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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 insulinomas (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[2] 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 ≤80% of MEN1-patients who harbor *MEN1* germ-line mutations on chromosome 11q13 (2). Although *MEN1* gene mutations are also 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 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-^{33}P]dCTP (ICN, Meckenheim, Germany) used these primers: p16F (5′-CAACGCACCGAATAGTTACG-3′), p16R/p19R (5′-GGTACCGTGCGACATCGCGAT-3′), p15F (5′-ATGCGCGAGGAGAACAAGGGCAT-3′), p15R (5′-GGGCGGCTGGGGAACCTGGGCG TCA-3′), p19F (5′-AGTGGCGCTGCTCACCTC-3′), p18F (5′-ATGGCCGAGCCTTGGGGGAACG-3′), p18R (5′-TTATTGAAGATTTGTGGCTCC-3′), p21F (5′-CCATGTCAGAACCGGCTGGGG-3′), p21R (5′-TTAGGGCTTCCTCTTGGAGA), p27F (5′-ATGTCAAACGTGCGAGTGTC-3′), and p27R (5′-TTACGTTTGACGTCTTCTGAG-3′). Integrity of the RNA isolated from the samples was verified by β-actin amplification (11). Different amplicons served as internal controls

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[2] Abbreviations: GEP, gastroenteropancreatic; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; Rb, retinoblastoma; CDK, cyclin-dependent kinase; 5-Aza-CdR, 5-aza-2-deoxycytidine; STS, sequence-tagged site; SSCP, single-strand conformation polymorphism; DAPI, 4′,6-diamidino-2-phenylindole; BrdUrd, 5-bromo-2′deoxyuridine.

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. 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 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/p16M_r 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 determined 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; 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 *CDKN2D/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*$^+$/*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, *Lane 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.

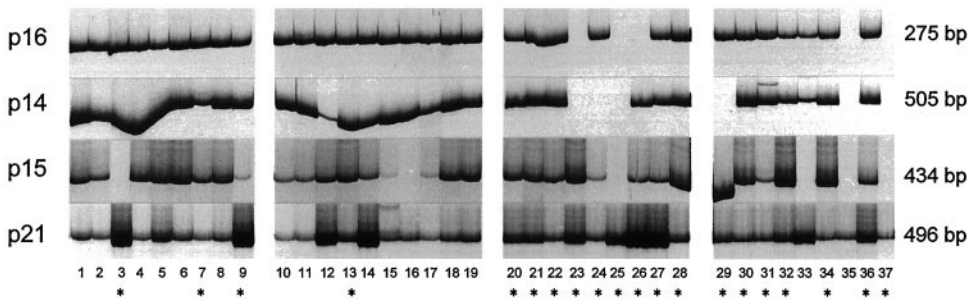

Fig. 1. Expression pattern of *CDKN2A/p16*, *CDKN2B/p15*, *CDKN2D/p14*, and *CDKN1A/p21* as determined by RT-PCR in subsets of primary neuroendocrine GEP tumors. Gastrinomas (*Lanes 1–9*), insulinomas (*Lanes 10–19*), small intestinal carcinoids (*Lanes 20–28*), nonfunctional tumors of the pancreas (*Lanes 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.

Table 1 *CDK inhibitor expression in neuroendocrine GEP tumors*

	Loss of gene expression			
	Σ[a]	*CDKN2B/p15*	*CDKN2A/p16*	*CDKN2D/p14*
Gastrinomas	2/9 (22%)	2/9 (22%)	0/9	0/9
No metastases		0/3		
Metastases		2/6 (33%)		
Insulinomas	3/10 (30%)	3/10 (30%)	0/10	0/10
No metastases		3/9 (33%)		
Metastases		0/1		
Small intestinal carcinoids	4/9 (44%)	2/9 (22%)	3/9 (33%)	3/9 (33%)
Metastases				
Functionally inactive tumors	4/9 (44%)	4/9 (44%)	2/9 (22%)	3/9 (30%)
Pancreas	4/7 (57%)	4/7 (44%)	2/7 (28%)	3/7 (43%)
No metastases		2/2 (100%)	1/2 (50%)	1/2 (50%)
Metastases		2/5 (40%)	1/5 (20%)	2/5 (40%)
Stomach	0/1			
Metastases				
Small intestine	0/1			
Metastases				

[a] No. (percentage) of all tumors in a subgroup with lost transcript expression of at least one of the tumor suppressor genes.

