**Tumor Suppressor Genes in the 9p21 Gene Cluster Are Selective Targets of Inactivation in Neuroendocrine Gastroenteropancreatic Tumors**

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**ABSTRACT**

Functional inactivation of the Rb and p53 pathways appears to be a rite of passage for all cancerous cells. However, p53 and Rb alterations are rare events in neuroendocrine gastroenteropancreatic (GEP) tumors. The CDKN2 locus on chromosome 9p21 sits at the nexus of both pathways harboring tumor suppressor genes, which restrain cell growth by affecting the function of pRb and p53. Therefore, we analyzed the implication of their inactivation in 37 primary neuroendocrine GEP tumors and two cell culture models. RT-PCR analysis revealed loss of expression of at least one of the tumor suppressor genes CDKN2A/p16, CDKN2B/p15, and CDKN2D/p14 with distinct genetic profiles, most frequently in nonfunctional pancreatic tumors (57%) and small intestinal carcinoids (44%), and less commonly in insulomas (30%) and gastrinomas (22%). DNA analysis and methylation-specific PCR attributed loss of expression to either homozygous deletion or 5’ CpG island hypermethylation. 5-Aza-2-deoxycytidine treatment reversed CDKN2A/p16 and CDKN2B/p15 silencing with concurrent growth restraint. Thus, tumor suppressor genes localized in the 9p21 gene cluster are specific targets of inactivation in neuroendocrine GEP tumors, and demethylating agents might hold promise for selective therapy.

**INTRODUCTION**

Neuroendocrine GEP tumors arise from the diffuse neuroendocrine system and are rare neoplasms with an incidence of 1–2 cases/100,000 people (1). They are classified according to their site of origin and by their secretory product into functionally active or inactive tumors. Approximately half of neuroendocrine GEP tumors are functionally active, with increased release of tumor-specific hormones leading to clinical presentation with characteristic hyperfunctional syndromes. Tumors with primary localization in the small intestine or stomach and secretion of serotonin and other neurotransmitters and polypeptides are classified as carcinoids. Other functional tumors, which are primarily localized in the pancreas or duodenum, include insulinomas and gastrinomas and, less frequently, VIPomas and glucagonomas. Nonfunctional neuroendocrine tumors mostly produce inactive peptides and present without clinical syndrome (1).

Neuroendocrine GEP tumors have attracted much scientific interest during the past decade. Although detailed information is now available on the morphofunctional profile of these tumors (1), little is known about the cellular and molecular basis of tumor initiation and progression. Neuroendocrine pancreatic tumors can be detected in a subgroup of sporadic neuroendocrine tumors, genetic constellations besides MEN1 gene mutations are thought to be required for endocrine tumor development (3). However, no pathogenetic role has been found in such tumors for the Rb susceptibility gene Rb, tumor suppressor gene p53, or the most commonly mutated oncogenes (4, 5), indicating that the tumorigenic pathways of adenocarcinomas and neuroendocrine tumors of the GEP system are divergent. However, mice deficient for both the Rb and p53 genes were highly susceptible to neuroendocrine tumor development, supporting the concept that functional inactivation of such genes is a prerequisite for all of the cancerous cells (6). The CDKN2A locus at 9p21 has proven to be a unique regulatory system controlling both the Rb and p53 pathways by generating two gene products: the p16 protein functions upstream of pRb, and the p14 protein blocks MDM2 inhibition of p53 activity (7). The CDKN2D/p14 gene, which shares a portion of the CDKN2A/p16 coding region, has a unique first exon 1B originating ~20 kb centromerically to CDKN2A/p16 exon 1a. This exon, under the control of its own promoter, splices onto exon 2 of CDKN2A/p16 in an alternative reading frame, allowing the production of two totally unrelated proteins (8). Whereas CDKN2A/p16 mutations selectively inactivate the Rb pathway, deletion of the CDKN2A locus impairs both the Rb and p53 pathways. Deletion of the CDKN2A locus also frequently affects the CDKN2B locus, which encodes p15, an important mediator of the antiproliferative effect of TGFβ (9). Recently, Muscarella et al. (10) reported CDKN2A/p16 inactivation in a limited number of gastrinomas and two nonfunctional neuroendocrine tumors. However, these results were not confirmed by evaluating CDKN2A/p16 expression, because RNA from these tumors is rarely available, and studies on paraffin-embedded tissues are difficult to perform with the p16 antibodies available. Moreover, the question of genetic alterations in small intestinal carcinoids and the role of the tumor suppressor genes CDKN2D/p14 and CDKN2B/p15 in neuroendocrine tumors has not been addressed to date. Therefore, we performed a comprehensive analysis of a series of neuroendocrine GEP tumors to elucidate specific targets and aberrations affecting this subchromosomal region.

