Molecular-genetic analysis of two cases with retinoblastoma: benefits for disease management

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Abstract

Effective counselling and management of retinoblastoma families using genetic information is presently practised in many parts of the world. We studied histopathological, chromosomal and molecular-genetic data of two retinoblastoma patients from India. The two patients, one with bilateral and the other with unilateral retinoblastoma, underwent complete ophthalmic examination, cytogenetic study, retinoblastoma gene (*RB1*) mutational analysis and *RB1* promoter region methylation screening. In the bilateral retinoblastoma patient deletion of chromosome region 13q14 in peripheral blood lymphocytes and a hemizygous novel 8-bp deletion in exon 4 of *RB1* in tumour sample were observed. In the unilaterally affected patient CGA to TGA transition protein truncation mutations were observed in exons 8 and 14 of *RB1*.

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Introduction

Retinoblastoma susceptibility gene (*RB1*), a tumour suppressor gene that regulates cell division (Chen *et al*. 1989), is deleted or mutated in retinoblastoma (Motegi 1982; Gallie *et al*. 1999). Tumour develops when both alleles of the gene are inactivated in embryonal retinal cells (Knudson 1971; Cavenee *et al*. 1983; Friend *et al*. 1986). In the two-step inactivation mechanism, the first mutation, inherited via a germ cell, is present in all somatic cells and the second mutation occurs in a somatic cell, leading to the disease; this form is hereditary. If both mutations occur in the same somatic cell the disease is nonhereditary (Dunn *et al*. 1989; Yandell *et al*. 1989). Familial and bilateral retinoblastoma patients should necessarily have an *RB1* germline mutation (Dunn *et al*. 1989; Yandell *et al*. 1989).

Retinoblastoma gene inactivation is due to chromosomal deletion, single-nucleotide alteration, microdeletion, loss of heterozygosity, or methylation of the promoter region. Usually alleles with premature termination of coding sequence lead to complete penetrance and bilateral retinoblastoma, while missense alterations, substitutions in the promoter region and some splice-site mutations cause incomplete penetrance and reduced expressivity. In bilateral retinoblastoma patients most of the mutations result in truncated gene product and such patients on an average have more than three tumours per eye (Munier *et al*. 1994; Lohmann *et al*. 1996). Single-base substitutions (40–50%) and small-length mutations (25–30%) were the majority of mutations observed by Lohmann *et al*. (1996). In a study by Lohmann *et al*. (1997), loss of constitutional heterozygosity (LOH) at intragenic loci was observed in 71% of tumours from retinoblastoma patients, and mutations were detected in 21 out of 23 tumours with LOH. Mosaicism was suggested in two patients who had multifocal and bilateral retinoblastoma but no detectable mutation in peripheral blood. In another study mutations were found in only 7.1% and 36% of patients with unifocal isolated

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and hereditary retinoblastoma respectively (Blanquet *et al.* 1995). Lohmann (1999) reported that, of 368 known distinct somatic and germline mutations analysed, 227 were single-base substitutions in the ORF, splice sites or promoter region; 134 were frameshift insertions or deletions; and seven were complex insertion/deletion mutations. In a Spanish study of 43 hereditary retinoblastoma patients (Alonso *et al.* 2001), 29 mutations were observed of which 62% had not been reported earlier. Of the 29 mutations, 69% were nonsense (mostly CpG transition) mutations and frameshifts that resulted in truncated RB protein; these mutations were associated with early age at diagnosis (8.7 months average). The Spanish group reported frameshift insertions/deletions in 41% of observed mutations, nonsense mutations in 27%, and splice junction changes in 31%. Richter *et al.* (2003) used a combination of molecular techniques and detected 452 *RB1* mutations from 378 patients.

In our hospital, Sankara Nethralaya, a tertiary referral centre for ophthalmic disorders in India, nearly 50 fresh cases of unilateral or bilateral retinoblastoma are diagnosed every year (Harini *et al.* 2001). Establishing DNA diagnostic services is essential for these patients and their families and such a service will help the ophthalmologist to decide whether the next child or a close relative should have constant ophthalmic surveillance. This will also save considerable money and time, and reduce psychological stress for these families (Noorani *et al.* 1996). We looked at molecular-genetic data for two patients and were able to discuss future reproductive options in both the families. The present report is part of a larger molecular-genetic screening programme currently in progress at this hospital.

