Loss of pRb Expression in Pituitary Adenomas Is Associated with Methylation of the RB1 CpG Island


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Abstract

We recently showed loss of pRb in a proportion of pituitary tumors that was not associated with loss of heterozygosity of an RB1 intragenic marker. To further define the mechanism responsible for loss of retinoblastoma protein (pRb) expression, we have investigated the methylation status of the CpG island contained within the promoter region of the RB1 gene, together with sequence analysis of the essential promoter region and exons coding for the protein-binding pocket domain. Methylation of the CpG island within the RB1 promoter region was detected in 6 of 10 tumors that failed to express pRb. In contrast, 18 of 20 tumors and all six histologically normal postmortem pituitaries that expressed pRb were unmethylated. No inactivating mutations were found within the RB1 promoter region in the four unmethylated tumors that failed to express pRb. However, one or more exons comprising the coding region for the protein-binding pocket domain were shown to be homozygously deleted in three of four tumors available for analysis. This study describes an additional tumor type, in addition to retinoblastoma, in which methylation of the RB1 promoter is associated with loss of pRb expression. Furthermore, we show that in addition to methylation of the RB1 promoter region, deletion within the protein-binding pocket domain is associated with a loss of detectable pRb expression. The reactivation of tumor suppressor genes, silenced through methylation, represents a promising therapeutic target in sporadic pituitary adenomas.

Introduction

The retinoblastoma susceptibility gene (RB1), located on the long arm of chromosome 13, was the first TSG isolated (1–3). The RB1 gene, spread over 200 kb, encodes a nucleoprotein (pRb) that is phosphorylated and dephosphorylated synchronously with the cell cycle (4, 5). The phosphorylation of pRb by the cyclin D1/cyclin-dependent kinase 4/6 complex in late G1 results in the release of nuclear proteins and transcription factors, including the E2F family, thereby initiating the expression of genes critical for transition into the S phase of the cell cycle. Because both cyclin D1 and p16 have the potential to complex with cyclin-dependent kinase 4, they function as positive and negative regulators of pRb phosphorylation, respectively.

In addition to retinoblastoma (1–3), loss of RB1 function has been described in numerous other tumor types (Ref. 6 and references therein). A significant association between LOH of RB1 intragenic markers and an absence of pRb expression has been observed in a number of tumor types, including bilateral and unilateral retinoblastoma (7), bladder carcinomas (8), and malignant neuroendocrine lung carcinomas (9). However, several studies have shown loss of pRb expression, for example in breast (10), prostate cancer (11), and pituitary tumors (12, 13), that is not associated with LOH of RB1 intragenic markers. Although earlier studies (14–16) reported frequent LOH of RB1 intragenic markers in human pituitary tumors, these tumors were unscreened in terms of clinical behavior. However, more recent reports (12, 13, 17) have described an increased frequency of LOH, within the chromosome 13q14 region, in invasive pituitary tumors compared with their noninvasive counterparts. In addition, two of these studies (12, 13) described loss of pRb protein expression that was not associated with loss of an RB1 intragenic marker. Additional mechanisms responsible for loss of pRb protein expression include point mutations and microdeletions. Mutation (18) and microdeletion (19) of the RB1 promoter region are frequently associated with an absence of pRb expression in hereditary retinoblastomas and prostate carcinomas, respectively. In several tumor types, including retinoblastomas (20), small cell lung cancers (21, 22), and prostate carcinomas (23), both of these aberrations (mutation and microdeletion) have been shown to target the protein-binding pocket domain (exons 20–24).

More recently, methylation errors resulting in the de novo methylation of CpG islands not methylated in normal DNA have been shown to contribute to the progressive inactivation of TSGs (reviewed in Refs. 24 and 25). The RB1 gene harbors a small (600-bp) CpG island that encompasses the essential promoter region, which remains unmethylated in all tissues during development (25). However, aberrant methylation of the CpG island within the RB1 promoter region has been described in unilateral retinoblastoma (26–29), which is associated with a loss of pRb expression. Furthermore, Ohtani-Fujita et al. (30) have demonstrated that in vitro methylation of the RB1 promoter region dramatically reduced pRb expression.

To further define the mechanism(s) responsible for loss of pRb expression in sporadic human pituitary adenomas, we investigated the methylation status of the CpG island contained within the promoter region of the RB1 gene. In addition, we examined the essential promoter region and exons 20–24, coding for the protein-binding pocket domain of the RB1 gene, for the presence of inactivating mutations or microdeletions.