**MATERIALS AND METHODS**

**Tumor Samples, Cell Lines, and RT-PCR.** RNA extraction and cDNA synthesis was performed on 37 primary neuroendocrine GEP tumors (9 gastrinomas, 10 insulinomas, 9 small intestinal carcinoids, 7 nonfunctional pancreatic tumors, 1 nonfunctional small intestine, and 1 nonfunctional stomach tumor) from cell lines BON, QGP-1 (human neuroendocrine pancreatic tumor), PC44, MiaPaCa-2 (human pancreatic adenocarcinoma), and HeLa (cervical carcinoma) as described (11, 12). PCR with [α-32P]dCTP (ICN, Meckenheim, Germany) used these primers: p16F (5′-CAACGCGACGATAGCTACG-3′), p16R/p19R (5′-GGTAGCTGGAGCACGATCCGAT-3′), p15F (5′-ATMCGGAGGAAAGAACAAG-GG-3′), p15R (5′-GGGGCTTGGGAAGAAGGCTC-3′), p19F (5′-AGTTGAGCCTGTCCTCACC-3′), p18F (5′-GGTTACAGTGGCGCTGCTCACCTC-3′), p19F (5′-ATGGCCGAGCCTTGGGG-3′), p19R (5′-TTTTGAGATTTGTGGCTCC-3′), p21F (5′-CATCATGAGCCCGTGGG-3′), p21R (5′-TTTACGTTCCAATTCTGAG-3′), p27F (5′-ATCTACACAGCTGCTGATTGC-3′), and p27R (5′-TTTACGTTCG-3′). Integrity of the RNA isolated from the samples was verified by β-actin amplification (11). Different amplicons served as internal controls.
CDK INHIBITOR INACTIVATION IN NEUROENDOCRINE GEP TUMORS

To confirm that each tumor template could be amplified efficiently, PCR products were eluted and confirmed by sequence analysis (ABI PRISM 310; Perkin-Elmer).

SSCP and Mutation Analysis. After DNA extraction (QiAmp Tissue kit; QiAgen) CDKN2A/p16 mutations were evaluated using SSCP in five separate PCR amplifications (13). PCR products were visualized by autoradiography. Aberrant bands were reamplified and directly sequenced. Genomic DNA from pancreatic carcinoma cell lines with known CDKN2A/p16 mutation status served as controls.

MSP. Cells were grown in the absence or presence of the methyltransferase inhibitor 5-Aza-CdR (Sigma Chemical Co.) to a final concentration of 1 μM. Medium was renewed every 24 h. The DNA methylation pattern in the 5′ CpG island of the CDKN2A/p16 gene was determined as described (14). In brief, 200–300 ng of DNA were treated with 3 M sodium bisulfite to convert unmethylated cytosine residues to uracil. Seminested PCR was performed as a diagnostic test using primers p16 M1/p16 M2 for the first and p16 M1/p16M2 for the second amplification. Amplification of unmethylated-specific alleles served as a control for the efficiency of cytosine conversion and DNA quality. As an internal control, a STS marker on chromosome 7 (D7S494) was coamplified (15).

Immunohistological Analysis of p16. Protein lysates were separated by 14% SDS polyacrylamide gels and electrophoresed to nitrocellulose membranes (Schleicher and Schuell, Inc.). After blocking (3% milk powder/Tris-buffered saline Tween 20) primary antibody p16 (sc-9968; Santa Cruz Biotechnology) was applied (1:200) and, subsequently, an antibody tagged to horseradish peroxidase (sc-2005; Santa Cruz Biotechnology) for chemiluminescence (enhanced chemiluminescence; Amersham).

Cell Morphology and Proliferation Assay. For evaluation of growth kinetics, cells were harvested by trypsinization, collected by centrifugation, and counted in the Neubauer-hematocytometer. Cell morphology was analyzed by microscopy, DAPI was applied for DNA staining. DNA replication was assessed by measuring BrdUrd incorporation (16).