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. 2*A*). These expression patterns, $CDKN2A^{-}/CDKN2D^{+}$ 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. 2*A*). 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. 2*B, 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. 2*B, Lanes 1* and *5*). The aberrant bands in exon 2b (Fig. 2*B, 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. 2*C, 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. 2*C, 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. 3*A*) 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

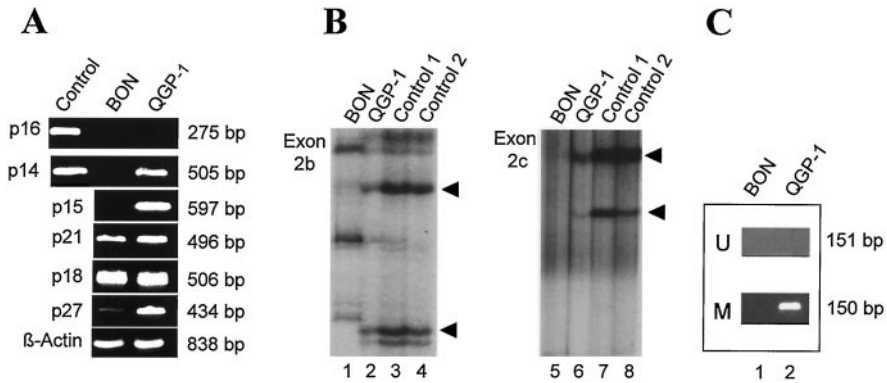

Fig. 2. *A*, RT-PCR analysis of *CDKN2D/p14, CDKN2B/p15, CDKN2A/p16, CDKN2C/p18, CDKN1A/p21,* and *CDKN1B/p27* in neuroendocrine BON and QGP-1 cells. *Left,* transcript description; *right,* size of the PCR products. *B,* SSCP analysis of *CDKN2A/p16* exons 2b and 2c. Autoradiographs show an intact *CDKN2A/p16* gene (◀) in QGP-1 cells (*Lanes 2* and *6*) and controls (*Lanes 3–4, 7–8*). BON cells (*Lanes 1* and *5*) demonstrate loss of *CDKN2A/p16* (aberrant bands in *Lane 1* represent unspecific amplifications). *C,* detection of *CDKN2A/p16* 5′CpG island methylation in BON and QGP-1 cells by MSP. The 151-bp PCR product (*U*, primers for bisulfite-modified unmethylated DNA) indicates the presence of an unmethylated allele; the 150-bp PCR product (*M*, primers for bisulfite-modified methylated DNA) indicates the presence of a methylated allele. BON cells (*Lane 1*) showed no PCR products confirming homozygous deletion of the *CDKN2A locus*. Hypermethylation in QGP-1 cells (*Lane 2*) is indicated by the methylated 150-bp PCR product.