Materials and methods

***Clinical methods*:** Complete eye examination was done, including visual acuity, external examination and fundus examination. In the younger child, who did not allow examination, the examination was done under general anaesthesia. A and B scan ultrasound and CT scan orbit and brain were done to confirm the diagnosis and also to rule out extraocular or intracranial extension of the disease. Tumours were graded according to the Reese–Ellsworth classification (Ellsworth 1977).

Eyes with large tumour mass and no visual potential were enucleated; then DNA was extracted from the tissue for analysis. Histopathological examination was done on the enucleated eyes, specifically looking for the type of differentiation of the tumour, optic nerve or choroidal invasion, presence of tumour cells at the surgical cut end of the optic nerve stump, and any extraocular extension. Pedigree was ascertained and peripheral blood cytogenetic analysis was done (Rooney and Czepulkowski 1992). Patients were followed up every three months in the first year; every six in the second, and yearly thereafter.

Laboratory methods (Rooney and Czepulkowski 1992; Harini *et al.* 2001): Venous blood sample and family information for the present investigations were obtained with informed consent of the parent. This project has been approved by the institutional ethics review board.

***Cytogenetic analysis*:** Laboratory methods for cytogenetic study were as described by Harini *et al.* (2001).

***Mutation screening*:** Mutation screening through automated sequencing of the *RB1* gene was carried out on both blood and tumour DNA. Exons 2–27 of *RB1* were PCR amplified in individual reactions containing 100–150 ng DNA, 4–6 pM primers, 160 nM dNTPs, 1× PCR buffer and 0.6 U *Taq* DNA polymerase in a 20-µl reaction. Exon fragments from each group were coamplified from 100 ng of genomic DNA extracted from peripheral blood by a standard phenol–chloroform protocol (Wolff and Gemmil 1998) using specific primers flanking the exonic regions obtained from the Genome Data Bank (http://www.gdb.org). PCR was carried out in a PE 2700 thermal cycler. Thirtyfive normal unrelated individuals were also sequenced and analysed as controls. The amplified products were electrophoresed in 2% agarose with ethidium bromide stain, visualized using Amersham Pharmacia gel documentation system, and analysed using Image Master VDS. Multiplexed PCR amplified products were ethanol precipitated overnight at – 20°C. The products were washed twice with 70% alcohol, air dried, and dissolved in 20 µl of heat-inactivated autoclaved milliQ water and stored at – 20°C. These products were used for cycle sequencing PCR with fluorescent dNTPs (Ready Reaction Mix) and then sequenced in the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Reference sequence from GenBank (http://www.ncbi.nlm.nih.gov) was used for comparison to identify mutations.

***Methylation analysis of RB1 promoter region*:** Methylation analysis of the promoter region was done by sodium bisulphite modification followed by methylation-specific PCR (MS-PCR) using primers specific for methylated and unmethylated DNA (Frommer *et al.* 1992; Simpson *et al.* 2000). A 25-µl reaction was set up using 2 µl of bisulphite-modified DNA, 0.3 µl of respective primers (300 ng/µl), 2.0 µl dNTPs, 2.0 µl magnesium chloride (25 mM), 2.0 µl PCR buffer, 0.4 µl *Taq* DNA polymerase (1.25 U/µl). A hot start PCR was done for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and finally 4 min at 72°C. The PCR products were run on acrylamide gel, stained with silver stain, and analysed using Image Master VDS gel documentation system.

Results

Table 1 gives a summary of the findings.

