Brief papers

Identification of RB1 germline mutations in Argentinian families with sporadic bilateral retinoblastoma

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Abstract

Hereditary predisposition to retinoblastoma is caused by germline mutations in the RB1 gene. Most of these mutations occur de novo and differ from one patient to another. DNA samples from 10 families with a child presenting sporadic bilateral retinoblastoma have been analysed for the causative mutation. Using intragenic DNA polymorphisms we detected large deletions in two patients. Heteroduplex and DNA sequence analysis of PCR products from each exon and the promoter region showed small mutations in four patients: a C to T transition in exon 18; 1 bp and 2 bp deletion in exons 20 and 19 re-

spectively; and a 4 bp insertion in exon 7. All these mutations are likely to result in premature termination of transcription. In one of these families, an unaffected carrier was detected. This emphasises the importance of detection of the causative mutation for predictive diagnosis in families with sporadic bilateral retinoblastoma.

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Retinoblastoma (RB) is a malignant childhood tumour, which occurs in a hereditary (40% of cases) and non-hereditary form (60% of cases).¹ It is caused by the loss of function of both copies of the RB1 gene, which encodes a cell cycle regulatory protein.²

In familial retinoblastoma, identification of mutant gene carriers is often achieved by segregation analysis of polymorphic loci within the RB1 gene.^{3,4} However, most of the germline mutations underlying hereditary retinoblastoma are de novo mutations. In these cases, the chromosome carrying the mutant allele cannot be identified by segregation analysis unless tumour material is available and found to be hemi- or homozygous for the germinal mutation. Therefore, identification of the causative mutation is the method of choice for predictive testing in subjects at risk. Since the mutations described previously show no preferential localisation in the gene (hot spots), in each new patient all 27 exons of the RB1 gene and regulatory sequences need to be searched for mutations. Gross structural alterations are detectable by Southern blot hybridisation, but occur in only 16% of patients with bilateral RB.⁵ Small structural alterations can be identified by sequencing of PCR products.⁶ A variety of prescreening methods, such as single strand conformation polymorphism analysis,⁷ heteroduplex analysis,⁸ and denaturing gradient gel electrophoresis⁹ can be used for rapid identification of the mutated region.

We used a sequential approach to the analysis of germline mutations in the RB1 gene. In a first step affected subjects were screened for gross structural alterations. Patients with no detectable abnormalities were analysed by het-

Figure 1 Southern blot analysis in family Rb10. (A) Hybridisation with the p123M1.8 probe, which recognises RFLPs near the 5' end of the RB1 gene. Each lane was loaded with BamHI digested DNA from family members denoted by the symbols of the pedigree in the upper part. Two different alleles are recognised by this probe, 4.4 kb and 2.3 and 2.1 kb (size indicated on the right). The patient shows a new fragment of about 3.8 kb and no paternal allele. (B) Hybridisation with the p68RS2.0 probe, which recognises VNTR in intron 17 of the RB1 gene. RsaI digested DNA from each family member was loaded in the same order as in (A). Numbers on the right indicate fragment size in kb.

eroduplex analysis to detect small mutations. We selected this screening method because it is a fast and non-radioactive technique, which does not require special equipment. Using this two step strategy, we have detected the genetic defect in six of 10 Argentinian patients.

Materials and methods

PATIENTS

Families with one child affected by bilateral retinoblastoma were referred by the Department of Ophthalmology, Buenos Aires Children Hospital. All cases were sporadic, that is, no other case of retinoblastoma was known in the family history. Diagnosis was established by current ophthalmological and histological criteria.

DNA SAMPLES AND ANALYSIS OF GROSS REARRANGEMENTS

Genomic DNA was isolated from leucocytes of peripheral blood according to standard methods.¹⁰ When available, tumour material was obtained at the time of enucleation and stored at -70°C until DNA extraction.

Southern analysis was performed by DNA digestion with the appropriate restriction endonuclease, *Bam*HI, *Hind*III, *Rsa*I, *Xba*I, or *Tth*111I, separation by electrophoresis, transfer onto a membrane, and hybridisation with one of the following probes: p123M1.8, 3.8 kb cDNA, p68RS2.0, p88PRO.6, or p35R0.6 (provided by Dr Dryja, Boston), and 9D11 (provided by Dr Cavenee, San Diego).

Analysis of the RB 1.20 polymorphism was carried out as described.¹¹ Briefly, genomic DNA spanning this VNTR sequence was amplified by PCR, cleaved with *Bst*NI, electrophoresed on an 8% polyacrylamide gel, and stained with silver nitrate.