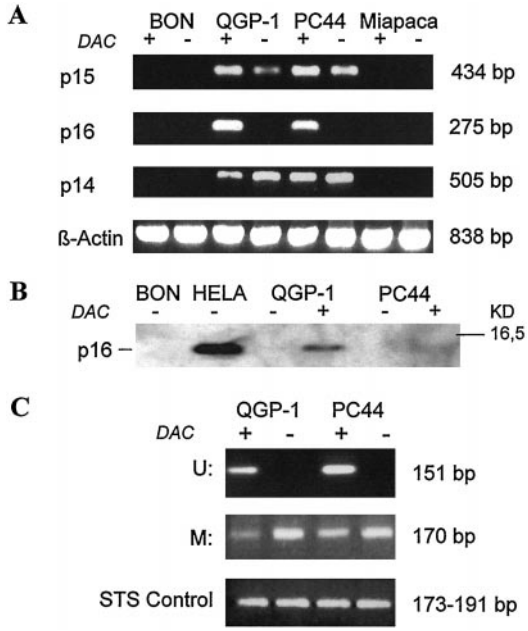

Fig. 3. Effect of 5-Aza-CdR on tumor-suppressor expression. *A*, RT-PCR analysis of *CDKN2B/p15*, *CDKN2A/p16*, and *CDKN2D/p14* expression in pancreatic tumor cell lines in the presence (+) or absence (−) of 1.0 μM 5-Aza-CdR (*DAC*) for 96 h. RNA integrity was verified by β-actin amplification. BON and QGP-1, neuroendocrine pancreatic cell lines; PC44 and Miapaca-2, ductal pancreatic carcinoma cell lines. *B*, immunoblot analysis of p16 expression dependent on 5-Aza-CdR (*DAC*) treatment. Equivalent amounts of protein from treated (+) and untreated (−) cells were analyzed for comparison. HeLa cells were used as positive control for p16 protein expression. *C*, analysis of DNA methylation of *CDKN2A/p16* 5′CpG island before (−) and after (+) 5-Aza-CdR (*DAC*) treatment by MSP. *M*, primers for bisulfite-modified methylated DNA. *U*, primers for bisulfite-modified unmethylated DNA. A STS amplicon on chromosome 7 (D7S494) served as control.

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. 3*B*, 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. 3*B*). 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. 3*C*, 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. 4*A*). Moreover, cells exposed to 5-Aza-CdR adopted an enlarged and flattened shape characteristic of a senescent phenotype (Fig. 4*B*). 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/p19* (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 1α 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 G_1 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-

