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

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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 oncoproteins (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 1β originating ~20 kb centromerically to CDKN2A/p16 exon 1α. 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'-CAACGACCGATACTTACC-3'), p16R (5'-GGTGACCCTGACATCGCAT-3'), p15F (5'-ATGGGCGAGGAGAACAAGGCCCAT-3'), p15R (5'-GGGCGGGCTGGGAACTGCGTCGA-3'), p19F (5'-AGTGCGGCTGGTCTACCTC-3'), p18F (5'-ATGGCCGGAGCTGAATGGGG-3'), p18R (5'-TATTTAAGGATTTGTTGCTCC-3'), p21F (5'-CTATGGACTACGCACTCCCA-3'), p21R (5'-TTAGGCTTCCTCTTGAGGA-3'), p27F (5'-ATGCATAACGGTTGCGAGTCTG-3'), and p27R (5'-TTCGTTTCTGACTGCTT-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 PRISM310; Perkin-Elmer).

SSCP and Mutation Analysis. After DNA extraction (QIAamp Tissue kit; QIAGen) CDKN2A/p16 mutations were evaluated using SSCP in five separate PCR amplifications (13). PCR products were visualized by autoradiography. Absent 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 nonmethylated cytosine residues to uracil. Seminested PCR was performed as a diagnostic step using primers p16 Mf/p16 M2r for the first and p16 Mf/p16 nonmethylated cytosine residues to uracil. Seminested PCR was performed as a diagnostic step using primers p16 Mf/p16 M2r for the first and p16 Mf/p16 Mf, for the second amplification. Amplification of nonmethylated-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).

Immunoblot Analysis of p16. Protein lysates were separated by 14% SDS polyacrylamide gels and electrotransferred 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 their expression profiles in two human cell culture lines with known CDKN2A/p16 mutation status served as controls.

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 23–25 and 28), less frequently in insulinomas (3 of 10 tumors; 30%; Fig. 1, Lanes 12 and 15–17) and gastrinomas (2 of 9 tumors; 22%; Fig. 1, Lanes 3 and 9). To evaluate independent expression, the unique first exon 1α was amplified for CDKN2A/p16 and exon 1β for CDKN2D/p14. None of the gastrinomas or insulinomas revealed loss of CDKN2A/p16 expression. With the exception of one insulinoma (Fig. 1, Lane 3), both tumor types also revealed strong CDKN2D/p14 expression. The weakly expressed CDKN2D/p14 PCR product in one insulinoma (Fig. 1, Lane 12) could be attributable to amplification from contaminating normal cells, despite loss of CDKN2B/p14 in tumor cells. This would increase the percentage of genetic alterations in the insulinomas to 44%. However, only complete or almost complete loss was assessed for transcriptional alteration. Gastrinomas and insulinomas revealed preferential loss of CDKN2B/p15 expression in 2 of 9 (22%; Fig. 1, Lanes 3 and 9) and 3 of 10 tumors (30%; Fig. 1, Lanes 15–17), respectively. Interestingly, only gastrinomas with metastases to the liver revealed CDKN2B/p15 alteration. Some reduction of CDKN2B/p15 expression was noted in one additional gastrinoma (Fig. 1, Lane 2) and two insulinomas (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+ and 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.
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 locus, whereas the QGP-1 cell line transcribed no detectable CDKN2A/p16 transcript, weak CDKN2B/p15 mRNA signal, and intact CDKN2D/p14 mRNA (Fig. 2A). These expression patterns, CDKN2A "/CDKN2B " and CDKN2A "/CDKN2D ", resembled those detected in the primary neuroendocrine pancreatic and small intestinal tumors analyzed previously (Fig. 1). Whereas the CDK inhibitor CDKN1B/p27 showed higher expression levels in QGP-1 cells, CDKN1A/p21 and CDKN2C/p18 were equally expressed in both cell lines (Fig. 2A). To evaluate the CDKN2A/p16 genomic status we performed SSCP analysis and revealed an intact CDKN2A/p16 gene in QGP-1 cells without indication of loss or gene mutation (Fig. 2B, Lanes 2 and 6 depict the exon 2 amplification). No specific CDKN2A/p16 PCR products were generated in BON cells, indicating homozygous deletion of the CDKN2A locus (Fig. 2B, Lanes 1 and 5). The aberrant bands in exon 2b (Fig. 2B, Lane 1) were confirmed as unspecific amplifications by sequence analysis. Because QGP-1 cells revealed an intact CDKN2A/p16 genomic sequence, epigenetic changes, particularly de novo methylation, were suspected to be associated with gene inactivation. The prevalence of CDKN2A/p16 promoter methylation was determined using MSP, which distinguishes unmethylated from hypermethylated alleles in a given gene based on sequence changes produced after bisulfite treatment of the DNA and subsequent PCR. Using primers specific to either methylated or unmethylated DNA, MSP displayed de novo methylation of the CpG island of CDKN2A/p16 in QGP-1 cells (Fig. 2C, Lane 2), explaining the selective transcriptional block of the CDKN2A/p16 gene. 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 CDKN2A/p16 could also be demonstrated in the ductal pancreatic carcinoma cell line PC-44 but not in the 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 in-tragenic 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 insulinomas.
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 effectively 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 lesions might drive specific genetic lesions in cancers (33). This has also been suggested for microsatellite instability and double-strand breaks in DNA, which occur as a consequence of promoter hypermethylation of the MSH2 locus. Demethylating drugs such as 5-Aza-CdR have already been subjected to clinical scrutiny; thus, much is known about their pharmacokinetics for future clinical trials (37). Identification of the critical molecular events in initiation, progression, and latency of neuroendocrine GEP tumors is an important future challenge, which could enable selection of patients on the basis of histological characteristics and lead to novel clinical therapeutic applications.

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