

Retinoblastoma in India

Microsatellite Analysis and its Application in Genetic Counseling

Vedam L. Ramprasad,¹ Jagadeesan Madhavan,¹ Sakthivel Murugan,¹ Jagadeesh Sujatha,² Seshadri Suresh,³ Tarun Sharma⁴ and Govindasamy Kumaramanickavel¹

- 1 SN ONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Sankara Nethralaya, Chennai, India
- 2 Department of Clinical Genetics, Fetal Care Research Foundation, Chennai, India
- 3 Department of Ultrasonography, Prenatal Diagnosis and Therapy, Fetal Care Research Foundation, Chennai, India
- 4 Shri Bhagwan Mahavir, Vitreoretinal Services, Medical Research Foundation, Sankara Nethralaya, Chennai, India

Abstract

Objectives: This study was conducted with two objectives. The first was to estimate the frequency of loss of heterozygosity (LOH) of the *RB1* gene as a mechanism in disease causation in tumors of patients from India. The second objective was to employ *RB1* molecular deletion and microsatellite-based linkage analysis as laboratory tools, while counseling families with a history of retinoblastoma (RB).

Methods: DNA was extracted from peripheral blood and tumors of 54 RB patients and their relatives. Eight fluorescent microsatellite markers, both intragenic and flanking the *RB1* gene, were used. After PCR amplification, samples were run on an ABI PRISM® 310 genetic analyzer for LOH, deletion detection, and haplotype generation.

Results: LOH was found in conjunction with tumor formation in 72.9% of RB patients (39/54 patients; $p = 0.001$; 95% CI 0.6028, 0.8417); however, we could not associate various other clinical parameters of RB patients with the presence or absence of *RB1* LOH. Seven germline deletions (13% of RB patients) were identified, and the maternal allele was more frequently lost ($p = 0.01$). A disease co-segregating haplotype was detected in two hereditary autosomal dominant cases.

Conclusion: LOH of the *RB1* gene could play an important role in tumor formation. Large deletions involving *RB1* were observed, and a disease co-segregating haplotype was used for indirect genetic testing. This is the first report from India where molecular testing has been applied for RB families in conjunction with genetic counseling. In tertiary ophthalmic practice in India, there is an emerging trend towards the application of genetical knowledge in clinical practice.

Background

Retinoblastoma (RB) is the most common malignant intraocular childhood tumor, having an estimated incidence of 1 in 15 000–20 000 live births.^[1] The tumor is retinal in origin and is usually fatal if untreated. However, with early detection and current methods of treatment the survival rate is >90%. Studies of RB tumors revealed that both hereditary and non-hereditary tumors are initiated by the loss of both alleles of the tumor suppressor gene *RB1*.^[2] Family history of RB is seen in <25% of all

cases.^[3] The rate of familial RB in India is not available. In familial RB, susceptibility is transmitted as an autosomal dominant trait due to mutations in the *RB1* gene. Offspring of patients with a family history of RB or bilateral tumors have a 50% risk of inheriting the mutant allele, and a 45% risk for developing RB. In the somatic cells (retinoblasts) of an individual with an already existing germline defect (first hit), there is one functionally inactive tumor suppressor allele of germline origin and one normal allele encoding a functional RB1 protein. The subsequent second hit will be a somatic event, leading to a defective RB1 protein,

resulting in the formation of RB. The second hit may occur through several mechanisms to inactivate the *RB1* gene, and the defective gene could be in a heterozygous, homozygous, or hemizygous state. The molecular mechanism (loss of heterozygosity [LOH], a single event for *RB1* gene inactivation) could be a result of deletion, mitotic recombination, non-disjunctional chromosome loss with or without reduplication, gene conversion, point mutation, or epigenetic allelic inactivation.^[4] LOH is the most common mechanism for tumor formation and hence could be part of RB molecular diagnostics.

In familial RB, molecular testing could differentiate those who are at risk of developing the disease from those who are not, with the exception of low penetrance RB. The risk for offspring of the 15% of unilateral RB patients carrying a germline mutation is 45% (for 90% penetrance), as in bilateral RB patients.^[5] Timely and sensitive molecular diagnosis of *RB1* gene defects enables early intervention that could lower the risk for mortality and morbidity in these patients. These tests could also help families to make informed family planning decisions, and the cost of the test for the family would be less than the conventional clinical surveillance.^[6,7] Molecular testing in familial/hereditary cases could be either direct or indirect; in the latter, linkage analysis using tightly linked microsatellite markers to *RB1* are used.