Patient 1

A 21-month-old boy presented with a history of white reflex in the right eye. He was the second child of a second-degree consanguineous marriage. On examination, he had an exotropia of 30 degrees, and leukocoria in the right eye. Fundus examination of the right eye revealed a large tumour mass (Grade 5a of Reese–Ellsworth) with secondary retinal detachment, and the left eye showed presence of two tumours (four and three disc diameters in size) inferior and inferonasal to the disc (Grade 1b). Ultrasound A and B scan and CT scan confirmed the diagnosis and suggested no extraocular or intracranial extension. Histopathological examination of the enucleated right eye revealed partially differentiated retinoblastoma with no choroidal or optic nerve invasion (figure 1). This patient was disease free at the end of four-year follow-up. In this patient, a novel hemizygous 8-bp deletion in *RB1* exon 4 (TTACTAAA; nucleotides 41945–41952) was identified in the tumour DNA that was not present in normal DNA (figure 2). Cytogenetic analysis revealed deletion of chromosome region 13q14 (location of *RB1*) in peripheral blood leucocytes. *RB1* gene promoter region was unmethylated.

Patient 2

A five-year-old boy presented with a history of white reflex in the left eye of 20 days duration. On examination he had a left exotropia of 15 degrees and had searching eye movements of the left eye on covering the right eye. Anterior segment examination and fundus examination with scleral depression of the right eye were within normal limits. Left eye on fundus examination showed a white creamy mass filling two-thirds of the vitreous cavity (Grade 5a). Ultrasound A and B scan showed presence of a mass lesion in the vitreous cavity with low to medium surface reflectivity and low to medium reflective point-like internal echoes filling the vitreous cavity. CT scan showed calcification in the mass lesion suggestive of retinoblastoma (figure 3), but there was no evidence of

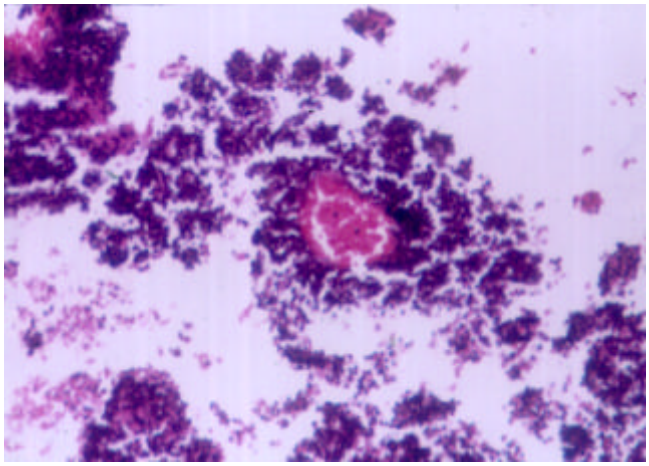

Figure 1. Partially differentiated retinoblastoma of the bilaterally affected patient where a novel mutation in *RB1* exon 4 has been detected.

Table 1. Clinical and genetic analysis of the two patients with retinoblastoma.

	Patient 1	Patient 2
Clinical examination		
Sex	Male	Male
Age of onset	1 year 9 months	5 years
Laterality	Bilateral	Unilateral
Visual acuity	Difficult to assess owing to young age	Right, 6/24 (20/80) Left, no perception of light
Fundus examination	Large tumour mass in right eye (RE grade 5a); two foci of tumours in left eye (RE grade 1b)	White creamy mass filling two-thirds of the vitreous cavity (RE grade 5a)
Calcification (CT scan)	+	+
Enucleation	Right eye	Left eye
Contralateral eye status	Left eye had two tumour masses which regressed after treatment over four years of follow-up	Right eye normal over three years of follow-up
Consanguinity	+	–
Histopathology	Partially differentiated	Undifferentiated
Genetic analysis		
13q14 deletion	+	–
Mutations	8-bp (TTACTAAA) deletion in *RB1* exon 4	Arginine to stop codon mutation in *RB1* exons 8 and 14
Methylation of *RB1* promoter	–	–

extraocular or intracranial extension. Left eye was enucleated, as there was no visual potential in the eye. Histopathological examination of the enucleated eye showed undifferentiated tumour with areas of necrosis and calcification. No choroidal or optic nerve invasion was found. In the three-year follow-up period, the patient had no involvement of the right eye with best-corrected visual acuity of 6/6 (20/20) and there was no orbital recurrence of the tumour in the left enucleated socket. In this patient CGA → TGA mutations were identified in exons 8 (figure 4) and 14. These mutations had occurred at nucleotides 59,673 and 76,430 respectively of these exons, resulting in change from arginine codon to stop codon (R251X and R445X). These changes were observed in the tumour tissues and not in peripheral blood. The mutations in exons 8 and 14 were not detected in the 35 healthy controls tested. Cytogenetic analysis revealed normal male karyotype. The proband was the fourth among six children of whom the eldest had died at the age of five from an unknown illness (figure 5). In this patient too the *RB1* gene promoter region was unmethylated.

