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Research Article

Targeted Screening of RB1 Exon 18 in Sudanese Children with Retinoblastoma: A Molecular and Bioinformatics Study.

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Abstract:

Background: Retinoblastoma (RB) is the most common intraocular malignancy in early childhood. It is most often related to mutations in the RB1 gene, with an incidence of 3% of all pediatric tumors.

Methods: Thirty-one (n=31) clinically and histopathological diagnosed cases of RB attending Makkah Eye Complex Orbit clinic (Khartoum, Sudan) were included in this Molecular Genetic RB Analysis. Fresh blood samples were collected, from seven RB patients and 15 close families, for DNA extraction and PCR products were sent for Genetic Sequencing and Bioinformatics approach for "Exon 18 mutations" which is a known mutational hotspot globally.

Results: Most patients (41.9%) were under five years old. Females were 58.1% and males were 41.9%. Leukocoria was the most common sign at presentation (41.9%). RB unilaterality was 77.4% while bilaterality was 19.4%. Both eyes were equally affected (50% each). The age at the time of diagnosis ranged from seven days to five years old. Consanguinity of parents was very high (85.7%); 1st degree cousins were much lower (28.6%) than 2nd degree cousins (57.1%). The patients' ethnic background and geographical area were from seven different tribes; all belong to Western Sudan. The Functional Analysis and Single Nucleotide Polymorphisms (SNPs) prediction study of exon 18 from NCBI data base showed that the various computational approaches used (SIFT, PolyPhen-2, I-mutant and Project hope) identified 16 reported mutations worldwide, three of which (rs137853292, rs375645171 and rs772068738) are major nsSNPs (non-synonymous) which might contribute to native RB1 protein malfunction and ultimately causing carcinoma (Additional file 2). Our molecular genetic study showed that no mutations were detected in exon 18 among the Sudanese RB patients and their relatives

Conclusion: RB mainly affected children under five years of age, and both sexes were equally affected. Unilaterality was predominant. Consanguinity plays a role in inheritance and most patients were from Western Sudan. The most detected deleterious mutations worldwide in exon 18 were not found in the Sudanese studies samples. Further studies targeting other frequently mutated exons (e.g., exons 8, 10, and 14) or employing next-generation sequencing (NGS) are recommended. Bioinformatics tools are useful in studying the functional analysis of SNPs.

Key words: molecular genetics; retinoblastoma; PCR, exon 18; sequencing; single nucleotide polymorphisms (snp); bioinformatics; sift; polyphen-2; i-mutant and project hope

Introduction

Retinoblastoma (RB) is a rare embryonal tumor of retinal origin and represents the most common intraocular tumor in children, with a 3% incidence of all pediatric tumors and a frequency averaging 1:20,000 live born in different populations (Nag and Khetan, 2024, Gu et al., 2021). It is estimated that 8,000 worldwide new cases of RB of the developing retina are diagnosed each year (Lukamba et al., 2018). Approximately 28% of the global RB population is found in sub-Saharan Africa. It has a favorable prognosis if diagnosed early as in some developed countries as in USA and Canada, however, delayed diagnosis significantly increases mortality risk as in African countries. This is due to a lack of awareness of the clinical outcomes and severity of the disease, preference to seek alternative medicine by some parents, difficulty in accessing referral centers and financial burden (Essuman et al., 2023, Beukman, 2024). Since the condition is lethal, there is a global survival rate of less than 30% if left untreated (Rathore et al., 2023). The most common presenting sign is Leukocoria, followed by strabismus (squint) (Fabian et al., 2020). There are large populations and high birth rates in Asia and Africa. Thus, both bear the most significant burden of RB as well as carry the highest mortality rate of 40 to 70%, compared to 3-5% in the USA, Europe, and Canada (Gu et al., 2021).The RB1 gene (OMIM 614041/ OMIM 180200), located on chromosome 13q14 (Retinoblastoma - GeneReviews® - NCBI Bookshelf, Entry - #180200 - RETINOBLASTOMA; RB1 - OMIM - (OMIM.ORG), consists of 27 exons (Byroju et al., 2023). It is the first tumor suppressor gene to be discovered. The lack of functional RB protein (pRB) in RB1 mutation results in uncontrolled cell division (Nag and Khetan, 2024, Gu et al., 2021). About one-third of RB tumors are hereditary and bilateral, with a median age of one year at diagnosis. It is caused by an RB1 constitutional mutation (M1) on one allele followed by a somatic RB1 mutation on the other allele (M2), leading to loss of function of the RB protein and tumor initiation. Knudson first hypothesized this 'two-hit' mechanism in 1971 (Norrie et al., 2021, Soliman et al., 2017). The other two-thirds of these tumors are unilateral, mostly nonhereditary (85%), and have somatic inactivation of both RB1 alleles with a median age of two years at diagnosis. The remaining 15% carry a germlineRB1 mutation and are heritable RB. Heritable tumors have autosomal dominant inheritance, and these patients can transmit the mutation to their offspring and are at increased risk for secondary tumor development (Nag and Khetan, 2024, Gu et al., 2021).