In our tertiary referral eye hospital, we see ≈60 new cases of RB every year, and molecular testing is becoming very important in the timely and efficient management of the patient and their family. Hence, we studied the frequency of LOH/*RB1* deletion and also applied haplotype-based family linkage analysis as a part of

predictive genetic testing in unilateral, bilateral, and familial cases of RB in the Asian Indian population while counseling the family.

Materials and Methods

Subjects

Diagnosis of RB was made based on complete ophthalmic examination, including visual acuity, slit lamp biomicroscopy fundus examination done under anesthesia, and histological criteria. Tumors were graded according to Reese-Ellsworth classification.

Blood samples were collected from 54 probands during enucleation or during a visit to the genetics department for counseling. Blood samples were also collected from the parents and all the family members in familial cases. Pre-test genetic counseling was given, where explanation about LOH, molecular deletion, linkage analysis, and also implications and limitations of the tests were given to the parents of RB patients in lay terms. All the investigations were performed after informed consent was obtained from the parents. The institutional review board approved this study and protocols were as per the Helsinki declaration.

DNA Extraction and Purification

DNA from the venous blood of RB patients and their family members and tumor DNA from the enucleated eyeball was isolated by phenol-chloroform extraction. Briefly, tissue was digested using digestion buffer (1M Tris, 200μL; Triton, 50μL; Milli Q water 740μL and proteinase K 10μL [15mg/mL]) and incubated at 60°C for 1 hour for blood samples, and 37°C for 48 hours for tumor samples. After digestion an equal volume of phenol was added, mixed, and centrifuged at 2500 rpm for 10 minutes. The aqueous phase was washed with an equal volume of phenol : chloroform : isoamyl alcohol, until the interphase was minimum. To the final aqueous layer, a chloroform : isoamyl alcohol mixture (24 : 1) was added and centrifuged for 10 minutes at 2500 rpm. DNA was precipitated by the addition of 1/10 volume of saturated NaCl and two volumes of chilled ethanol, this was then kept at -20°C for 16 hours. Finally, the tube was brought to room temperature and centrifuged at 2500 rpm for 10 minutes. The pellet was washed with 70% ethanol and dried at 37°C, then dissolved in 500μL of Tris-EDTA buffer (Tris, Triton, and proteinase K were obtained from Bangalore Genei, Bangalore, India; all other reagents obtained from SISCO Research Laboratories Pvt. Ltd, Mumbai, India).

Table I. Details of microsatellite DNA markers in the 13q14-q21 chromosomal region flanking and within the *RB1* gene, used for loss of heterozygosity, molecular deletion, and linkage analysis in retinoblastoma

S.no	Microsatellite marker	Mapping information (marker position in cM/mbp)		
		Genethon map ^[9] (cM)	Marshfield map ^[10] (cM)	Ensembl data ^[11] (mbp)
1	D13S263	40.4	38.32	40.9
2	D13S153 (RB i.2)	47.5	45.55	47.78
3	RBI.20	47.5	45.55	48
4	D13S262	47.5	45.55	50.15
5	D13S284	47.6	45.55	Not mapped
6	D13S1320	50.10	49.38	62.70
7	D13S1296	53.5	52.64	69.5
8	D13S156	57.3	55.85	73.55

cM = centiMorgan; **mbp** = mega base pairs; **S.no** = serial number.