Materials and Methods

Patient Material. Twelve sporadic, nonfunctional pituitary tumors (4 noninvasive and 8 invasive) and 18 somatotrophinomas (11 noninvasive and 7 invasive) with matched blood samples were obtained from patients who had undergone hypophysectomy. Tumor tissues were collected retrospectively after standard histological assessment and graded as described previously (12, 31).

We have reported previously (13) chromosome 13q deletion mapping and immunohistochemical status of pRb in both sporadic nonfunctional adenomas and somatotrophinomas. Sufficient tumor material was available from 30 of...
these tumors (see above) for DNA analysis and reassessment of immunohistochemical status, which is described in this report. In addition, six histologically normal postmortem pituitary and matched spleen samples were obtained simultaneously, within 12 h of death, and stored at −20°C.

**Tissue and DNA Preparation.** Ten 5-μm sections were taken from tumor and postmortem pituitary that had been formalin fixed and paraffin embedded. A single section was subjected to H&E staining, allowing tumor identification and subsequent microdissection from the remaining unstained sections. DNA was extracted by prolonged (3 days) proteinase K (0.2 mg/ml) digestion in 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 0.5% Tween 20. Samples were heated to 99°C for 10 min and subjected to brief centrifugation. Supernatants were removed and stored at 4°C. Leukocyte DNA was extracted from matched blood samples using commercially available reagents (Nucleon I; Scotlab, Strathclyde, Scotland).

**pRb Immunohistochemistry.** Archival sections were deparaffinized, rehydrated, and underwent antigen retrieval by pressure cooker/microwaving on full power in a 750-W microwave oven in a citrate buffer (pH 6.0) with 5 min at full pressure. The primary antibody (Rh-clone IF8; Novacstra Labs, Newcastle-upon-Tyne, United Kingdom) was used at 1:50 dilution and incubated with tissue sections for 1 h. The secondary antibody and peroxidase steps were carried out using a commercially available kit according to the manufacturer’s protocol (LSAB2 kit; Dako Ltd., Buckinghamshire, United Kingdom). Sites of binding were visualized using 3′,3′-diaminobenzidine as chromogen. A malignant melanoma was used as positive control. Negative controls were the substitution of mouse immunoglobulin for the primary antibody and the omission of the primary antibody. Only nuclear positivity was assessed; cytoplasmic staining was regarded as nonspecific (32). Tumors were scored positive if all cells were stained or the staining pattern was heterogeneous with a portion of the cells showing immunonegativity in surrounding or infiltrating normal cells. Tumors were scored pRb negative only if all of the malignant cells showed no pRb staining in the presence of nuclear immunoreactivity in surrounding or infiltrating normal tumor cells. Tumors were scored without knowledge of tumor subtype, behavior, or methylation status.

**Sodium Bisulfite Modification.** DNA (200 ng) was denatured with NaOH (final concentration, 0.2 M) in a total volume of 10 μl for 10 min at 37°C. One μg of salmon sperm DNA was added as a carrier prior to denaturation. Sodium bisulfite solution (250 μl; 0.5 M hydroquinone, 2.2 M sodium bisulfite) at pH 5, freshly prepared, was added and mixed. To ensure full denaturation and deamination, DNA samples were denatured at 95°C for 5 min and further incubated at 55°C for 55 min; this was repeated for a total of six cycles (33). DNA samples were then purified using the Wizard purification resin according to the manufacturer’s protocol (Promega, United Kingdom) and eluted in 50 μl of water. Modification was completed by desulfonation with NaOH (final concentration, 0.3 M) for 15 min at 37°C. DNA was ethanol precipitated and resuspended in 10 μl of water. Samples were stored at 4°C.

**Methylation Status by Methylation-sensitive PCR.** Oligonucleotides were designed according to criteria described previously (34). Briefly, oligonucleotide sequences were designed to a region of the Rb1 essential promoter element shown previously to be methylated in hereditary and unilateral sporadic retinoblastoma (26, 28, 29). Oligonucleotides were designed for regions containing frequent cytosines (to distinguish between modified and unmodified DNA) and contained CpG dinucleotides at the 3′ end (to provide maximal discrimination between methylated and unmethylated DNA). Oligonucleotide sequences are shown in Table 1. To ensure PCR amplification of methylated Rb1 promoter sequence after modification, we methylated genomic DNA in vitro with the CpG methylase enzyme SssI. This DNA was then subjected to sodium bisulfite modification as described above and served as a positive control.

