Original Research Article

Mutation Analysis of the RB1 Gene in Nigerian Children with Retinoblastoma

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Abstract

Retinoblastoma is the most common intraocular malignancy in infancy and childhood. The incidence varies in different populations. Mutations of the retinoblastoma susceptibility gene have been implicated in malignant retinoblastoma. This research seeks to identify any mutations that could be present in exon 19 and have implications in the pathogenesis of retinoblastoma among children in Calabar. Children (9) with retinoblastoma attending the University of Calabar teaching Hospital (UCTH), Dept of Opthalmology. 30 unrelated and unmatched controls with no history of retinoblastoma were recruited into the study. 2- 3mls of blood was collected from each child, genomic DNA was extracted from blood, PCRs and sequencing were performed on exon 19. The nucleotide sequences of the RB_1 gene were decoded from the chromatogram using Bioedit software and aligned manually. Multiple sequence alignment was performed using CLUSTAL W. The RB1 gene mutation frequencies were 2(22.2%) and 3(33.3%) for missense mutations and deletions respectively. Sequencing revealed two missense mutations namely: g.98A>G (p.Y33C) and g.154A>G (p.I52V) in a male patient and a female patient. Deletions include: g.1delC, g.1-12delCAGGAAAACCA, g.45-46delAA, g.14-21delTTATTAAA and g.1-55delCAGGAA.....TTC were all observed in male (bilateral) cases of retinoblastoma 3(11.1%). These mutations were absent in 6(66.6%) children and the control subjects. Two missense and five deletions were observed in four males and a female patient. The RB1 gene mutation frequency was low among the retinoblastoma children, implying that these mutations were not directly responsible for retinoblastoma, the main causal mutation may be present in other exons. Exon 19 needs to be investigated in a larger population.

Keywords: RB1 gene, Mutations, Retinoblastoma, Calabar, children.

INTRODUCTION

Background

Retinoblastoma is a rare eye tumor of childhood that arises in the retina. It is the most common intraocular malignancy of infancy and childhood with varying incidence depending on the population investigated [1]. Retinoblastoma is bilateral in about 25-35% of cases and unilateral cases being diagnosed at around 24 months [2]. Retinoblastoma (Rb) is the first disease for a genetic etiology of cancer that has been described and the first tumor suppressor gene identified [3]. It is inherited when a patient carry a germline inactivated RB1 allele present in all cells in the body and a somatic loss of the second allele in retinal cells. Germinal Rb1 mutations with a high penetrance rate of 90% were observed in patients with bilateral retinoblastoma and approximately 15% in unilateral form of the disease [4]. In non-hereditary cases (45% of all patients) both RB1 alleles are inactivated somatically in a single developing retinal progenitor cell and the disease is always unilateral and unifocal [5, 6]. RB1 encodes the nuclear phosphoprotein pRB, which plays a prominent role during the G1/S phase transition [7]. In knock-out mouse model of retinoblastoma, Zhang *et al.*, [8] demonstrated that pRB is required for appropriate exit from the cell cycle of retinal progenitor cells and for rod development. Mutation of both alleles of RB1 is required for tumor initiation. Numerous studies indicated that other molecular events, in addition to the loss of pRB, are necessary for tumorgenesis like chromosomal gain +q, +6p; chromosomal loss -16, -16q, -17q, [9, 5]. Laboratory testing of RB1 mutations will be feasible in determining the severity of retinoblastoma and counseling of patients [10].

Retinoblastoma is brought about by biallelic inactivation of human retinoblastoma susceptibility gene, located on chromosome 13q14 that codes for the RB protein. The cytogenetic deletions examined in retinoblastoma patients have assigned the genetic locus

of the disease to q14 of chromosome 13 linked with the polymorphic marker gene enzyme esterase D as documented by Friend *et al.,* [11]. Predisposition to retinoblastoma usually segregates as an autosomal dominant trait with high penetrance [6]. Precise identification of the RB1 gene mutation will help in enhancing the clinical management of patients, relatives at risk [12]. Establishing DNA diagnostic services is imperative for these patients and their families and as such will help ophthalmologist to decide whether the next child or a close relative should have constant ophthalmic surveillance. Thus we looked at RB1 gene mutation for retinoblastoma patients, their parent and siblings to deduce further reproductive options for the disease management, counseling and better visual outcome in affected individuals. The aim of this research is to identify any mutations present in exon 19 and its flanking intronic sequences of RB1 gene that is implicated in the molecular pathogenesis of retinoblastoma in Calabar.

MATERIALS AND METHODS

All participants were recruited from the Department of Ophthalmology, University of Calabar Teaching Hospital (UCTH), Calabar, Cross River State, Nigeria after obtaining full approval from the Institutional Ethics Review Board. Nine retinoblastoma children as patients and 30 unrelated, unmatched children as controls for this pilot study. All participants recruited as controls were individuals who attend the UCTH eye clinic for general eye examinations who did not have retinoblastoma or any other eye disorder and their consent were obtained accordingly. Each participant had undergone complete eye examinations by more than one ophthalmologist and the diagnosis was reviewed independently by the retinoblastoma consultant. Venous blood samples of 2-3mls were collected from each retinoblastoma patients and controls. The samples were stored in separate EDTA bottles neatly labeled and kept in deep freezers at a temperature of about- 20°C. DNA extractions, PCRs were carried out at the Department of Virology and Molecular Diagnostic Units. IITA, Nigeria. DNA sequencing was performed at the DNA Facility Laboratory, Iowa State University, Ames, USA. DNA was extracted using the procedure as previously reported by Kooffreh *et al.*, [13]. PCR amplification of the targeted RB1 gene on exon 19 and intron-exon boundary was undertaken using previously described primers [14] and the primers sequences are as follows: The forward primer was 5^{1} – AGGCAGTAATCCCCAGGAAAAGCC- 3^{1} and the reverse primer was 5^{1}- CACAGAGATATTAAGTGACTTGCC- 3^{1}. The PCR were performed in 50µl cocktail containing 4µl of genomic DNA, 10µl of PCR buffer, 3µl of MgCl$_2$, 1.0µl of dNTPs, 1.0µl of each primer (forward and reverse primer), 29.76µl of nuclease free water, and 0.24µl of Taq DNA polymerase.

Cycling Conditions

Initial denaturation step at 95°C for 3 minutes. Then 35 cycles of denaturation at 95°C for one minute, annealing at 58°C-62°C for one minutes, and elongation at 72°C for one minute. Then a final extension step of 10 minutes at 72°C. About 5µl of the amplicons was observed on agarose gel electrophoresis for PCR amplification. The method for purifying the amplicons was carried out according to Bejjani *et al.*, [15]. The purified amplicons were suspended in double distilled water and stored in the freezer until packaging and transportation to DNA Facility Laboratory, Iowa State University, USA for bidirectional sequencing on all PCR products.

Mutational analysis of sequenced RB1 gene

The amplicons were screened for RB1 gene mutation on exon 19 and intron-exon boundary using ABI 3730XL sequence. The nucleotide sequences of the CYP1B1 gene were decoded from the chromatograms using Bioedit software. The nucleotide sequence of the targeted gene from patients, controls were compared with the published RB1 sequence on NCBI gene bank to query for similarity on the database. Multiple sequence alignment was performed using CLUSTAL W software in MEGA 6.06 [16]. The statistical analyses were carried out using SPSS version 20.0. Quantitative variable were compare using chi-square (X^2) and simple percentage. