DOI: https://doi.org/10.4038/jcosl.v29i2.80

Research article

A study on genetic variants associated with retinoblastoma in a cohort of Sri Lankan patients

R. S. Gama Athige[1], T. K. Wetthasinghe[2], M. M. Dissanayake[3], D. Irugalbandara[4]

Journal of the College of Ophthalmologists of Sri Lanka 2023; 29: 54-63

Abstract

Introduction: *Retinoblastoma is the most common intraocular malignancy of infancy and childhood (<5yrs) with 8000-9000 cases detected every year. The mean age of diagnosis is 18 months. Symptoms of retinoblastoma range from Leukocoria, red eye, poor vision and squint to glaucoma and proptosis in advanced cases. Different genes such as KIF14, E2F3, DEK, MYCN and CDH11 have been found to be related to this cancer, but the RB1 gene shows the most significant association throughout the world. The RB1 gene which was the 1st tumor suppressor gene to be ever recognized is located at chromosome 13q 14.2 locus and consists of 27 exons and 27 introns. When a single RB1 allele is having a variant, the individual will be exposed to Retinoblastoma and when the balanced normal allele is lost as well for a cancer predisposing variant, the Retinoblastoma will develop. Retinoblastoma cases are reported throughout the entire region across Asia with the highest incidence in India. But still the Asian region is having limited information and poor Retinoblastoma recognition leading to increased mortality rates in the developing countries. This study aims to figure out genetic variants associated with Retinoblastoma in Sri Lankan population in order to support the screening/ testing and early recognition by molecular genetic testing/ screening which will help to reduce the mortality rate and save the vision of Sri Lankan Retinoblastoma patients.*

Materials and methods: *The genetic variants associated with Retinoblastoma were identified through a comprehensive literature review and they were filtered for Asia/ South Asia. Most significant 02 variants were selected out of them (rs121913300 and rs1131690863) and their allele frequencies were manually computed based on the genotyping results reported in RB1 studies across Asia/South Asia. In line with literature review, the availability of resources and the meeting of research criteria, Tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) was determined as the most effective genetic assay for this study. Primers were designed accordingly for the selected 02 variants. After optimizing the PCR protocol, 59 anonymous retinoblastoma patient-blood samples obtained from the Human Genetics Unit, Faculty of Medicine, University of Colombo, Sri Lanka were genotyped and a descriptive study was carried out. The genotyping results were further validated by Sanger Sequencing.*

Results: *All the Retinoblastoma patients were homozygous for the ancestral allele in terms of both the variants rs121913300 and rs1131690863. The manually computed minor allele frequencies determined that both the variants were rare in the Sri Lankan community. Their genotype distributions were in Hardy-Weinberg Equilibrium; hence will continue the same across generations.*

Discussion and conclusion: *The study found that rs121913300 and rs1131690863 were rare among the Sri Lankan patients and therefore these 02 variants would be less important in developing screening tests for the Sri Lankan community.*

Key words: Retinoblastoma, Retinoma, RB1 gene, T-ARMS-PCR, minor allele frequency, genetic screening

Introduction

Retinoblastoma is the most common intraocular cancer of infancy and childhood (<5 years). Around 9000 Retinoblastoma cases are diagnosed every year worldwide[1,2]. During the very early stage of Retinoblastoma, when the tumor is still in the globe will present with a variety of symptoms such as leukocoria, red eye, poor vision and squint. Delay in diagnosis and treatment will cause spreading of the tumor from the globe to the periorbital region and even into the brain, reducing the chance of survival. Proptosis and glaucoma are among other signs and symptoms in the late stage of Retinoblastoma[2].

[1] *MSc Student, Human Genetics Unit, Department of Anatomy, Genetics and Biomedical Informatics, Faculty of Medicine, University of Colombo, Sri Lanka,* [2]*Professor and Medical Geneticist, Department of Anatomy, Genetics and Biomedical Informatics, Faculty of Medicine, University of Colombo, Sri Lanka,* [3]*Professor and Consultant Ophthalmologist, Department of Anatomy, Genetics and Biomedical Informatics, Faculty of Medicine, University of Colombo, Sri Lanka,* [4] *Consultant Eye Surgeon, Eye Unit, Lady Ridgeway Hospital for Children, Colombo, Sri Lanka.*

The *RB1* gene

The *RB1* gene located at chromosome 13q14.2 locus was the 1st tumor suppressor gene ever to be discovered. As per Knudson's 2 hit theory, loss of a single *RB1* allele / 1st hit leads to a predisposed condition to Retinoblastoma. When the 2nd allele is mutated as well in a developing retinal cell (2nd hit), the *RB1* gene completely loses its tumor suppressor ability and Retinoblastoma is initiated[2,3].