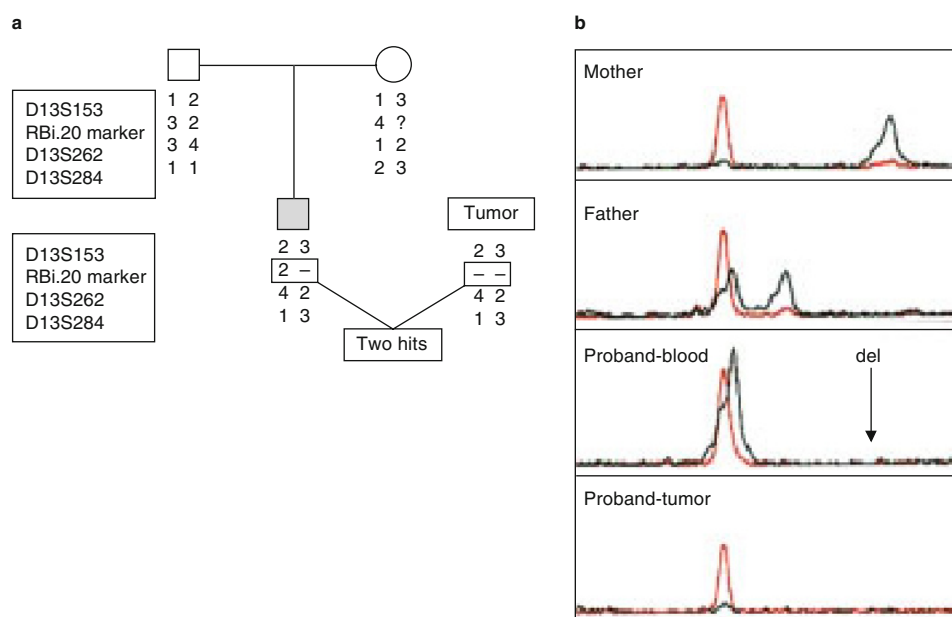


Fig. 1. Analysis of a case (G688/02) showing loss of the maternal allele at the RBi.20 microsatellite marker. Pedigree (**a**) and fluorescent microsatellite analysis of RBi.20 marker (**b**) for a patient (G688/02) with a bilateral tumor that was noticed 15 months after birth. On the left eye, the multifocal tumor involved the whole of the vitreous cavity by 3 months after birth with no choroidal or optic nerve extension. (**a**) The pedigree shows the haplotypes for the indicated microsatellite markers (boxed at left) in both parents and the proband, as well as in tumor DNA from the proband. The proband carries a deletion allele (-) at the RBi.20 marker, with a second deletion occurring in the tumor. The mother had an uninformative allele (indicated by '?') at this marker. (**b**) Fluorescent microsatellite analysis of the RBi.20 marker in the proband and his parents, showing allelic peaks in green (VIC dye). The arrow in the proband-blood panel indicates a peripheral blood deletion of the maternal allele. Tumor DNA shows no allelic peak, indicating loss of heterozygosity. The red peaks show the ROX 500HD size standard.

Amplification of Microsatellite Markers

DNA was PCR amplified using fluorochrome-labeled microsatellite primers (Applied Biosystems, Foster City, CA, USA). A total of eight fluorescent microsatellite markers at the 13q14-q21 region flanking and within the *RB1* gene (linear chromosomal sequence cen-D13S263, D13S284, D13S153 [a repeat in intron 2 (RBi.2)], a tetranucleotide repeat in intron 20 [RBi.20], D13S262, D13S1320, D13S1296, D13S156-tel [Applied Biosystems]) were used.^[8] Initially, D13S284, D13S153, RBi.20, and D13S262 were used for genotyping. However, additional microsatellite markers (D13S263 and D13S1320) were also used (see table I) in case of non-informativeness and to exclude recombination. The primers were labeled with FAM, VIC, or NED fluorescent dyes. The loci were selected from the Genethon human genetic linkage map,^[9] based on chromosomal locations and heterozygosity. All markers were part of ABI linkage panel version 2.5, except D13S262, RBi.20, and D13S284. In deletion detection, a microsatellite marker on a different chromosome (i.e. not chromosome 13) was amplified as a control for PCR amplification.

PCR was carried out as 5μL reactions in 0.2mL tubes with 20ng of DNA, 0.02μM of each primer, 10× PCR buffer, 1.5mM MgCl₂,

0.3 units of Taq polymerase, and 40nM deoxynucleotide triphosphates (Bangalore Genei, Bangalore, India). PCR was standardized to 5μL reactions in order to reduce the amount of DNA that was used, as tumor DNA is precious. For microsatellite markers D13S263, D13S153, D13S1320, D13S1296, and D13S156, PCR was performed with an initial denaturation at 95°C for 12 minutes; 10 cycles consisting of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 30 seconds; and 30 cycles consisting of denaturation at 89°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds. These markers were part of ABI PRISM® Linkage Mapping set, version 2.5 (panels 17, 18, 19, and 67). Markers D13S284, RBi.20, and D13S262 were custom made (primer sequence according to Alonso et al.^[5]) and the PCR was standardized with an initial denaturation at 94°C for 2 minutes; 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. After PCR amplification, 1.25μL of amplified product was mixed with 12μL of injection mix. The injection mix consisted of 11.5μL of deionized formamide and 0.5μL of ROX-500 HD size standard (Applied Biosystems, Foster City, CA, USA). Samples were denatured at

