealed *TIMP-3* alterations, as did 6 of 10 tumors without allelic loss of 22q12.3. *TIMP-3* promoter methylation leading to loss of expression was detected in four tumors with and four tumors without LOH on 22q12.3. Table 2 summarizes all *TIMP-3* alterations identified.

Analysis of the clinical-genetic data revealed that PETs with lymph node and/or liver metastasis had a significantly higher rate of *TIMP-3* alterations (11/14, 79%) than PETs without metastases (1/7, 14%; $P < 0.02$).

Discussion

As shown previously, PETs reveal allelic losses of 31% to 59% at chromosomal band 22q12.3 (17–20). *TIMP-3*, colocalized with these markers, therefore appears to be an attractive candidate gene for genetic analysis in PETs to clarify its role for the tumorigenesis of these neoplasms. *TIMP-3* is a member of the family of secreted inhibitors that blocks the activity of MMPs, therefore regulating matrix composition, cell growth, invasion, and migration, and has been shown to be implicated in tumor progression, including the development of metastasis (21–25, 30). A significant finding of our study is that methylation-associated silencing of *TIMP-3* is prevalent in 44% of human PETs, which corresponds to loss of TIMP-3 protein expression. This effect is tumor-specific in the majority of PETs, because this epigenetic change was restricted to tumors in 17 of 18 matched normal/tumor pairs. Only the corresponding pancreatic tissue of vipoma 20/96 showed a weak methylated *vs.* a strong unmethylated MSP product. Low frequencies of methylation of the *TIMP-3* promoter in corresponding normal tissue have also been described for some lung cancers (31) and may be due to contamination with adjacent tumor tissue. Methylation-associated loss of TIMP-3 expression was also identified in 78% of kidney, 27% of colon, and 19% of lung cancers (26). Notably, normal islet cells of the pancreas showed a strong level of TIMP-3 expression. It is, therefore, conceivable that TIMP-3 expression may be critical for the normal growth of these cell populations. The high frequency of promoter methylation in PETs may provide a mechanism for loss of such control, further indicating a tumor suppressor role for *TIMP-3*, as recently suggested (26, 31, 32).

However, the epigenetic phenomenon of promoter hypermethylation is the major mode for loss of TIMP-3 expression in PETs and seems not to be associated with LOH on 22q12.3, because four tumors with LOH and four tumors without LOH revealed this alteration. Thus, *TIMP-3* might not be the main target of the relatively high frequency of allelic losses, suggesting the presence of another tumor suppressor gene in this region. Concordantly, homozygous deletions of *TIMP-3*, another important mechanism of tumor suppressor gene inactivation, were detected in only one tumor (166/98). The PCR-based detection of homozygous deletion is complicated in primary tumor specimens by the presence of admixed nonneoplastic tissue. We therefore minimized contamination from nonneoplastic tissue by microdissecting the tumors. Although we achieved a neoplastic cellularity of at least 85% in all tumor samples, the remaining nonneoplastic cell fraction (up to 15%) might have precluded a valid assessment of

