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Advances in Brief

Genetic Alterations in Gastrinomas and Nonfunctioning Pancreatic Neuroendocrine Tumors: An Analysis of *p16/MTS1* Tumor Suppressor Gene Inactivation[1]

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Abstract

Neoplasms of the endocrine pancreas are extremely rare, and molecular mechanisms influencing their development are poorly understood. Nevertheless, gastrinomas have become a paradigm for the study of hormonally active tumors. In the present study, 12 gastrinoma and nonfunctioning pancreatic neuroendocrine tumor specimens were evaluated for genetic alterations of the *p16/MTS1* tumor suppressor gene. DNA extracted from microdissected portions of paraffin-embedded tumor sections were examined for mutations and homozygous deletions using "Cold" single-strand conformation polymorphism and semiquantitative PCR-based analyses, respectively. Samples were also analyzed for the presence of 5′ CpG island hypermethylation using methylation-specific PCR. The *p16/MTS1* gene was found to be homozygously deleted in 41.7% of tumors and methylated in 58.3%, but no mutations were identified by single-strand conformation polymorphism analyses. Overall, 91.7% of the specimens demonstrated inactivating alterations in *p16/MTS1*. These data suggest that transcriptional silencing of *p16/MTS1* is a frequent event in these rare and poorly understood tumors.

Introduction

Gastrinomas and nonfunctioning pancreatic neuroendocrine tumors arise from the pancreatic islet cells and are associated with the MEN1[3] syndrome of parathyroid hyperplasia, pituitary adenomas, and pancreatic islet cell tumors. Tumors of the endocrine pancreas account for approximately 1% of pancreatic tumors but are now being diagnosed more frequently because of increased recognition by physicians. Originally described by Zollinger and Ellison in 1955 (1), gastrinomas have become a paradigm for the study of hormonally active tumors (2). Despite the numerous studies concerning the pathophysiology of gastrinomas, relatively little is understood about the molecular mechanisms involved in gastrinoma tumorigenesis. HER-2/*neu* gene amplification (3) and loss of heterozygosity at 11q13 (4–6), the location of the recently cloned *MEN1* gene (7), have been described in sporadic gastrinomas, but mutations in K-*ras* (3, 8, 9) and *p53* (3) do not appear to be involved.

Inactivation of the *p16/MTS1* tumor suppressor gene has been demonstrated in a wide variety of human malignancies, including those of the exocrine pancreas (10–13). Mechanisms of inactivation include homozygous deletion, mutation, and aberrant methylation of the *p16/MTS1* 5′ CpG island. There are presently no published studies evaluating *p16/MTS1* inactivation in gastrointestinal neuroendocrine tumors. The purpose of this study was to determine the involvement of *p16/MTS1* inactivation in gastrinomas and NPNTs.

Materials and Methods

Tumor Specimens and DNA Extraction. Specimens included eight gastrinomas and four NPNTs that were obtained at the time of surgical resection at The Ohio State University Medical Center. Following resection, the tissues were paraffin-embedded according to routine protocol. The diagnosis of neuroendocrine tumors was made by histological analysis, and patients were considered to have gastrinomas if they had either elevated fasting serum gastrin levels or paradoxical rises in serum gastrin levels in response to provocative testing with secretin. Patients were considered to have NPNTs if they clinically demonstrated no evidence of hormonally active tumors in the presence of a pancreatic mass with neuroendocrine characteristics by histopathological analysis. All of these tumors were sporadic.

For DNA extraction, serial 5-μm sections were cut from each tissue block and applied to slides without coverslips. The middle slide from each specimen was stained with H&E. Foci of tumor cells were identified by light microscopy, and corresponding tissue was excised from three to five adjacent slides using #10 scalpel blades. A new scalpel blade was used for each sample to avoid cross-contamination. The tissues were placed into 1.7-μl tubes and deparaffinized with *n*-octane and ethanol. Genomic DNA was prepared by digesting the samples in proteinase K/Tween 20 solution for 48 h at 55°C.

Semiquantitative PCR-based Deletion Analysis. To avoid the "plateau effect" associated with an increased number of PCR cycles, the number of PCR cycles was selected to ensure that all amplifications were in the linear range. These were determined by cycle curves performed on representative samples for each primer pair (data not shown). A portion of *p16/MTS1* exon 2 and an internal control marker, *HPRT*, were amplified simultaneously in individual reactions. The *HPRT* gene served as an internal quantitative control and also confirmed the existence of viable template DNA for each PCR reaction. The *p16/MTS1* primer sequences have been published (14). The *HPRT* sequences were as follows: forward, 5′ ACG TCT TGC TCG AGA TGT GA 3′; and reverse, 5′ CCA GCA GGT CAG CAA AGA AT 3′.