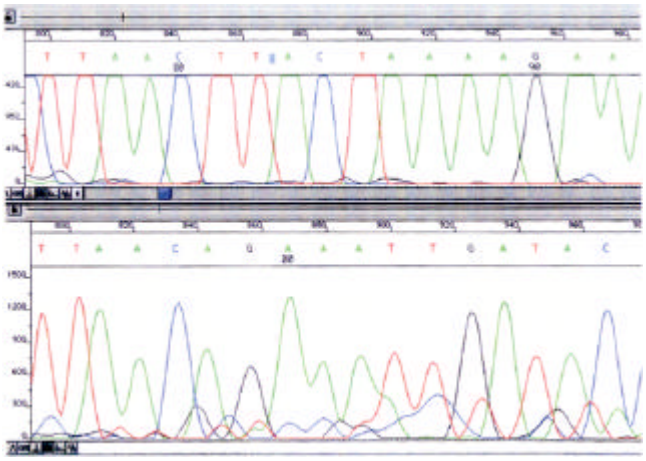

Figure 2. *RB1* exon 4 wild-type sequence using forward primer (*top*), and *RB1* exon 4 in bilateral patient using forward primer, indicating 8-bp TTACTAAA deletion (*bottom*).

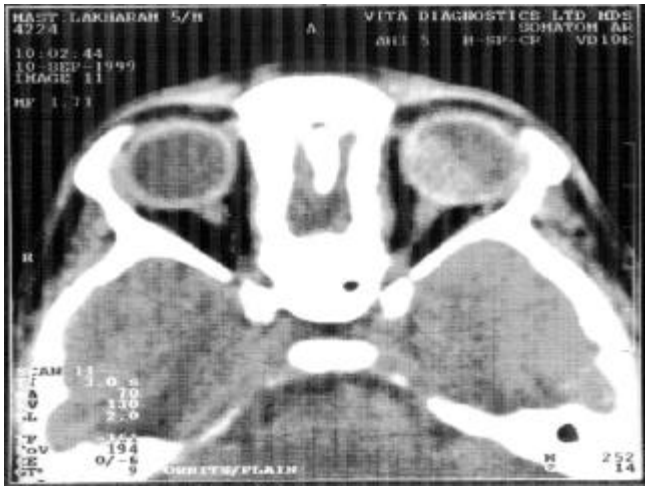

Figure 3. Computerized tomography scan showing the tumour mass of the unilaterally affected patient.

Discussion

RB1 encodes a 110-kDa protein that is important in controlling cell division (Zheng and Lee 2001). Multiplex PCR is a necessity for mutation screening of the 27 exons of *RB1* (Scheffer *et al*. 2000), and we adopted this methodology to screen our patients. The amino-terminal region of the RB protein contains six consensus CDK phosphorylation sites, which may play a role in regulating the cell cycle (Knudsen and Wang 1997). Amino-terminal-deficient mutant RB protein is insufficient for RB function and tumour suppression (Dryja *et al*. 1993; Riley *et al*. 1997). In consonance with these observations, we speculate that the novel 8-bp deletion mutation in *RB1* exon 4, which is more towards the amino terminal part of the protein, seen in the bilaterally affected patient could be a low-penetrance mutation, but why it caused bilateral and early-onset disease is, however, not clearly understood. This patient also had deletion of chromosome region 13q14 and this could be the reason why the phenotype was severe. There was no protein product from the chromosome carrying the deletion and the 8-bp-deletion allele produced a defective protein, resulting in nil tumour suppressor function. However, the patient is doing well after the four-year follow-up period.

