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Retinoblastoma Mutational Screening in India: Opportunities and Challenges in Clinical Decisions and Genetic Counselling

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Abstract

Background: India accounts for 20% of the global retinoblastoma (RB) burden. Existing data is sparse on *RB1* gene germline mutations and its influence on clinical decisions is minimally explored.

Methods: Fifty children with RB underwent complete clinical examination and appropriate multidisciplinary management. Screening of germline *RB1* gene mutations was performed through next-generation sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. The mutation and non-mutation groups were compared for clinical parameters especially severity and recurrence.

Results: Twenty-nine patients had bilateral RB (BLRB) and 21 had unilateral RB (ULRB). The genetic analysis revealed 20 *RB1* variations in 29 probands (79%), inclusive of 3 novel mutations, previously reported 16 mutations and heterozygous whole gene deletions. The mutation detection rate (MDR) was 86.2% in BLRB and 19% in ULRB. Associations of disease recurrence (p=0.021), progression (p=0.000) and higher percentage of optic nerve invasion, subretinal seeds and high-risk pathological factors were observed in the mutation group. Clinical management was influenced by the presence of germline mutations, particularly while deciding on enucleation, frequency of periodic follow up and radiotherapy.

Conclusions: We identified novel *RB1* mutations and our mutation detection rate was at par with previous robust global studies. Genetic results influenced clinical management and we suggest that it should be an essential and integral component of RB-care in India.

Background

Retinoblastoma (RB) (OMIM#180200) is the commonest childhood intraocular tumor, with a global estimated annual incidence of 1 in 15,000 to 20,000 live births(1). India accounts for the highest global burden having one out of every five RB children with an estimated annual incidence of 1500 RB children(2-4). RB occurs due to the two-hit hypothesis of Knudson, which is because of loss-of-function of the tumour suppressor *RB1*gene, owing to homozygous allelic mutations, loss of heterozygosity mechanism or gene silencing(5). *RB1* is a nuclear phosphoprotein, essential for G1/S check point during the cell cycle regulation, while in a dephosphorylated state binds to mitotic agents like E2F, viral particles and other factors, but releases them during mitosis when phosphorylated. *RB1* gene is located on chromosome band 13q14.2, consisting of 27 exons, which encodes a 4.7 kb mRNA. So far, 1748 unique *RB1* variants in 3366 individuals have been identified and summarized in the Leiden Open Variation Database (LOVD)(6). Most of the *RB1* mutations are unique and found in exon, splicing introns and untranslated regions[5-8]. Interestingly, *RB1* exon deletions are seen not only in RB but also less frequently in breast cancer, osteosarcoma and lung cancer.

Usually, in any given population, there are more children with unilateral RB (ULRB-60%) than bilateral (BLRB-40%) and a clinician has to keep in mind that a majority of those with BLRB and a small proportion of those with ULRB might have germline *RB1* mutations, who may need genetic screening and

counselling(2). Genetic screening could play a vital role in management of RB which could influence various crucial clinical decisions (7).

Unless genetic testing is available, the minority of unilateral hereditary cases, fail to get the desirable clinical management decisions, genetic counselling and frequent clinical surveillance. Hereditary RB tends to be early in onset, bilateral and multifocal, hence needs continuous surveillance for early detection, effective management, which could reduce health costs and morbidity. Apart from retinoblastoma, these children have higher predisposition to other cancers (osteosarcoma) which warrant close monitoring and appropriate lifetime support. All cases with mutation, as mentioned earlier, have a lifetime risk for osteosarcoma, soft tissue sarcoma, malignant melanoma or multiple brain tumours. Hence they need lifelong follow-ups, as opposed to sporadic cases, which may not have genetic predisposition(8)(8). Between 1905-2005 about 199 RB survivors were retrospectively analysed for second primary tumours (SPT) and found that 44 of them developed SPT(9). Any form of radiation for investigation (like X-ray, CT scan) or treatment has to be preferably avoided in all germline cases, due to probable increased risk of second malignancies. Besides North America and Germany, *RB1* mutations have been reported from various populations around the world like, Argentina(10), Brazil(11), China(12, 13), Colombia(14), Ecuador(15), Egypt(16), India(17-21), Iran(22), Israel(23, 24), Italy(25), Korea(26), Netherlands(27), Spain(28, 29), Malaysia(30), Mexico(31), Morocco(32), New Zealand(33), Pakistan(34), Swiss(35), Tunisia(36) Singapore(37) Thailand(38) and United Kingdom (39). Out of five earlier studies from India, stratifying genetic tests is an option suggested by Thirumalairaj et al (40).