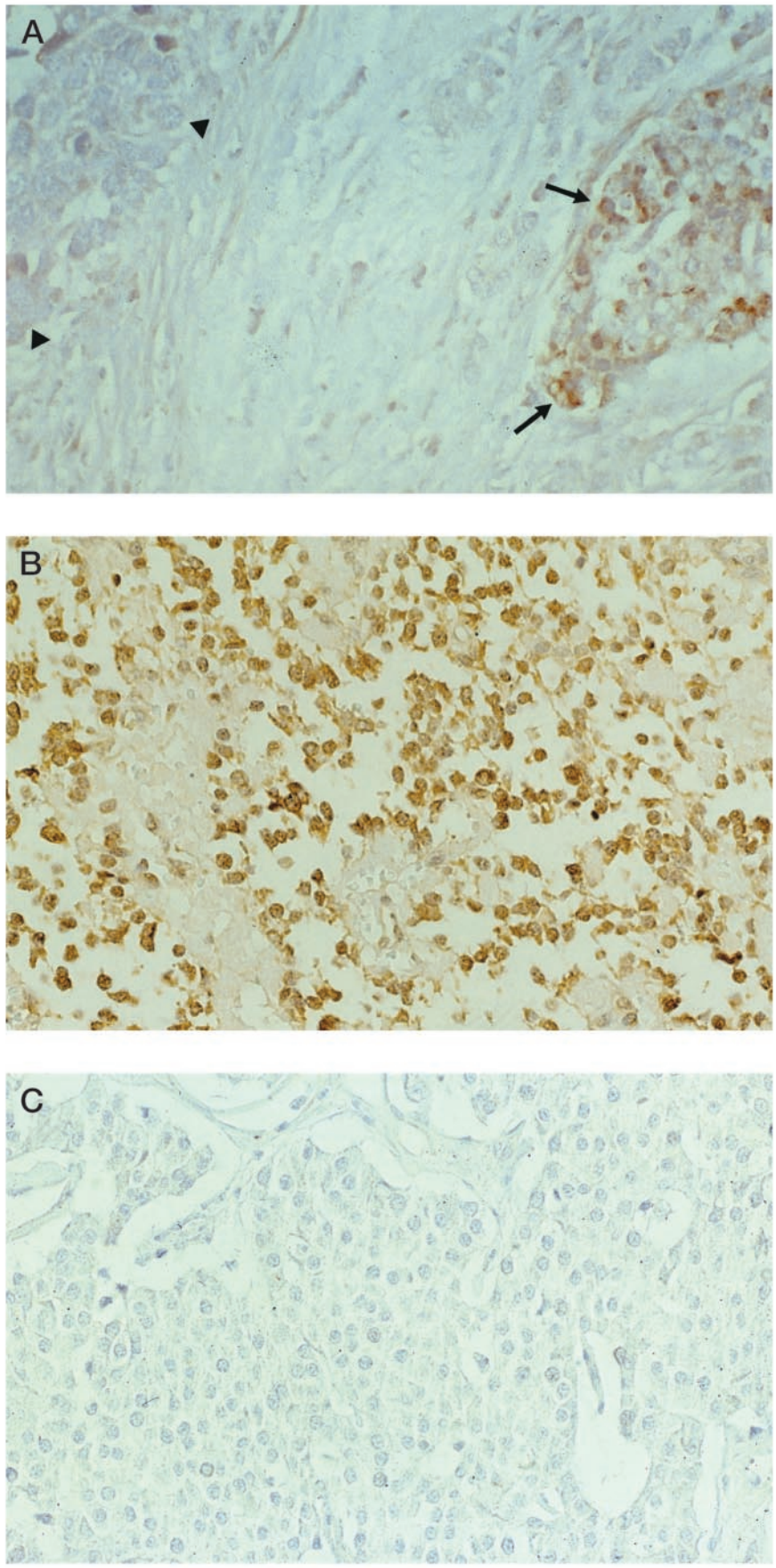

FIG. 2. Representative examples of TIMP-3 immunohistochemistry. A, Gastrinoma 167/98T (*arrowheads*) that is characterized by hypermethylation of *TIMP-3* does not express the TIMP-3 protein. In contrast, normal pancreatic islet cells (*arrows*) as internal positive control reveal strong expression of TIMP-3. B, Insulinoma 90 revealing no aberrant promoter methylation of *TIMP-3* shows strong TIMP-3 expression. C, NNPC 155/98 with promoter hypermethylation of *TIMP-3* has no detectable TIMP-3 immunoproduct. Magnification, −400; counterstain with hematoxylin.