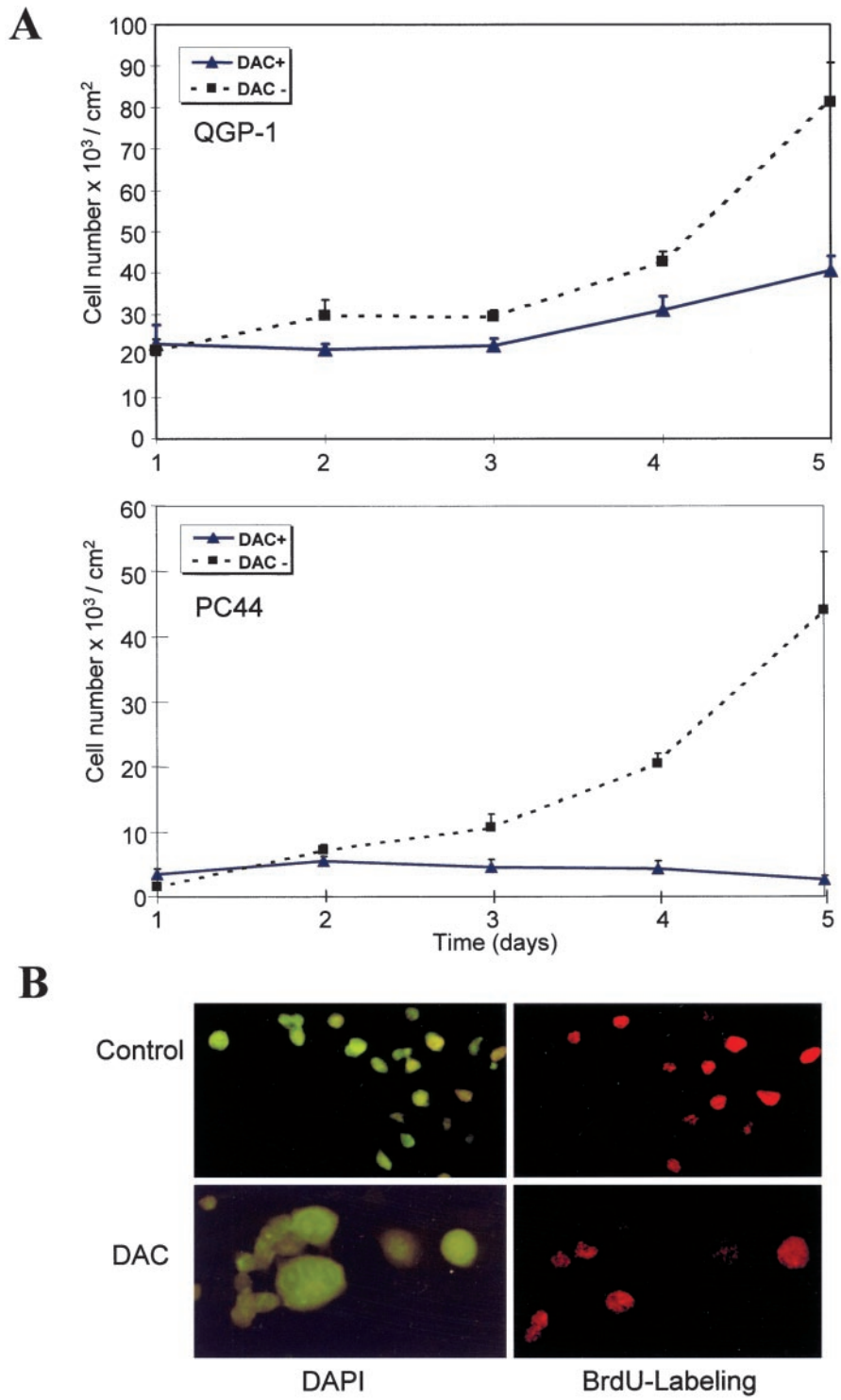

Fig. 4. *A*, effect of 5-Aza-CdR (*DAC*) on growth of QGP-1 and PC44 cells in culture. Exponentially growing cells were treated with (*DAC+*) or without (*DAC−*) 1.0 μM 5-Aza-CdR for 96 h. Growth inhibition was determined every 24 h. *Symbols*, averages of three counts; *bars*, ± SD. *B*, effect of 5-Aza-CdR (*DAC*) on the morphology of PC44 cells. Representative sections showing DAPI staining (*left*) and immunofluorescent staining of BrdUrd-labeled cells (*right*) in the absence (*control*) or presence (*DAC*) of 1.0 μM 5-Aza-CdR for 72 h. The percentage of S-phase (BrdUrd-positive) nuclei relative to the total number of nuclei revealed no significant difference in the treated *versus* untreated cells.

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 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 DNA mismatch repair gene *hMLH1* or the familial breast cancer gene *BRCA1*, respectively (36). Thus, it is conceivable that *de novo* methylation of tumor suppressor genes, *e.g., CDKN2A/p16* or *CDKN2B/p15* in neuroendocrine GEP tumors, might precede irreversible genomic mutations, *e.g.*, homozygous deletion of the *CDKN2A/CDKN2D* 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 methylation defects and lead to novel clinical therapeutic applications.