RESULTS

Preferential Loss of Expression of Tumor Suppressor Genes at 9p21 in Subtypes of Neuroendocrine GEP Tumors. The expression levels of CDKN2A/p16, CDKN2D/p14, CDKN2B/p15, and CDKN1A/p21 mRNA transcripts were assessed in 37 neuroendocrine GEP tumors by RT-PCR analysis. Absent expression of at least one tumor suppressor gene was observed in 57% of the nonfunctional neuroendocrine pancreatic tumors (4 of 7 tumors; Fig. 1, Lanes 29, 33, 35, and 37) and 44% of the small intestinal carcinoids (4 of 9 tumors; Fig. 1, Lanes 23–25 and 28), less frequently in insulinomas (3 of 10 tumors; Fig. 1, Lanes 10 and 11). In contrast with the gastrinomas and insulinomas, small intestinal carcinoids and nonfunctional pancreatic tumors demonstrated very low or absent expression of all of the three tumor suppressor genes analyzed, either alone or in combination. The data suggested four expression patterns for the CDKN2A/CDKN2D locus: (a) CDKN2A+/CDKN2D- (Fig. 1, Lanes 20–22, 27–28, 30–34, and 36); (b) CDKN2A+/ CDKN2D (Fig. 1, Lanes 24 and 29); (c) CDKN2A+/CDKN2D- (Fig. 1, Lanes 26); and (d) CDKN2A-/CDKN2D- (Fig. 1, Lanes 23, 25, 35, and 37). Both tumor suppressor genes CDKN2A/p16 and CDKN2D/p14 were independently altered in three tumors (Fig. 1, Lanes 24, 26, and 29), although more alterations affected both genes (Fig. 1, Lanes 23, 25, 35, and 37), suggesting homozygous deletion. Almost half of the nonfunctional pancreatic tumors (44%) revealed absent or very low levels of the CDKN2B/p15 transcript (Fig. 1, Lanes 29, 33, 35, and 37). One additional nonfunctional pancreatic tumor (Fig. 1, Lane 31) revealed a reduced PCR product, suggesting that the number of CDKN2B/p15 alterations in nonfunctional tumors might be even higher than revealed by expression loss. Concomitant loss of CDKN2A/p16, CDKN2D/p14, and CDKN2B/p15 was observed in one small intestinal carcinoid (Fig. 1, Lane 25) and two nonfunctional pancreatic tumors (Fig. 1, Lanes 35 and 37), indicating homozygous deletion of the CDKN2A/CDKN2D/CDKN2B gene locus. The nonfunctional tumors of the small intestine and stomach showed unaltered expression profiles (Fig. 1, Lanes 32 and 34). The expression profiles are summarized in Table 1. Because of the potential for contamination of normal cells in the primary tumors to give false negatives, the results obtained from RT-PCR analysis in the primary tumors represent a minimum frequency of expression loss or reduction. Although expressed at varying levels, the CDK inhibitor CDKN1A/p21 was expressed in all of the tumor specimens.

Inactivation of CDK Inhibitors CDKN2A/p16 and CDKN2B/p15 in Neuroendocrine Tumor Cells Is Attributable to Homozygous Deletion or Aberrant Promoter Methylation. To elucidate the underlying molecular mechanisms of altered CDK-inhibitor expression, we assessed their expression profiles in two human cell culture models derived from neuroendocrine pancreatic tumor specimens.

Fig. 1. Expression pattern of CDKN2A/p16, CDKN2B/p15, CDKN2D/p14, and CDKN1A/p21 as determined by RT-PCR in subsets of primary neuroendocrine GEP tumors. Gastrinomas (Lanes 1–9), insulinomas (Lanes 10–19), small intestinal carcinoids (Lanes 20–28), nonfunctional tumors of the pancreas (Lanes 29–31, 33, 35–37), the stomach (Lane 32), and small intestine (Lane 34). Tumors with complete lack or strongly diminished transcript expression were assessed. Left, transcript description; right, size of the PCR product.

*+, tumors metastasized at the time of surgery. Lane 29 of the CDKN2B/p15 analysis revealed an unspecific PCR product on sequencing.

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RT-PCR analysis of BON cells revealed concomitant loss of CDKN2A/p16, CDKN2B/p15, and CDKN2D/p14 expression, suggesting homozygous deletion of the CDKN2A/CDKN2B/CDKN2D gene loci. In contrast, BON cells revealed no PCR products in either the unmethylated or methylated specific PCR, in accordance with homozygous deletion of the CDKN2A locus (Fig. 2C, Lane 1).