Though enormous number of studies are available on *RB1* gene mutations across the globe, including India, there is limited information on how the genetic result could influence clinical management. Hence, we undertook this study to describe and associate to correlate the genetic and clinical parameters of 50 RB patients from India. We also examined the opportunities and challenges in clinical decisions and genetic counselling which were influenced through *RB1* gene screening in a developing country scenario.

Methods

Patient recruitment and clinical examination

Fifty (48 unrelated and two related siblings) RB patients (aged 0.2 yrs-5.3 yrs) with various clinical presentations, from the Department of Paediatric Ophthalmology, Narayana Nethralaya, Bangalore, India were recruited from June 2014-Feb 2015. Among these twenty-nine were BLRB and twenty-one had ULRB. A complete clinical examination was carried out under general anaesthesia which included dilated retinal evaluation, imaging of retina using wide field fundus camera (Retcam), measurement of intraocular pressure, anterior segment evaluation by handheld slit lamp. Also, magnetic resonance imaging (MRI) of the orbits and brain, B scan ultrasonography of the eye, cerebrospinal fluid analysis and bone marrow analysis were performed when indicated. The clinical disease was classified as per the AJCC TNM classification for RB, as well as the International Classification of Intraocular Retinoblastoma(41). The study was approved by the Institutional Ethical Committee, which followed the

Tenets of the Declaration of Helsinki. After ascertaining pedigree and written informed parental consent, five ml of blood sample was obtained in EDTA coated vacutainer tubes from patients (during examination under anaesthesia) for genetic analysis. For clinical analysis the cohort was divided into two groups - those with and without *RB1* mutations.

NGS target sequencing of RB1 gene analysis:

Genomic DNA was used for targeted gene capture using a custom capture kit. Briefly, 1ug of DNA was subjected to fragmentation resulting in an average size of 150bp followed by end repair, adenylation, adaptor ligation and amplification to obtain whole genome libraries using the Kapa DNA library preparation kit v2.14. These libraries were then hybridized to biotinylated probes (NimbleGen, Roche) specific to *RB1*gene for 72hours and extracted using streptavidin beads, washed and normalized. The libraries were then sequenced to mean >80-100X coverage on Illumina sequencing platform (HiSeq 2500). The sequences obtained are aligned to human reference genome (GRCh37/hg19) using BWA program (42), (43) and analyzed using Picard and GATK-Lite tool kit (44) (45) to identify variants relevant to the clinical indication. Annotations of the variants were performed against the Ensembl release 75 gene model (46). Clinically relevant mutations were annotated using published variants in literature and a set of variant databases including ClinVar, OMIM, GWAS, HGMD and SwissVar (47-54).

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis:

In order to detect large deletions/duplications in the *RB1* gene, we performed Multiplex Ligation-dependent Probe Amplification (MLPA). SALSA MLPA kit P047 *RB1* (Amsterdam, Netherlands) was used as per manufacturer's recommendations.

Statistical analysis:

Multivariate analysis for genotype phenotype correlation was done using Pearson Chi square test, SPSS software. Clinical factors like sub retinal seeds, optic nerve invasion, pathological high-risk factors (HRF), tumour recurrence, tumor resistance to treatment, need for 2nd line drugs like topotecan and need for radiotherapy were analysed in the mutation versus no mutation groups.

Results

Of 50 RB patients, 29 had BLRB (average age at presentation of 1.8 years) and 21 had ULRB (average age at presentation of 2.3 years). A family history of RB was observed in two patients. In the BLRB group, 25 out of 29 probands (86.2%) had a germline mutation whereas in the ULRB group, 4 out of 21 (19%) had a mutation. NGS and MLPA analyses revealed total of 20 *RB1* gene variations in 29 probands, inclusive of three novel mutations (3 probands, 6% - c.1050-8_1050-2delTTATTTA (intronic splice variant), Q444P and S567P)), previously reported 16 mutations (22 probands: 44%) and heterozygous deletion of whole *RB1* gene (3 BLRB, 1 ULRB, 8%). The types of mutations were, non-sense being the maximum (13),

followed by missense (7), splice site (4), whole gene deletions (4). One proband had frameshift (Table 1 and Fig 1).

