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Advances in Brief

Frequent Loss of the $P16^{INK4a}$ Gene Product in Human Pituitary Tumors[1]

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Abstract

Pituitary tumors develop at a high frequency in *retinoblastoma (Rb)*-knockout mice; however, defects in the *Rb* gene are not common in human pituitary tumors. The inverse correlation of *Rb* and *p16* defects in certain human tumors has led us to investigate the expression of p16 in human pituitary tumors as an indirect mechanism of Rb inactivation. By Western blot analysis, the *p16* gene product was undetectable in 25 human pituitary tumors, whereas high levels of p16 could be demonstrated in 10 normal human pituitary specimens under the same conditions of protein extraction and immunoblotting. Similar results were obtained at the mRNA level with low to undetectable levels of p16 mRNA in 13 of 14 pituitary tumors relative to 5 normal pituitary specimens. Single-strand conformation polymorphism analysis of *p16* exons 1 and 2 revealed no mobility shifts in 25 tumors; however, a quantitative differential PCR analysis revealed diminished amplification of *p16* relative to a control gene in 3 of 25 tumors, suggesting homozygous *p16* gene loss. We conclude that altered expression of the *p16* gene product occurs at a high frequency in human pituitary tumors. This altered expression is not associated with frequent *p16* mutation or gene loss, suggesting that alternative mechanisms of gene inactivation and/or altered regulation occur in the majority of these tumors.

Introduction

In 1990, studies using X-allelic inactivation determined that human pituitary tumors are monoclonal in origin (1). Although mutations in the *gsp* oncogene have been reported for a subset of GH[3]-secreting pituitary tumors (2), this subset represents only a small fraction of all pituitary tumors, and subsequent studies over the past several years have yielded limited information on the origins of the majority of human pituitary tumors. The high incidence of pituitary tumors that develop in heterozygous *Rb*-knockout mice has implicated the Rb pathway in pituitary tumorigenesis (3). These mice develop pituitary tumors at the frequency of 100% associated with loss of the wild-type *Rb* allele in the pituitary tumor tissue. Previously, our laboratory and others have reported that LOH at the *Rb* locus is not common in human pituitary tumors (4, 5). Although Pei *et al.* (6) have reported LOH at the *Rb* locus in rare human pituitary carcinomas and in a subset of aggressive benign pituitary tumors, they found that this allelic loss was not associated with loss of Rb at the protein level, and they concluded that a locus close to *Rb* is likely to be important in these tumors (6). In a more extensive analysis, our laboratory has reported the absence of *Rb* mutations in exons 20–24 of the *Rb* gene and the expression of Rb protein by immunoblotting in all 24 human pituitary tumors examined (7). Altogether, these studies suggest that the *Rb* gene itself is not an important target in human pituitary tumorigenesis.

Recently, several CDK inhibitors have been identified, which have important roles in controlling the cell cycle and have been found to be disrupted in a high number of human tumors (reviewed in Ref. 8). p16 is a specific inhibitor of CDK4, the CDK that phosphorylates Rb to its inactive form. p16 functions in a feedback regulatory loop with the Rb protein, and its disruption represents an important indirect mechanism of Rb inactivation (9). *p16* has rapidly come under intense investigation as a tumor suppressor gene, because it has been found to be inactivated in a wide variety of human tumor-derived cell lines (10) and several specific types of primary human tumors (11, 12). This inactivation occurs by homozygous deletions and point mutations (10–12), as well as by alternative mechanisms such as gene methylation (13, 14). Interestingly, alterations of *p16* and *Rb* appear to have an inverse correlation in certain types of tumors, such as lung carcinoma, in that wild-type p16 protein accumulates to high levels in tumor cells with *Rb* disruptions, whereas *p16* disruptions (and undetectable p16 protein) occur in tumor cells expressing wild-type Rb (15, 16). These reports have led us to speculate on the status of p16 in human pituitary tumors.

Materials and Methods

Tumor Specimens. Specimens were collected in accordance with a protocol approved by an institutional review board. Pituitary tumor specimens were obtained from 25 patients at the time of transphenoidal resection and were snap-frozen at −70°C until the time of protein, RNA, and DNA isolation. Normal postmortem pituitary specimens were obtained from the National Hormone and Pituitary Program (Bethesda, MD). Tumors were classified according to their immunohistochemical staining for adrenocorticotropic hormone, GH, prolactin, follicle-stimulating hormone β, luteinizing hormone β, and TSH-β using an avidin-biotin method. Tumors were also classified as invasive if they demonstrated invasion of the cavernous sinus, sphenoid bone or sinus, or cranial nerves, or if they infiltrated blood vessels or venous sinuses. NCI-H69 cells, used as a positive control for p16 expression, were obtained from the American Type Culture Collection (Bethesda, MD).