Heritable Retinoblastoma patients already carry a germline variant (1st genetic hit) in the *RB1* gene at birth (found in all cells). There's a 50% probability that this germline variant could be transmitted (as autosomal dominant) to the offspring. Afterwards when the 2nd hit occurs as a somatic variant in retinal cells, the Retinoblastoma tumor will progress. When the 2nd hit occurs as a non-ocular somatic variant, secondary non-ocular cancers like osteosarcoma, melanoma, brain tumors, etc. will develop later. Meanwhile, non-heritable (unilateral sporadic) Retinoblastoma shows a normal *RB1* gene at birth and both mutations (hits) occur as somatic variants as the retina develops suggesting a no risk of secondary non-ocular cancers[3].

Mostly the *RB1* variants occur as de novo mutations; more commonly during spermatogenesis than during oogenesis [due to highly active cell division in spermatogenesis]. This makes men of advanced age highly exposed to the risk of Retinoblastoma. Although the de novo *RB1* variants will typically be unique to that respective family line, some recurrent *RB1* variants have been observed in unrelated personnel as well which provides the rationale to this research study. Moreover, *RB1* variants could also be induced by X-rays, smoking, DNA viruses, UV irradiation etc[4].

The *RB1* gene codes for the pRB which involves controlling cell division and cell proliferation by regulating the transcription factors[5]. Majority of *RB1* variants (i.e., frameshift, nonsense) make the pRB completely inactive[2].

Retinoma

Retinoma is typically an underlying condition (a precursor) of Retinoblastoma. Retinoma occurs as the *RB1* gene loses its both alleles. Unlike in retinoblastoma, Retinoma comprises non-proliferative cells and is usually associated with low levels of genetic instabilities and elevated expression of senescence-associated proteins. Therefore, Retinoma is typically a benign condition[6]. Retinoma would transform quickly into Retinoblastoma with the accumulation of genetic variants which is why Retinoblastoma is associated with a high degree of genomic instability[7].

More than 90% of the global Retinoblastoma patients live in developing countries worldwide while 43% of them are from 6 Asian countries including India, Pakistan, Bangladesh, Indonesia[1]. Meanwhile 40-70% of Retinoblastoma patients die in Asia and in Africa while the mortality rate is limited only to 3-5% in Europe, USA and Canada. More than 6 months delay in diagnosis since the 1st clinical sign is leading to the higher percentage of mortality in developing countries[2]. Retinoblastoma outcome is poor in developing nations due to social and technological factors such as lack of Retinoblastoma awareness, inadequate infrastructure, socioeconomic factors and lack of national screening program[1,4]. Furthermore, the treatments available for late Retinoblastoma presentations are expensive and are a burden to the developing economies of the world. Therefore, this study aims to establish cost-effective genetic screening tests for selected Retinoblastoma-related genetic variants to support early diagnosis and genetic counseling in order to reduce mortality.

Materials and methods

A detailed literature review was carried out to analyze the genetic variants associated with Retinoblastoma. It was determined that the *RB1* gene variants were mostly related. The *RB1* variants were filtered for the highest frequency and the geographical region and 2 significant variants were selected for the research study (Table 1).

Table 1. The selected variants of significance for the study[8,9,10]

rs ID	*Protein*	*DNA*	*cDNA*	*Exon*	*Significance*
rs121913300	p.R320X	g.64348C>T	c.958C>T	10	Globally most frequent, Recurrent in India / Asia
rs1131690863	p.R251X	g.59683C>T	c.751C>T	08	Globally 3rd most frequent, Recurrent in India / Asia

This study was conducted as a retrospective study to determine genetic variants associated with Retinoblastoma in a cohort of Sri Lankan patients at the Human Genetics Unit, University of Colombo, during the period of September 2021 to September 2022. During this study, *RB1* variants of interest were genotyped and gel electrophoresis was done. Furthermore, variants were checked for the Hardy Weinberg equilibrium to determine their possible significance in Retinoblastoma testing in the Sri Lankan population. The adequate sample size for this research study was calculated by using the following formula.