Table II. Results of loss of heterozygosity (LOH) analysis and associated phenotype in patients with retinoblastoma (RB)

Case no.	Case reference	Sex	Age at diagnosis (mo)	Laterality of RB	Differentiation	LOH ^a
1	G95/04	Female	36	BL	UD	+
2	G234/01	Male	18	UL	WD	+
3	G21/00	Male	10	BL	UD	+
4	G311/97	Male	36	BL	WD	+
5	G22/99	Male	6	UL	WD	+
6	G435/99	Female	10	BL	WD	+
7	G557/99	Female	11	UL	WD	–
8	G320/00	Male	15	UL	UD	+
9	G520/99	Male	48	BL	WD	+
10	G492/99	Male	21	BL	WD	+
11	G842/02	Male	3	UL	UD	+
12	G223/00	Male	11	BL	WD	+
13	G96/99	Male	5	BL	WD	+
14	G600/99	Female	36	BL	UD	+
15	G803/00	Male	12	BL	WD	+
16	G844/02	Male	10	BL	WD	+
17	G840/03	Male	36	BL	UD	–
18	G560/02	Male	22	UL	UD	+
19	G368/02	Male	1	BL	WD	+
20	G452/03	Male	1	BL	WD	–
21	G283/00	Male	12	BL	WD	+
22	G591/99	Female	6	BL	UD	–
23	G266/97	Female	21	UL	UD	+
24	G773/00	Male	18	BL	WD	+
25	G688/02	Male	18	BL	UD	+
26	G38/03	Female	2	BL	WD	+
27	G800/03	Female	84	UL	UD	+
28	G658/03	Male	24	BL	WD	+
29	G670/03	Female	12	BL	WD	–
30	G213/03	Female	10	UL	UD	+
31	G821/03	Female	78	UL	UD	– (0.07) ^b
32	G606/02	Male	54	BL	WD	–
33	G73/02	Female	60	UL	WD	+
34	G132/02	Female	20	UL	UD	+
35	G637/02	Female	8	BL	UD	+
36	G804/02	Male	12	UL	WD	–
37	G146/00	Male	30	BL	WD	+
38	G131/00	Male	36	BL	UD	–
39	G81/04	Male	60	BL	UD	– (3.0) ^b
40	G862/03	Female	14	BL	UD	+

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Table II. Contd

Case no.	Case reference	Sex	Age at diagnosis (mo)	Laterality of RB	Differentiation	LOH ^a
41	G184/02	Female	60	UL	UD	+
42	G765/00	Male	42	UL	WD	–
43	G46/01	Male	48	BL	UD	+
44	G27/01	Female	12	UL	UD	+
45	G350/02	Female	24	BL	UD	–
46	G193/02	Male	11	BL	WD	+
47	G183/00	Female	36	UL	UD	–
48	G141/01	Female	42	UL	UD	–
49	G223/99	Male	5	BL	WD	+
50	G426/99	Male	60	UL	UD	+
51	G138/03	Female	2	BL	WD	+
52	G112/04	Female	2	BL	No viable cells	–
53	G28/04	Female	30	UL	UD	+
54	G296/03	Male	7	UL	WD	–

a Total loss of the allele unless otherwise indicated.

b Allelic imbalance value calculated.

BL = bilateral; **UD** = undifferentiated; **UL** = unilateral; **WD** = well differentiated; + indicates present; – indicates absent.