REFERENCES

1. Rindi, G., Capella, C., and Solcia, E. Cell biology, clinicopathological profile, and classification of gastroenteropancreatic endocrine tumors. J. Mol. Med., *76:* 413–420, 1998.
2. Bartsch, D., Kopp, I., Bergenfelz, A., Rieder, H., Münch, K., Jäger, K., Deiss, Y., Schudy, Y., Barth, A., Arnold, R., Rothmund, M., and Simon, B. *MEN1* gene mutations in 12 MEN1 families and their associated tumors. Eur. J. Endocrinol., *139:* 416–420, 1998.
3. Görtz, B., Roth, J., Krähenmann, A., Krijer, R. R., Muletta-Feurer, S., Rütimann, K., Saremaslani, P., Speel, E. J., Heitz, P. U., and Komminoth, P. Mutations and allelic deletions of the *MEN1* gene are associated with a subset of sporadic endocrine pancreatic and neuroendocrine tumors and not restricted to foregut neoplasms. Am. J. Pathol., *154:* 429–436, 1999.
4. Yoshimoto, K., Iwahana, H., Fukuda, A., Sano, T., Saito, S., and Itakura, M. Role of p53 mutations in endocrine tumorigenesis: mutation detection by polymerase chain reaction-single strand conformation polymorphism. Cancer Res., *52:* 5061–5064, 1992.
5. Chung, D. C., Smith, A. P., Louis, D. N., Graeme-Cook, F., Warshaw, A. L., and Arnold, A. Analysis of the retinoblastoma tumor suppressor gene in pancreatic endocrine tumors. Clin. Endocrinol., *47:* 523–528, 1997.
6. Harvey, M., Vogel, H., Lee, E., Bradley, A., and Donehower, L. Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. Cancer Res., *55:* 1146–1151, 1995.
7. Haber, D. A. Splicing into senescence: the curious case of p16 and p19ARF. Cell, *91:* 555–558, 1997.
8. Serrano, M., Lee, H. W., and Chin, L. Role of the INK4a locus in tumor suppression and cell mortality. Cell, *85:* 27–37, 1996.
9. Hannon, G. J., and Beach, D. p15INK4B is a potential effector of TGF-β-induced cell cycle arrest. Nature (Lond.), *371:* 257–261, 1994.
10. Muscarella, P., Scott, M., Fisher, W. E., Foor, J., Ellison, E. C., Herman, J. G., Schirmer, W. J., Hitchcock, C. L., DeYoung, B. R., and Weghorst, C. M. Genetic alterations in gastrinomas and nonfunctioning pancreatic neuroendocrine tumors: an analysis of *p16/MTS1* tumor suppressor gene inactivation. Cancer Res., *58:* 237–240, 1998.
11. Wulbrand, U., Wied, M., Zofel, P., Goke, B., Arnold, R., and Fehmann, H. Growth factor receptor expression in human gastroenteropancreatic neuroendocrine tumours. Eur. J. Clin. Investig., *28:* 1038–1049, 1998.
12. Simon, B., Bartsch, D., Barth, P., Prasnikar, N., Munch, K., Blum, A., Arnold, R., and Goke, B. Frequent abnormalities of the putative tumor suppressor gene *FHIT* at 3p14.2 in pancreatic carcinoma cell lines. Cancer Res., *58.* 1583–1587, 1998.
13. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., and Dracopoli, N. C. Germline p16 mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.
14. Kersting, M., Friedl, C., Kraus, A., Behn, M., Pankow, W., and Schuermann, W. Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers. J. Clin. Oncol., *18:* 3221–3229, 2000.
15. Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morisette, J., and Weissenbach, J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature (Lond.), *380:* 152–154, 1996.
16. Ellwart, J., and Dörmer, P. Effect of 5-fluoro-2′deoxyuridine (FdUrd) on 5-Bromo-2-Deoxyuridine (BrdUrd) incorporation into DNA measured with a monoclonal BrdUrd antibody and by the BrdUrd/Hoechst quenching effect. Cytometry, *6:* 513–520, 1985.
17. Serrano, M. The tumor suppressor protein p16INK4a. Exp. Cell Res., *237:* 7–13, 1997.
18. Liggett, W. H., and Sidransky, D. Role of p16 tumor suppressor gene in cancer. J. Clin. Oncol., *16:* 1197–1206, 1998.
19. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., and Baylin, S. B. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 6870–6875, 1998.
