https://doi.org/10.4038/jcosl.v28i2.60

Research article

Designing and implementation of a T-ARMS-PCR assay to genotype genetic variants associated with retinoblastoma in a cohort of Sri Lankan population

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The Journal of the College of Ophthalmologists of Sri Lanka 2022; 28: 63-70

Abstract

Retinoblastoma (RB) is the most common intraocular malignancy, characterized by high mortality if not detected early and treated promptly. The rare childhood malignancy retinoblastoma serves one of the most important models in modern cancer genetics. Since the study of its familial and sporadic occurrence has led to the identification of the first tumor suppressor gene RB1. Mutations screening is important for risk assessment in future siblings and offspring of RB patients.

The aim of this study was to design and implement a novel genetic assay to identify genetic variants associated with retinoblastoma in a cohort of Sri Lankan patients.

Materials and methods: *A prospective descriptive study was carried out with 59 patients referred to the Eye Unit of the Lady Ridgeway Hospital. Genomic DNA of 59 patients were genotyped using primers designed for Tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR).*

Results: *The median age at diagnosis was 2 years and 7 months. Female to male ratio was 3:2. Out of which, 63% had unilateral retinoblastoma and 36% had bilateral retinoblastoma. Family history of RB was seen in 6.78% patients. Most cases were advanced group D at presentation. As the final result, all patients tested homozygous for the ancestral allele for both rs587776789 and rs121913305 variants of the RB1 gene.*

Discussion and conclusion: *The discovery of germ-line mutations in unilateral patients is valuable since they can be segregated based on their mutational status and this would impact the genetic counselling given to them as they age. In conclusion, this assay can be introduced as a sensitive, specific and simple diagnostic technique for screening related genetic variants for retinoblastoma in the Sri Lankan population.*

Key words: retinoblastoma, novel variants, *RB1* gene mutation, genotype, allele frequency

Introduction

Retinoblastoma is an early childhood intraocular cancer which develops in the retinal cell and predominantly occurs in young children below 5 years. The reported incidence of Rb is constant worldwide at one case per 16,000 - 18,000 live births. Moreover, the highest disease prevalence is recorded in areas with high birth rates, which is the case of many low- and middle-income countries (LMICs). Out of which, Asia-Pacific region bears a significant global burden of retinoblastoma (RB), therefore understanding RB in Asia-Pacific region is important. Based on the year 2013 population estimates, 43% (3452 of 8099 children) of the global burden of RB lives in 6 countries of Asia Pacific region: 1486 children in India, 1103 children in China, 277 children in Indonesia, 260 children in Pakistan, 184 children in Bangladesh, 142 children in Philippines[4]. Although there is no valuable figure on the incidence of retinoblastoma in Sri Lanka and also found in the literature review, leucocoria is the most common presenting sign in many populations worldwide.

It had been well known before the discovery of the disease-causing gene that retinoblastoma can be inherited hereditary form or occur sporadically (nonhereditary form). These two forms were explained by Knudson's two-hit hypothesis in 1970s that retinoblastoma occurs with at least two mutational events in the disease-causing gene. In hereditary form, the first mutation is inherited via the germline cells and the second occurs in somatic

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cells, while both mutations occur sporadically in somatic cells in nonhereditary form. It should be noted that hereditary patients have higher probability to develop more tumors than nonhereditary patients. All bilateral and multifocal unilateral tumors are hereditary. Even in cases of hereditary form of bilateral retinoblastoma, the majority of children do not have a family history of the disease, and which is a result of de novo mutations in the gene that primarily occur during spermatogenesis. This can be a reason for the Mosacism. By recognizing the inheritance pattern, the familial risk estimates can be made.

According to the reports having been identified that several genes are likely to be associated with retinoblastoma, such as MDM2, MDM4, *RB1*, Tp53, MTR and p21. Despite that the *RB1* gene is the commonest one, most significantly associated with Asian population amongst all other genes.