95°C for 5 minutes, cooled on ice and run on the ABI PRISM® 310 genetic analyzer (Applied Biosystems). Analysis was performed using GeneScan software (version 2.1). In cases of allelic imbalance, LOH was evaluated according to the GeneScan reference guide of ABI PRISM® 310 genetic analyzer. The mathematical calculation is $LOH = \text{height of tumor allele two} / \text{height of normal allele one} / \text{height of normal allele two} / \text{height of tumor allele one}$. An LOH value ≤ 0.5 indicates that the tumor sample shows significant loss of the longer allele, whereas an LOH value ≥ 1.5 indicates that the tumor sample shows significant loss of the shorter allele.^[8]

Results and Discussion

Loss of Heterozygosity

LOH was studied in 54 patients; 21 with unilateral RB tumors and 33 with bilateral RB tumors. LOH was noticed in 39 patients (72.2%), including 14 (36%) with unilateral tumors, and 25 (64%) with bilateral tumors. Analysis of a case showing LOH in a patient with bilateral tumors is shown in figure 1. Of the patients without LOH (n = 15), 7 had unilateral RB, and 8 had bilateral RB. LOH could play an important role in tumor formation in the tumors of our patients (p = 0.001; 95% CI 0.6028, 0.8417 [‘Z’ test for proportions]). The mean age at diagnosis for LOH-positive patients was 22.94 months (range = 1–84 months) and for LOH-

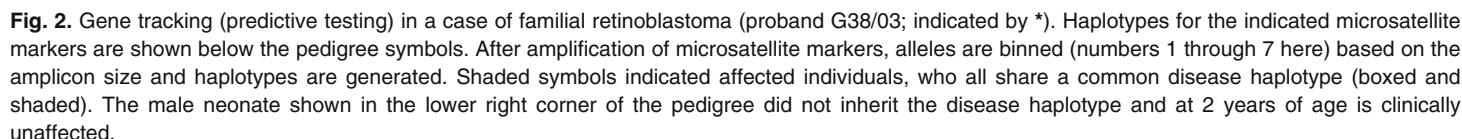
negative patients was 24.4 months (range = 1–78 months). However, in the present study, age at diagnosis, laterality, and tumor differentiation showed no statistically significant difference between LOH-positive and LOH-negative groups (p = 0.60, p = 0.68, and p = 0.93, respectively) [table II].

LOH has been shown to be an indicator for bad prognosis in osteosarcoma.^[5] Previous studies have reported an association between LOH at the *RB1* locus and slow tumor growth, absence of choroidal invasion, tumoral differentiation, and younger age at diagnosis.^[12] Kato et al.^[13] reported that LOH-negative tumors develop earlier than LOH-positive tumors in hereditary cases of RB; however, we did not find any such association in the present study.

RB1 Germinal Deletions

Among the seven germinal deletions identified in the current study, the paternal allele of the *RB1* gene was lost in only one case. In the remaining six cases, the maternal allele was lost (see figure 1), which was statistically significant (p = 0.01; Fisher exact test for two proportions). This observation contradicts that of earlier reports showing that germline mutations in familial RB preferentially involve paternal alleles.^[5,14,15]

Large deletions in the *RB1* locus have been associated with poor prognosis in osteosarcoma patients.^[5,16] As most of the large *RB1* deletions involve introns 2 and/or 20, a similar percentage should be detected with the microsatellite methodology using



(case 24; G773/00 [table II]), although a segregating disease haplotype was detected in the affected members, indirect testing was not immediately helpful because of homozygosity of two *RB1* intragenic marker alleles (data not shown).

In the fourth familial case (case no. 26; G38/03 [table II]), linkage analysis was also useful. The proband was an affected 4-year-old girl who had bilateral RB, which had been diagnosed elsewhere. The right eye had been enucleated at the age of 2 months, and the tumor in the left eye was treated with chemotherapy, cryotherapy, laser, and radiotherapies. The patient was later referred to our hospital because of the development of a new tumor mass in the left eye. An ophthalmologist at our hospital, having noticed the positive family history for the disease, referred the case for genetic counseling. Five members in this three-generation affected family had RB (figure 2). A specific haplotype (1-1-5-5-6-1) co-segregating with the disease was detected in all of the affected members, with no recombination of intragenic markers. As there was an urgency to perform predictive testing on a neonate whose father was affected, we generated the haplotype of the neonate and compared this with the disease haplotype (1-1-5-5-6-1). The disease haplotype was not detected in the

Linkage Analysis

Linkage analysis (indirect testing) was performed on five familial RB cases. In the first familial case (G101/2004), the haplotype was not conclusive because of suspected low penetrance RB (data not shown). In the second familial case (case no. 1; G95/04 [table II]), where the proband and proband's mother were affected, genotyping revealed a molecular deletion of one of the intragenic markers in affected members (data not shown). This information was helpful in counseling the family. In the third case

newborn, but a normal paternal haplotype (4-6-4-2-4-2) was seen. Following this, the parents of the child were counseled and informed that the child had a very low risk of developing RB, which was a relief for the affected family. To date, the 2-year-old child is clinically unaffected.