Demethylation Reverses Transcriptional Silencing of CDKN2B/p15 and CDKN2A/p16 in Neuroendocrine Tumor Cells and Restrains Tumor Cell Growth. To support the data that silencing of the CDKN2A/p16 gene in QGP-1 cells was a functional consequence of aberrant gene methylation, QGP-1 and BON tumor cells were treated with the methyltransferase-inhibitor 5-Aza-CdR, an agent that resulted in DNA demethylation. After a 4-day treatment, 5-Aza-CdR substantially restored not only CDKN2A/p16 but also CDKN2B/p15 expression in QGP-1 cells (Fig. 3A) but not in BON cells. This indicated that aberrant methylation of 5’CpG islands in the QGP-1 cells participated in the transcriptional inactivation not only of the CDKN2A/p16 gene but also of the CDKN2B/p15 gene. Demethylation-induced restoration of CDKN2Ap16 could also be demonstrated in the ductal pancreatic carcinoma cell line PC-44 but not in MiaPaCa-2 cell line, known to harbor a homozygous deletion of the CDKN2A/CDKN2D locus. Interestingly, 5-Aza-CdR demonstrated a reduced CDKN2D/p14 transcript level in QGP-1 cells. These data show that hypermethylation is an alternative mechanism for
CDKN2A/p16 and CDKN2B/p15 inactivation in neuroendocrine pancreatic tumor cells besides homozygous deletion. To determine whether reexpression of the CDKN2A/p16 transcript was accompanied by reexpression of the protein, we performed immunoblot analyses of cell lysates from 5-Aza-CdR-treated or untreated tumor cells. As shown in Fig. 3B, 5-Aza-CdR-treated QGP-1 cells demonstrated a clear induction of CDKN2A/p16 protein in contrast with untreated cells, whereas the drug marginally increased p16 protein expression in PC44 cells (Fig. 3B). To confirm that reexpression of p16 after 5-Aza-CdR treatment was attributable to demethylation of a methylated 5′CpG island of CDKN2A/p16, its methylation status in the cell lines before and after drug treatment was analyzed by MSP. As shown in Fig. 3C, 5-Aza-CdR treatment was associated with partial demethylation and reappearance of unmethylated CDKN2A/p16 alleles in both cell lines, QGP-1 and PC44, but they could not be detected before drug exposure. These data confirm that reexpression of CDKN2A/p16 was, in fact, attributable to demethylation of cytosines in the promoter region of the CDKN2A/p16 gene. To determine whether methylation directly affected cellular growth, we tested QGP-1 and PC44 cells for their proliferative potential in the presence or absence of 5-Aza-CdR. Significant growth inhibition was observed in both cell lines treated with 1 μM of 5-Aza-CdR (Fig. 4A). Moreover, cells exposed to 5-Aza-CdR adopted an enlarged and flattened shape characteristic of a senescent phenotype (Fig. 4B). Thus, besides its influence on proliferation, demethylation may also exert effects on cellular differentiation. Although the growth-suppressive effects of 5-Aza CdR could be attributable to up-regulation of p16 and p15 in QGP-1 cells, demethylation-associated phenotypic changes are unlikely to be solely attributable to their restored function but rather a consequence of reexpression of additional genes silenced by aberrant de novo methylation.

DISCUSSION

In the present study we provide experimental evidence for genetic and epigenetic inactivation of the tumor suppressor genes CDKN2A/p16, CDKN2D/p14, and CDKN2B/p15 in neuroendocrine GEP tumor cells. The CDK inhibitor gene CDKN2A/p16 represents a major target in human carcinogenesis and has been shown to be functionally inactivated in a wide variety of malignancies (17, 18). In our study, loss of CDKN2A/p16 expression was detected exclusively in small intestinal carcinoids and nonfunctional neuroendocrine pancreatic tumors, not in gastrinomas or insulinomas. Cell culture models revealed the underlying mechanisms for CDKN2A/p16 expression loss as either homozygous deletion of the CDKN2A locus or transcriptional silencing because of CDKN2A/p16 promoter hypermethylation. The results after partial demethylation with 5-Aza-CdR treatment suggest that bi-allelic hypermethylation is required for CDKN2A/p16 inactivation, which has also been demonstrated as a prerequisite for hMLH1 promoter hypermethylation in colorectal cancers (19). Because recent experimental evidence implicated a role for p16 in fundamental cellular processes as well as cell cycle control (18, 20–24), restoration of normal p16 function after exposure to 5-Aza-CdR might alone restore growth control in QGP-1 cells, although other growth-regulatory genes silenced by de novo methylation are also likely to be involved. Muscarella et al. (10) reported the presence of CDKN2A/p16 hypermethylation and homozygous deletion in a limited number of gastrinomas and two nonfunctional pancreatic tumors. However, these data were not confirmed by evaluating CDKN2A/p16 expression, and tumor specimens analyzed by this group would, in fact, be expected to show an intact CDKN2A/p16 transcript because: (a) MSP demonstrated strong expression of unmethylated PCR products; and (b) specimens thought to harbor homozygous deletions for CDKN2A/p16 surprisingly showed prominent unmethylated CDKN2A/p16 PCR products.