The *RB1* gene is located in chromosome 13 at q arm region 14 from 48,877,887bp to 49,056,122bp. It spans for about 180 kb in length, having 27 exons. Transcription of *RB1* results in a 4.8 kb mRNA that encodes a 110 kDa ubiquitously expressed nuclear phophoprotein, pRB containing 928 amino acid residues[2], pRB, which plays a role in the cell cycle regulation, binds to E2F transcription factors to repress genes related to cell proliferation.

It has also been discussed whether single nucleotide polymorphisms (SNP) in *RB1* may have a major predisposing effect in Sri Lankan population. As according to the research, It was found that, the most prevalent *RB1* gene variant associated with retinoblastoma is the stop gained mutation (rs 121913305) which accounts for majority of cases among Sri Lankan population. Another gene also associated with *RB1* gene, known to have a higher allele frequency than rs 121913305 is the splice doner variant rs587776789. The above mentioned variants identified at the HGU database and selected for primer designing.

Scientific data on retinoblastomas in Sri Lanka is limited as this is the initial study investigating the molecular level presentations, heredity phenotypes and outcome of retinoblastomas in Sri Lankan children. Therefore, it is important that novel variants should be discovered, and assays should be implemented for screening and diagnosing the patients and family members prior to symptoms. Identifying novel genomic biomarkers associated with retinoblastoma would deliver targeted therapies for individuals with complicated retinoblastoma and to identify patients at risk for increased morbidity and significant mortality as a consequence of complications of the disease by mutation screening.

Method

An extensive literature review was carried out through online software tools to design the four specific primers to genotype each variant. NCBI, dbSNP database, serial cloner 2.0, primer Z gene pipe, SNPedia, primer 1, primer 3 and NCBI primer blast were the major tools used to design the primers and crosscheck their specificity to find single nucleotide variants relatively subject to cause retinoblastoma in recent researches at the global, Asian level to discover the most relevant SNPs concerned with the disease.

Sample collection

A prospective descriptive study was conducted, including 59 patients with retinoblastoma referred to Eye Unit of the Lady Ridgeway Hospital after obtaining informed consent from the parents of the patients between August 2021 and June 2022 also the recruitment of patients was carried out after getting necessary approval from the Ethical Review Committee of the University of Colombo and receiving authorization from the Eye Unit of the Lady Ridgeway Hospital, Colombo.

In this study setting, patients who had first treatment elsewhere was excluded as this would not give a true reflection of time to diagnosis and outcome. All retinoblastoma patients referred for RB screening at LRH were selected. A 3ml of peripheral blood sample from patients were collected in EDTA tube at LRH with their parents' consent and stored at the HGU at -8°C.

Genotyping

Genomic DNA was extracted from peripheral blood according to the manufacturers protocol (QIAGEN, 2016). *RB1* gene polymorphisms genotyping was carried out by T-ARMS PCR assay by using primer designed primers (two non-allele specific primers and two allele specific primers) for the PCR optimization. PCR amplification was performed in a total of 25µl reaction mixture for SNP1: (rs587776789): 5mM PCR Buffer, 2.0 mM MgCl2, 1.5 mM of dNTP mixture, 0.3U Taq DNA polymerase, primers (1 µM of outer forward, 0.5 µM outer reverse, 0.4 µM inner forward and 1 µM inner reverse) 30 ng of genomic DNA, and sterile distilled water to a final volume of 25mL. The amplification started with an initial denaturation of 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, annealing for 55.1°C for 30 sec and 72°C for 1 min for extension, ended by a final extension for 5 min at 72°C and infinite hold for 4°C.