Reactions were carried out in 25 μl containing 5 μl of genomic DNA, 2 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Corp., Arlington Heights, IL)), 1× PCR buffer (Life Technologies, Inc., Gaithersburg, MD) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ (3.0 mM for *HPRT* reactions), 200 μM each deoxynucleotide triphosphate, 1 unit Taq DNA polymerase (Life Technologies, Inc.), 5% DMSO, and 0.1 μM each primer. A melanoma cell line, SK-MEL-44, in which the *p16/MTS1* gene is known to be homozygously deleted, was used as a homozygous deletion-positive control. DNA obtained from the pancreas, liver, and small bowel of four otherwise healthy trauma patients were used as wild-type controls. A no-template control was also run. The PCR reactions were carried out in a Perkin-Elmer DNA thermocycler 9600 (Perkin-Elmer, Norwalk, CT) at 94°C for 50 s, 64°C (56°C for *HPRT*) for 50 s, and 72°C for 50 s, for 30 cycles, followed by an incubation for 7 min at 72°C.

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[3] The abbreviations used are: MEN1, multiple endocrine neoplasia type 1; NPNT, nonfunctioning pancreatic neuroendocrine tumor; HPRT, hypoxanthine ribosyl transferase; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity.

Table 1 *Tumor samples, characteristics, and results of p16/MTS1 tumor suppressor gene inactivation analysis*

Tumor	Type	Primary *vs.* metastatic	Location	Homozygous deletion	Mutation analysis	Methylation status
1	Gastrinoma	primary	pancreas	−	−	+
2	Gastrinoma	metastatic	lesser omental node	−	−	+
3	Gastrinoma	metastatic	liver	−	−	+
4	Gastrinoma	metastatic	inf. peripanc node	−	−	+
5	NPNT	primary	pancreas	−	−	+
6	NPNT	metastatic	liver	+	−	−
7	Gastrinoma	primary	duodenum	+	−	−
8	NPNT	primary	pancreas	−	−	+
9	Gastrinoma	metastatic	liver	+	−	+
10	Gastrinoma	primary	gastroduodenal jxn	+	−	−
11	NPNT	primary	pancreas	+	−	−
12	Gastrinoma	metastatic	liver	−	−	−

Seven μl of both PCR products for each specimen were loaded onto 10% precast polyacrylamide gels (Novex, San Diego, CA) and separated by electrophoresis at a constant voltage of 270 V for 30 min. The gels were directly exposed to Hyperfilm (Amersham) for 30 min for purposes of documentation. Quantitative determination of [α-^{32}P]dCTP incorporation was determined by liquid scintillation counting of bands excised from the gels. Following exposure, the gels were stained with ethidium bromide, and the *p16/MTS1* and *HPRT* bands were visualized in UV light. Gel slices containing the *HPRT* and *p16/MTS1* bands were excised separately for each sample. Care was taken to assure that the *p16/MTS1* and *HPRT* band containing gel slices were approximately the same size. The gel slices were placed into 10 ml of liquid scintillation cocktail and counted using an LS600 liquid scintillation counter (Beckman Instruments, Fullerton, CA). Gel slices from the no-template lane were considered to be background.

[α-^{32}P]dCTP incorporation for the *p16/MTS1* fragments were normalized by dividing them by the corresponding *HPRT* [α-^{32}P]dCTP incorporation values. Our evaluation of this semiquantitative PCR-based data was based on the premise that most tumors are heterogeneous in nature. If homozygous deletion of the *p16/MTS1* gene occurred within a tumor, sufficient copies of wild-type *p16/MTS1* may be present within infiltrating "nontumor" cells to allow for PCR amplification. With this in mind, a sample was considered to contain a deletion if the *p16:HPRT* [α-^{32}P]dCTP incorporation ratio was <50% of the ratio for wild-type controls (14). Optimal SSCP buffer temperatures were maintained using the Novex Thermoflow SSCP System (Novex, San Diego, CA).