Precise identification of RB1mutations in affected families can significantly improve the quality of clinical management of the affected patient and relatives at risk (Soliman et al., 2017). Children at risk undergo series of clinical examinations, including examination under anesthetic (EUA), to early diagnose and treat tumors (Nag and Khetan, 2024, Gu et al., 2021). Siblings of an individual with heritable retinoblastoma should be tested for germline mutation, its presence or absence would determine the future risk which is very similar to the general population if no mutation is found. Whereas those with mutations are considered at high risk and this necessitates periodic surveillance (Byroju et al., 2023, Nag and Khetan, 2024). Thus, cost-effective and sensitive genetic testing is essential to determine the presence of a germline mutation before routine implementation in clinical practice (Gudiseva et al., 2019). Genetic counseling and prenatal testing for pregnancies at risk are thus necessary (Nag and Khetan, 2024, Gu et al., 2021). Therefore, a good understanding of RB genetics is important to support optimal care for children with RB and their families (Soliman et al., 2017). Next-generation sequencing (NGS) strategies are becoming essential in the management and counseling of these patients (Lukamba et al., 2018). In Africa, much research has been conducted regarding incidence, inheritance pattern, treatment outcomes and associated cancers with RB (Hill et al., 2016, Parkin and Stefan, 2017, Essuman et al., 2023). Fewer research has been conducted regarding mutational analysis of the RB1 gene in Arabian African countries (El Amrani et al., 2022). Very few research has been published in Sudan regarding RB from the year 2006 to 2024. A recent study conducted by Abdalla and Mohammed showed that RB has been ranked 4th (n = 410; 9.4%) among the most common cancers and the 2nd most frequent solid tumor in a 15-year retrospective study in Khartoum Oncology hospital, Sudan, from the year 2005 to 2019. The patients were mostly from central Sudan (Khartoum and Al Jazeera) while one third originated from Western Sudan (Kordofan and Darfur). There was no significant difference between boys and girls (Abdalla and Mohammed, 2024). Retinoblastoma incidence in Sudan is not well known and is believed to be underestimated. It is estimated to be around 40-50 cases per year (unpublished data by other colleagues). No complementary updated follow up research was conducted by the Institute of Nuclear Medicine in Gezira regarding the incidence of retinoblastoma and other childhood cancers. To date, no study regarding molecular analysis of RB1 gene mutations in patients with Retinoblastoma has been published in Sudan. By searching the database, there are couple of papers published regarding molecular studies of RB1 gene in tumors other than RB, the recent one was conducted by Elagali et al, 2021 regarding breast cancer patients in Sudan. (Elagali et al., 2021). RB1 gene is well known for its CpG islands dispersed across several exons, exon [8, 10, 11, 14, 15, 17, 18 and 23]. Recurrent mutations in these locations represent mutational hotspots (Marković et al., 2023). Therefore, this study aims at screening for the most deleterious mutations reported worldwide in exon 18 (rs137853292, rs375645171, and rs772068738) (Additional files) in Sudanese families with positive RB member/s from the same ethnic background. To our knowledge, this study is the first to screen RB1 exon 18 in Sudanese RB cases.