SCREENING FOR SMALL MUTATIONS BY HETERODUPLEX AND DNA SEQUENCE ANALYSIS

All exons of the RB1 gene as well as the promoter and poly(A) signal sequences were amplified by PCR as described.⁸ Briefly, the PCR reactions contained 66 ng of genomic DNA, 0.25 $\mu\text{mol/l}$ of each primer, 0.2 $\mu\text{mol/l}$ of each dNTP, and 1.25 units of *Taq* polymerase (Perkin Elmer), using standard buffer conditions (total volume 26.6 μl). Thermal cycling (denaturing for 15 seconds at 94°C , annealing for 15 seconds at 52 to 62°C , and extension for 30 seconds at 72°C) was performed on a PE 9600 thermocycler (Perkin Elmer). The annealing temperature varied depending on the primer pair used.⁸ Thirty-five cycles were preceded by four minutes at 94°C and followed by an additional four minutes at 72°C . The PCR products were treated with T4 DNA polymerase. Some fragments longer than 300 bp were cleaved with an appropriate restriction enzyme: the promoter-exon 1 fragment with *Alu*I; exon 15-16, *Cfo*I; exon 17, *Dra*I; exon 22, *Dde*I. The processed PCR products were investigated by heteroduplex analysis. This method allows the detection of double stranded DNA fragments containing local mismatches based on their differential migration when separated in a mildly denaturing gel.¹² The electrophoretic pattern consists of bands that correspond to homoduplexes (resulting from reassociation of complementary strands of one allele) and heteroduplexes (formed between two strands from different alleles). To enhance heteroduplex formation the amplified DNA samples were denatured at 95°C for one minute and reannealed by cooling to 40°C during 60 minutes. The samples were electrophoresed at 5 W at room temperature on 1 x MDE gel (AT-Biochem) containing $0.6 \times \text{TBE}$ buffer and 1.4 mol/l urea. DNA was visualised by silver staining.¹³

The nucleotide sequence of DNA samples showing an altered electrophoretic behaviour was determined by direct sequencing. DNA was amplified by PCR as described above and purified from unincorporated primers and dNTPs by ultrafiltration using a microcon 100 filtration unit (Amicon). Both DNA strands were sequenced using the *Taq* cycle sequencing dye terminator protocol (Applied Biosystems). Sequencing reactions were analysed on an Applied Biosystems 373A Sequencer.

Figure 2 RB1.20, p88/*Xba*I polymorphism and H3.8/*Hind*III cDNA analysis in family Rb19. Family members are indicated in the pedigree on the upper part. (A) Genomic DNA from each family member was amplified using appropriate primers and the DNA fragments were electrophoresed on an 8% polyacrylamide gel and silver stained. (B and C) Southern analysis with the probes 3.8 cDNA, 9D11 (as internal standard), and p88. Restriction enzymes and size of DNA fragments in kb are indicated.

Results

IDENTIFICATION OF LARGE RB1 GENE DELETIONS
Abnormal Southern patterns were observed in two of 10 patients, Rb10 and Rb19. Analysis of the 5' region of the RB1 gene with probe p123M1.8 showed a new fragment of 3.8 kb and the absence of a paternal 2.3 and 2.1 kb allele in patient Rb10 (fig 1A). Hybridisation with a probe for the VNTR polymorphism in intron 17 also showed the maternal allele only (fig 1B). Similar results were obtained with the RB1.20 polymorphism (data not shown). These results suggest a deletion in one allele with one breakpoint close to the p123/BamHI site. RFLP studies in Rb19 were not informative, but reduced hybridisation intensity was observed in the patient's DNA with several probes (fig 2B, C). The presence of a deletion was confirmed with the RB1.20 polymorphism, which showed the absence of a paternal allele in the patient (fig 2A). Since this patient was heterozygous for the p123/BamHI polymorphism at the 5' end of the RB1 gene (data not shown) the results indicate a partial deletion in one allele of the RB1 gene, involving the 3' end.

DETECTION OF SMALL RB1 GENE MUTATIONS

In order to identify small mutations we used heteroduplex analysis. All DNA samples, including those with a large deletion, were ana-

lysed exon by exon. We identified an altered electrophoretic pattern, owing to the presence of a heteroduplex, in DNA samples from four patients. All family members of these four patients were also analysed (fig 3). The mutations were confirmed by sequencing.