Protein Preparation. Tissues were lysed in a lysis buffer that contained 150 mM NaCl, 25 mM Tris (pH 7.5), 0.02% sodium azide, 1% NP40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A. An equal volume of 2× SDS loading buffer [100 mM Tris (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol] was added, and the samples were boiled for 10 min. Protein was quantitated by a modified Lowry method.

Western Blots. One hundred μg total protein were loaded onto SDS-polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electrotransfer. Membranes were blocked for 60 min in 5% dry milk, probed for 1 h with primary antihuman p16 antibody (15126E or 13251A; PharMingen, Inc., San Diego, CA), and then probed with sheep antimouse secondary antibody. The antibody reaction was revealed by chemiluminescence detection, according to the manufacturer's recommendations (Amersham, Arlington Heights, IL). After exposure, the blots were stripped and reprobed with anti-β-actin antibody (Ab-1; Oncogene Science, Manhasset, NY) to confirm protein integrity and equal loading.

Preparation of Total RNA. Tissues were homogenized in a lysis buffer containing 0.3 M sucrose, 10 mM Tris, 1.5 mM $MgCl_2$, 0.5% NP40, and 0.25% sodium deoxycholate and then layered onto a cushion buffer containing 0.4 M sucrose, 10 mM Tris, and 1.5 mM $MgCl_2$. The lysed cells were centrifuged

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[3] The abbreviations used are: GH, growth hormone; SSCP, single-strand conformation polymorphism; Rb, retinoblastoma; LOH, loss of heterozygosity; CDK, cyclin-dependent kinase; TSH, thyrotropin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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through the gradient at a speed of 3000 rpm for 10 min. The cytoplasmic fraction was removed and treated with 120 μg proteinase K in 1% SDS, 10 mM Tris, and 5 mM EDTA for 1 h. The cytoplasmic RNA was treated with 10 units of RNase-free DNase I for 15 min at 37°C. After phenol-chloroform extraction, the RNA was precipitated with 100% ethanol. Quantitation of total RNA was performed by spectrophotometry using absorbance at 260 nm.

RT-PCR. Ten to 15 μg total RNA isolated from pituitary tumors and normal pituitary specimens were subjected to RT using oligodeoxythymidylic acid, followed by PCR amplification using the p16 primer and GAPDH primer sets listed in Table 1. Thirty to 40 cycles of the reaction were performed at 94, 55, and 72°C for 1, 2, and 3 min, respectively in a Power Block thermal cycler (Ericomp, San Diego, CA). Quantitation was performed by PCR incorporation of [^{32}P]dCTP as well as blotting of PCR products and hybridization to cDNA probes, followed by phoshphorimager analysis. For each sample, values were expressed as the signal ratio p16:GAPDH.

Genomic DNA Preparation. Genomic DNA was prepared by incubating tissue fragments in 500 μl isolation solution containing 0.1 M NaCl, 0.001 M Tris, 0.001 M EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K for 4 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellet was reconstituted in 1× TE [10 mM Tris (pH 8.0) and 1 mM EDTA] and quantitated using absorbance at 260 nm.

SSCP Analyses. Primers used for SSCP are listed in Table 1. DNA prepared from LD600 cells, containing a known *p16* mutation (17), was used as a control. Normal and tumor DNA samples were subjected to PCR containing 10 pmol/μl of each respective primer, 10 mmol each of four deoxynucleotides, 50–200 ng genomic DNA, 5 units *Taq* Polymerase (Promega Corp., Madison, WI), 5 μl 10× *Taq* buffer, and 1 μl [^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml; DuPont New England Nuclear, Boston, MA). Twenty-five to 30 cycles of the reaction were performed using similar conditions as described above. Two μl reaction mixture were mixed with 18 μl denaturing buffer (20 mmol EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol, and 95% formamide) and heated to 95°C for 5 min. One to 2 μl/lane were applied to a 6% polyacrylamide sequencing gel containing 90 mmol Tris-buffered EDTA, 4 mmol EDTA, and 10% glycerol. Electrophoresis was performed at 30 W for 17 h. The gel was dried and exposed to X-ray film at −70°C for 12 h.