TABLE 1: TYPE OF GENETIC ABNORMALITIES

Sr no	Genetic Abnormality	Number of mutations	Number of patients	COMPONENT of Novel mutation	No of pts with novel mutation	Unilateral /**bilateral**
1	WHOLE GENE DELETION	NA	4	NA	NA	1:3
2	MISSENSE MUTATION	7	7	2	2	3:4
3	FRAME SHIFT	1	1	NIL	NIL	0:1
4	SPLICE SITE	3	4	1	1	0:4
5	NON-SENSE	10	13	NIL	NIL	0:13
TOTAL		19	29	3	7	4:25

Genotype to clinical analysis revealed that there was no direct correlation between age of presentation and disease severity between the groups. Clinical features, age of presentation and high-risk features like optic nerve invasion in the groups have been listed in Table 2. Mutation group had more patients with increased severity requiring enucleation (95.23%), optic nerve invasion (64.7%), sub-retinal seeds (68%) and pathological high-risk factors (73.9%). The disease severity factors like average clinical TNM and pathological TNM were stratified as per the mutation type (splice site, missense, termination and whole gene deletion) and the findings are listed in Table 3. In the current cohort, splice site mutation had the highest average clinical and pathological TNM, as well as the youngest average age of enucleation. Disease recurrence and disease progression correlated significantly with mutation group ($p = 0.021$ and $p = 0.000$ respectively). Notably, of the total 10 recurrences in the current cohort, 9 patients had the mutation (Table 4). The mutation detection rate (MDR) was 86.2% in BLRB (25 out of 29) and 19% in ULRB (4 out of 21), which was better than many other global studies and comparable to some of the recent robust ones (Table 5).

TABLE 2 CLINICAL PRESENTATION

	MUTATION	NO MUTATION
AVERAGE AGE OF DIAGNOSIS	1.82 YRS	2.08YRS
NEED FOR ENUCLEATION	23 OF 29(79.31%)	20 OF 21(95.23%)
AVERAGE AGE OF ENUCLEATION	2.08YRS *	2.05 YRS #
OPTIC NERVE INVASION	11 of 17 (64.7%)	8 of 19(42.1%)
SUB RETINAL SEEDS	17 OF 25(68%)	4 OF 20(20%)
PATHOLOGICAL HIGH RISK FACTORS	17 OF 23(73.9%)	9 OF 19 (47.36%)

(* Average age of enucleation calculated after excluding 1 patient who was enucleated at 13.77 yrs # INCLUDES ONE PT WHO PRESENTED AT 5.13 YRS AND WAS NOT ENUCLEATED) **BL detected early and therefore managed early compared to UL which is late.**

TABLE 3: CORRELATING MUTATION VERSUS CLINICAL DISEASE SEVERITY

	SPLICE SITE MUTATION	MISSENESE MUTATION	TERMINATION	WHOLE GENE DELETION
Total	4	7	14	4
U:B	0:04	3:04	0:14	1:03
Age(yrs)	1.45	1.81	1.28	4.09
Enucleation	4(100%)	7(100%)	10(71.4%)	3 (75%)
Average Age at enucleation(yrs)	1.83	1.95	2.02	1.98 *
Avg Pathological TNM	2.6	2.14	1.85	2.3
Avg Clinical TNM	3.3	2.7	2.38	2.75

(* Average age of enucleation calculated after excluding 1 patient who was enucleated at 13.77 years)

TABLE 4: CORRELATION OF GENOTYPE WITH HIGH RISK PHENOTYPE

PHENOTYPIC FEATURES	MUTATION PRESENT n(%)	NO MUTATION n(%)	Pearson Chi-Square tests	
			Chi sq	Significance (p value)
OPTIC NERVE INVASION Y N	 11 6	 8 11	1.83	0.575
RECURRENCE Y N	 9 20*	 1 20	5.25	0.021
PROGRESSION Y N	 16 13	 1 20	13.79	0.000
NEED FOR TOPOTECAN Y N	 7 22	 1 20	3.40	0.065
NEED FOR RADIOTHERAPY Y N	 3 26	 2 19	0.091	0.923