Primer output: Forward inner primer (T allele): ATCACATTTTTATTAGCTAAGGT

Reverse inner primer (G allele): GCATTTAATAAA TATAATGAACTCAC

Forward outer primer (5′-3′): AAGACATAGAAACAT TAAATGAAT

Reverse outer primer (5′-3′): TGTTTCTAGTACC AGAATTATAGG (Product size for T allele: 189 product size for G allele: 295 product size of two outer primers: 435)

PCR master mix preparation for SNP11: (rs121913305): 5mM PCR buffer, 1.5 mM $MgCl_2$, 1.5 mM of dNTP mixture, 0.3U Taq DNA polymerase, primers (0.3 µM of outer forward, 0.3 µM outer reverse, 0.3 µM inner forward and 1.2 µM inner reverse) 30 ng of genomic DNA, and sterile distilled water to a final volume of 25mL. The amplification started with an initial denaturation of 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, annealing for 58°C for 30 sec and 72°C for 1 min for extension, ended by a final extension for 5 min at 72°C and infinite hold for 4°C.

Primer output: Forward inner primer (C allele): TGATCTTATTAAACAATCAAAGGCCC

Reverse inner primer (T allele): TGATCAGTTGG TCCTTCGCA

Forward outer primer (5′-3′): 90 TCAATTGGGAATTT CGAAGTAGAGA

Reverse outer primer (5′-3′): 428 AAATCTATTTGC AGTTTGAATGGTCA (Product size for C allele: 154 product size for T allele: 231 product size of two outer primers: 339)

Next step, about 5µl of the amplicons was observed on agarose gel electrophoresis for PCR amplification. As the final step, sequencing of randomly selected amplified products were carried out for confirming genotypic data with an ABI PRISM 3100 automated platform (Applied Biosystems, Foster City, CA).

Statistics

All statistical analysis was performed using the SPSS 23 (IBM) statistical software. The strength of associations was evaluated using a 95% confidence interval. All continuous data were presented as mean + standard deviation. The statistical associations between categorical variables were found using the Chi squared test. A p value less than 0.05 ($p<0.05$) was considered statistically significant. The major and minor allele frequencies were not obtained using the Hardy Weinberg equation shown below due to the patient samples test results represent homozygous wildtype.

[P 2 + 2pq + q2 = 1] In this equation P stands for the frequency of the homozygous dominant genotype, 2pq is the frequency of heterozygous genotype and q2 frequency of homozygous recessive genotype.

Results

Demographic data of the study population

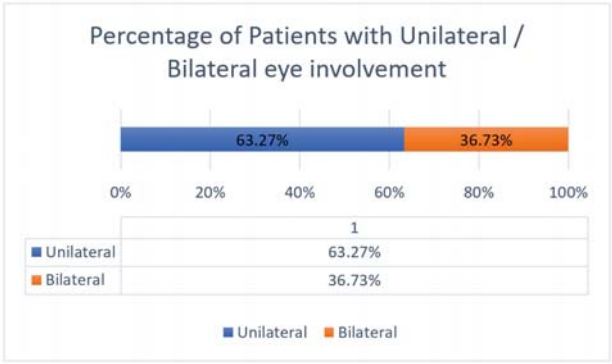


Figure 1. *Distribution of bilateral and unilateral eye involvement in the pediatric patients and the collective cohort.*

Figure 2. *Number and percentages of patients depending on the family history positivity.*

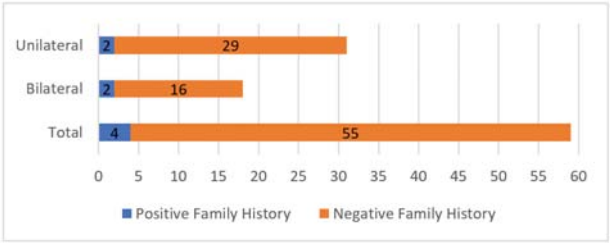


Figure 3. *Family history positivity in total bilateral and unilateral RB percentage.*

Retinoblastoma patients' samples were genotyped by the developed optimized AS- PCR assay protocols for the *RB1* rs 587776789 and *RB1* rs121913305 variant. Out of which, there were 34 female (57.63%), 25 male (42.37%). Age of the patients were within a range of 2 years to 13 years.

