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Detection of Mutations of the *RB1* Gene in Retinoblastoma Patients by Using Exon-by-Exon PCR-SSCP Analysis

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Summary

Most sporadic cases of retinoblastoma, malignant eye tumor of children, may require the identification of a mutation of the retinoblastoma gene (*RB1* gene) for precise genetic counseling. We established a mutation detection system of and screened for the *RB1* gene mutation in 24 patients with retinoblastoma—12 bilateral patients and 12 unilateral patients. Mutation analysis was performed by PCR-mediated SSCP analysis in the entire coding region and promoter region, as an initial screening method, followed by direct genomic sequencing. Possible oncogenic mutations were identified in 14 (58%) of 24 tumors, of which 6 were single base substitutions, 4 were small deletions, 3 were small insertions, and 1 was a complex alteration due to deletion-insertion. A constitutional somatic mosaicism was suggested in one bilateral patient. A majority (57%) of mutations were found in E1A binding domains, and all were presumed to truncate the normal gene products. The mutation analysis presented here may provide a basis for the screening system of *RB1* gene mutations in retinoblastoma patients.

Introduction

Retinoblastoma is a malignant eye tumor that arises almost exclusively in children under age 4 years. Two forms of this disease, hereditary and nonhereditary (Vogel 1979), are known. Approximately 35% of all retinoblastoma patients are hereditary cases, defined by either familial occurrence or the presence of bilateral and multifocal tumors. The remaining 65% have no family history and present solitary unilateral tumors. A gene responsible for the development of this tumor has been identified (Friend et al. 1986); namely, the *RB1* gene, which spans 180 kbp on the chromosome 13q14 locus (McGee et al. 1989; T'Ang et al. 1989) and encodes a 105-kD nuclear phosphoprotein (Buchkovich et al. 1989). Several reports have identified germ-line and somatic mutations of the *RB1* gene in hereditary and nonhereditary retinoblastomas (Friend et al. 1986; Dunn et al. 1989; Yandell et al. 1989; Hogg et al. 1993). Somatic mutations of the *RB1* gene have also been found in several other types of tumors (Friend et al. 1987; Toguchida et al. 1988; Yokota et al. 1988; Lee et al. 1989; Reissmann et al. 1989; Horowitz et al. 1990).

An identification of the responsible gene allowed us to make a DNA-based diagnosis in each retinoblastoma patient. In most of the familial cases where DNA from patients and affected relatives is available, the analysis of RFLPs in the *RB1* gene is useful to identify the allele that carries a mutation (Wiggs et al. 1988). However, such an approach is not suitable for patients with new germ-line mutations, who represent about 80% of all hereditary cases, including unilaterally affected patients with germ-line mutations, and the direct identification of the mutation is inevitably required for these cases. Since only 15% of mutations are detectable by Southern analysis (Kloss et al. 1991), most efforts have been devoted to identify subtle mutations, such as point mutations. Various approaches have been reported, including RNase protection assay (Dunn et al. 1989),

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direct genomic sequencing (Yandell et al. 1989), high-resolution gel electrophoresis (Lohmann et al. 1992), SSCP analysis (Hogg et al. 1992), and reverse transcriptase–based PCR (RT-PCR) analysis (Kato et al. 1994).

In the present study, we established a system to detect the *RB1* gene mutation by using SSCP analysis as the initial screening method and direct genomic sequencing to identify mutations. The system was tested for its sensitivity and feasibility in 24 retinoblastoma patients.

Material and Methods

Samples

In a consecutive study on 48 retinoblastoma tumors, consisting of 5 familial, 22 sporadic bilateral, and 21 sporadic unilateral tumors, we previously found (*a*) constitutional 13q deletions in 3 cases (1 sporadic unilateral and 2 sporadic bilateral tumors) and (*b*) gross structural changes of the *RB1* gene, detectable by Southern blot analysis using cDNA probe, in 1 familial tumor and 1 sporadic unilateral tumor. RFLP analysis revealed loss of heterozygosity (LOH) on chromosome 13 in 30 of the remaining 43 cases (Kato et al. 1993). In the present study, a total of 24 cases—12 bilateral (2 familial and 10 sporadic) and 12 sporadic unilateral patients—were randomly selected from these 30 patients whose tumor DNA showed LOH on chromosome 13.

Fibroblast cultures were established from skin biopsy material in α-modified minimum essential medium (Irvine) supplemented with 10% fetal bovine serum (Hyclone). The tumor tissues were maintained in L-15 medium (Irvine) during transportation from the hospital.

DNA Isolation

High-molecular-weight DNA was isolated from tumors and fibroblast cells according to standard protocols (Sambrook et al. 1989).

SSCP Analysis

Oligonucleotide primer pairs were designed to amplify fragments containing each of the *RB1* gene exons 1–26, the coding region in exon 27, and sequence 5′ to exon 1 which contained sequences with promoter activity (Sakai et al. 1991). Primer sequences were designed according to the sequence data of McGee et al. (1989), with some modifications based on recent data on the total genomic sequence (Toguchida et al. 1993). The PCR fragments included at least 25 bp of the flanking intron regions surrounding each exon. To increase the sensitivity of the SSCP analysis, the PCR fragments were further digested by appropriate restriction endonucleases, to reduce the size of the PCR fragments to <230 bp. Primers and restriction enzymes are shown in table 1. Amplification from genomic DNA was performed in a 50-μl mixture containing 20 μM of dATP, dTTP, and dGTP; 2 μM dCTP, 1.0–3.5 mM $MgCl_2$; 20 pmol of each primer; 20 mM Tris (pH 8.4 or 8.6); 50 μM KCl; 50 μg bovine serum albumin/ml; 0.5 unit of *Taq* polymerase (Perkin-Elmer Cetus); and 0.1 μl (7 nM) [α-^{32}P] dCTP (3,000 Ci/mmol). PCR was performed with 40 cycles for 1 min at 94°C, 1 min at 50°C–60°C, and 0.5 min at 72°C, on a programmable thermal controller (ATTO). An aliquot of 5 μl of amplified product was diluted with a mixture of 10–20 μl of 0.1% sodium dodecyl sulfate and 10 mM EDTA, followed by 1:1 dilution with a 95% formamide, 2 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol loading solution. Diluted samples were heat denatured at 95°C for 5 min and then were loaded on 6% nondenaturing polyacrylamide gels with or without 10% glycerol. Electrophoresis was performed at 22°C with constant power of 25 W for 1.5–4 h. Autoradiography was carried out for 12–48 h at −70°C with intensifier screen.

Sequence Analysis

Direct genomic sequencing of double-stranded PCR fragments was performed as described elsewhere (Yandell et al. 1989). Amplified PCR products were purified by SUPREC-02 (Takara) and combined with 7 nM of ^{32}P-end-labeled primer. Primer and template mixtures were heat denatured, and sequencing reaction was carried out with a BcaBEST sequencing kit (Takara). Electrophoresis was performed on 6% denaturing polyacrylamide gel. Autoradiography was carried out for 12–48 h at room temperature.

Results

By using exon-by-exon analysis, the *RB1* mutations were first screened in tumor DNA and then in DNA from skin fibroblasts. In 24 tumor samples studied, possible oncogenic mutations were found in 14 (58.0%) cases. Of 14 mutations, 12 were found in coding regions, and 2 were located in exon-intron junctions.

Examples of each germ-line and somatic mutation are shown in figure 1, where the SSCP analysis of exon 13 shows an abnormal banding pattern in tumor DNA of patients RB303 and RB226. Sequence analysis of

Table 1

Oligonucleotides Used to Amplify Genomic *RB1* Sequence

RB1 Exon Amplified	Oligonucleotides[a]	Restriction Enzyme	Size of Fragments (bp)
Promotor	5'CTGGACCCACGCCAGGTTTC 5'ATTGGTACCCGACTCCCGTTACAAAAT	*Apa*I	95 and 144
1	5'ACGTGCGCGCGTCGTCCT 5'AACTGCACCTGTCACTTCGC	*Hha*I	7, 19, 215, 31, 49, and 139
2	5'TTGATTTATAAGTATAGCC 5'TTTTGTATAGTGATTTCGC	*Hinc*II	153 and 94
3	5'AGTTTTAACATAGTATCCAG 5'TTTCCTTTTATGGCAGAGGC	*Hinf*I	103 and 125
4	5'GAATTGAAATATCTATGATT 5'CTAATTGTGAACAATGACAT	*Rsa*I	128 and 137
5	5'TACTATGACTTCTAAATTA 5'CTAACTATCAAGATGTTTGA	No digestion	176
6	5'TGGAAAACTTTCTTTCAGTG 5'GAATTTAGTCCAAAGGAATG	No digestion	203
7	5'CCTGCGATTTTCTCTCATAC 5'GCAACTGCTGAATGAGAAAG	No digestion	226
8	5'ATTTTATATGATGGATGTAC 5'ATCTAAATCTACTTTAACTG	*Taq*I	84 and 159
9	5'AGTCAAGAGATTAGATTTTG 5'CAATTATCCTCCCTCCACAG	No digestion	202
10	5'GACATGTAAAGGATAATTGT 5'AGCTAAAGACTATATAATCT	*Bgl*II	87 and 142
11	5'GATGCATAAAGCACAAATTG 5'CTGAAACACTATAAAGCCAT	No digestion	205
12	5'CTCCCTTCATTGCTTAACAC 5'TTTCTTTGCCAAGATATTAC	No digestion	182
13	5'GATTACACAGTATCCTCGAC 5'CGAACTGGAAAGATGCTG	*Eco*RI	172 and 61
14	5'AAACAGTGAGACTCCATCTC 5'AGGATGATCTTGATGCCTTG	*Msp*I	185 and 85
15 and 16	5'CAATGCTGACACAAATAAC 5'GAAACACACACATTTTAAC	*Hha*I	225 and 94
17	5'TTCTTTGTCTGATAATAAC 5'CTCTCACTAACAATAATTTGTT	*Taq*I	187, 70, and 90
18	5'AAATTATGCTTACTAATGTG 5'TCTTTATAGAATGTTACATT	*Pst*I	154 and 89
19	5'TGTACAACCTTGAAGTGTAT 5'TCAGCCTAGTTTCAGAGTC	*Bgl*II	96 and 177
20	5'GGGGAAAGAAAAGAGTGG 5'AGGAGAGAAGGTGAAGTG	*Dde*I	198 and 123
21	5'ATTCTGACTACTTTTACATC 5'TTATGTTATGGATATGGAT	No digestion	196
22	5'ATATGTGCTTCTTACCAGT 5'TTGGTGGACCCATTACATTA	*Dde*I	131 and 171
23	5'TCTAATGTAATGGGTCCACC 5'TCAAAATAATCCCCCTCTCA	*Taq*I	95 and 225
24	5'GAATGATGTATTTATGCTCA 5'TTCTTTTATACTTACAATGC	No digestion	165
25	5'CTAACTATGAAACACTGGCA 5'CAGATGACCATCTCAGCTAC	*Eco*RI	40 and 203
26	5'TCCATTTATAAATACACATG 5'TAACGAAAAGACTTCTTGCA	No digestion	167
27	5'TACCCAGTACCATCAATGCT 5'TCCAGAGGTGTACACAGTG	No digestion	141

[a] Top = sense; bottom = antisense.

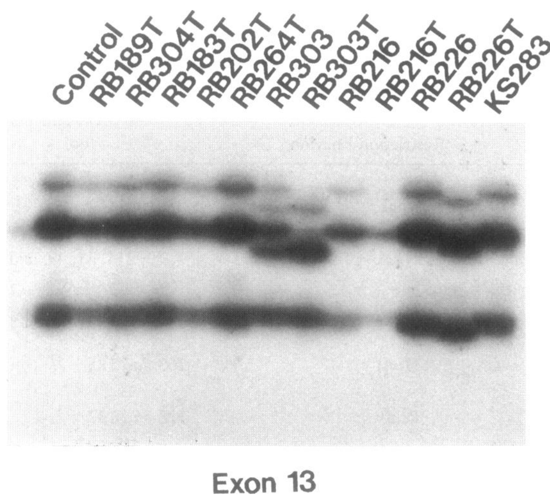

Figure 1 SSCP analysis of exon 13 of the *RB1* gene. Abnormal band patterns are evident in RB303, RB303T, and RB226T. T denotes tumor tissue.

exon 13 in tumor tissue from RB303 revealed a homozygous single-base deletion at codon 444, which presumably creates a stop codon downstream at codon 456. This patient was bilaterally affected, and the identical mutation was observed in the DNA of fibroblasts of this patient along with a normal allele. One A deletion in a stretch of four A's spanning codons 412–413 was detected in a tumor from patient RB226, creating a premature stop codon in exon 14. The patient was unilaterally affected, and this deletion mutation was not present in fibroblasts, indicating somatic origin of the mutation. In 12 bilateral cases, 6 mutations were found in tumors, of which 5 mutations were also present as a heterozygote with a normal allele in fibroblasts of each patient, which is consistent with the germ-line origin of mutation in bilaterally affected patients.

The dissociation of mutation between tumor and fibroblasts was found in one bilateral case (RB247). The tumor showed a complex deletion-insertion mutation in exon 17, where 7 bases were replaced by 21 bases of tandem duplication of sequences (TCTCAAG(A)A), which were homologous to the sequence flanking the deletion breakpoint (fig. 2). However, this mutation was not detected in DNA from his fibroblasts by either SSCP or direct sequence analysis. None of eight mutations found in unilateral tumors was present in the fibroblasts of patients.

Types of mutations are listed in table 2. Of 14 mutations detected in this study, 12 were found in coding regions, and 2 were located in exon-intron junctions. No mutations were found in the promoter region. Mutations in the coding regions were single-base substitutions in four cases, microdeletions in four cases, simple insertions in three cases, and a complex deletion-insertion mutation in one case. They were either nonsense mutations that directly create stop codons or frameshift mutations that would create downstream stop codons. Two mutations in the exon-intron junction were A-to-G transitions at the second nucleotide from the 5′ end of exon 11 in one case and a T-to-G transversion in the sixth nucleotide from the 3′ end of exon 17 in an-

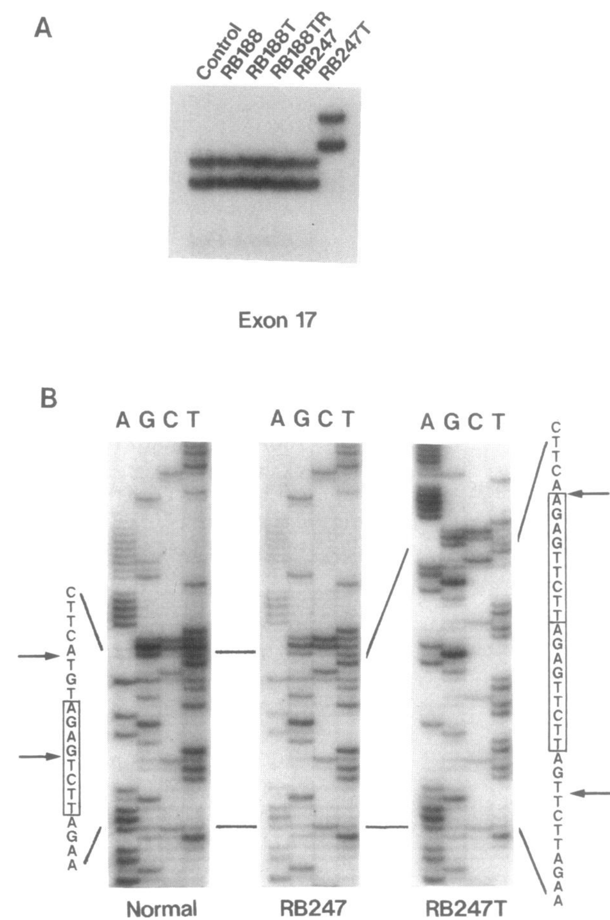

Figure 2 *A*, SSCP analysis of exon 17 of the *RB1* gene. Abnormal banding pattern is evident in the tumor DNA of patient RB247, (lane RB247T), and is absent in his normal skin fibroblasts (RB247). TR denotes tumor of recurrence. *B*, Sequence analysis of exon 17 of the *RB1* gene (antisense strand). In tumor tissue, 7 bases are replaced by 21 bases of tandem duplication of the sequence that is homologous to the sequence flanking the breakpoint. Arrows show the deletion breakpoints. Duplicated sequences are boxed.