* Of the 20 without recurrence and with mutation, 7 had disease progression and 13 had 'none'

TABLE 5: Mutation detection rates in unilateral and bilateral RB patient groups studies across the globe

S.NO	Author	Country	Type of mutations	Mutation detection rate BLRB	Mutation detection rate ULRB	Year of study
02	Mohd Khalid, M.K., et al.	Malaysia	Nonsense, Frame shift, Splice site & De-novo origin	100%	25%	2015
05	Grotta, S., et al	Italy	Point mutations, Frame shift, Large deletions	96.5%	22%	2015
09	Chen, Z., et al	USA	Nonsense, Splice, Frameshift	97%	18%	2014
07	Price et al	United Kingdom	Point mutation, deletions, missense, splice site mutations	96%	9.5%	2014
10	Seo, S.H., et al	Korea	Missense, nonsense, frameshift and splice	94.5%	none	2013
11	Ottaviani, D., et al	Argentina	Nonsense, frameshift, missense, deletions	94%	-	2013
08	Dommering, C.J., et al	Netherland	Nonsense, frameshift, splice, large indel, missense, chromosomal deletions and promoter.	92%	10%	2014
01	Frenkal.S*et al*	France	Stop codon, Splice site and large deletions	90%	19.8%	2016
15	Macias, M., et al	Mexico	Nonsense, Splice, Frameshift	76.9%	34.8%	2008
16	Abouzeid et al	Switzerland	Nonsense, frameshift, missense, deletions	73%	10.7%	2007
03	Zhang, L., et al	China	Nonsense, Splice, Frameshift	65%	35%	2015
06	Devarajan et al	India	Nonsense, Frame shift, Splice site & Denovo origin	63%	37%	2015
04	Kalsoom, S., et al	Pakistan	Null mutation, deletions, missense, splice site mutations	45.7%	54.3%	2015
12	Barbosa, R.H., et al	Brasil	Nonsense, Splice, Frameshift	42.2%	56.3%	2013
14	Abidi et al.,	Morocco	Duplication, Deletion, Splice, Frameshift	40%	None	2011
17	Choy et al	Hong kong& China	Nonsense, Splice, Frameshift	38%	19%	2002
13	Ahani et al	Iran	Missense, frameshift and splice site	16.6%	18.2%	2013
14	Present study - Himika, Malaichamy, et al	India	Missense, frameshift, gene deletions	86.2%	19%	2020

A nonsense mutation, c. 233G>A (p.W78Ter) was identified in two unrelated patients with bilateral RB. The novel nucleotide changes include two missense substitutions - c.1699T>C (p.S567P), c.1331A>C (p.Q444P) and one splice site variation (c.1050-8_1050-2delTTATTTA). Bioinformatics prediction analysis of SIFT, PolyPhen–2, Provean, showed that the missense substitutions (p.S567P, p.Q444P) had deleterious effect which may affect the functional properties of the protein and both the missense variations are present in the retinoblastoma-associated protein A domain of the RB1 protein. All novel mutations were in BLRB. One of the BLRB patients, who presented at 2.5 years of age, had a p.W78X

mutation and he was diagnosed to have pinealoma and was a case of trilateral RB. Another proband, presented with bilateral disease at 1.5 years with a positive family history. The father had regressed tumour, both the daughter and father carried the same familial mutation, c.1789C>T (Q597Ter). Interestingly, the half sibling of this proband, who was of the same father, presented at the age of 2.3 years with BLRB and had the same mutation c.1789 C>T (Q597Ter). Another interesting aspect was the varied clinical spectrum presentation and outcome of our four BLRB probands with the same termination mutation c.1333C>T (p.R445Ter) (Fig. 2).