Methods

Editorial Policies and Ethical Considerations

Demographic data and the clinical information about each child and his family were collected through a close ended Questionnaire. A family pedigree was constructed for each family and was included in the questionnaire (Additional file 1). All patients' guardians were informed and consented in writing to participate in the study before collecting the samples for publication. Ethical approval was obtained from the ethics committee of Makkah Eye Complex.

Study area

This study was carried out in Khartoum state at Makkah Eye Complex, the largest hospital in Khartoum that provides eye care services for people from all parts of Sudan.

Sampling

Out of all Sudanese patients diagnosed with RB (31 patients) attending Makkah Eye Complex during the year 2017, seven patients were selected randomly for genetic sequencing and analysis according to budget limitations. Three healthy family members with no RB have been added as controls. Blood specimens were collected using EDTA-vacutainer

tubes from the selected patients and controls. The specimens were preserved at −20 °C.

DNA extraction

DNA extraction was carried out using a kit from iNtRON Biotechnology for both patients and controls. Genomic DNA extraction was confirmed by agarose gel electrophoresis, and the DNA was kept at −20 °C until use.

PCR amplification

Samples from seven patients and 15 relatives were amplified using the primer set in (Table 1) targeting RB1 gene exon 18. Primers were synthesized and purchased from Macrogen Incorporation (Seoul, South Korea). The annealing temperature was adjusted using Maxime PCR PreMix Kit (i-Taq) 20µl (INTRON Biotechnology, South Korea) on several runs of PCR. The adjusted temperatures are described in (Table 1). Amplification for the targeted region was done after the addition of 14ul distilled water, 1ul DMSO, 3ul DNA sample, and 1ul of each of the forward and reverse primers to the ready-to-use master mix volume. The PCR mixture was subjected to an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95 °C for 45 s, primer annealing at 55.6 °C for 60 s, followed by a step of elongation at 72 °C for 60 s, and the final extension was at 72 °C for 7 min (Elimam et al., 2017). PCR was repeated twice to yield enough DNA samples. The PCR products were checked and analyzed by 2% agarose gel electrophoresis at 100 V for 30 to 45 min, then, an automated gel photo documentation system visualized the bands. [Figure 1] The seven patients and the three controls were subsequently selected for sequencing by the Sanger sequencing technique.

Primer's nucleotide sequence	PL*	AT*	Am*
F: 5' dTGACTTTATTTGGGTCATGTACCTT 3'	25	55.6	360
R: 5' dGCCACTGTCAATTGTGCCTA 3'	20	55.6	

Table 1: Primers used to amplify RB1geneexon 18.

PL* Primer length in base pair, AT* Annealing Temp, Am* Amplicon size (bp)

Sequencing of RB1 gene exon 18

Sanger sequencing was performed for the selected PCR products. Both DNA strands were sequenced by Macrogen Company (Seoul, South Korea).

Bioinformatics analysis

FinchTV program version 1.4.0 was used to view and check the quality of each sample's two purified chromatogram (forward and reverse) nucleotide sequences. NCBI offers the BLAST (Basic Local Alignment Search Tool) family of programs (blast.ncbi.nlm.nih.gov) to detect similarities between the database sequences and a query sequence (Sayers et al., 2019). Thus, NCBI Nucleotide database was searched for reference sequences for Retinoblastoma gene. The RB1 nucleotide sequence (gene ref_ NG 009009.1) was obtained (Homo sapiens RB transcriptional corepressor 1 (RB1), RefSeqGene (LRG_5 - Nucleotide - NCBI) and exon 18 was analyzed accordingly using nucleotide BLAST. BioEdit software was used to find any apparent changes within the tested sequences through multiple sequence alignment. It is available for download at https://bioedit.software.informer.com/7.2/.