PCR-"Cold" SSCP. Three PCR fragments, generated from all 12 samples, were screened for mutations using the highly sensitive technique of "Cold" SSCP (15). Together, these three fragments contain the entire coding portions of exons 1 and 2. The terminal 11 coding bp lying within exon 3 were not evaluated. Primers, PCR conditions, and SSCP conditions are published elsewhere (14). Fragments known to contain mutations and normal fragments were run alongside the samples as controls.

Methylation-specific PCR. All 12 samples were evaluated for the presence of 5′ CPG island methylation using the recently described method of methylation-specific PCR (16). This assay exploits the ability of sodium bisulfite to convert unmethylated, but not methylated, cytosine residues to uracil. PCR amplification is then carried out using primers specific for both methylated and unmethylated DNA.

Results

Twelve tumor specimens from patients with gastrinomas and NPNTs were evaluated for *p16/MTS1* deletion, mutation, and 5′ CpG island methylation using the techniques described above (Table 1). Of the eight gastrinomas, three were primary lesions and five were metastatic (lymph nodes or liver metastases). Three of the four NPNTs were primary pancreatic lesions, and one was a liver metastasis.

Five specimens consistently exhibited *p16:HPRT* [α-^{32}P]dCTP incorporation ratios <50% of the representative control ratio in duplicate experiments and were considered to contain homozygous deletions of *p16/MTS1* (Table 2). Of the samples found to have a *p16/MTS1* deletion, three were gastrinomas and two were NPNTs. The wild-type control *p16:HPRT* ratios ranged from 0.917 to 2.478. The most conservative ratio (0.917) was chosen as the representative control to minimize false positives. The deletion control sample, SK-MEL-44, was found to be deleted by our analysis. Autoradiographs of the samples are shown in Fig. 1.

"Cold" SSCP analysis was performed on PCR-generated fragments comprising the entire coding sequence of exons 1 and 2 for all samples. Positive and negative controls were run on each gel. Visible SSCP bands were identified for all samples, even for those later found to be homozygously deleted by deletion analysis. These bands were most likely due to amplification of infiltrating "normal" DNA and the higher cycle number used in these PCR reactions. No mobility shifts were identified in any of the samples for all three fragments, suggesting that no mutations were present.

Visible bands were identified in 7 of 12 samples PCR-amplified

Table 2 *Semiquantitative PCR-based deletion analysis data*

[α-^{32}P]dCTP incorporation is measured by cpm. Samples were considered to be homozygously deleted if the normalized *p16/HPRT* ratio was <50% of the representative normal, control ratio.

Sample	*p16*[α-^{32}P]dCTP incorporation (cpm)	*HPRT*[α-^{32}P]dCTP incorporation (cpm)	*p16:HPRT* ratio	Sample/control	Homozygous deletion
1	1938	2958	0.655	0.714	−
2	5403	7049	0.766	0.836	−
3	8583	8713	0.985	1.074	−
4	5321	5597	0.950	1.037	−
5	1592	2094	0.760	0.829	−
6	2827	6945	0.407	0.444	+
7	372	1522	0.244	0.266	+
8	2339	3487	0.670	0.731	−
9	1314	3845	0.341	0.373	+
10	1052	2777	0.378	0.413	+
11	754	2385	0.316	0.345	+
12	7339	7181	1.022	1.114	−
SK-MEL-44	846	4464	0.189	0.207	+
Normal	2891	3152	0.917	1.000	NA[a]

[a] NA, not applicable.

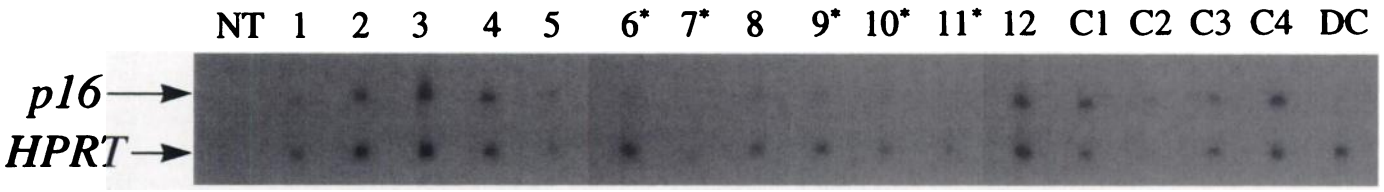

Fig. 1. Semiquantitative PCR-based *p16/MTS1* deletion analysis of gastroma and nonfunctioning pancreatic neuroendocrine tumor samples. Autoradiograph demonstrates [α-^{32}]dCTP incorporation for both *p16/MTS1* and *HPRT* PCR product. NT, no template control; 1–12, tumor samples; C1-C4, normal tissue control samples; DC, *p16/MTS1* deletion control cell line. *, samples determined to contain deletions after quantitation by liquid scintillation.

with methylation-specific primers after treatment with bisulfite (Fig. 2). It has been shown that this method is a highly sensitive technique for the detection of aberrantly methylated DNA (16); therefore, these six samples were considered to be positive for *p16/MTS1* 5′ CpG island methylation. Five methylated specimens were from gastrinomas and two from NPNTs.