$N= [Z^2 P (1-P)] / d^2$. It was considered P in the equation as expected prevalence, d as precision, Z as the statistic corresponding to the confidence level and N as the sample size[11]. When a confidence level of 95%, a P value of 5% and a 0.05 precision value was considered, the sample size / N was calculated as 25. A total of 59 peripheral blood samples collected from Retinoblastoma patients were obtained from the Human Genetics Unit for this research study.

Suitable genetic assay to genotype the *RB1* variants of interest

By considering a range of factors like cost effectiveness, simplicity and availability of resources, Tetra-Primer Amplification Refractory Mutation System (T-ARMS-PCR) was selected for this research study. It was understood that T-ARMS-PCR would employ 02 outer primers and 02 inner primers whereas the outer primers would amplify a region in which the expected variant is located and the variant allele specific and the wild type allele specific inner primers would anneal and amplify only if the respective allele was present. The DNA band amplified by the 02 outer primers was known to act as the control for the T-ARMS-PCR.

Primer designing

Ensemble genome browser was browsed for the *RB1* variant table[12] where the rs IDs were retrieved for the 02 variants of interest. The dbSNP database[13] was browsed with the rs IDs for the FASTA sequence views on which the variants were located. Proper templates were selected for the 02 variants separately and Primer 1 tool was used for primer designing. The NCBI primer blast[14] was used to check for the specificity of the designed primers. The primers designed for the rs121913300 *RB1* variant have been depicted in Table 2. The primers designed for the rs1131690863 RB1 variant have been shown in Table 3.

Table 2. Primers for rs121913300 *RB1* variant

Primer	*Melting Temperature [°C]*
Forward inner primer (T allele): 270 CAAGGTTGAAAATCTTTCTACAT 292	54
Reverse inner primer (C allele): 314 TTAAGATAAATTTCTTCGTAGCG 292	55
Forward outer primer (5' - 3'): 53 TTCTATGCACGAAATAGACCT 73	54
Reverse outer primer (5' - 3'): 399 ATACCATGTGCAATACCTGTC 379	54

Product size for T allele: 131

Product size for C allele: 262

Product size of two outer primers: 347

Table 3. Primers for rs1131690863 *RB1* variant

Primer	*Melting Temperature [°C]*
Forward inner primer (C allele): 410 GCTGTTATACCCATTAATGGTTCACATC 437	63
Reverse inner primer (T allele): 454 ACCTCGCCTGGGTGTGCA 437	66
Forward outer primer (5' - 3'): 75 CAGAAAACTTTTTTTTCCTCTTGCAGAC 102	64
Reverse outer primer (5' - 3'): 658GCACTAATTACAAAAAAAAACATGCTCATAACA 627	64

Product size for C allele: 250

Product size for T allele: 380

Product size of two outer primers: 584

Genotyping

The Eppendorf tubes, PCR tubes and micropipette tips were autoclaved. DNA was extracted from the 59 peripheral blood samples extracted from a Retinoblastoma cohort in a Sri Lankan population which were handed over as anonymous samples by the Human Genetics Unit of Faculty of Medicine, University of Colombo; the Qiagen DNA extraction protocol[15] was followed. The extracted DNA samples were quantified by using a fluorometer through the Promega Quanti Fluor® dsDNA System. The primers were then optimized for the *RB1* variants. The optimized protocols are shown in Tables 4, 5, 6 & 7.