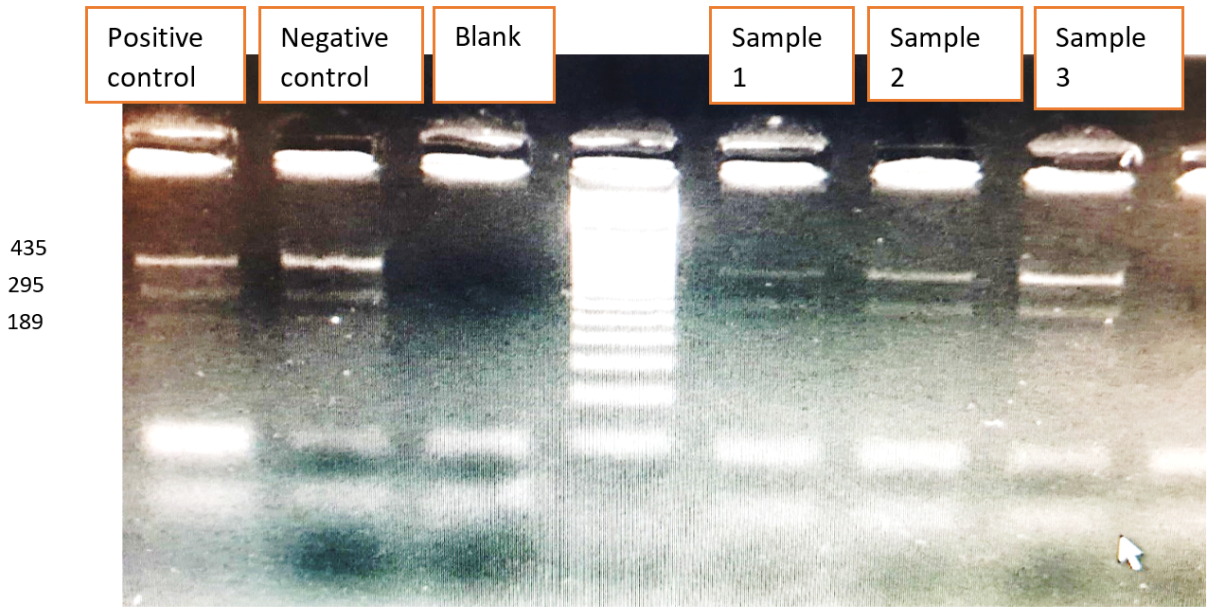


Figure 4. *2% agarose gel interpretation for optimized protocol of rs 587776789 variant.*

Two bands were there as expected from homogenized ancestral sample which means without the variant. The two bands in above found in patient's samples were control band with 435 bp and the below bands were ancestral allele band with 295 bp length. The mutant band to the variant allele (189 bp) not shown in patient samples. Genotyping results are 100% homozygote wildtype.