Family Rb15

Heteroduplex analysis of exon 7 PCR products showed several abnormal bands in the patient (fig 3A). Direct sequencing showed an insertion of 4 bp, which was a duplication of the sequence GTTG, located in codons 233-234 of one allele (table). As a result of this insertion an alteration of the reading frame occurs and produces a premature stop codon in the junction of exons 7 and 8 (table). The mutation was not detected in the parents of the patient, thus it is a de novo mutation.

Family Rb1

Analysis of exon 18 PCR products showed a slower migrating heteroduplex in addition to the normal homoduplex band in the patient (fig 3B). Direct sequencing showed heterozygosity for a C to T transition (data not shown). The mutation is 40 bp from the 5' end of exon 18 and converts codon 579 from an arginine (CGA) to a stop codon (TGA) (table). Therefore, this mutation results in premature termination at amino acid 578. The mutation

Figure 3 Heteroduplex analysis of leucocyte DNA in four families and of tumour DNA in one of these families. The pedigree of each family is indicated above the electrophoretic pattern of heteroduplex analysis. Exons 7, 18, 19, and 20 were analysed in families Rb15 (A), Rb1 (B), Rb51 (C), and Rb60 (D) respectively. PCR products of each exon were electrophoresed on an MDE gel and visualised by silver staining. Heterozygosity for the mutation is indicated by the slower migration heteroduplex DNA. This DNA is shown as one band (B and D) or as two separated bands (A and C) of two heteroduplexes, one with the mutated sense strand and the other with the mutated antisense strand. The lower two bands in the patients' DNA in (A) and (C) are the normal and the mutated homoduplex. The tumour DNA is visualised as one band of mutated homoduplex, which indicates a loss of heterozygosity.

Summary of small mutations in the RB1 gene

Patient	Exon	Mutation	Sequence	Resulting change
Rb15	7	4 bp insertion	mut wt CTCCCATGTTGGTTGCTC CTCCCATGTTGCTC	Frameshift in exon 7 leads to a new stop codon in the junction of exons 7 and 8
Rb 1	18	C to T transition	mut wt CGACTGAGA GGACCGAGA	Single base change causes arginine codon to become a new stop codon
Rb51	19	2 bp deletion	mut wt TC TTTCACT TCTCTTTCACT	Frameshift in exon 19 leads to a new stop codon nearby in exon 19, codon 651
Rb60	20	1 bp deletion	mut wt ATGAG GACA ATGAGAGACA	Frameshift in exon 20 leads to a new stop codon in exon 21, codon 704

mut and wt: mutant and normal sequence respectively. The codon number is above the sequence. The base changed is indicated as a bold letter, the insertion is underlined, and the deletion is indicated by a line.

was not identified in either parent (fig 3B). Therefore, it is a new germline mutation.

Family Rb51

Heteroduplex analysis of exon 19 PCR products showed several abnormal banding patterns in this family (fig 3C). Two heteroduplexes (upper bands) and two homoduplexes (lower bands) are visible in the DNA of the patient (lane II·1) instead of one homoduplex band present in the normal DNA (lane I·2). Analysis of the tumour DNA showed only one, the mutated homoduplex band, of faster migration than the normal homoduplex, indicating loss of constitutional heterozygosity for the exon 19 mutation in this tumour. The faster migration of the mutated homoduplex compared with the normal DNA suggested that the mutation is a deletion. Analysis of DNA from the mother of the patient also showed an abnormal banding pattern, similar to that of the patient. Sequence analysis of tumour DNA showed a 2 bp deletion of nucleotides TC, 124 bp from the 5' end of exon 19 (data not shown). However, owing to the presence of a direct repeat of TC it is not possible to determine which nucleotides have been lost. Sequence analysis of leucocyte DNA showed the same mutation, a TC deletion at position 122 or 124 in one allele. Alteration of the reading frame results in the generation of a new stop codon at codon 651 of exon 19 (table). The same alteration was found in the mother of the patient (fig 3C), which, however, did not have visible abnormalities on ophthalmological examination. These results suggest that she is a non-penetrant carrier of the mutation that gave rise to tumour development in her child.

Family Rb60

Heteroduplex analysis of exon 20 PCR products showed an additional, slower migrating band in the patient's DNA, compared with the samples from his parents (fig 3D). Direct sequencing showed a 1 bp deletion of nucleotide A at codon 696 (table). This 1 bp deletion produces a frameshift which gives rise to a new stop codon at codon 704 of exon 21. The mutation was not detected in the parents of the patient (fig 3D) and thus is a new germinal mutation.