Overall, 91.7% of the samples demonstrated *p16/MTS1* homozygous deletion (41.7%) and/or 5′ CpG island methylation (58.3%). Of the gastrinomas, *p16/MTS1* was methylated solely in 50.0% of the tumors, and in 25.0% of the tumors, *p16/MTS1* was singularly deleted. One gastrinoma specimen (a metastatic liver lesion) demonstrated both methylation and deletion. All of the NPNTs demonstrated *p16/MTS1* abnormalities (50.0% deleted and 50.0% methylated).

Discussion

Knowledge of the genetic changes associated with the initiation and progression of gastrinomas and other endocrine tumors of the pancreas is limited. Published studies include small numbers of samples, and few have demonstrated positive results. The most well-described change is related to LOH at 11q13, the chromosomal location of the recently cloned *MEN1* gene (7). Eubanks *et al.* (4) have reported LOH at 11q13 in 8 of 22 patients with sporadic gastrinomas and 0 of 2 patients with NPNTs. One patient with an MEN1-associated gastrinoma did not demonstrate LOH at 11q13 (4). Other studies have demonstrated LOH at 11q13 in 0 of 7 (5) and 5 of 11 (6) sporadic gastrinomas. One MEN1-associated NPNT did demonstrate LOH at 11q13. Overall, 13 of 40 (32.5%) sporadic gastrinomas and 0 of 2 sporadic NPNTs have demonstrated LOH at 11q13. It is likely that inactivation of *MEN1* is related to familial gastrinoma development; however, the one specimen studied did not demonstrate LOH at 11q13.

Based on a study of 11 gastrinomas, Evers *et al.* (3) have suggested that amplification of the HER-2/*neu* proto-oncogene may be involved in the pathogenesis of gastrinomas. These data are based on the results of semiquantitative PCR analyses of genomic DNA. There are no published data, however, demonstrating elevated levels of HER-2/*neu* mRNA or protein in gastrinoma specimens. Immunohistochemical analysis of 20 gastrinomas and NPNTs performed in our laboratory failed to demonstrate membranous staining with antibody to the HER-2/*neu* gene product (data not shown). This suggests that genomic amplification of HER-2/*neu* may not have functional significance. K-*ras* mutations, associated with up to 95% of exocrine pancreatic tumors, do not appear to play a role in endocrine tumors of the pancreas. Several studies have demonstrated no mutations in groups of 11, 11, and 33 tumors (3, 8, 9). Mutations of the *p53* tumor suppressor gene were not identified in 11 specimens evaluated by direct sequencing of genomic DNA (3).

The *p16/MTS1* tumor suppressor gene, located in chromosome region 9p21, codes for a regulatory protein known to inhibit the cell cycle at the G_1-S junction (17). Mutations and deletions of this gene have been reported in a number of cell lines and primary tumors, including those of the exocrine pancreas (10–13). Analyses of primary exocrine pancreatic carcinomas demonstrate *p16/MTS1* mutations in 38% of specimens and deletions in 41% (10). CpG island methylation is a well described alternative mechanism for *p16/MTS1* inactivation. Aberrant methylation of the 5′ CpG island of *p16/MTS1* is known to occur in 31% of breast tumors and 40% of colon tumors (18). Studies of transitional cell carcinomas of the bladder indicate that *p16/MTS1* methylation correlates with loss of expression (19). Recent data demonstrate that hypermethylation of *p16/MTS1* occurs in seven of eight (88%) exocrine pancreatic tumors not inactivated by mutation or deletion. Overall, 98% of exocrine pancreatic tumors demonstrate abrogation of the Rb/p16 tumor-suppressive pathway (20). This figure is comparable with the data for endocrine pancreatic tumors found in the present study, except that mutational inactivation of *p16/MTS1* does not appear to play a role in endocrine tumors of the pancreas.