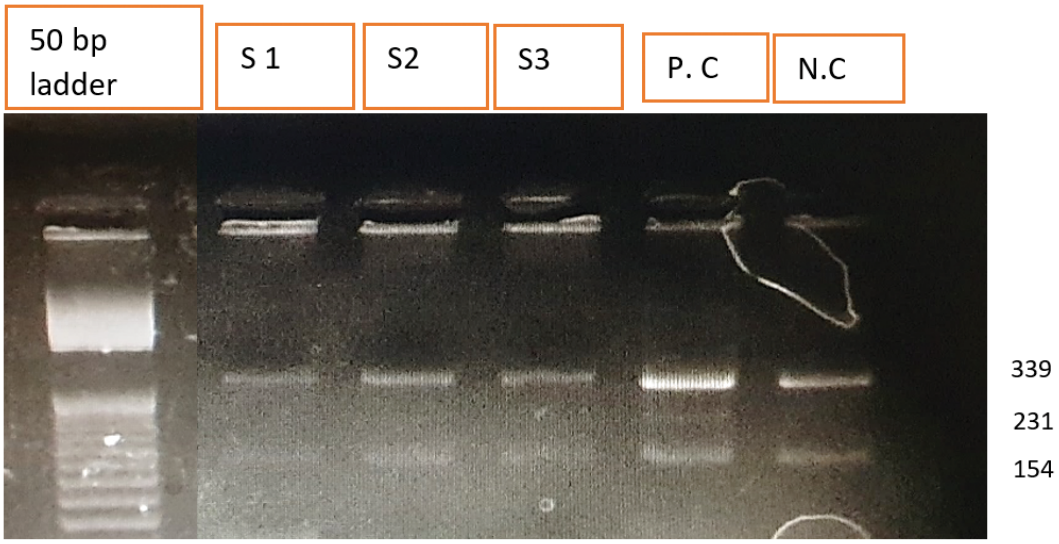
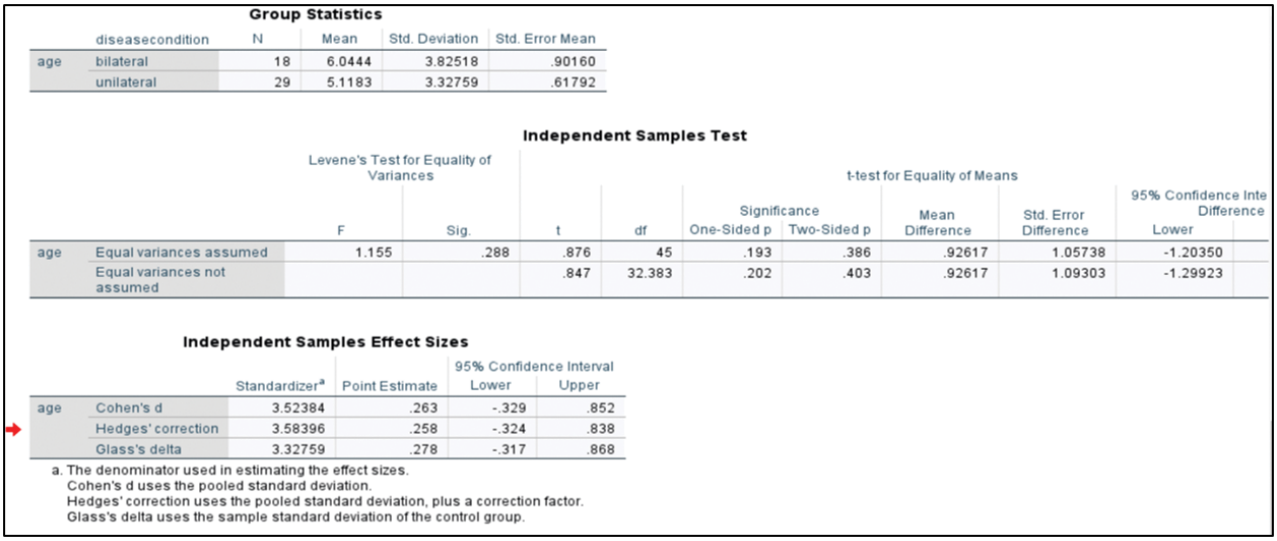


Figure 5. *Agarose gel interpretation for optimized protocol of rs121913305 variant.*

The two bands in above found in patient's samples were control band with 339 bp and the below bands were ancestral allele band with 154 bp length. The mutant band to the variant allele (231 bp) not shown in patient samples. Genotyping results are 100% homozygote wildtype.