Both the transition mutations of the unilateral case occurred in cytosine of CGA codons in exons 8 and 14.

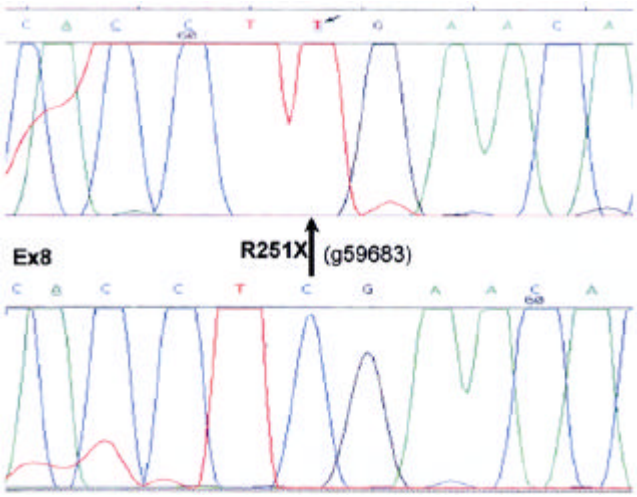

Figure 4. *RB1* exon 8 transition mutation in tumour DNA of unilateral retinoblastoma patient. *Top*, *RB1* exon 8 sequence in patient using forward primer, indicating C → T transition. *Bottom*, *RB1* exon 8 wild-type sequence using forward primer.

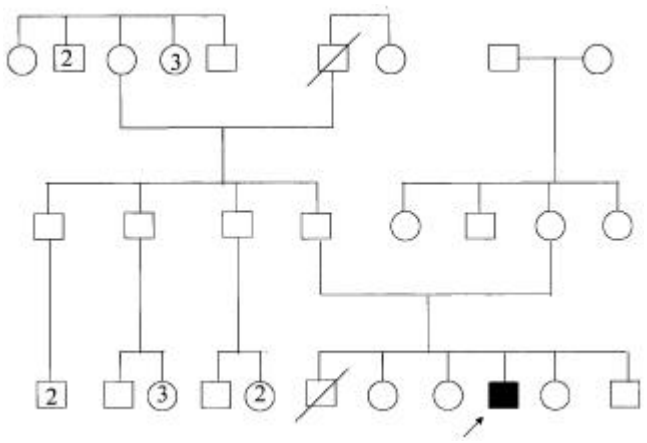

Figure 5. Pedigree of the unilaterally affected retinoblastoma patient.

Most of the nonsense mutations reported in *RB1* are recurrent transitions in CGA codons within the open reading frame. The predisposition for deamination of cytosine is the cause of recurrent transitions at CpG sites (Harbour 1998). The CpG site in exon 14 of *RB1* is one among the CpG sites where frequent transition mutations are reported. The Arg → Stop mutations could have resulted in a truncated protein curtailing its normal activity, but how this high-penetrance mutation could have caused a unilateral late-onset disease is not clear. Both the mutations could not be observed in peripheral blood DNA and this could be because of mosaicism (Munier *et al*. 1998). Understanding that these mutations were not constitutional in this child helped us to infer that the risk for future progeny and first-degree relatives in the family was not significant.

Cost comparison of conventional clinical and molecular screening methods has clearly shown that the latter is comparatively a cheaper approach (Noorani *et al*. 1996). Molecular screening would help to determine the penetrance or expressivity and help decisively in clinical management (Harbour 1998; Munier *et al*. 1998; Otterson *et al*. 1999; Alonso *et al*. 2001). In our study, a bilateral patient had a novel 8-bp deletion in the retinoblastoma tumour which could have resulted in dysfunctional protein; he also had a constitutional 13q14 deletion. The tumour from a unilateral patient showed a mutation that changed an arginine codon to a stop codon (CGA → TGA) in exon 8 as well as in exon 14. This again could have resulted in a truncated protein. Understanding the molecular-genetic basis of the disease in these cases helped in counselling the families much more effectively and calculating the risk for future progeny.

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