Discussion

The 8th AJCC has incorporated "H" or hereditary into the TNM staging of RB, thus envisaging the role of genetics in management - H1 carry the RB1 cancer-predisposing gene; H0 are tested and proven to have normal Rb alleles and HX carry unknown risk(55). In the current era of personalized medicine and cancer-care accessibility, availability and reliability - NGS/MLPA techniques have revolutionised the genetic diagnostic scenario of RB-care globally and also selectively in India [22-26]. Incorporating genetic testing as part of RB-care has significant advantages - these opportunities and challenges are highlighted in the current study. For example, our four ULRB cases who would have otherwise not been monitored closely post treatment completion with the mutation were switched to 3-6 monthly surveillance, like any other BLRB patient with an RB1 mutation in our study cohort. With the advent of ophthalmic artery chemotherapy, intra vitreal and intracameral chemotherapy, globe salvage in RB has reached a paradigm shift (56),(57), thus availability of tumour tissue for pathology studies now is a rarity and in such situations an objective test like NGS/MLPA serves as a useful clinical marker tool. Although, ophthalmic artery chemotherapy involves targeted chemotherapy to just the affected eye and obviates need of systemic chemotherapy, this modality is best reserved for non germline cases. Hence, before using this supra-selective modality in a unilateral disease, the clinical team needs to be sure that the other eye is not at-risk due to an inherited mutation(58). Genetic test as a prognostic marker has been applied in medulloblastoma, paediatric gliomas (59, 60) and breast cancer(61). However, in comparison, clinical adoption of RB genetic diagnostics is poor amongst the clinicians in India and other developing countries.

The mutation detection rates across countries in BLRB varied from 100 to 16.6% and in ULRB from 56.3 to 9.5% (Table 5), the wide variation could be due various reasons inclusive of the fact that the studies were performed prior to highly sensitive NGS/MLPA tests era. Price et al., in United Kingdom studied 403 unrelated patients, 209 blood and 194 tumour samples and identified 533 variations, including RB1 gene mutations and loss of heterozygosity (LOH)(39). In another Netherlands large cohort study, 529 RB patients were screened with a 92% detection rate in BLRB and 10 % in ULRB, the latter being the lowest in the mutation spectrum(27). In the largest mutation meta-analysis of 932 RB patients, it was found that globally the most frequent mutations reported were R320X (nearly 50 times), R579X (nearly 40 times) and R251X (nearly 30 times)(62). All the studies uniformly found deletions, duplications, missense, nonsense, splice and frameshift mutations, once again establishing that *RB1* gene has no hotspot (10-13, 16, 17, 20-24, 26, 30-32, 34-36, 63-66). In our study, we found 20 *RB1* gene variations in 29 probands (79%),

inclusive of three novel mutations, 16 were previously reported mutations, four heterozygous deletions of the whole *RB1* gene. We had one case each of frameshift and commonly reported R251X and R320X and it is to be noted that those with the arginine/termination mutations have a risk to develop SPT (15). In our study, we identified mutations in 86% of BLRB patients and 19% in ULRB – which is comparable to other global studies, however we could not find any mutation in 4 BLRB patients and this could be because of various reasons including mosaicism and *MYCN* gene mutations, which we did not study. Mosaicism is a tricky issue in RB diagnostics and prenatal genetic counselling, hence may go unnoticed suggests Rushlow et al(63, 67).

RB1 mutations have shown variable genotype-phenotype correlations - Taylor et al (68), observed complete penetrance in nonsense, frameshift mutations and large rearrangements, whereas missense mutations showed heterogenous phenotypes and low penetrance, which was contradicted by Zhang et al (69). Lidderman et al, suggested that frameshift, nonsense and aberrant splice mutations result in hereditary RB, whereas missense, inframe and promoter mutations result in variable phenotype with reduced penetrance, (70) similar to Lohman and Gallie and Harbour (71, 72). RB1 gross alterations were found in 15% of 433 BLRB and 6.5% of 262 ULRB patients – these patients developed fewer tumours compared to those with null mutations and interestingly, those with cytogenetic or sub-microscopic whole gene deletions often had ULRB, however all those with gross deletions with one breakpoint inside the RB1 gene had BLRB (73)Notably, in our cohort all cases of ULRB, irrespective of their mutation type, had optic nerve invasion and were severe enough to warrant enucleation. Prior knowledge of mutation may influence enucleation decisions in the subset of ULRB patients, who all had the mutation, the other eye is also ‘at risk’ and must be treated potentially as a ‘bilateral’ case. In the four c.1333C>T (p.R445Ter) BLRB patients, three had disease progression despite treatment, in one bilateral globe salvage was successful by using plaque brachytherapy, two needed unilateral enucleation and one case needed bilateral enucleation due to progressive disease unresponsive to multimodality treatment (Fig. 2). The variable clinical phenotype and response to treatment despite the same mutation, could be due to epigenetic molecular events in the tumor (74). In pineal cyst, a pre-malignant form of pinealoblastoma, BLRB is more common than ULRB where germline mutations are invariably identified (75) and we had a patient with pinealoma, trilateral RB who had the pW78X mutation.