In addition to the p16 protein, other members of the INK4 family also play minor roles in tumorigenesis (25). In our study, some neuroendocrine GEP tumors demonstrated an expression pattern implicating CDKN2D/p14 independently in neuroendocrine tumorigenesis. A recent gene-targeting study has confirmed the importance of p14 as a potent growth- and tumor-suppressor when acting alone (26). Mice with disrupted CDKN2D/p14 (mouse homologue to CDKN2D/p14) but intact CDKN2A/p16 expression revealed a susceptibility to cancer quite similar to that of mice with a deletion of the entire CDKN2A/CDKN2D locus (17, 26). Moreover, recent studies in colorectal cancer cell lines demonstrated that the CDKN2D/p14 promoter can become methylated de novo and transcriptionally silenced despite an adjacent unmethylated CDKN2A/p16 promoter (27). Because intragenic CDKN2D/p14 mutations have not been reported, aberrant CDKN2D/p14 promoter methylation is the likely mechanism of selective CDKN2D/p14 silencing in a small subset of primary GEP tumors. Demethylation of QGP-1 cells with restoration of CDKN2A/p16 expression was accompanied by reduced CDKN2D/p14 transcript level. This is in accordance with observations that have indicated a direct relationship between methylation of the CDKN2A exon 1a CpG island and expression of the CDKN2D/p14 transcript, where compaction of the chromatin around the promoter is facilitated, thus favoring initiation of the CDKN2D/p14 transcript (28). Loss of p14 function might explain unresponsiveness of some GEP tumors to IFN-α, which induces G1 phase arrest through up-regulated expression of p14 and p21 (29).

A recent study provided evidence for the action of p15 as a tumor suppressor by inhibition of cellular transformation by Ras and other oncogenes (30). The expression data in some GEP tumors indicate an independent role of p15, most notably in gastrinomas and insulino-
mas. Besides the homozygous deletion detected in BON cells, 5-Aza-CdR-induced up-regulation of CDKN2B/p15 in QGP-1 cells suggests transcriptional repression in conjunction with aberrant CDKN2B/p15 hypermethylation. The promoter region of CDKN2B/p15 contains a CpG island, which is hypermethylated in many hematological malignancies, and CDKN2B/p15 inactivation independent of the CDKN2A/p16 gene has been demonstrated in acute myelogenous leukemia and high-risk myelodysplastic syndromes (31).

Despite their histological similarities, the subset-specific tumor-suppressor expression patterns in the GEP tumors support the concept that GEP tumors are characterized by divergent molecular profiles. This is consistent with our previous work demonstrating DPC4/Smad4 gene alterations in nonfunctional pancreatic tumors but not in insulinomas or gastrinomas (32). Thus, genetic profiling of GEP tumors could provide sensitive molecular markers to refine diagnosis and improve therapeutic decisions.

Analysis of fresh neuroendocrine GEP tumor samples by RT-PCR presented a challenge, because contamination with residual normal cells might override the putative negative signals from the neoplastic cells. Thus, the actual frequency of alterations in the tumors might be...
even higher than that detected in our study. Unfortunately, DNA from the primary tumors was not available to allow confirmation of these findings. However, diagnostic assays for molecular profiling would be best based on the absence of a gene product rather than on the detection of structural gene alterations.

It has been recognized that the CpG islands of a growing number of genes involved in carcinogenesis are methylated in many types of cancer (33). Genes silenced by methylation error are very sensitive to inhibitors of DNA methylation. The neuroendocrine GEP tumors are attractive targets for such treatment, because the growth-regulating genes are not initially mutated or lost, and their function can still be restored. In rats, 5-Aza-CdR was demonstrated to reactivate effectivelv a dormant CDKN2A/p16 gene in vivo in bladder tumors with concomitant growth restraint (34). Suppression of intestinal neoplasia by DNA hypomethylation has been shown in MIN mice, where 5-Aza-CdR had a dramatic effect on the number of intestinal polyps but required early administration for this effect (35). Thus, epigenetic alterations in gastrinomas and nonfunctioning pancreatic neuroendocrine tumors: an update. J. Clin. Endocrinol.


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