Results

Study population characteristics

Patient characteristics & clinical parameters

Thirty-one patients (n=31) diagnosed with RB attended Makkah eye complex (MEC) for the year 2017. The majority of patients (41.9%), when attended the Orbit clinic and diagnosed with RB, were below the age of 5 years (2-5 years), next age group were 1-2 years old (29.0%), followed by below 1 year old group (19.4%) and only 3 patients (9.7%) were above the age of 5 years. [Table 2] Females (58.1%) slightly dominating males (41.9%). [Table 3] Despite the incomplete records for all patients (n=15), Leukocoria seems to be the most common sign at presentation (41.9%), while Phthisis, Enophthalmos and Retinal detachment were one case each. [Table 4] Unilaterality of RB (n=30) were described in 77.4% of patients while Bilaterality in 19.4% (one missing record). [Table 5] Right and left eyes were equally affected, 50% each. [Table 5]. Regarding the seven RB cases, the demographic, characteristics and clinical features are summarized in Table 6. Their age at the time of diagnosis ranged between seven days (0.02 year) to five years (mean age of 2 years ± 0.71 years). Again, most patients (six patients) were below the age of five years, only one patient (14.3%) was above the age of five years. There were two males (28.6%) compared to five females (71.4%). Leukocoria was found in most patients (5 females/ 71.4%). Regarding tumor site, unilaterality was found in85.7% of patients. Five patients have a unilateral sporadic tumor (i.e. no family history), one patient with unilateral familial tumor (affected cousin) and one patient (14.3%) with bilateral sporadic tumor (no family history at that time). The patient with the unilateral familial RB had a family history of a cousin diagnosed with an eye tumor at the age of seven months and died shortly at the age of 10 months. [Figure 2] Regarding consanguinity of parents, it was very high. It was found in six out of seven cases (85.7%), in which two patients (28.6%) have parents who are 1st degree cousins, whereas four patients (57.1%) have parents who are 2nd degree cousins. Only one patient (14.3%), his parents are not relatives. [Table 6] No distant metastasis was reported in our samples but there were four female recurrences out of the seven samples (57.14%). Regarding ethnic background and geographical area, patients were from seven different tribes, all of them (100%) belong to Western Sudan [Baramka (Kurdofan/Darfor), Rezaigat (Kurdofan/Darfor), Jammoeyya (Central/Kurdofan),Zaghawa (Darfor), Fallata (Darfor and Western Africa), Mesaireya (Kurdofan/Darfor) and Kawahla (Kurdofan), [Table 6] Geographical area is not definite as most of these tribes are nomadic with known habits of moving across the country with their animals. [Table 6]

PCR products of exon 18

The PCR products (360 nucleotides long) of the seven afflicted children with RB (P1, 5, 7, 9, 12, 15, 20) and their relatives as Controls (C2-C22) are shown in figure 1A. All 22 samples yielded sufficient quality bands (sample #19 did not show a PCR product in this gel picture but gave a clear band in another run. Picture is not shown).

Bioinformatics result analysis

The sequencing data was checked for consistency and quality by FinchTV as shown in figure 1B. Our result showed consistency and good quality for all 10 sequences. By using the multiple sequence alignment tool BioEdit, the analysis of seven tested patients and three family controls compared to NCBI reference sequence RefSeq (NG 009009.1) revealed no nucleotide change as shown in figure 1C.

Age at diagnosis (years)	No. of patients	%
0-1	6	19.4
>1-2	9	29
>2-5	13	41.9
>5	3	9.7
Total	31	100%

Table 2: Age distribution at first RB diagnosis in MEC (n=31).

Note: RB: Retinoblastoma; MEC: Makkah Eye Complex.

Sex	No. of patients	%
M	13	41.9
F	18	58.1
Total	31	100%

Table 3: Gender of RB patients attended MEC.

Note: RB: Retinoblastoma; MEC: Makkah Complex Eye.

First signs at diagnosis	No.
Leukocoria	13
Phthisis eye diagnosed as Enophthalmos	1
Retinal detachment	1
Total	15

Table 4: Distribution of RB First sign at diagnosis in MEC (n=15).

Note: RB: Retinoblastoma; MEC: Makkah Eye Complex

Laterality	No.	%	OD	%	OS	%
Unilateral	24	77.4	12	50	12	50
Bilateral	6	19.4	-	-	-	-
Total	30	96.8%				

Table 5: Laterality Distribution of RB in MEC (n=30).