The clinical and pathological factors that are considered as predictors of poor outcome, are the presence of subretinal seeds for recurrence anterior segment, choroidal, optic nerve involvement (76) and glaucoma (77). Pathological high-risk factors have been known to be strong predictors of metastasis in RB and in our study the mutation group had statistically significant progression, recurrence and higher percentage of optic nerve invasion, subretinal seeds and high-risk pathological factors but lower percentage of enucleation compared to the non-mutation group. Those with germline mutations have an 8% lifetime risk of developing osteosarcomas, apart from small cell sarcomas of the lung, and urinary bladder cancers. Radiotherapy is contraindicated in patients with germline mutations and this valuable information could help the clinician to modify treatment options. There are studies describing ill effects of radiation on RB, which however do not have the mutation data (78). Interestingly, analysis of radiation effects based on genetic susceptibility and age clearly shows that radiation induced side effects occur in

patients who are genetically predisposed (79). Rarely the hereditary retinoblastoma could be a part of chromosome 13q deletion syndrome, such children additionally suffer from developmental delay, mental retardation and craniofacial abnormalities, thus warranting active surveillance and rehabilitation from the beginning (80), With the advent of preimplantation genetic diagnosis for retinoblastoma, the avenues for families of hereditary retinoblastoma have tremendously improved (81). This further envisages the importance of genetic analysis in comprehensive retinoblastoma care.

Testing the RB1 gene for mutation is a challenging task, owing to its size, heterogeneity of mutations (with 200 reported), lack of hotspot and the variable intronic lengths (82). In our study, about three patients were exclusively referred for mutation analysis from outside our organisation and they continued receiving clinical care for the RB elsewhere. This envisages the fact that clinical management of RB is well addressed across the country, however the same level of care doesn't exist for genetic testing, even though CAP certified private companies provide NGS/MLPA RB testing at a cost. Moreover, established RB guidelines specifies the role of genetic testing in RB care (8). Centres for RB care without a genetic support, must be aware of this need and should sensitize the family on the role and usefulness of genetic testing and also inform them of the additional cost of care to the family which is not covered by insurance (83). The conclusions made in the study was based on small size and the techniques failed to detect mutations in all the BLRB patients.

Conclusion

In summary, 50 RB patients were screened for *RB1* mutations using targeted NGS and MLPA methodologies, which found detection rates at par with robust global studies. Comparing case-wise genetic findings with various clinical parameters and mutations found that there was clinical phenotypic and allelic heterogeneity and the mutation group had a higher clinical risk of recurrence, which needed additional clinical care. *RB1* mutation screening is an important tool in RB-care globally, irrespective of the socio-economic status of the family and developed or developing country.

Abbreviations

RB- Retinoblastoma, RB1- Retinoblastoma gene, NGS- Next Generation Sequencing, MLPA- Multiplex Ligation dependent Probe Amplification, ULRB- Unilateral Retinoblastoma, BLRB- Bilateral Retinoblastoma, MDR- Mutation Detection Rate, AJCC- American Joint Committee on Cancer, TNM- Tumor Node Metastasis, DNA-Deoxyribo Nucleic Acid, OMIM- Online Mendelian Inheritance in Man, GWAS-Genome Wide Association Study, HGMD- Human Gene Mutation Database, SPSS- Statistical Package for the Social Sciences, LOVD- Leiden Open Variation Database, BWA- Burrows Wheeler Aligner, EDTA- Ethylenediaminetetraacetic acid, LOH- Loss of Heterozygosity, SPT- Secondary Primary Tumors, CAP- Certified Analytics Professional

Declarations

Ethics approval and consent:

The ethics approval and consent for the study have been approved by the Institutional Ethics Committee of Narayana Nethralaya (EC Ref No: C/2013/03/02) and the study have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.Consent for publication:

Written informed parental consent were obtained for publication

Data availability:

The mutation datasets generated and analysed during the study are available in MedVarDb internal database. The clinical & genetic datasets used and analysed during the current study are available from corresponding author on reasonable request.

Competing Interests:

The authors declare that they have no competing interests.