PCR reactions contained oligonucleotide sets specific for methylated or unmethylated Rb1 promoter region (2 pmol of each primer), 1.5 mM MgCl₂, and 200 ng of modified template DNA. Reactions were hot started (96°C for 5 min) before the addition of 1 unit of Taq DNA polymerase and 200 μM each of dATP, dGTP, dTTP, and dCTP. PCR was carried out for 30 cycles (55°C for 30 s, 72°C for 30 s, and 94°C for 1 min). Products were run on 8% nondenaturing polyacrylamide gels, fixed in 10% methanol:methylated 0.5% acetic acid for 6 min, and then incubated in 0.1% aqueous silver nitrate for 15 min. After two brief washes in distilled water, products were visualized by development in 1.5% sodium hydroxide/0.1% formaldehyde.

**Sequence Analysis of the Rb1 Promoter Region and Exons 20–24.** To investigate the possibility of inactivating mutations or microdeletions, tumors that were unmethylated and failed to express detectable levels of pRb were subjected to sequence analysis of the essential promoter region (encompassing nucleotides −300 to −174) and the protein-binding pocket domain (exons 20–24). Specific oligonucleotides were designed to encompass the region of the Rb1 promoter examined for methylation (Table 1). PCR amplification was achieved at an annealing temperature of 58°C. PCR conditions were as described above. Exons 20–24 were subjected to PCR amplification and sequence analysis using oligonucleotides designed to encompass specific exons and surrounding intronic sequences. Amplification of exons 22 and 23 was achieved by designing oligonucleotides to the 5′ and 3′ regions, resulting in the amplification of two overlapping PCR products (Table 1).

**Multiplex Analysis of the Protein-binding Pocket Domain.** Tumor and matched blood DNA were subjected to PCR amplification of exons 20–24 of the...

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**Table 1 Oligonucleotides used for PCR amplification**

<table>
<thead>
<tr>
<th>Oligonucleotide set</th>
<th>Sense 5′–3′</th>
<th>Antisense 5′–3′</th>
<th>Genomic position</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. MSP-PCR&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RB1M</td>
<td>GGGGATTTGCGGACCTGTTGAGG</td>
<td>−286 to −267</td>
<td>ACCGTCGAAACAGCCTCACCCG</td>
<td>−114 to −141</td>
</tr>
<tr>
<td>RB1U</td>
<td>GGGGATTTGCGGACCTGTTGAGG</td>
<td>−286 to −267</td>
<td>ACCGTCGAAACAGCCTCACCCG</td>
<td>−114 to −141</td>
</tr>
<tr>
<td>B. Essential promoter region sequence analysis</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SBR1</td>
<td>CGCCCCGGTTTCCCCACAGA</td>
<td>−319 to −301</td>
<td>GCCAACTGACGCGCCGCTT</td>
<td>−156 to −173</td>
</tr>
<tr>
<td>C. Sequence and multiplex PCR analysis&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RB20</td>
<td>TCTACTTGTGAATCTCCTCATAC</td>
<td>145377–154600</td>
<td>GGAAGAGCTAGTGTTGTTGAT</td>
<td>154807–15787</td>
</tr>
<tr>
<td>RB21</td>
<td>ATTCGCTACTTTCATTTATC</td>
<td>158636–158566</td>
<td>TATATTCAGTGTTGATATAGT</td>
<td>158828–158899</td>
</tr>
<tr>
<td>RB22.1</td>
<td>ATAGTGCTCTCCTACAGG</td>
<td>159802–159822</td>
<td>CACGTTGATGTTGCTGAGAGA</td>
<td>159950–159930</td>
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<tr>
<td>RB22.2</td>
<td>CCTGACAGATCTAAACCTGTG</td>
<td>159929–159949</td>
<td>TTGTGATGAGGCCATACATACATT</td>
<td>160104–160084</td>
</tr>
<tr>
<td>RB23.1</td>
<td>TAAATGTAAGGCGTTCACACCA</td>
<td>160084–161040</td>
<td>TATAGTCCTCCCTCGGAGA</td>
<td>160235–160215</td>
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<tr>
<td>RB23.2</td>
<td>ACAACCTTGTCTGTCCTTTGCCC</td>
<td>160187–162007</td>
<td>TCATTAAATGCTTCTCCTCAAC</td>
<td>160362–160342</td>
</tr>
<tr>
<td>RB24</td>
<td>GAATGATATTTATGATCTCA</td>
<td>168232–168252</td>
<td>TTCCTTTATATCTCACAATGCTC</td>
<td>168397–168377</td>
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<tr>
<td>D. Housekeeping gene&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>GAPDH</td>
<td>CGTGTCAGTGCTGGCCACGTCG</td>
<td>4357–4357</td>
<td>AAGAGCCGTCCTCGGCCACGCA</td>
<td>4589–4564</td>
</tr>
</tbody>
</table>

<sup>4</sup>Oligonucleotide sequences that are specific for methylated (RB1M) and unmethylated (RB1U) DNA after sodium bisulfite modification. Sequence differences between oligonucleotides specific for methylated and unmethylated DNA after sodium bisulfite modification, compared with the RB1 unmethylated sequences, are underlined.

<sup>5</sup>Oligonucleotide sets specific for exons 20–24 are shown. RB1 exons amplified by each oligonucleotide set are indicated. Amplification of exons 22 and 23 was achieved by designing oligonucleotides to the 5′ (RB22.1 and RB23.1) and 3′ (RB22.2 and RB23.2) regions, resulting in the amplification of two overlapping PCR products.

<sup>6</sup>Woloschak et al. (15)

<sup>7</sup>The sequence of the oligonucleotide set specific for the amplification of the GAPDH housekeeping gene is shown. The genomic positions of each oligonucleotide are numbered according to its Genbank accession (accession no. J04038).

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the \( \text{RB1} \) gene using specific oligonucleotides. In addition, the housekeeping gene \( \text{GAPDH} \) was coamplified as an internal control for DNA integrity (Table 1). Tumor and matched blood DNA were serially diluted prior to PCR amplification. PCR amplification was carried out as described above; however, PCR cycles were limited to 26 to ensure amplification remained in the exponential phase. PCR products were run on 8% nondenaturing polyacrylamide gels, and products were visualized as described above. On the resulting gel, a tumor lane was chosen in which the intensity of the \( \text{GAPDH} \) signal was similar to that of \( \text{GAPDH} \) signal in the matched blood DNA. Homozygous deletion of the \( \text{RB1} \) coding region was confirmed by the absence of the expected \( \text{RB1} \) PCR product in the tumor DNA (see Fig. 3).

Results

**pRb Immunohistochemistry**

Expression of the \( \text{RB1} \) gene was examined by immunohistochemical localization of pRb. Of 11 tumors shown previously not to express pRb, 10 tumors (7 somatotrophinomas and 3 nonfunctional adenomas) were available for reassessment of their pRb expression status. Loss of pRb was confirmed in all 10 tumors. In addition, a heterogeneous positive staining pattern for pRb was confirmed in 20 (11 somatotrophinomas and 9 nonfunctional) tumors and 6 histologically normal postmortem pituitaries (Table 2), confirming our previous findings (13). Fig. 1 shows the representative staining pattern seen in normal pituitary (Fig. 1A), a tumor showing immunopositivity for pRb (Fig. 1B), and a tumor that failed to express detectable levels of pRb (Fig. 1C and summarized in Table 2).

Table 2 also shows the LOH status of the 30 tumors and 6 histologically normal postmortem pituitaries investigated. The data, taken from our previous study (13), show that 2 of 30 tumors had evidence of LOH of the \( \text{RB1} \) intragenic marker \( \text{D13S153} \). In both tumors (nos. 20 and 253), LOH was associated with an absence of pRb. However, in all other cases, loss of detectable pRb expression, as assessed by immunohistochemistry, was not associated with LOH at \( \text{D13S153} \).

**Methylation of the \( \text{RB1} \) Promoter**

The MSP-PCR technique uses bisulfite-induced modification of DNA, under conditions whereby cytosine is converted to uracil, but 5-methylcytosine remains nonreactive (35). Because LOH of the \( \text{RB1} \) gene was infrequent in the tumor cohort, we examined the methylation status of the \( \text{RB1} \) promoter region in the 30 tumors (12 nonfunctional and 18 somatotrophinomas) available together with matched blood DNA and six histologically normal postmortem pituitaries. CpG dinucleotides within the promoter region were examined for methylation using the MSP-PCR technique (34). Oligonucleotides were designed that were specific for the promoter region despite detectable pRb expression. However, because of the heterogeneous staining pattern in both the unmethylated tumors and normal pituitaries, it was not possible to discern any difference in pRb expression in these two tumors. Fig. 2 shows the staining status of representative tumor samples and normal pituitary using the MSP-PCR technique. Table 2 summarizes the methylation status of the \( \text{RB1} \) promoter region in the tumor cohort and histologically normal postmortem pituitaries examined.

**\( \text{RB1} \) Sequence Analysis**

**Promoter Region.** Four tumors (nos. 2, 159, 162, and 253), which were unmethylated and failed to express detectable levels of pRb, were available for sequence analysis of the \( \text{RB1} \) promoter region. No inactivating mutations of the \( \text{RB1} \) core promoter were found in any of the tumors examined (Table 2).

**Protein-binding Pocket Domain.** Three tumors (nos. 2, 159, and 162) were available for further analysis of the exons 20–24. In one of those tumors (no. 159), it was possible to sequence exons 20–23. No inactivating mutations or microdeletions were detected in any of these coding exons or the surrounding intronic sequences in this tumor.
However, we were unable to sequence exon 24 for inactivating mutations or deletions because it was not possible to amplify this region (see below). In the remaining tumors (nos. 2 and 162), we failed to generate a PCR product for any of the coding exons comprising the protein-binding pocket domain.

### Multiplex PCR Analysis

Because it was not possible to amplify one or more exons, comprising the region coding for the protein-binding pocket domain, from unmethylated tumors that failed to express detectable pRb (nos. 2, 159, and 162), we examined exons 20–24 for homozygous deletion using multiplex PCR analysis. Two tumors (nos. 2 and 162) demonstrated homozygous deletion of exons 20–24, whereas the remaining tumor (no. 159) had sustained a deletion of exon 24 only (Fig. 3). Under the same conditions, we were able to PCR amplify exons 20–24 from all matched blood DNA samples, histologically normal postmortem pituitary, methylated tumors that failed to express pRb, and unmethylated tumors that continued to express pRb (Table 2). In addition, to show a retained region of the RB1 gene in those tumors with ostensible deletions of the protein-binding pocket domain, Fig. 3 also shows the PCR amplicon resulting from the amplification of the RB1 essential promoter region.

### Discussion

Two recent studies (12, 13) have shown loss of pRb expression in sporadic human pituitaries that was not associated with LOH of an RB1 intragenic marker. To identify the mechanisms responsible for loss of pRb expression, we investigated the methylation status and carried out sequence analysis of the CpG island contained within the RB1 promoter region. In addition, we have examined exons 20–24 comprising the protein-binding pocket domain for inactivating mutation and deletion.

In this study, we show a significant association between loss of pRb expression and methylation of the CpG island within the RB1 promoter region. Previous studies using methylation-sensitive restriction enzyme digest techniques have shown that methylation of RB1 alleles (promoter region and exon 1) is associated with a reduced level of RB1 transcript in sporadic retinoblastoma (28). These studies were extended by Stirzaker et al. (26), who reported that methylation was not confined to single CpG dinucleotides but extended to essentially all CpG dinucleotides spanning the RB1 CpG island, including the core promoter region in unilateral retinoblastoma. Using a model system, Ohtani-Fujita et al. (30) have shown that transcription factors, important in activating the RB1 promoter, are unable to bind when CpG dinucleotides within their recognition sequences are methylated. A recent study (36) has shown that within the RB1 promoter region, the same promoter element can act as a binding site for the E2F transcription factor or the methylcytosine-binding protein 2, depending on its methylation status. Methylcytosine-binding protein 2 bound to the methylated E2F element may recruit proteins that mediate histone deacetylation and may change the nucleosomal organization, thereby contributing to the transcriptional repression of the RB1 gene.

Although for several TSGs there is ample evidence that methylation is causal in gene silencing (reviewed in Ref. 25), this question has not been directly assessed for RB1. However, the available data (see above) would suggest a causal link between methylation and gene silencing. Ideally we would have wished to address this question directly by attempting to reexpress the RB1 gene product in primary tumors after treatment with cytosine nucleotide analogues. However, the difficulty in propagating primary human pituitary tumors in vitro precluded these types of studies.