To our knowledge, this is the first reported analysis of *p16/MTS1* genetic alterations in gastrointestinal neuroendocrine tumors. Our data suggest that transcriptional silencing, secondary to homozygous deletion and 5′ CpG island methylation, are frequent events in these increasingly recognized tumors and that mutations do not play a significant role. It would be gratifying to confirm our findings by evaluating *p16/MTS1* expression, but this is difficult, because RNA isolation requires snap-frozen tissue; consequently, gastrinoma RNA is rare. In addition, the interpretation of immunohistochemistry studies performed on paraffin-embedded tissues is difficult with presently available antibodies to p16.

The evaluation of primary tumor specimens for homozygous deletions is complicated by the presence of infiltrating "normal" DNA. We attempted to minimize contamination by dissecting only grossly visible tumor, but it is impossible to totally eliminate contamination.

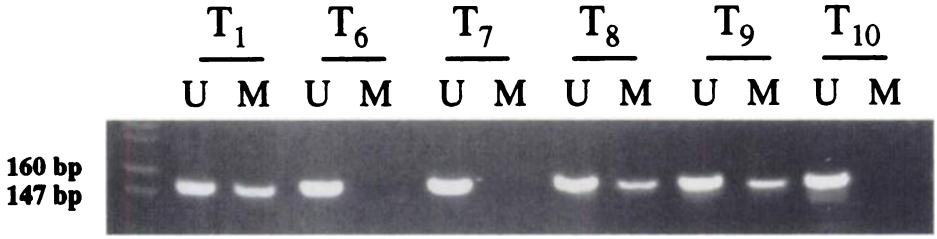

Fig. 2. Methlation-specific PCR. Gel electrophoresis of modified-unmethylated (U) and modified methylated (M) PCR products for representative tumor (*T*) samples indicates that *p16/MTS1* CpG island methylation is present in samples 1, 8, and 9.

Amplification with the internal control, *HPRT*, was performed to assign a semiquantitative value to the amount of [α-^{32}P]dCTP incorporation in the *p16/MTS1* PCR reactions. Cycle curves were performed prior to the experiment to ensure that all amplifications were within the linear range, and the most conservative wild-type control *p16:HPRT* ratio was used to minimize false positives. We believe that sample-to-control *p16:HPRT* ratios <50% are most likely explained by homozygous deletions within the majority of tumor cells, whereas those with >50% are less discernible. Homozygous deletions within a minority of tumors cells or LOH within a majority of tumor cells, complicated with the presence of infiltrating wild-type cells, could yield similar *p16:HPRT* ratios >50%.

"Cold" SSCP has been shown to be a highly sensitive method for detecting mutations in PCR-generated fragments (15). The inherent weakness in this technique, however, is that mutations may be missed because they do not lead to conformational shifts. We deem this unlikely in our study, because *p16/MTS1* inactivation could be explained by alternative mechanisms in 91.7% of the specimens. Mutational inactivation would, therefore, be redundant. These data are also consistent with recent studies on squamous cell carcinomas of the head and neck, which demonstrate high rates of *p16/MTS1* deletion and methylation and lower identified mutations (21).

Methylation-specific PCR is a highly sensitive technique used for the detection of methylated DNA in otherwise normal specimens (16). This is particularly useful when evaluating specimens potentially contaminated with unmethylated DNA. Curiously, one gastrinoma specimen demonstrated both methylation and deletion. This seems unlikely because a completely deleted gene could not possibly be methylated. We believe that this tumor was heterogeneous in nature and was composed of at least two populations of cells containing either *p16/MTS1* deletions or methylated *p16/MTS1* DNA. It has been suggested that methylation may, in fact, predispose to allelic loss; several lines of evidence support this hypothesis. For example, in renal tumors, hypermethylation of chromosome 17p precedes allelic loss (22), and in rat lung, tumors homozygous deletions were demonstrated in four cell lines derived from primary tumors that exhibited *p16/MTS1* methylation (23).

Although the number of samples in this study is limited, it is the first demonstration of *p16/MTS1* tumor suppressor gene alterations in these rare and poorly understood gastrointestinal tumors. Furthermore, our data suggest that these *p16/MTS1* molecular events leading to inactivation may be the most frequent molecular events yet described in gastrinomas and NPNTs, and may have clinical implications for potential pharmacological or gene therapies.

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