Differential PCR Analysis. The ability to amplify *p16* exon 2 was compared with the ability to amplify a region of the human *β-actin* gene. PCR was performed using 100 ng genomic DNA, primers listed in Table 1, and similar conditions as described above. PCR products were analyzed by electrophoresis through 1% agarose, followed by blotting to nylon membranes and hybridization to ^{32}P-labeled *β*-actin and p16 DNA probes, labeled by random priming. Quantitation was performed by phosphorimager analysis of the dried membranes. Positive samples were repeated three times.

Results

Twenty-five human pituitary tumors were examined by Western blot analysis. All tumor samples were found to express undetectable levels of the p16 protein relative to high levels detected in protein samples prepared from normal human pituitary specimens and control NCI-H67 cells under the same conditions of protein extraction and immunoblotting (illustrated in Fig. 1). Probing with anti-*β*-actin antibody was performed in all cases to control for protein loading and integrity. These results were confirmed in more than 10 Western blots using 25 pituitary tumor specimens, 10 normal pituitary specimens, and 2 different antihuman p16 antibodies. The conditions of the antibody incubations were varied (including antibody concentrations and lengths of incubation), as were the methods of protein preparation and quantities of total protein loaded per lane (up to 200 μg/lane). Of the 25 tumors, 14 were considered nonfunctioning and did not stain by immunohistochemistry; 3 stained for adrenocorticotropic hormone; 3 stained for prolactin; 2 stained for GH; 1 stained for TSH-*β*; and 2 stained for follicle-stimulating hormone *β*, luteinizing hormone *β*, and TSH-*β*. Twelve were considered macroadenomas, and 5 were classified as invasive based on criteria described in "Materials and Methods." There were no notable differences in any of these tumors with respect to levels of p16 protein detected.

Table 1 *PCR primers*

Name	Primer sequence (5′ to 3′)	Use
p16X	ATGGAGCCTTCGGCTGACT	RT-PCR
p16Y	GAGCCTCTCTGGTTCTTTCA	RT-PCR
GAPDH1	CCACCCATGGCAAATTCCAT	RT-PCR
GAPDH2	TCTAGACGGCAGGTCAGGTCCACC	RT-PCR
p16a1	GAAGAAAGAGGAGGGGCTG	SSCP
p16b1	GCGCTACCTGATTCCAATTC	SSCP
p16a2	CATTCTGTTCTCTCTGGCAG	SSCP
p16b2	CTCAGATCATCAGTCCTCAC	SSCP
p16X2a	TCTGACCATTCTGTTCTCTC	Differential PCR
p16X2b	CTCAGCTTTGGAAGCTCTCA	Differential PCR
β-actin1	GACGAGGCCCAGAGCAAGAGA	Differential PCR
β-actin2	ACGTACATGGCTGGGGTGTTG	Differential PCR

To further investigate this alteration in p16 expression in these tumors, RNA was prepared from 14 pituitary tumors and 5 normal pituitary specimens and subjected to quantitative RT-PCR analysis in three separate experiments to determine the relative levels of p16 mRNA in these samples. Fig. 2*A* illustrates an analysis in which equal amounts of total RNA prepared from pituitary tumors (*Lanes T1-T4*) and normal pituitary samples (*Lane N1*) were subjected to RT using oligodeoxythymidylic acid, PCR using p16-specific primers, and blotting and hybridization to a p16 cDNA probe. In all experiments, p16 mRNA levels were normalized to the level of GAPDH mRNA detected in each sample by the same method. Shown in Fig. 2*B*, p16 mRNA was undetectable in six pituitary tumors, whereas in seven other tumors, levels ranged from 0.07 to 0.35 of the levels detected in normal pituitary specimens. In one nonfunctioning macroadenoma, the level of p16 mRNA was comparable to levels detected in the normal pituitary, although p16 protein was undetectable in this tumor.

The strikingly reduced levels of p16 protein and mRNA detected in human pituitary tumors led us to investigate these tumors for primary defects in the *p16* gene. *p16* gene defects reported in human tumors include point and missense mutations as well as homozygous deletions (10–12). The human *p16* gene structure comprises three exons, with exons 1 and 2 constituting 97% of the coding region (9). To identify *p16* gene mutations in these tumors, SSCP analyses of exons 1 and 2 were performed for 25 pituitary tumors. Fig. 3*A* illustrates an exon 1 SSCP analysis of several pituitary tumor DNA samples (*Lanes T1-T8*). In this figure, each sample was loaded in two adjacent lanes for more accurate identification of a mobility shift relative to normal DNA (*Lane N1*). Twenty-five pituitary tumors were examined by SSCP analysis for exon 1 and 2 mutations, and no mobility shifts were detected in any of these samples.