Table 4. Components for rs1131690863 PCR Master mix and their respective volumes

Component	*Volume *1 (ul)*
5* Buffer (Promega, USA)	5.0
dH2O	10.3
25mM MgCl2 (Promega, USA)	2.0
2.5mM dNTP (Promega, USA)	1.5
25mM FI primer (Table 3)	1.0
25mM RI primer (Table 3)	1.0
25mM FO primer (Table 3)	1.0
25mM RO primer (Table 3)	1.0
Taq (Promega, USA)	0.2
DNA	2.0
Reaction Volume	25.0

Table 5. Optimized PCR protocol for rs1131690863 variant

Initial Denaturation	95°C	5 min	
Denaturation	95°C	1 min	
Annealing	61.5°C	30 seconds	30 Cycles
Extension	72°C	1 min	
Final Extension	72°C	5 min	
Holding	4°C	∞	

Table 6. Components for rs121913300 PCR Master mix and their respective volumes

Component	*Volume *1 (ul)*
5* Buffer (Promega, USA)	5.0
dH2O	6.3
25mM MgCl2 (Promega, USA)	2.0
2.5mM dNTP (Promega, USA)	1.5
25mM FI primer (Table 2)	2.0
25mM RI primer (Table 2)	2.0
25mM FO primer (Table 2)	2.0
25mM RO primer (Table 2)	2.0
Taq (Promega, USA)	0.2
DNA	2.0
Reaction Volume	25.0

Table 7. Optimized PCR protocol for rs121913300 variant

Initial Denaturation	95°C	5 min	
Denaturation	95°C	1 min	
Annealing	60.0°C	30 seconds	30 Cycles
Extension	72°C	1 min	
Final Extension	72°C	5 min	
Holding	4°C	∞	

T-ARMS-PCR was carried out for all 59 DNA samples for the both rs1131690863 and rs121913300 variants and the PCR products were electrophoresed in 2% Agarose gel at 85V for 40 minutes and were observed under UV light with the help of Mega-Capt software. Figure 1 and 2 illustrate the gel interpretation of the rs121913300 and rs1131690863 PCR products respectively.

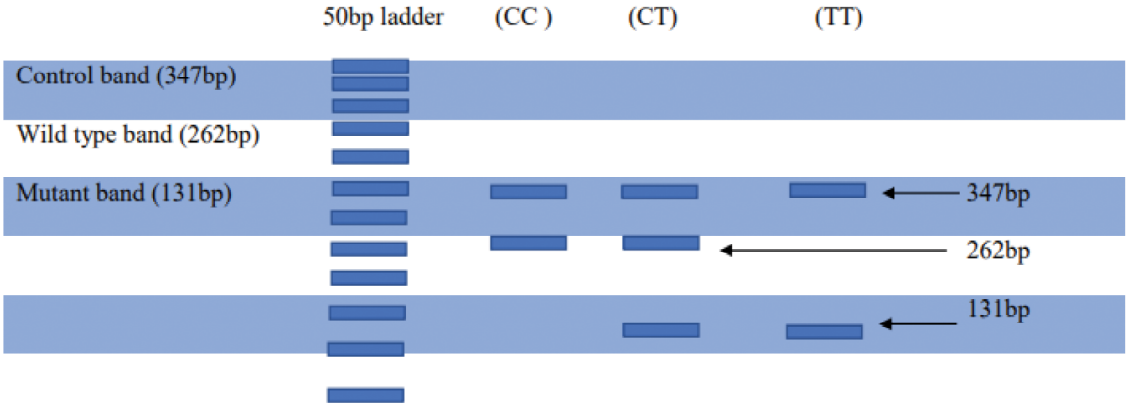

Figure 1. *Expected gel image of rs121913300 PCR products; Lane 01: 50bp step ladder, lane 02: Homozygous for wild type, lane 03: Heterozygous, lane 04: Homozygous for mutant type*