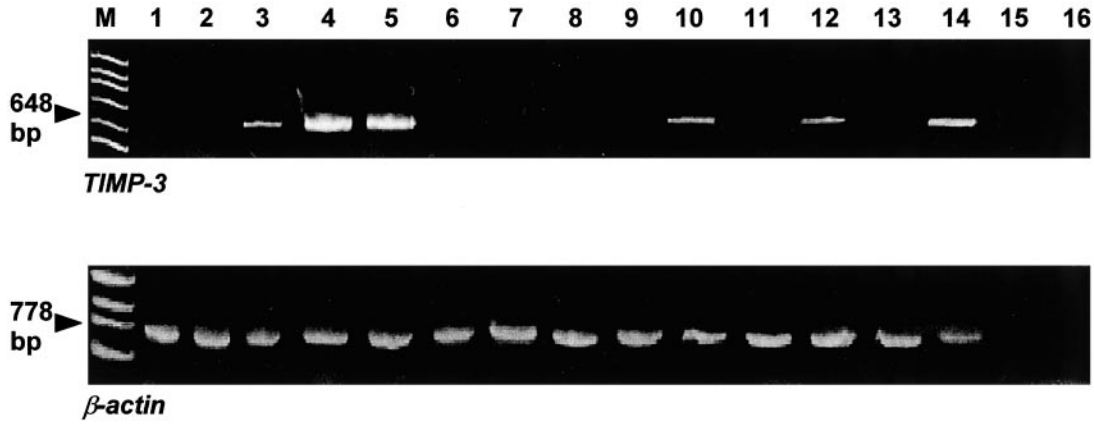

FIG. 3. Expression analysis of *TIMP-3* by RT-PCR in human PETs. Expression of β-actin was performed for all samples to ensure the integrity of the RT reactions. Lanes 1–16 contain RT-PCR products of total RNA from tumor tissues and control samples for TIMP-3 (648 bp) or β-actin (778 bp) mRNA expression, respectively. Lane 1, 155/98T; 2, 166/98T; 3, 172/98T; 4, normal pancreatic islet cells; 5, normal kidney as a positive control; 6, 122T; 7, 164/98T; 8, 167/98T; 9, 74/96T; 10, 141/98T; 11, SW15122T; 12, positive control; 13, 26/96T; 14, 90T; 15, negative control for DNA contamination; and 16, water blank. M, DNA size standard (50-bp ladder).

homozygous deletions in some of our cases. Intragenic mutations are another major mode of tumor suppressor gene inactivation but have not yet been described in *TIMP-3*, despite some germline mutations causing Sorsby fundus dystrophy (33, 34). We could not detect any *TIMP-3* mutations screened by SSCP in 21 PETs. SSCP analysis as used in this study has a sensitivity of 80% for the identification of mutations in PCR-generated fragments (11, 35, 36). However, certain mutations, especially large intragenic deletions may not be detected using this method. This might be the case for the three tumors with loss of TIMP-3 expression but no detectable mutation or hypermethylation. In addition, other more distantly located regulatory elements may be sites of pathological mutation, or another gene product that regulates TIMP-3 expression might be affected. Nevertheless, intragenic mutational inactivation of *TIMP-3* seems to be an infrequent event in PETs as it has been shown for the $p16^{INK4a}$ tumor suppressor gene (8–10).

The only unequivocal criteria of malignancy in PETs are metastases or infiltrating growth in adjacent tissues. The availability of molecular markers for the distinction between benign and malignant forms of PETs would have great clinical relevance as a predictor of prognosis. Some previous studies suggested that allelic losses of chromosomes 1, 3p, 10q, 11q13, and 22q are associated with a more aggressive phenotype in PETs (12–14, 16, 20), whereas other groups could not confirm these associations (37, 38). In the present study, *TIMP-3* alterations seem to be associated with a more aggressive tumor phenotype, because alterations occurred significantly more frequently in PETs with metastases than in PETs without metastases (79% *vs.* 14%; $P < 0.02$). This clinical association may support the recent hypothesis that reduced expression of *TIMP-3* contributes to the metastatic process (22–25), as it has been shown for gastric cancer (32). In the future, carefully designed, controlled, large-scale multicenter studies are required to further evaluate this potential prognostic association.

In summary, the presented data indicate that methylation-associated inactivation of *TIMP-3* is one of the most common genetic events yet identified in PETs. This finding should stimulate further research using this tumor type as a model system to investigate the potential tumor suppressive function of *TIMP-3*, helping to refine our picture of the role *TIMP-3* is playing in tumorigenesis. Large-scale multicenter studies are needed to further evaluate whether *TIMP-3* alterations may be a useful prognostic marker for PETs.