For certain tumor samples, the ability to amplify *p16* bands was noted to be diminished using quantities of genomic DNA less than 200 ng in the PCR reaction (Fig. 3*A*, *Lane T1*). To investigate the possibility of homozygous deletions in these specimens, a quantitative differential PCR analysis was performed in which the ability to amplify a region of *p16* exon 2 was compared quantitatively with the ability to amplify a region of the *β-actin* gene for normal and tumor DNA samples. This method has been successfully used to estimate the frequencies of homozygous *p16* deletions in DNA derived from tumor cell lines and primary human tumors (18, 19). Fig. 3*B* shows an example of this analysis, in which 100 ng genomic DNA from tumor specimens (*Lanes T1-T4*) and a normal DNA sample (*Lane N*) were used to amplify regions of the *p16* and *β-actin* genes. PCR products were blotted and hybridized to p16 and *β*-actin cDNA probes, and bands were quantitated by phosphorimager analysis. The same normal samples were used as standards for comparison on each gel to control for differences in the amounts of probe used, efficiency of cross-linking, and other possible variables inherent to these experiments. Intensity ratios were calculated as the ratio of *p16* tumor sample:*p16* normal standard divided by the ratio of *β-actin* tumor sample:*β-actin*

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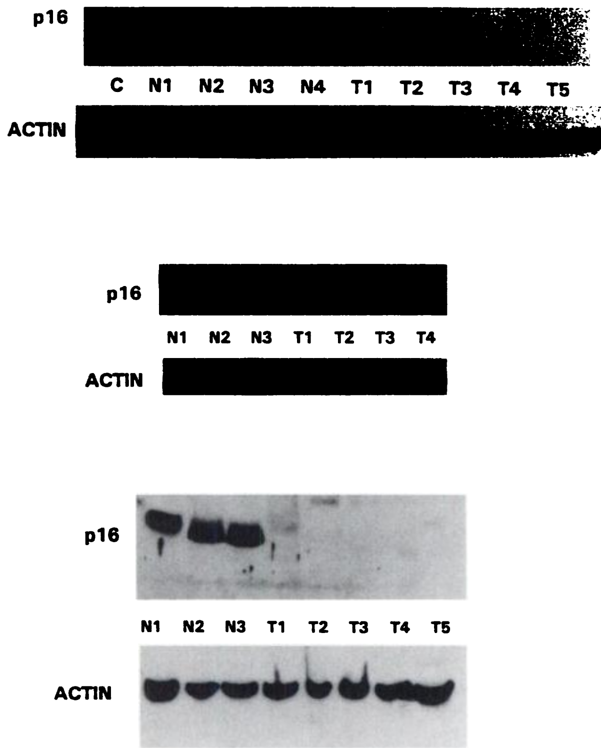

Fig. 1. Protein samples prepared from 25 human pituitary tumors and 10 normal pituitary specimens were examined in more than 10 Western blots using two different antihuman p16 antibodies. In all cases, p16 protein was undetectable in pituitary tumors (lanes labeled *T*), whereas high levels of p16 were detected in normal pituitary samples (lanes labeled *N*) and control NCI-H69 cells (*Lane C*). Probing with anti-β-actin antibody was performed in all cases to control for protein loading and integrity.

normal standard, as described previously by Churchill *et al.* (20). Of 25 tumors, 3 were found to demonstrate diminished amplification of *p16* relative to *β-actin,* with intensity ratios less than 0.44. This value has been previously determined for primary tumor DNA samples to correlate with homozygous deletion at a 95% confidence level in mixing experiments of normal and *p16*-deleted genomic DNA (19). Relative intensities of *p16* bands for other specimens in this analysis ranged from 0.65 to 1.4. Positive samples were repeated three times.