20. Harada, H., Nakagawa, S., Iwata, S., Saito, M., Kumon, Y., Sakaki, K., Sato, K., and Hamada, K. Restoration of wild-type *p16* down-regulates vascular endothelial growth factor expression and inhibits and angiogenesis in human gliomas. Cancer Res., *59:* 3783–3789, 1999.
21. Chintala, S. K., Fueyo, J., Gomez-Manzano, C., Venkaiah, R., Bjerkvig, W. K., Yung, R., Sawaya, A. P., Kyritsis, A. P., and Rao, J. S. Adenovirus-mediated $p16^{INK4a}$/CDKN2 gene transfer suppresses glioma invasion *in vitro*. Oncogene, *23:* 2049–2057, 1997.
22. Fahraeus, R., and Lane, D. P. The $p16^{INK4a}$ tumor suppressor protein inhibits $\alpha_v\beta_3$ integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of $\alpha_v\beta_3$ to focal contacts. EMBO J., *18:* 2106–2118, 1999.
23. Naruse, I., Heike, Y., Hama, S., Mori, M., and Saijo, N. High concentrations of recombinant adenovirus expressing $p16^{INK4a}$ gene induces apoptosis in lung cancer cell lines. Anticancer Res., *18:* 4275–4282, 1998.
24. Plath, T., Detjen, K., Welzel, M., Marschall, Z., Murphy, D., Schirner, M., Wiedenmann, B., and Rosewicz, S. A novel function for tumor suppressor $p16^{INK4a}$: induction of anoikis via up-regulation of the $\alpha_5\beta_1$ fibronectin receptor. J. Cell Biol., *150:* 1467–1477, 2000.
25. Ruas, M., and Peters, G. The p16INK4a/CDKN2A tumor suppressor gene and its relatives. Biochim. Biophys. Acta, *137:* 115–177, 1998.
26. Kamijo, T., Zindy, F., and Roussel, M. F. Tumor suppression at the mouse *INK4a* locus mediated by alternative reading frame product $p19^{ARF}$. Cell, *91:* 649–659, 1997.
27. Esteller, M., Tortola, S., Toyota, M., Capella, G., Peindo, M. A., Baylin, S. B., and Herman, J. G. Hypermethylation-associated inactivation of *p14*(ARF) is independent of *p16*(INK4a) methylation and *p53* mutational status. Cancer Res., *60:* 129–133, 2000.
28. Larsen, C. J. Contribution of the dual coding capacity of the p16INK4a/MTS/CDKN2 locus to human malignancies. Prog. Cell Cycle Res., *3:* 109–124, 1997.
29. Matsuoka, M., Tani, K., and Asano, S. Interferon-α-induced G1 phase arrest through up-regulated expression of CDK inhibitors, $p19^{INK4D}$ and $p21^{CIP1}$ in mouse macrophages. Oncogene, *16:* 2075–2086, 1998.
30. Malumbres, M., Perez de Castro, I., Hernandez, M. I., Jimenez, M., Corral, T., and Pellicer, A. Cellular response to oncogenic ras involves induction of the cdk4 and cdk6 inhibitor $p15^{INK4b}$. Mol. Cell. Biol., *20:* 2915–2925, 2000.
31. Uchida, T., Kinoshita, T., Hotta, T., and Murate, T. High-risk myelodysplastic syndromes and hypermethylation of the p15INK4B gene. Leuk. Lymphoma, *32:* 9–18, 1998.
32. Bartsch, D., Hahn, S. A., Danichevski, K., Ramaswamy, A., Bastian, D., Galehdari, H., Barth, P., Schmiegel, W., Simon, B., and Rothmund, M. Mutations of the *DPC4/Smad4* gene in neuroendocrine pancreatic tumors. Oncogene, *18:* 2367–2371, 1999.
33. Baylin, S. B., and Herman, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet., *16:* 168–174, 2000.
34. Bender, C. M., Pao, M. M., and Jones, P. A. Inhibition of DNA methylation by 5-aza-2′deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res., *58:* 95–101, 1998.
35. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A., and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. Cell, *81:* 197–205, 1995.
36. Esteller, M. Epigenetic lesions causing genetic lesions in human cancer promoter hypermethylation of DNA repair genes. Eur. J. Cancer, *36:* 2294–3000, 2000.
37. Wijermans, P., Lubbert, M., Verhoef, G., Bosly, A., Ravoet, C., Andre, M., and Ferrant, A. Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J. Clin. Oncol., *18:* 956–962, 2000.

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

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