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Authors Contributions:

HG - Writing up clinical part of the manuscript, phenotype and genotype analysis

SM- Writing up the manuscript, laboratory work and analysis

AM- Clinical analysis of the patients, reviewing the manuscript

SM -laboratory critical analysis, guidance, reviewing the manuscript

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Figures

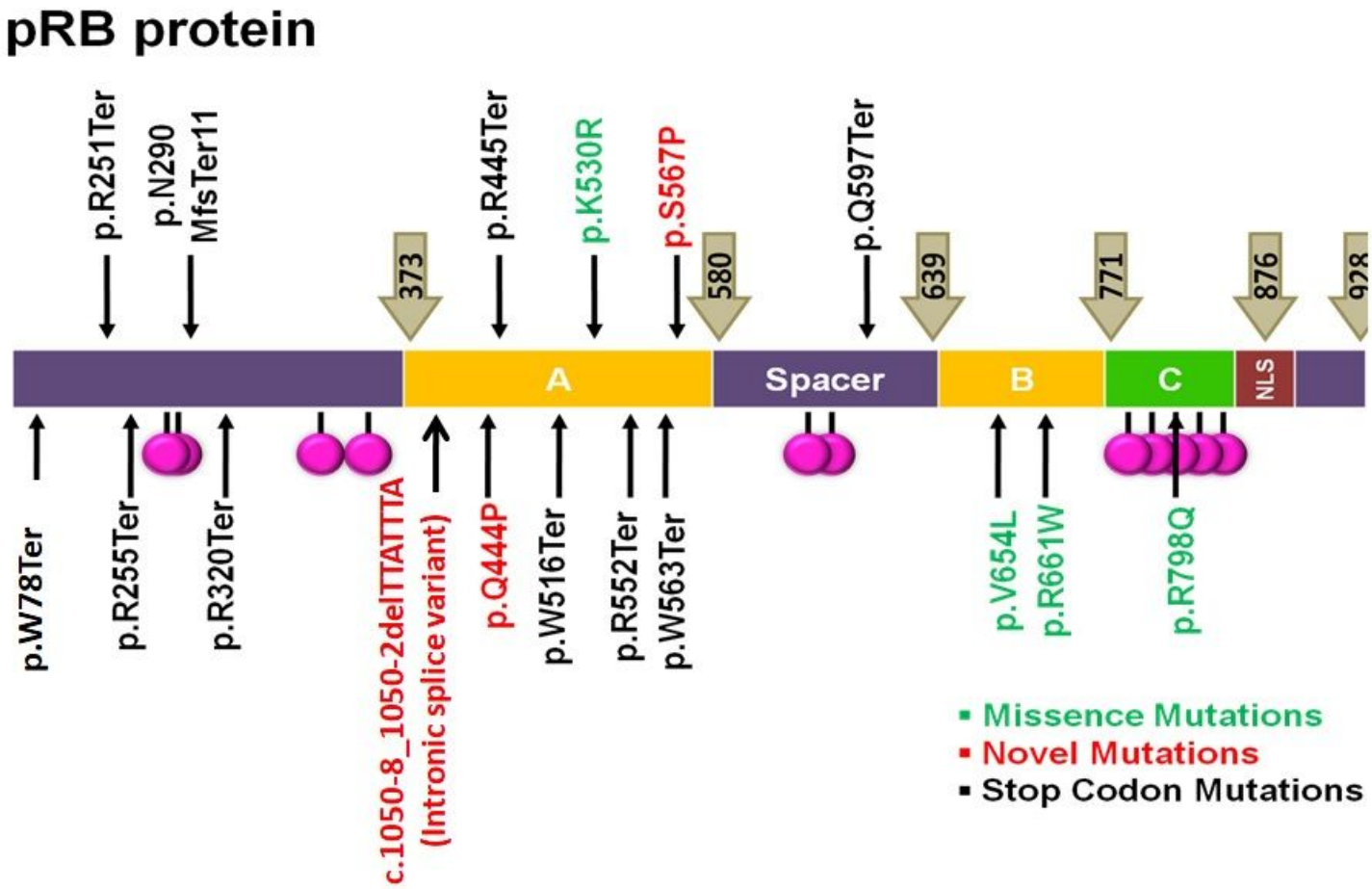

Figure 1

Represents the mutations identified in RB patients distributed across the RB protein structure.