Note: RB: Retinoblastoma; MEC: Makkah Eye Complex

OD: Oculus Dextrus (right eye); OS: Oculus Sinister (left eye)

Variable		Frequency (%)
Onset	≤1 year	4 (57.1%)
	1-2 years	1 (14.3%)
	2-5 years	1 (14.3%)
	>5	1 (14.3%)
Sex/Gender	Males	2 (28.6%)
	Females	5 (71.4%)
Leukocoria	Males	2 (28.6%)
	Females	5 (71.4%)
Tumor site	Unilateral	6 (85.7%)
	Bilateral	1 (14.3%)
Family history	Ocular cancer	1 (14.3%)
	Other cancer	0 (0%)
Consanguinity of parents	1st degree cousins	2 (28.6%)
	2nd degree cousins	4 (57.1%)
	None	1 (14.3%)
Recurrence	Male	0 (0%)
	Female	4 (57.1%)
Tribe	Baramka (Kurdofan/Darfor)	1 (14.3%)

	Rezaigat (Kurdofan/Darfor)	1 (14.3%)
	Jammoeyya (Central/Kurdofan)	1 (14.3%)
	Zaghawa (Darfor)	1 (14.3%)
	Fallata (Darforand Western Africa)	1 (14.3%)
	Mesaireya (Kurdofan/Darfor)	1 (14.3%)
	Kawahla (Kurdofan)	1 (14.3%)
Geographical region	Central Sudan [1]	0
	Western Sudan	7 (100%)
	Northern Sudan	0
	Eastern Sudan	0

Table 6: The demographic, characteristics and clinical features of the seven RB patients attended MEC (2017).

Note: RB: Retinoblastoma; MEC: Makkah Eye Complex [1] Comprising both Khartoum and Al Gezira