Table 2

Mutation Identified in the *RB1* Gene

Sample ID	Tumor Site[a]	Sex	Exon	Codon	Nucleotide Change[b]	Amino Acid Change
RB247	SB	M	17	502–504	GAAGT ACAT CTC AGAATCTT→ GAAGT TCTCAAGAAT CTCAAGAAT CA AGAATCTT	Stop in exon 17
RB266	SB	M	11	Acceptor site	tagTT→tggTT	
RB299	SB	M	17	Donor site	Agtaagta→Agtaagga	
RB303	SB	F	13	444	TCA C AG→TCA AGgt	Stop in exon 14
RB313	SB	M	12	384	CAA→TAA	Gln→stop
RB357	SB	M	24	833	CTTA G TAT→CTTA TAT	Stop in exon 25
RB151	SU	F	14	455	CGA→TGA	Arg→stop
RB202	SU	F	2	71	GTC AGAGA→GTC G AGAGA	Stop in exon 3
RB226	SU	F	13	412	CC AAAA GAA→CC AAA GAA	Stop in exon 13
RB231	SU	F	16	489	TGC→TGA	Cys→stop
RB232	SU	F	12	389	TTA→TAA	Leu→stop
RB264	SU	F	22	771	CAG TATGC→CAG T TATGC	Stop in exon 23
RB270	SU	F	15	472	AAAAT TTT→AAAAT AAAT TTT	Stop in exon 16
RB272	SU	F	19	646–647	C CTCTCT TT→C CTCT TT	Stop in exon 19

[a] SB = sporadic bilateral; SU = sporadic unilateral.
[b] Changes in nucleotides are shown by underlined characters.

other. All of the deletions were flanked by short, direct repeats.

Figure 3 illustrates the distribution of the mutations along the *RB1* gene in 14 retinoblastoma patients. Although mutations were found in a broad region from exon 2–24, the majority (8 of 14) of mutations were found in E1A binding domains (Hu et al. 1990).

In addition to these mutations, aberrant SSCP bands were also found for the intron regions only in the tumor DNA. Mutations occurred in a stretch of T's at ≥8 bases away from the exon-intron junction. One was a T insertion in intron 15 in tumor of bilateral patient RB247, and the other was a single T deletion in intron 14 in tumor DNA of a unilateral patient. Because of the uncertainty in their biological significance, these mutations were not included in the oncogenic mutations.

Discussion

Here we presented the results of a screening for germ-line and somatic mutations of the *RB1* gene in 24 unrelated retinoblastoma patients, by analyzing the entire coding region and promoter region. Six mutations were found in bilaterally affected patients. Identification of these mutations will facilitate the genetic counseling of family members of these patients. In one bilateral case (BR247), however, a mutation observed in the tumor tissue was not detected in the fibroblasts of the patient. We have no definite explanation for this result at present. Somatic mosaicism is not uncommon in the case of chromosome aberrations in retinoblastoma (Ribeiro et al. 1988), although the PCR method is supposed to detect the mutant allele, even when the mu-

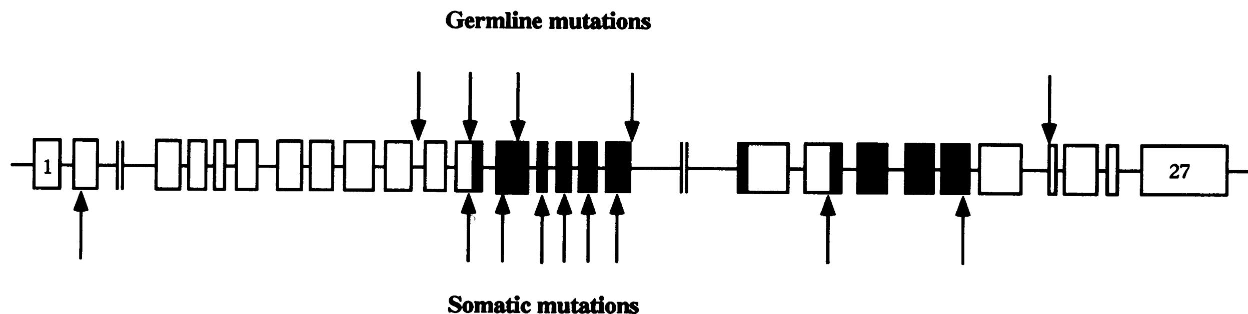

Figure 3 Distribution of germ-line and somatic mutations of *RB1* gene. Downward arrows and upward arrows show the sites of germ-line and somatic mutations, respectively. Blackened areas in exons represent adenovirus E1A-binding sites (codons 393–572 and 646–672).

tant allele represents only a small proportion of the total DNA. This mutation might have arisen as a somatic mutation at very early stage of the retinal development.