Statistical analysis:



Group Statistics

	diseasecondition	N	Mean	Std. Deviation	Std. Error Mean
age	bilateral	18	6.0444	3.82518	.90160
	unilateral	29	5.1183	3.32759	.61792

Independent Samples Test

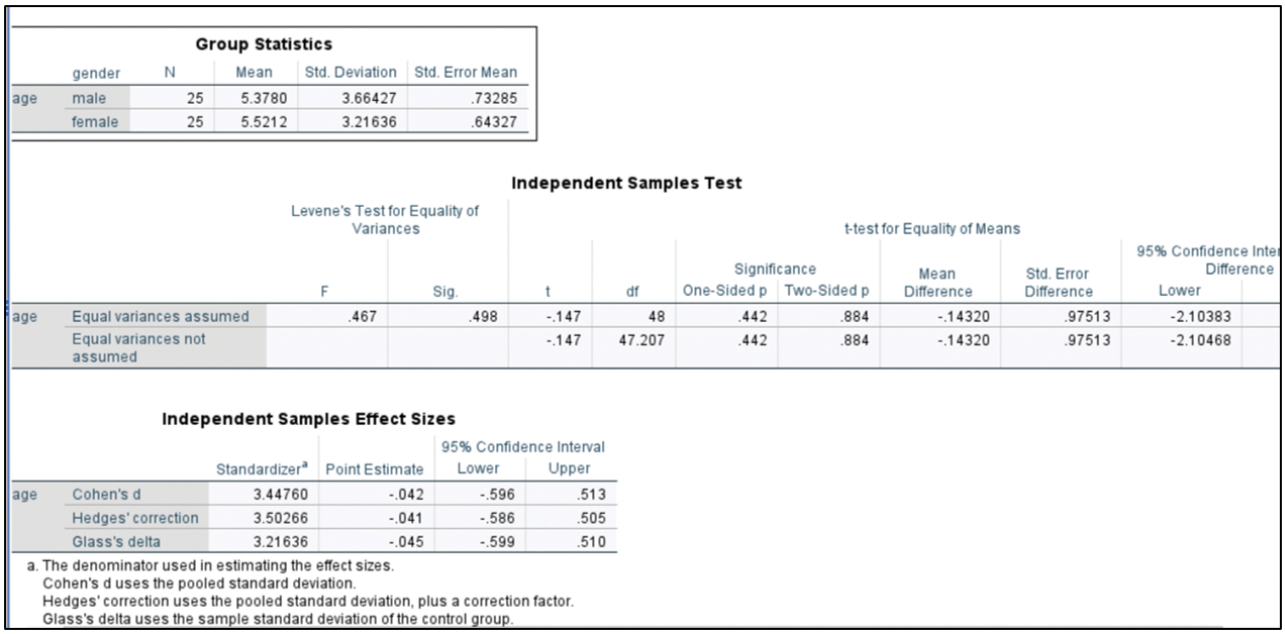
		Levene's Test for Equality of Variances		t-test for Equality of Means					
						Significance			95% Confidence Inte Difference
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference	Lower
age	Equal variances assumed	1.155	.288	.876	45	.193	.386	.92617	1.05738	-1.20350
	Equal variances not assumed			.847	32.383	.202	.403	.92617	1.09303	-1.29923

Independent Samples Effect Sizes

		Standardizer[a]	Point Estimate	95% Confidence Interval Lower	Upper
age	Cohen's d	3.52384	.263	-.329	.852
	Hedges' correction	3.58396	.258	-.324	.838
	Glass's delta	3.32759	.278	-.317	.868

a. The denominator used in estimating the effect sizes.
Cohen's d uses the pooled standard deviation.
Hedges' correction uses the pooled standard deviation, plus a correction factor.
Glass's delta uses the sample standard deviation of the control group.

Figure 6. *The p-value is 0.288 which is greater than the ref value (P<0.05). So, there is no significant difference in the age with the disease condition.*



Group Statistics

	gender	N	Mean	Std. Deviation	Std. Error Mean
age	male	25	5.3780	3.66427	.73285
	female	25	5.5212	3.21636	.64327

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
						Significance			95% Confidence Inte Difference
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference	Lower
age	Equal variances assumed	.467	.498	-.147	48	.442	.884	-.14320	.97513	-2.10383
	Equal variances not assumed			-.147	47.207	.442	.884	-.14320	.97513	-2.10468

Independent Samples Effect Sizes

		Standardizer[a]	Point Estimate	95% Confidence Interval Lower	Upper
age	Cohen's d	3.44760	-.042	-.596	.513
	Hedges' correction	3.50266	-.041	-.586	.505
	Glass's delta	3.21636	-.045	-.599	.510

a. The denominator used in estimating the effect sizes.
Cohen's d uses the pooled standard deviation.
Hedges' correction uses the pooled standard deviation, plus a correction factor.
Glass's delta uses the sample standard deviation of the control group.