Discussion

Identification of mutations in patients with heritable RB enables reliable diagnosis of carriers of germline mutations in families and therefore allows the detection of unaffected gene carriers and presymptomatic diagnosis in newborns. Using intragenic DNA polymorphisms and a combination of PCR and heteroduplex analysis we have been able to detect heterozygous mutations in constitutional cells of six out of 10 patients with bilateral RB.

Gross rearrangements were found in two patients. This frequency is in agreement with previously published data.⁵ In one case (family Rb10), a deletion which includes polymorphisms from the 5' end and intron 17 of the RB1 gene was shown by Southern analysis. This deletion was not found in the parents of the patient. Analysis of the two RFLPs, which resulted in the lack of a paternal allele in the patient's DNA, allowed us to determine that this mutation is of paternal origin, which is most frequently observed (90%).^{14,15} Densitometric analysis indicated a probable deletion in the patient Rb19. The presence of a deletion was corroborated by segregation analysis of the intragenic RB1.20 VNTR polymorphism. The absence of a paternal allele in the patient's DNA again indicated the paternal origin of this deletion, which was a de novo mutation.

We detected an altered banding pattern by heteroduplex analysis in four out of eight samples without gross alterations. Sequencing of these samples showed one C to T transition, two deletions, and one insertion. The C to T transition occurred in a CpG dinucleotide, which is the most frequently observed single base pair substitution causing human genetic diseases and may be the result of spontaneous deamination of 5-methyl cytosine. This mutation changed the arginine CGA codon to a stop codon TGA, which is a very common finding and has been reported for different CGA codons in the RB1 gene.^{6,16}

Small deletions, 1 to 20 bp, represent the second most common form of mutation in the RB1 gene.^{6,9,13,17,18} The 1 bp (A) and 2 bp (TC) deletions found in exon 20 (Rb60) and exon 19 (Rb51) were part of short direct repeats, GAGAGA and CCTCTC respectively. Similar deletions were reported for several genes implicated in genetic diseases.¹⁹ For example,

1 bp (A) deletion, included in the GAGAG sequence, was observed in the antithrombin III gene, and 2 bp (TC) deletion was shown as a part of CCTCTC sequence in the 1-antitrypsin gene. The same 2 bp deletion (TC) in exon 19 of the RB1 gene was also found in an RB tumour by Hogg *et al.*¹⁶ As discussed by these authors, these deletions may arise as a result of a "slipped mispairing" mechanism. During the replication process, the DNA duplex becomes single stranded at the replication fork permitting a mispairing of one direct repeat with its displaced homologue. As a result, the base or bases of one direct repeat loop out, and the next round of replication would generate one deleted and one wild type duplex.

Insertions causing human genetic disease are relatively rare and few insertions ranging from 1 to 55 bp have been reported for the RB1 gene.^{9,16,17} The 4 bp insertion in exon 7 of the RB1 gene (Rb15) was a duplication of a symmetrical motif GTTG. The insertion was also flanked by direct repeats CTC (table). A similar insertion, but of 3 bp (TTG), included in a similar sequence, CCAGTTGTTGCT, was reported for the elliptocytosis SPTA gene.²⁰ Two deletions were found in the same symmetrical motif, GTTG, in the HPRT gene.¹⁹ Therefore, the identical mechanism of "slipped mispairing" may account for the generation of both deletions and insertions. An insertion will occur if the bases looping out are on the new synthesised strand, while a deletion will occur if the extrahelical bases are on the template strand.

All these small mutations generate premature stop codons in exons 7, 18, 19, and 20, which would be expected to result in a truncated protein of 240, 578, 650, and 703 amino acids in length respectively. The full length RB protein contains several functional domains. The "pocket" domain, which extends from residue 379 to 792, is necessary for T and E1A viral antigen binding, and a larger domain, from residue 379 to 928, is required for the growth suppression function.²¹ These functional domains would be disrupted in the truncated proteins synthesised from the RB1 genes carrying the mutations described in our study. All these mutations, including the gross rearrangements and the small length alterations, may therefore cause initiation of tumour development.

Using the heteroduplex analysis we were able to detect four out of eight mutations that were not identified by Southern blot hybridisation. Considering that this method is simple and, in contrast to SSCP or DGGE, no special equipment or isotopic labelling is needed, we think that our approach is suitable for identification of RB1 germline mutations in DNA from constitutional tissues. In one family, the unaffected mother of a patient was found to carry the mutation identified in her affected child. In the other families the parents have no

increased risk for having another child with RB, unless either of the parents is a genetic mosaic. The frequency of mosaicism, however, is low.²² Our results emphasise the importance of direct mutation detection in families with sporadic bilateral retinoblastoma.

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