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References

1. **Klöppel G, Intveld PA, Stamm B, Heitz PU** 1990 The endocrine pancreas. In: Kovacs K, Asa SL, eds. Functional endocrine pathology. Boston: Blackwell Scientific Publications; 396–421
2. **Zhuang Z, Vortmeyer AO, Pack S, Huang S, Pham TA, Wang C, Park WS, Agarwal SK, Debelenko LV, Kester M, Guru SC, Manickam P, Olufemi SE, Yu F, Heppner C, Crabtree JS, Skarulis MC, Venzon DJ, Emmert-Buck MR, Spiegel AM, Chandrasekharappa SC, Collins FS, Burns AL, Marx SJ, Lubensky IA** 1997 Somatic mutations of the MEN1 tumor suppressor gene in sporadic gastrinomas and insulinomas. Cancer Res 57:4682–4686
3. **Shan L, Nakamura Y, Nakamura M, Yokoi T, Tsujimoto M, Arima R, Kameya T, Kakudo K** 1998 Somatic mutations of multiple endocrine neoplasia type 1 gene in the sporadic endocrine tumors. Lab Invest 78:471–475
4. **Yoshimoto K, Iwahana H, Fukuda A, Sano T, Katsuragi K, Kinoshita M, Saito S, Itakura M** 1992 ras mutations in endocrine tumors: mutation detection by polymerase chain reaction-single strand conformation polymorphism. Jpn J Cancer Res 83:1057–1062
5. **Yashiro T, Fulton N, Hara H, Yasuda K, Montag A, Yashiro N, Straus 2nd F, Ito K, Aiyoshi Y, Kaplan EL** 1993 Comparison of mutations of ras oncogene in human pancreatic exocrine and endocrine tumors. Surgery 114:758–764
6. **Moore PS, Orlandini S, Zamboni G, Capelli P, Rigaud G, Falconi M, Bassi C, Lemoine NR, Scarpa A** 2000 Pancreatic tumours: molecular pathways implicated in ductal cancer are involved in ampullary but not in exocrine nonductal or endocrine tumorigenesis. Br J Cancer 84:253–262
7. **Yoshimoto K, Iwahana H, Fukuda A, Sano T, Katsuragi K, Saito S, Itakura M** 1992 Role of p53 mutations in endocrine tumorigenesis. Cancer Res 53: 1057–1062
8. **Muscarella P, Melvin WS, Fisher WE, Foor J, Ellison EC, Herman JG,**