Figure 7. *The p-value is 0.498 which is greater than the ref value (P<0.05). So, the mean age not significantly associated with the gender.*

Table 1. Chi-squared test was used to determine whether there is a significant difference between disease condition and the gender

Gender disease condition crosstabulation*					
			Disease condition		*Total*
			Unilateral	*Bilateral*	
Gender	Male	Count	15	10	25
		Expected count	15.8	9.2	25.0
	Female	Count	16	8	24
		Expected count	15.2	8.8	24.0
Total		Count	31	18	49
		Expected count	31.0	18.0	49.0

Chi-Square tests

	Value	*Df*	*Asymptotic significance (2-sided)*	*Exact sig. (2-sided)*	*Exact sig. (1-sided)*
Pearson Chi-Square	.234[a]	1	.628		
Continuity correction[b]	.035	1	.851		
Likelihood ratio	.235	1	.628		
Fisher's exact test				.769	.426
Linear-by-linear association	.229	1	.632		
N of valid cases	49				

[a] 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.82.

[b] Computed only for a 2×2 table

Chi-square test helps to determine whether the observed counts are different enough for the test to be significant for the association. According to our analysis, the disease condition is independent from gender as there is no difference between observed and the expected count, and also the p-value is greater than the ref p-value 0.628 (α<0.05).

Discussion

Positive family history was expected in most of the patients clinically diagnosed to have retinoblastoma, even though the family history positivity was found only in 4 patients. This could be explained genetically by occurrence of de novo mutations or germline mosaicism in parents or by two hit hypothesis and the negative family history might be clinically explained by reduced penetrance. Hence, molecular genetic testing of pathogenic mutations needs to be done for patient and the relatives in order to find out the origin of the genetic mutations and to guide diagnosing the pathogenic mutations through generations. Moreover, according to this study, the family history positivity in percentage was 6.78%, which almost similar to a study that was done in Tunisia (9.5%)[3]. Similarly, another study in Iran found to be 5% of the cohort[11].

According to our comparison between patients age-disease condition (P=0.288), age-gender (p=0.498) and gender-disease condition (P=628), no significant association was found between the conditions and development of retinoblastoma.

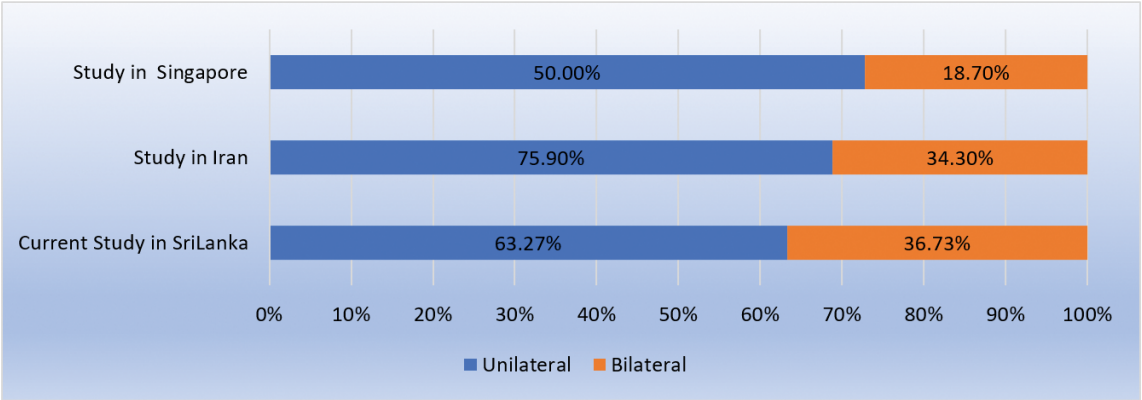


Figure 8. *A comparison of globe preservation rates in bilataral and unilatral retinoblastomas in different countries*[1,13].