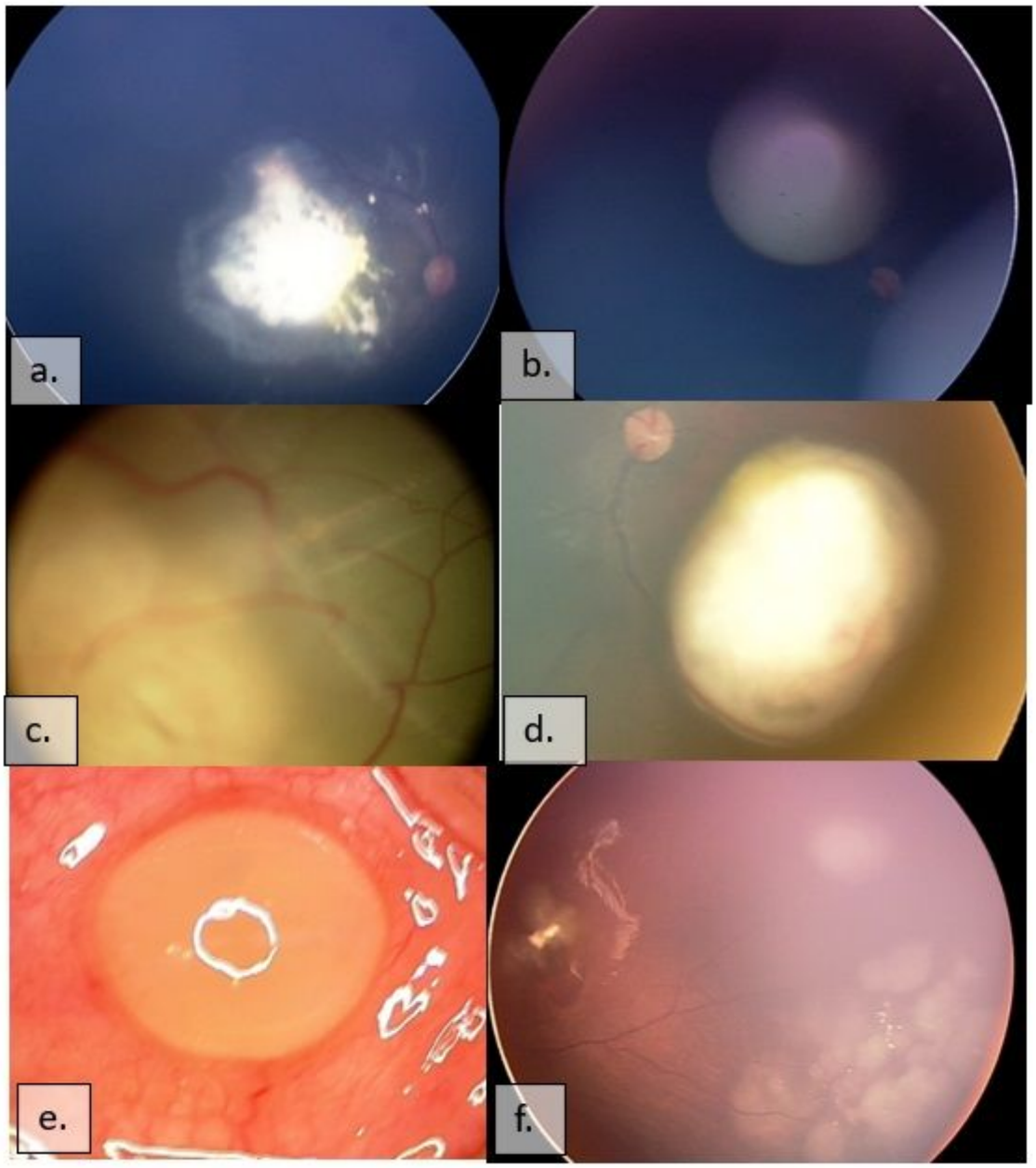

Figure 2

Variable phenotype of same genotype. Case75 (a) and (b) had mild disease in both eyes, bilateral globe salvage successful. Case 55 (c)one eye had severe disease needing enucleation, while other eye(d) had mild disease with successful globe salvage. Case 9 (e) one eye extensive tumor needing enucleation. Later the other eye (f) developed tumor which was nonresponsive to Rx and eventually needed enucleation.