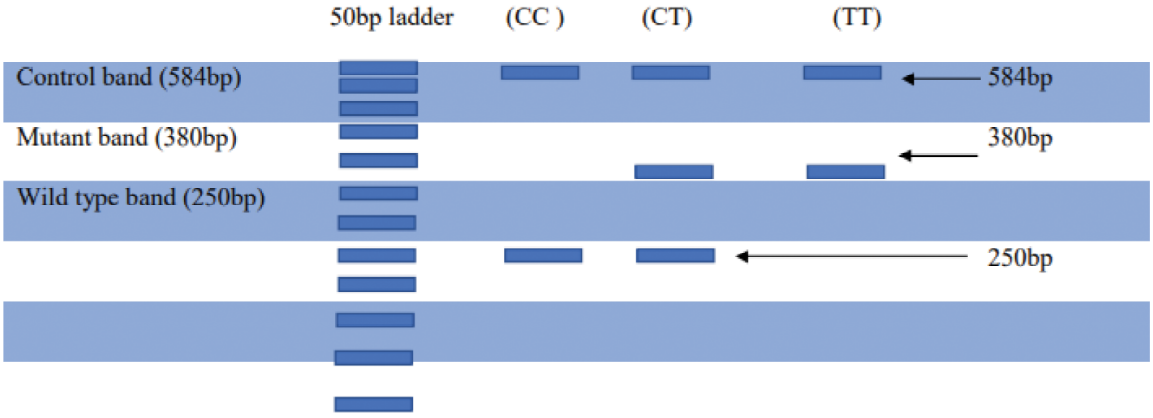

Figure 2. *Expected gel image of rs1131690863 PCR products; Lane 01: 50bp step ladder, lane 02: Homozygous for wild type, lane 03: Heterozygous, lane 04: Homozygous for mutant type.*

Sanger Validation

Sanger sequencing was done to confirm the availability of the variant of interest as a confirmation of the T-ARMS-PCR results. The Sanger sequencing results were read with 'Codon Code Aligner' software and the validity of the T-ARMS-PCR results were confirmed.

Statistical Analysis

Based on the genotyping results, the calculation of overall mutation detection rate and the calculation of allele and genotype frequencies for each genotyped variant were done manually; the calculated minor allele frequencies were used to determine if the variant was rare/common in Sri Lanka. Each variant was checked for the Hardy-Weinberg equilibrium in order to determine their possible significance in Retinoblastoma screening/testing in the Sri Lankan population. Chi square test was performed to check if the Retinoblastoma laterality was significantly related to the gender and disease stage in the study population.

Results

Genotyping results and statistical analysis

Figure 3 and Figure 4 show representative examples for the 2% Agarose gel interpretation for rs1131690863 and rs121913300 RB1 variants respectively.

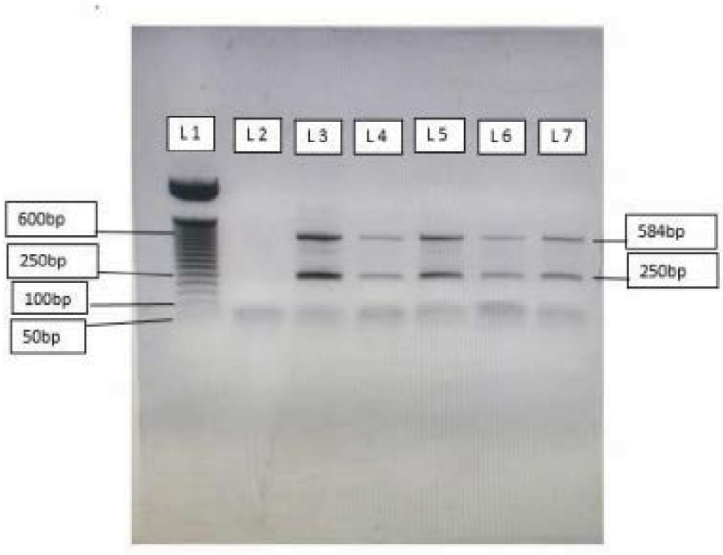

Figure 3. *Gel Interpretation;*
Allele Specific PCR for rs1131690863 variant followed by 2% Agarose gel electrophoresis; Lane 1 shows 50 bp step ladder, lane 2 shows 'blank' while lane 3 shows negative control. Lane 4-7 show RB patient samples which represent the wild type homozygous state (CC genotype) with 584bp control DNA band and 250bp wild type specific DNA band while the 380bp variant specific DNA band isn't visible in the gel image.