Discussion

We have studied human pituitary tumors for defects in p16 expression as an indirect mechanism of Rb inactivation in these tumors. The high frequency of pituitary tumors that develop in *Rb*-deficient mice, the lack of *Rb* abnormalities in human pituitary tumors, and several reports demonstrating an inverse correlation of *Rb* and *p16* defects in human tumors led us to this investigation. Of 25 human pituitary tumors examined by Western blotting, none were found to express detectable levels of the p16 protein, whereas p16 expression could be readily demonstrated in several normal human pituitary specimens in the same experiments. Similar findings were observed at the mRNA level in nearly all of the pituitary tumors examined. In contrast to these results, we previously reported that these pituitary tumors express high levels of the Rb protein and lack mutations in the "pocket" region of the *Rb* gene (7), underscoring an inverse correlation of p16 and Rb expression in these tumors. This inverse correlation was first reported in a large study of lung carcinoma cell lines by Otterson *et al.* (15), who demonstrated p16 protein only in tumors with absent or mutant Rb. More recently, this correlation was reported by Aagaard *et al.* (16) in a number of cell lines derived from a variety of different human tumors. The mutually exclusive appearance of p16 and Rb alterations in human tumors highly implies that both of these proteins function in the same cell cycle regulatory pathway, and that loss of either achieves the same biological effect on cell cycle control.

We have investigated a number of pituitary tumors for evidence of *p16* gene defects. In these studies, *p16* mutations in exons 1 and 2 were not detected in 25 human pituitary tumors. Although exons 1 and 2 constitute 97% of the coding region, our experiments would not have detected *p16* mutations if they occurred in exon 3 or in noncoding regions such as the promoter. Another important mechanism of *p16* gene inactivation is homozygous deletion. To estimate the frequency of this abnormality in pituitary tumors, we have used a quantitative differential PCR analysis, a technique that has been increasingly used for this determination. Recent experiments by Walker *et al.* (19) have demonstrated that the use of the limiting intensity ratio of 0.44 is more accurate in estimating homozygous gene loss than the arbitrary ratio of 0.2 previously used as the cutoff. Using this technique, 3 of 25 pituitary tumors were found to have intensity ratios indicative of homozygous *p16* gene loss. Interestingly, the three pituitary tumor specimens with this abnormality were macroadenomas, which perhaps contained lower relative amounts of normal pituitary margin tissue than those found in microadenoma samples. Our results may underestimate the occurrence of *p16* gene deletions in human pituitary tumors, because contamination from normal DNA may be a confounding factor in cases of microadenomas. To our knowledge, the frequency of LOH on chromosome 9p21 has never been reported for human pituitary tumors but clearly warrants investigation.

The general frequency of *p16* gene defects has been speculated to be lower in primary tumors than in cultured tumor cells (17), although recently, this view has been challenged (21). Despite the low fre-

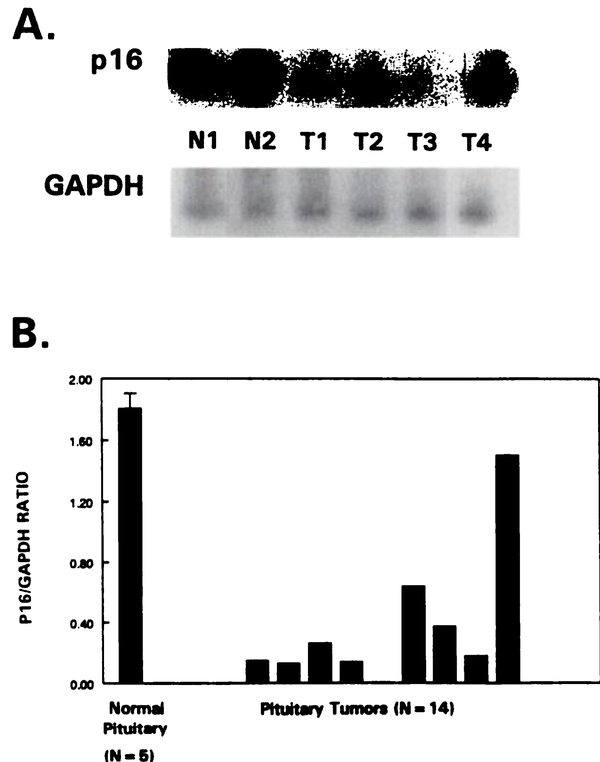

Fig. 2. By RT-PCR analysis, levels of p16 mRNA were undetectable or reduced in 13 of 14 pituitary tumor samples relative to 5 normal pituitary samples. *A*, equal amounts of RNA prepared from pituitary tumors (*Lanes T1-T4*) and normal pituitary samples (*Lanes N1* and *N2*) were subjected to RT-PCR, followed by Southern blotting of PCR products and hybridization to p16 and GAPDH cDNA probes. *B*, p16 mRNA levels were quantitated and expressed as ratios relative to respective levels of GAPDH mRNA in these samples.