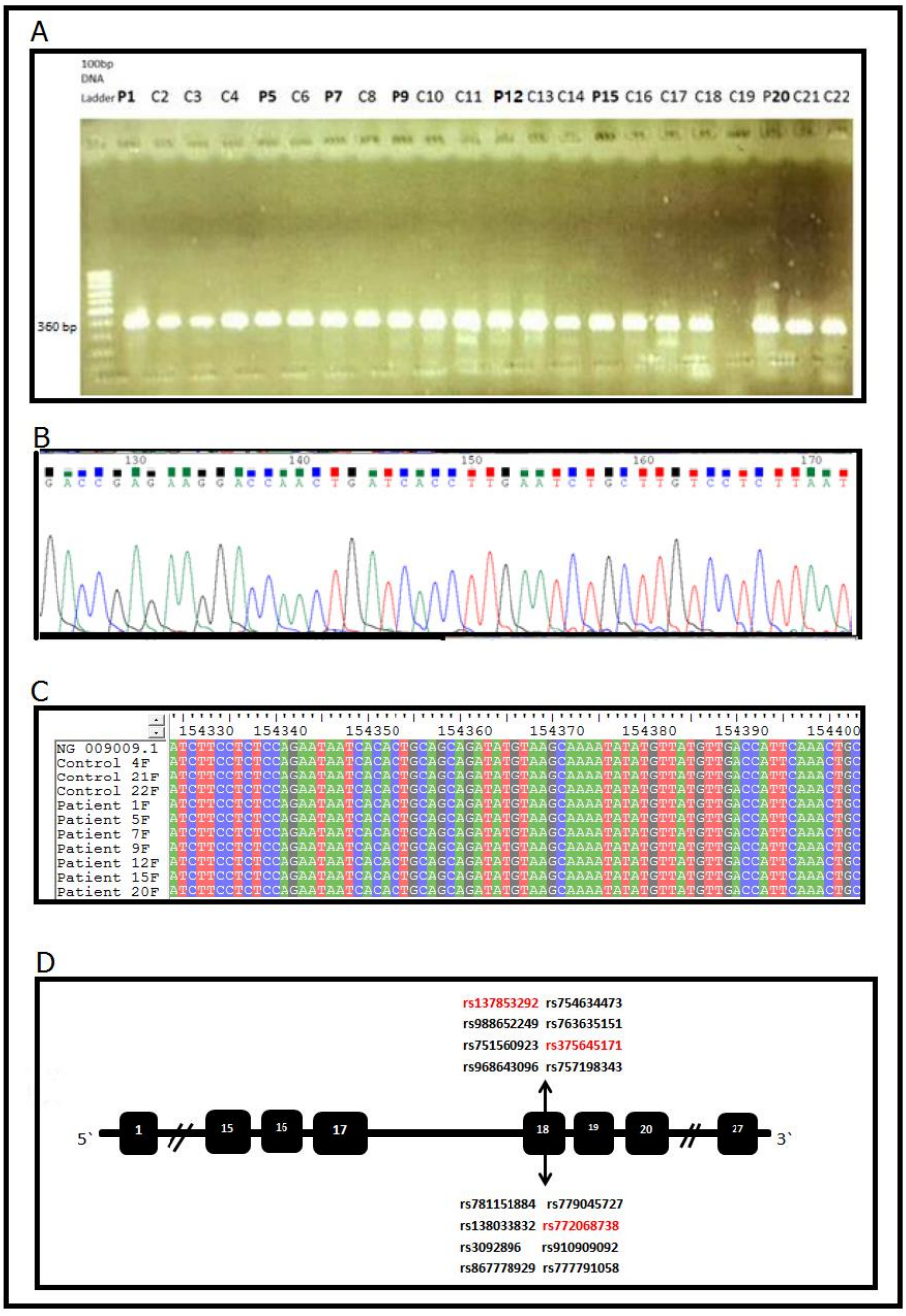

Figure 1: A-C: Results of the seven affected patients with RB. **D:** Reported mutations worldwide in exon 18.

A: PCR amplification products (360 bp) of the seven afflicted children with Retinoblastoma (P1,5,7,9,12,15,20) and their relatives as Controls (C2-C22). The band of C19 appeared in the second gel (data not shown). DNA ladder of 100 bp was used.

B: FinchTV result showing good DNA quality of exon 18 coding sequence.

C: BioEdit result showing alignment of the seven patients with their family controls compared to NCBI control. F: forward sequence. NG: genomic nucleotide reference sequence. No SNPs are found.

D: Identification of 16 reported mutations world-wide, three of which (red color) are major nsSNPs (non-synonymous) which contribute to native RB1 protein malfunction.

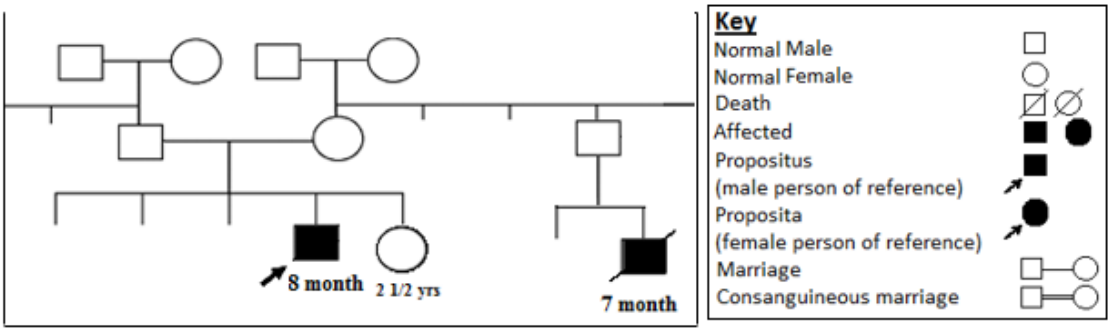

Figure 2: Pedigree of an 8-month-old RB patient with a unilateral familial RB who has a cousin that is diagnosed with an eye tumor at the age of seven months and died shortly after three months at the age of 10 months.