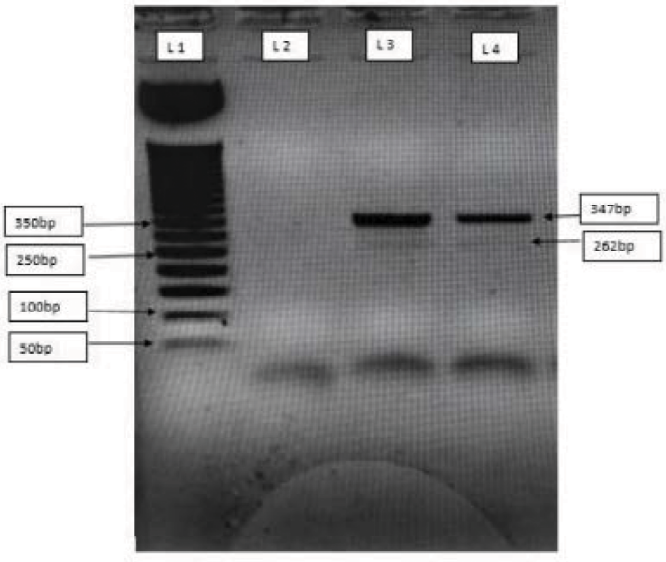

Figure 4. *Gel Interpretation;*
Allele Specific PCR for rs1131690863 variant followed by 2% Agarose gel electrophoresis; Lane 1 shows 50 bp step ladder, lane 2 shows 'blank' while lane 3 shows negative control. Lane 4-7 show RB patient samples which represent the wild type homozygous state (CC genotype) with 584bp control DNA band and 250bp wild type specific DNA band while the 380bp variant specific DNA band isn't visible in the gel image.

Observed				Expected				
	Unilateral	Bilateral	Total		Unilateral	Bilateral	df	1
Male	15	10	25	Male	15.81633	9.183673	p value	0.628449
Female	16	8	24	Female	15.18367	8.816327		
Total	31	18	49					

Figure 5. *Chi Square test to compare the gender with Retinoblastoma laterality;*
Chi-square test determines if the observed counts are deviated enough from the Expected counts for the test to be significant for the association. In line with this research study, the Retinoblastoma laterality is independent from gender as the generated p-value (0.628449) is greater than the ref p-value (0.05) which indicates that there is no significant difference in between observed and the expected count.

All the Retinoblastoma patients were homozygous for the wild type allele in terms of both *RB1* variants. Accordingly, the detection rates of both *RB1* variants are zero in the population. Both variants consisted of the ancestral allele (C) only, while the mutant allele (T) was completely absent. The minor allele frequencies of both variants being zero revealed that both these variants are rare in the population. Furthermore, the distributions of rs121913300 and rs1131690863 genotypes have been in Hardy-Weinberg equilibrium. Accordingly, genotypes of the two considered RB1 variants can continue to be rare in this population across generations unless for an external disturbance. The Chi Square tests determined that there were no significant differences in between the gender and Retinoblastoma laterality (Figure 5) and in between the Retinoblastoma laterality and disease stage (Figure 6).

Observed				Expected				
	Unilateral	Bilateral	Total		Unilateral	Bilateral		
group B	6	1	7	group B	4.8125	2.1875		
group C	6	2	8	group C	5.5	2.5		
group D	6	5	11	group D	7.5625	3.4375	df	3
group E	4	2	6	group E	4.125	1.875	p value	0.54621
Total	22	10	32					

Figure 6. *Chi Square test to compare the Retinoblastoma laterality with Retinoblastoma stage*
In line with this research study, the Retinoblastoma laterality is independent from disease stage as the generated p-value (0.54621) is greater than the ref p-value (0.05) which indicates that there is no significant difference in between observed and the expected count.

Discussion

This is the 1st research study in Sri Lanka which focused on rs121913300 and rs1131690863 *RB1* variants. It was conducted to determine if the rs121913300 and rs1131690863 RB1 variants could be used to develop Retinoblastoma screening tests in Sri Lanka in order to support the early detection of Retinoblastoma/its possible risk. This study has found that those variants are rare in the Sri Lankan community; hence they could be poorly considered and priority could be given for other Retinoblastoma-related genetic variants in developing Retinoblastoma screening tests. Furthermore, knowing the irrelevance of rs121913300 and rs1131690863 *RB1* variants will support genetic counseling while contributing to precise retinoblastoma management.

Although it was suggested that the genotype distributions would prevail the same across generations as the distributions were in Hardy-Weinberg

Equilibrium, still with time these variants might become significantly frequent due to external disturbing factors. This suggests repeating research studies for the prevalence of these 02 *RB1* variants which the optimized protocols will be needed later. In case if the variant turns out to be significant, an optimized protocol will be needed for screening, diagnosis and prognosis in future. This research is significantly beneficial in that case as it has optimized PCR protocols for rs121913300 and rs1131690863 RB1 variants which could be employed by future studies. Some studies have used the same or related methodologies as in our research where as one Sri Lankan study has employed T-ARMS-PCR to genotype genetic variants16 while some other Indian studies have used sequencing techniques during their studies[17,18].