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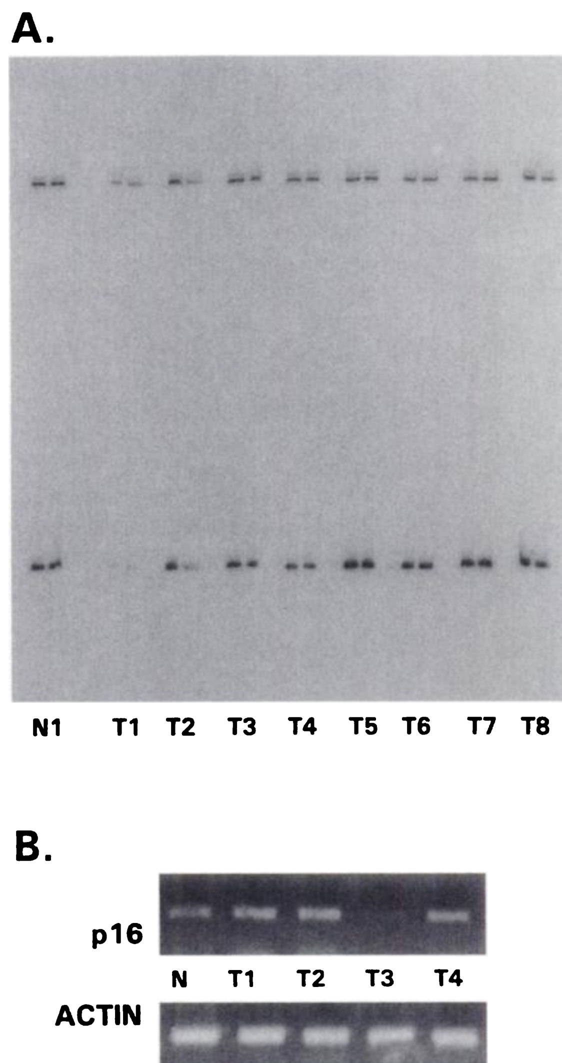

Fig. 3. *A*, SSCP analysis illustrating differential mobilities of *p16* exon 1 DNA fragments amplified from normal (*Lane N1*) and pituitary tumor DNA samples (*Lanes T1-T8*). Each sample is loaded in two adjacent lanes. Analysis of 25 human pituitary tumors for *p16* exon 1 and 2 mutations revealed no mobility shifts in any of the tumors. For certain tumor samples (*Lane T1*), the ability to amplify *p16* bands was diminished. *B*, quantitative differential PCR analysis was performed and demonstrated diminished amplification of *p16* relative to *β-actin* in 3 of 25 tumors (*Lane T3*), suggesting homozygous *p16* gene loss in these tumors.

quency of these lesions in primary pituitary tumors, we have demonstrated a clear loss of p16 expression in all pituitary tumors examined. In our study, as well as others, the use of immunoblotting has proven to be a highly effective means of screening tumor samples for p16 loss. Much attention has recently focused on the importance of alternative mechanisms of *p16* inactivation in primary tumors, particularly gene methylation. Herman *et al.* (13), for example, reported that *de novo* methylation of a 5′-CpG island of the *p16* gene associated with loss of transcription occurs at a high frequency in several different types of primary human tumors. This alteration was particularly striking for colon carcinomas, in which the rate of LOH on chromosome 9p21 is low, as is the frequency of homozygous *p16* deletion. Similarly, Gonzalez-Zulueta *et al.* (14) described the high rate of methylation of *p16* at 5′-CpG islands in transitional cell carcinoma of the bladder, in which *p16* mutations and homozygous deletions are also rare. Interestingly, a number of pituitary genes, such as the *prolactin* gene, are known to be regulated by methylation at sites in their 5′ regions (22), and the possibility of transcriptional silencing of *p16* in these tumors by gene methylation certainly requires investigation. Alternatively, the loss of p16 expression in human pituitary tumors may suggest a defect in regulation by another component in this cell cycle control pathway. An epigenetic event affecting such a component could then represent the fundamental lesion inactivating this pathway in these tumors. Clearly, further work is necessary to uncover the mechanisms underlying altered p16 expression in pituitary tumors and to define the effects of this alteration on cell cycle control that result in tumorigenesis.

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