Discussion

In our study, the number of RB patients attending Makkah Eye complex in 2017 was 31. Most of our patients were below the age of five years, and there is a slight predilection for the female gender with a male-to-female ratio of 1:1.4. Although some records were incomplete, leukocoria remained the most frequently reported presenting sign (13 patients). This finding is consistent with the result reported by K Shahraki et al. in Iran that Leukocoria represents the most significant complaint in RB patients (Shahraki et al., 2017). Regarding the laterality of the disease, our findings slightly diverge from global trends reported in the literature, where unilateral tumors occupied 77.4% and bilateral tumors 19.4% compared to ~ 60% unilateral and 40% bilateral worldwide (Nag and Khetan, 2024). For unilateral tumors, the right and left eyes are equally affected and have no preference to one side. Although most of our patients had unilateral sporadic disease (five out of seven), family history of an eye tumor was found in one case with a unilateral RB and whose parents are 2nd degree cousins. The patient has a cousin who had an eye tumor at the age of seven months and died shortly at the age of 10 months old (Figure 2). This supports the literature in that familial RB can presents as unilateral disease (Nag and Khetan, 2024). The other patient (two years old) with a bilateral sporadic tumor and whose parents are not relatives had a younger sibling who was one month old at the time of study, and thus, no family history was demonstrated at that time. This family needs follow-up for possible sibling affection with RB. Regarding consanguinity, our results showed that RB could occur in 2nd degree relatives as well as in non-relatives. This demonstrates that RB has no predilection to close relatives; it can occur in any family relationship. Regarding ethnic background and geographical area, all seven studied families were from Western Sudan. Although this is a small sample number, it is comparable with a study performed by a colleague (unpublished), which demonstrated that RB is present in Western Sudan (Kurdofan and Darfor) in a slightly higher percentage (40.6%) than in Central Sudan (Khartoum and Al Gezira) (34.4%). Recurrences occurred in four patients either immediately after six months of chemotherapy or later, indicating late presentation and spread of the disease or ineffectiveness of the chemotherapy. Due to budget constraints, only seven patients under active treatment and follow-up were selected for molecular analysis. Mutations in exon 18 in this study is one of the hotspots reported world-wide (Marković et al., 2023, Nguyen et al., 2018, Parma et al., 2017, Tomar et al., 2017). The various computational approaches used (SIFT, PolyPhen-2, I-mutant and Project hope) identified 16 reported mutations worldwide, three of which (rs137853292, rs375645171 and rs772068738) are major ns SNPs (non-synonymous) which might contribute to native RB1 protein malfunction and ultimately causing carcinoma (Figure 1D). In this study, these targeted mutations were absent in exon 18 of the RB1 gene among the Sudanese study sample. However, mutations in RB1 gene are random and could be found in other exons as well due to heterogeneity of the disease, and thus comprehensive screening of all RB1 exons is recommended. In addition, several previous studies from different countries agreed with our finding in which they revealed numerous mutations across RB1 gene of RB patients, but not in exon 18 (unilateral, bilateral, trilateral, sporadic and familial RB cases) (Kalsoom et al., 2015). Further investigations should focus on recurrent mutations in exons 8, 10, and 14 or employ next-generation sequencing (e.g. Gene Panel, whole-exome sequencing) to capture broader mutational landscapes.

To our knowledge, this is the first published study to screen RB1 exon 18 in Sudanese patients affected with RB.

Conclusion

This study reveals that retinoblastoma mostly affects Sudanese children under the age of five, with most cases being unilateral. A high rate of parental consanguinity was observed, specifically among diverse tribes in western Sudan, suggesting possible contribution to genetic predisposition. Notably, none of the widely known harmful variants in exon 18 of the RB1 gene were found in the Sudanese study sample. Further screening for the highly reported mutations in exons 8, 10 and 14 or NGS (whole-Exom sequencing) are recommended, ideally with a larger sample size and complete family trios (child, parents, and siblings). Clinically, the use of tailored molecular genetics diagnostics could improve early detection,

facilitate family counseling, and optimize surveillance techniques for at-risk families. To our knowledge, this is the first study performed to screen exon 18 in Sudanese patients.

List of Abbreviations

DNA: Deoxyribonucleic acid

MEC: Makkah Eye Complex

NCBI: National Center for Biotechnology Information

NGS: Next Generation Sequencing

nsSNPs: non-synonymous Single Nucleotide Polymorphisms

PCR: Polymerase Chain Reaction

RB: Retinoblastoma

Rs: reference SNP ID number or (“rs#”; "refSNP cluster")

SIFT: Sorting Intolerant from Tolerant

SNPs: Single Nucleotide Polymorphisms

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Additional Files

Additional file 1

Questionnaire (Closed-Ended) _Template.

This questionnaire used to collect demographic data, pedigree, and clinical information from each family.

Additional file 2

The sixteen nsSNPs of exon 18 Polyphen, and I-Mutant software were also identified.

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