The findings of this study are related to previously published research in several ways. India is the country with the highest number of Retinoblastoma cases.

Many related studies have been carried out in India as well. Nevertheless, there are certain Indian Retinoblastoma studies which do not report[17,18] the globally most frequent and globally 3rd most frequent *RB1* variants which were selected for this study. This agrees with the fact that both the variants were completely absent in the Sri Lankan cohort. But on the other hand, the genotypic results of this study are immensely in contrast to the arguments that have previously been made, such as rs121913300 is the most frequent and rs1131690863 is the 3rd most frequent *RB1* variant in the world[8]. But still it can be justified with the fact that they have drawn their conclusions after years of data collection over numerous populations across the world. This study reports 6.78% cases with positive RB family history; hence it is in line with another study[19] which reports only a 5% of cases with positive RB family history. Poor family history could be due to low penetrance, germline mosaicism or due to absence of the 2nd genetic hit in the proband's family members.

The allele frequencies of the current study are in line with the manually computed allele frequencies of other studies in Asia and South Asia whereas certain Indian studies report the same allele frequency for rs121913300 *RB1* variant[20] and some others report the same allele frequency for rs1131690863 RB1 variant[18]. A study in Pakistan Retinoblastoma population[21] and Malaysian Retinoblastoma population[22] have reported the same frequencies for rs121913300 *RB1* variant as in our study. The allele frequencies calculated from genotypic results of a Singaporean Retinoblastoma study[9] is in line with the allele frequencies of rs1131690863 *RB1* variant of this study. A study on the Thai population[6] reveals the same frequencies for both the variants as in this study. The allele frequency for the selected variants were not reported in the exome database of the Human Genetics Unit, Faculty of Medicine, University of Colombo, Sri Lanka. This could be due to the lack of research conducted on the selected 02 variants.

However, it is in line with this study's finding that rs121913300 and rs1131690863 *RB1* variants could be rare in the Sri Lankan population.

During the study, proper gel images were obtained with DNA bands of correct band size. The DNA ladder was properly separated and no contaminations were found. Non-specific DNA bands weren't observed as well. Furthermore, the Sanger sequencing chromatograms confirmed the T-ARMS-PCR results which validated the optimized PCR protocols. All these could be considered as criteria to determine the success of this research study.

There are a few limitations in this study. Global RB1 studies have revealed many RB1 variants which could give rise to retinoblastoma[6,21,18]. But this research study was limited to 02 selected *RB1* variants only whereas the cohort might be having variants which weren't considered for this research. Different Retinoblastoma studies[6] have detected novel variants in their studies on the *RB1* gene. The patients in the Sri Lankan retinoblastoma cohort might also have such novel variants. But the T-ARMS-PCR technique used in this study can detect known variants only, which is another limitation.

DNA was extracted from blood samples only, whereas tumor samples were not used; hence this study is limited to germline variants and does not test or include somatic variants. Positive controls were not available for both variants, therefore the PCR protocols had to be optimized with negative control only. It could be considered as a limitation as well; but unavailability of positive controls for the 02 variants agrees with the conclusion of this research study that both variants were rare in Sri Lanka.

Acknowledgements

We are grateful to Professor Vajira H. W. Dissanayake, Professor in Anatomy and Medical Geneticist, Director, Human Genetics Unit and Dean Faculty of Medicine, University of Colombo, and the staff of Human Genetics Unit and the Lady Ridgeway Hospital for their kind support in making this project a success.