Schirmer WJ, Hitchcock CL, DeYoung BR, Weghorst CM 1998 Genetic alterations in gastrinomas and nonfunctioning pancreatic neuroendocrine tumors: an analysis of p16/MTS1 tumor suppressor gene inactivation. Cancer Res 58:237–240
9. **Serrano J, Goebel SU, Peghini PL, Lubensky IA, Gibril F, Jensen RT** 2000 Alterations in the p16INK4a/CDKN2A tumor suppressor gene in gastrinomas. J Clin Endocrinol Metab 85:4146–4156
10. **Bartsch DK, Kersting M, Wild A, Ramaswamy A, Gerdes B, Schuermann M, Simon B, Rothmund M** 2000 Low frequency of p16(INK4a) alterations in insulinomas. Digestion 62:171–177
11. **Bartsch DK, Hahn SA, Danichevski KD, Ramaswamy A, Bastian D, Galedhari H, Barth P, Schmiegel W, Simon B, Rothmund M** 1999 Mutations of the DPC4/Smad4 gene in neuroendocrine pancreatic tumors. Oncogene 18:2367–2371
12. **Perren A, Komminoth P, Saremaslani P, Matter C, Feurer S, Lees JA, Heitz PU, Eng C** 2000 Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine pancreatic tumors compared to normal islet cells. Am J Pathol 157:1097–1103
13. **Ebrahimi SA, Wang EH, Wu A, Schreck RR, Passaro Jr E, Sawicki MP** 1999 Deletion of chromosome 1 predicts prognosis in pancreatic endocrine tumors. Cancer Res 59:311–315
14. **Chung DC, Smith AP, Louis DN, Graeme-Cook F, Warshaw A, Arnold A** 1997 A novel pancreatic endocrine tumor suppressor gene locus on chromosome 3p with clinical prognostic implications. J Clin Invest 100:404–410
15. **Nikiforova MN, Nikiforov YE, Biddiger P, Gnepp DR, Grosembacher LA, Wajchenberg BL, Fagin JA, Cohen RM** 1999 Frequent loss of heterozygosity at chromosome 3p14.2–3p21 in human pancreatic islet cell tumours. Clin Endocrinol (Oxf) 51:27–33
16. **Hessman O, Lindberg D, Einarsson A, Lillhager P, Carling T, Grimelius L, Eriksson B, Akerström G, Westin G, Skogseid B** 1999 Genetic alterations on 3p, 11q13, and 18q in nonfamilial and MEN 1-associated pancreatic endocrine tumors. Genes Chromosomes Cancer 26:258–264
17. **Rigaud G, Missiaglia E, Moore PS, Zamboni G, Falconi M, Talamini G, Pesci A, Baron A, Lissandrini D, Rindi G, Grigolato P, Pederzoli P, Scarpa A** 2001 High resolution allelotype of nonfunctional pancreatic endocrine tumors: identification of two molecular subgroups with clinical implications. Cancer Res 61:285–292
18. **Chung DC, Brown SB, Graeme-Cook F, Tillotson LG, Warshaw AL, Jensen RT, Arnold A** 1998 Localization of putative tumor suppressor loci by genome-wide allelotyping in human pancreatic endocrine tumors. Cancer Res 57:3706–3711
19. **Wild A, Langer P, Ramaswamy A, Chaloupka B, Bartsch DK** 2001 A novel insulinoma tumor suppressor gene locus on chromosome 22q with potential prognostic implications. J Clin Endocrinol Metab 86:5782–5787
20. **Wild A, Langer P, Celik I, Chaloupka B, Bartsch DK** 2002 Chromosome 22q in pancreatic endocrine tumors: identification of a homozygous deletion and potential prognostic associations of allelic deletions. Eur J Endocrinol 147:1–7
21. **Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP** 1997 Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 74:111–122
22. **Anand-Apte B, Bao L, Smith R, Iwata K, Olsen BR, Zetter B, Apte SS** 1996 A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. Biochem Cell Biol 74:853–862
23. **Chambers AF, Matrisian LM** 1997 Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89:1260–1270
24. **Ahonen M, Baker AH, Kahari VM** 1998 Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res 58:2310–2315
25. **Baker AH, George SJ, Zaltsman AB, Murphy G, Newby AC** 1999 Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. Br J Cancer 79:1347–1355
26. **Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB, Graff JR** 1999 Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res 59:798–802
27. **Langer P, Bartsch DK, Gerdes B, Schwetlick I, Wild A, Erley C, Lamberts R** 2002 Renin producing neuroendocrine pancreatic carcinoma–a case report and review of the literature. Exp Clin Endocrinol Diabetes 110:43–49
28. **Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB** 1996 Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826
29. **Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, Goggins M** 2000 Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 60:1835–1839
30. **Bian J, Wang Y, Smith MR, Kim H, Jacobs C, Jackman J, Kung HF, Colburn NH, Sun Y** 1996 Suppression of in vivo tumor growth and induction of suspension cell death by tissue inhibitor of metalloproteinases (TIMP)-3. Carcinogenesis 17:1805–1811
31. **Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD** 2001 Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 61:249–255
32. **Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG** 2001 CpG island methylation in premalignant stages of gastric carcinoma. Cancer Res 61:2847–2851
33. **Felbor U, Suvanto EA, Forsius HR, Eriksson AW, Weber BHF** 1997 Autosomal recessive Sorsby fundus dystrophy revisited: molecular evidence for dominant inheritance. Am J Hum Genet 60:57–62
34. **Ayyagari R, Griesinger IB, Bingham E, Lark KK, Moroi SE, Sieving PA** 2000 Autosomal dominant hemorrhagic macular dystrophy not associated with the TIMP3 gene. Arch Ophthalmol 118:85–92
35. **Prosser J** 1993 Detecting single-base mutations. Trends Biotechnol 11:238–247
36. **Nataraj AJ, Olivos-Glander I, Kusukawa N, Highsmith Jr WE** 1999 Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis 20:1177–1185
37. **Stumpf E, Aalto Y, Hoog A, Kjellman M, Otonkoski T, Knuutila S, Andersson LC** 2000 Chromosomal alterations in human pancreatic endocrine tumors. Genes Chromosomes Cancer 29:83–87
38. **Speel EJ, Scheidweiler AF, Zhao J, Matter C, Saremaslani P, Roth J, Heitz PU, Komminoth P** 2001 Genetic evidence for early divergence of small functioning and nonfunctioning endocrine pancreatic tumors: gain of 9Q34 is an early event in insulinomas. Cancer Res 61:5186–5192