In the fifth familial case (G-293/2000), the proband's father had a regressed tumor and the female offspring had bilateral RB (data not shown). The mother was pregnant when she came for consultation, and the couple were anxious to know the risk for their next child. As direct screening by mutational analysis is time consuming, an umbilical cord sample was collected and the haplotype was determined. In this case, the child inherited the paternal risk allele. We observed her through pre-natal ultrasound for intra-ocular tumors (figure 3). The infant is presently 6 months old with no signs of retinoblastoma and is under clinical observation.

Thus, in the given scenario, linkage analysis remains an indispensable tool for the identification of *RB1* deletions, which account for a critical 15% of all the mutations in RB in general. Using microsatellite markers as part of linkage analysis is a robust

and sensitive method for detecting whole gene deletions, and assists in the assessment of individuals at risk in families with a history of RB.^[5]

Even though indirect genetic testing by linkage analysis is relatively quick and simple, there are limitations with the test. One limitation is the availability of samples from family members for the determination of known meiotic phase. In addition, the DNA marker used for gene tracking is not the gene sequence that causes the disease, and hence could result in an incorrect prediction if a recombination separates the disease and the marker. Moreover, linkage-analysis based indirect testing can be done only in familial cases where there is more than one affected person in the pedigree, and the test cannot be applied in low penetrance RB cases. Thus, mutation analysis by direct sequencing remains the gold standard to detect defects in the *RB1* gene.

Conclusion

In the present study, a microsatellite-based genotyping methodology has identified that (i) LOH has an important role in tumor formation; (ii) deletions were observed; and (iii) a disease segregating haplotype was used for indirect genetic testing in a newborn and also for pre-natal diagnosis in another family. The studied method and protocol is simpler and faster than conventional Southern blot methodology. This is necessary in developing countries with high fertility rate, such as India, where there is a great need for molecular diagnostics in genetic diseases for application in counseling. This is the first report from India where we show that modern genetic laboratory tools were applied in clinical practice and counseling.

Acknowledgments

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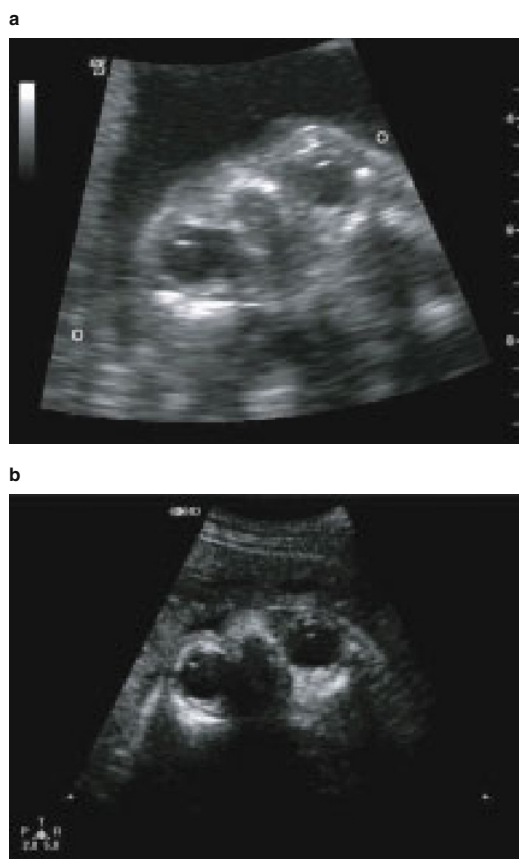


Fig. 3. Prenatal ultrasonographic picture of normal eyes of the fetus carrying the defective *RB1* gene in the peripheral blood. (a) 25–26 weeks of gestation. (b) 34–36 weeks of gestation.

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Correspondence and offprints: Dr *Govindasamy Kumaramanickavel*, SN ONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Sankara Nethralaya, 18 College Road, Chennai (Madras), 600 006, India.

E-mail: gkumarmvel@rediffmail.com