References

1. Jain M, Rojanaporn D, Chawla B, et al. Retinoblastoma in Asia. *Eye* 2018; **33**(1): 87-96.
2. Dimaras H, Kimani K, Dimba E, et al. Retinoblastoma. *The Lancet* 2012; **379**(9824): 1436-46.
3. Singh L, Kashyap S. Update on pathology of retinoblastoma. *International Journal of Ophthalmology* 2018; **11**(12): 2011-216.
4. Soliman S, Racher H, Zhang C, MacDonald H, Gallie B. Genetics and Molecular Diagnostics in Retinoblastoma - An Update. *Asia-Pacific Journal of Ophthalmology* 2017; **6**(2): 197-207.
5. Witkiewicz AK, Knudsen ES. Retinoblastoma tumor suppressor pathway in breast cancer: prognosis, precision medicine, and therapeutic interventions. *Breast Cancer Research* 2014; **16**(2).
6. Rojanaporn D, Boontawon T, Chareonsirisuthigul T, et al. Spectrum of germline *RB1* mutations and clinical manifestations in retinoblastoma patients from Thailand. *Molecular Vision* 2018; **24**: 778-88.
7. Dimaras H, Khetan V, Halliday W, et al. Retinoma Underlying Retinoblastoma Revealed After Tumor Response to 1 Cycle of Chemotherapy. *Archives of Ophthalmology* 2009; **127**(8): 1066-68.
8. Valverde J, Alonso J, Palacios I, Pestaña Á. RB1 gene mutation up-date, a meta-analysis based on 932 reported mutations available in a searchable database. *BMC Genetics* 2005; **6**(1).
9. Tomar S, Sethi R, Sundar G, et al. Mutation spectrum of RB1 mutations in retinoblastoma cases from Singapore with implications for genetic management and counseling. *PLoS ONE* 2017; **12**(6): e0178776.
10. Gupta H, Malaichamy S, Mallipatna A, et al. Retinoblastoma genetics screening and clinical management. *BMC Medical Genomics* 2021; **14**(1).
11. Pourhoseingholi MA, Vahedi M, Rahimzadeh M. Sample size calculation in medical studies. *Gastroenterology and Hepatology from Bed to Bench* 2013; **6**(1): 14-17. doi: 10.22037/ghfbb.v6i1.332.
12. EMBL-EBI. Gene: RB1 (ENSG00000139687) - Variant table - Homo_sapiens - Ensembl genome browser 105. 2021; [online] Asia.ensembl.org. Available at: [Accessed 28 February 2022].
13. Ncbi.nlm.nih.gov. Home - SNP - NCBI 2022; [online] Available at: [Accessed 28 February 2022].
14. Ncbi.nlm.nih.gov. Primer designing tool 2022; [online] Available at: [Accessed 28 February 2022].
15. QIAGEN. QIAamp DNA Mini and Blood Mini Handbook. *Qiagen* 2016; (5): 1-72. Available at: http://www.qiagen.com/knowledge-and-support/resource-center/resource-download.aspx?id=67893a91- 946f-49b5-8033-394fa5d752ea&lang=en.
16. Shathushika A, Wetthasinghe TK, Dissanayake MM, Irugalbandara D. Designing and implementation of a T-ARMS-PCR assay to genotype genetic variants associated with retinoblastoma in a cohort of Sri Lankan population. *The Journal of the College of Ophthalmologists of Sri Lanka* 2022; **28**: 63-70.
17. Devarajan B, Prakash L, Kannan TR, et al. Targeted next generation sequencing of RB1 gene for the molecular diagnosis of Retinoblastoma. *BMC Cancer* 2015; **15**(1).
18. Kiran VS, Kannabiran C, Chakravarthi K, Vemuganti GK, Honavar SG. Mutational screening of the *RB1* gene in Indian patients with retinoblastoma reveals eight novel and several recurrent mutations. *Human Mutation* 2003; **22**(4): 339-9.
19. Naseripour M, Nazari H, Bakhtiari P, et al. Retinoblastoma in Iran: outcomes in terms of patients' survival and globe survival. *Br J Ophthalmol* 2009; **93**(1): 28-32. doi: 10.1136/bjo.2008.139410.
20. Singh J, Mishra A, Pandian AJ, et al. Next-generation sequencing-based method shows increased mutation detection sensitivity in an Indian retinoblastoma cohort. *Molecular vision* 2016; **22**: 1036-47.
21. Kalsoom S, Wasim M, Afzal S, et al. Alterations in the *RB1* gene in Pakistani patients with retinoblastoma using direct sequencing analysis. *Molecular Vision* 2015; **2015**(21): 1085-92.
22. Mohd Khalid MK, Yakob Y, Md Yasin R, et al. Spectrum of germ-line *RB1* gene mutations in Malaysian patients with retinoblastoma. *Molecular Vision* 2015; **21**: 1185-90.