Although methylation of the RB1 promoter region examined was significantly associated with an absence of pRb, two tumors, which expressed detectable levels of pRb, also showed methylation of the RB1 promoter region. Jones (24) and others (25, 37) suggest that complete gene silencing is dependent on the density and extensiveness of methylation, which may vary with the developmental stage of the tumor. Partial methylation may result in the down-regulation of the RB1 gene and reduced pRb levels, whereas progressive methylation of the CpG island will lead to complete gene silencing. Indeed, increasing cycle numbers in the MSP-PCR analysis showed a faint but detectable band corresponding to unmethylated alleles in these tumors (data not shown). However, we consider that this most likely represents the fact that methylation at the CpG sites examined is heterogeneous within the tumor cell population.
Methylation in Pituitary Tumors

Two tumors harboring loss of the RB1 intragenic marker D13S153 failed to express detectable levels of pRb. MSP-PCR showed that one of these tumors had also sustained methylation of the intact RB1 allele. In this tumor, methylation of the intact allele may be functionally equivalent to an allele-specific mutation. In a recent review, Zingg and Jones (37) suggested that methylation may represent an epimutation demonstrating the same characteristics as a sequence mutation. Thus, the loss of one functional RB1 allele by deletion and the inactivation of the remaining allele through methylation fulfills the two-hit criteria for the inactivation of a TSG originally proposed by Knudson (38). Prompted by the findings of neurointermediate lobe tumors in "RB1 knockout-mice," early studies of the RB1 gene in human pituitary tumors appeared to discount a role for the RB1 TSG in human forms of this disease (reviewed in Ref. 39). However, the association of methylation with loss of pRb, if viewed as functionally equivalent to a mutation (38), may reconcile the earlier disparate findings between the mouse model and human pituitary tumors.

Of the cohort examined, four tumors (three somatotrophinomas and one nonfunctional) failed to express detectable pRb, despite an absence of methylation at the CpG sites examined. Because microdeletion of the RB1 promoter region (19) and point mutation of SP-1 transcription factor binding sites have been shown to be sufficient to inhibit pRb expression (18), we examined this region in detail using a direct sequencing approach in these four tumors; however, we found no inactivating mutations or microdeletions. Because no aberrations were found within the essential promoter region in these four tumors and several reports have shown mutation and microdeletion within the coding region (6, 7), in particular exons 20–24 are associated with an absence of pRb or absent/reduced RB1 transcript (7, 20–23), we examined this region in detail. Because it was not possible to amplify one or more of the exons coding for the protein-binding pocket domain from these tumors, the possibility of homozygous deletion of exons 20–24 was examined by multiplex PCR. Individual exons, comprising the coding region for the protein-binding pocket domain, were subjected to coamplification with the housekeeping gene GAPDH, while cycle numbers were limited to ensure that amplification remained in the exponential phase, as described previously (40). Two tumors showed large deletions encompassing exons 20–24, whereas a
single tumor had sustained a deletion confined to exon 24 (Table 2 and Fig. 3). However, insufficient DNA precluded Southern blot analysis of the RB1 gene to further define the extent of the deletion in these tumors. A previous study of human pituitary tumors (15) failed to find deletion or mutation in exons 20–24, as assessed by single-strand conformational polymorphism analysis. The absence of inactivating mutations or deletions in these pituitary tumors, as reported by Woloschak et al. (15), most likely reflects the fact that all tumors studied showed detectable pRB expression, as assessed by Western blot analysis. Thus, in keeping with previous studies (see above) for other tumor types, our data are consistent with deletion within this region, resulting in either absent or undetectable protein expression.

To our knowledge, this is the first report of methylation of the CpG island contained within the RB1 promoter region in a tumor type other than retinoblastoma. Furthermore, in the majority of tumors, which failed to express detectable pRB, we suggest two mutually exclusive mechanisms: (a) methylation of the RB1 CpG island; or (b) deletion of the protein-binding pocket domain, which may be responsible for the inactivation of the RB1 gene. Several studies have shown that demethylation of TSGs, using a nucleotide analogue, is capable of restoring expression of a methylated TSG (reviewed in Refs. 24, 25, and 37). Reactivation of methylated TSG such as RB1, with inhibitors of DNA methylation, may provide a target for therapeutic intervention in specific pituitary tumor subtypes.

Acknowledgments

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