In line with the above graphical representation, Sri Lanka and Iran consisting the higher rate of bilateral and unilateral cases compared to Singapore which directly indicating the better socioeconomic states in Singapore compared to Sri Lanka and Iran and availability of more advanced alternative therapy may be preserving good treatments in Singapore. Seeking health care at early stages in Singapore may also contribute for the better survival rates. The Lower socioeconomic status, lack of awareness about RB in general population – delay in seeking medical attention and lack of national screening program have been implicated as the reason for higher incidence of RB in developing countries[7]. Also, strabismus associated with leucocoria is the commonest presentation in Sri Lankan patients as leucocoria is acceptable to be the most common presenting sign in many populations worldwide.

According to the previous research articles, there is an increased occurrence of high-risk intraocular retinoblastoma with increased invasiveness of the tumor into the optic nerve, choroid, sclera, and extrascleral tissues in Asian Indians compared with the patients from the developed world[8].

Most specifically, T-ARMS PCR is the widely spread technique to exploit variations in homologous DNA sequences, which only requires basic equipment such as a conventional thermal cycler and a gel documentation system which are available in most genetic laboratories. It is cost-effective as it does not use fluorescent nucleic acid stains or hybridization probes, whilst retaining test sensitivity and specificity by the inclusion of positive and negative controls. This makes it suitable to be used in studies where lack of funding, equipment or expertise may be a factor and also is a highly sensitive mutation detection assay and has been used to identify low-level mosaic causing recurrent mutations over the past year studies[12]. However, the optimization step could be time-consuming. One of the common problems faced with T-ARMS PCR is the specificity. In the course of optimization, it was remarked that the slightest variation in the reagent content could result in a significant change in the final outcome, eg: Higher concentrations of $MgCl_2$ tends to result as nonspecific bands and it knew that the quantity and the quality of DNA is closely related with $MgCl_2$ concentration[10]. Therefore, the $MgCl_2$ concentration, primer ratio and dNTP and annealing temperatures are often tested during the optimization process.

The primer pairs, which selected are the allele specific primers which were designed according to the mismatch strategy. The aim of the mismatch strategy is to eliminate false positives and increases the specificity by destabilizing the base pairing of the primers with their non target template.

As suggested by most literature, we also note that the sanger sequencing cannot be replaced in the molecular laboratory as it remains the most preferred method to sequence the SNPs of routine samples. Also, it is an essential confirmatory and validation tool in the development of novel genetic assays or in the times of failure where expected yield was not given[6,9].

Conclusion

Globe preservation rate in Sri Lanka is parallel to most of the developing countries and has not yet achieved the Globe preservation rates of developed countries. Screening programs of retinoblastomas both genetically and clinically must be implemented to achieve early diagnosis and thereby reducing the mortality and morbidity rates.

The novel germline mutations in *RB1* arise on the paternally derived chromosome according to the recent past studies[5]. Therefore, the positive family history with the germline mutated unilateral and bilateral cases can be analyzed with the pedigree analysis to reduce the disease mortality and morbidity within the family.

Reduction in the amount of normal pRB that is produced (class 1 mutation) or result in a partially functional mutant pRB (class 2 mutation) often affected by unilateral retinoblastoma along with family's low penetrance. At present, tumor samples are not available for analysis due to logistic issues. Once this is resolved, analysis of tumor DNA can be performed in future studies. If the result determines each of the mutant allele have an *RB1* mutation then leucocytic DNA can be done, though it could also be present in the blood but in lower frequency due to germ-line mosaic mutations. Also, the discovery of germ-line mutations in unilateral patients is valuable because they can be segregated based on their mutational status, and this will impact the genetic counselling given to them as they age.

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