The identification of somatic mutations in eight unilaterally affected cases also has a significant meaning for these patients, because even if they are unilaterally affected, about 12% of these patients are presumed to be at risk for being a germ-line mutation carrier (Vogel 1979). In the remaining 10 (42.0%) cases, no abnormalities were detected in either the coding regions or the exon-intron junctions, with the detection rate being comparable to that for germinal mutations of the *APC* gene in familial adenomatous polyposis patients (Miyoshi et al. 1992). In contrast, Hogg et al. (1993) reported the presence of *RB1* mutations in all of 12 retinoblastoma tumors studied. Several possibilities may be considered to account for this discrepancy. We have no data concerning the sensitivity of the present method, and it might be possible that some mutations escaped detection by our initial screening method. The other possibility might be that some mutations exist outside the region that we have analyzed. Although the primer sets used in this study are designed to include at least 25 bp of intronic region adjacent to both ends of each exon, some genetic alterations outside these regions may have an effect in disturbing the production of RB1 protein through the disruption of the splicing process or suppression at the transcription level. Indeed, in the factor VIII gene of severe-type hemophilia, it has been suggested that approximately one-half of all mutations are located in the intronic regions away from the exon (Higuchi et al. 1991). Recently, intrachromosomal recombinations resulting in the inversion of the factor VIII gene were found in these cases (Lakich et al. 1993). Recently, Zacksenhaus et al. (1993) showed that the Sp1- and ATF-binding sites in the promoter region are critical for promoter activity. Although our analysis of promoter region included the −327-bp to −89-bp region, which covered the critical sites, we have not analyzed the methylation modification in this region, which has been shown to inhibit the transcriptional activity (Ohtani-Fujita et al. 1993). Alternative approaches, including the improvement of the efficiency of the present method, might be needed to fulfill the identification of mutations in the *RB1* gene.

Although the number of mutations found in this study is small, there are several characteristics of the mutations. All mutations were supposed to truncate the normal *RB1* transcript by either creating a premature stop codon or disrupting the normal splicing process. These mutations are expected to create nonfunctional protein, which is consistent with the studies in which retinoblastomas had no detectable *RB1* gene product. The dominance of the protein-losing type of mutation was reported in several other tumor-suppressor genes, including the *APC* gene (Nishisho et al. 1991; Miyoshi et al. 1992). Mutations we found were widely spread over the entire region of the *RB1* locus, from exon 2 to exon 24, which makes the detection of mutation time-consuming. Although no clustering of mutations was observed, a majority of mutations were found in adenovirus E1A protein-binding domains (Hu et al. 1990), which supports the functional significance of these domains.

We found two intronic mutations away from splice sites: one was a single-bp deletion in intron 15, and the other was a single-bp insertion in intron 14. The biological significance of these mutations is not clear. However, these alterations were not found in the constitutional cells of these patients and hence occurred as somatic mutations. Because, in RB247, we found another mutation in the coding region, it is likely that these alterations in the intronic region do not have a significant role in tumor development and may reflect the genomic instability in tumors.

In view of the recent advances in cancer treatment for retinoblastoma patients, the identification of the mutation in the *RB1* gene is increasingly important with respect to the genetic prognosis of the patients. The present study provides a basis for the genetic diagnosis. In our study population, 3 (10.7%) of 28 hereditary cases were identified to have microscopically recognizable 13q deletions. In the remaining 25 cases, 14 revealed LOH on chromosome 13, where the possible oncogenic mutations were evident in 6 of 12 cases, by PCR-SSCP analysis. Therefore, if we assume a similar detection rate for tumors that retained heterozygosity, the overall detection rate for germ-line mutations will be about 55%. The efficiency for the detection of the mutation is still unsatisfactory, when the time and costs for the analysis are considered, and further improvement of the system is obviously needed. While there are no apparent mutational hot spots, it is suggested that an initial screening for exons containing E1A-binding domains be performed.

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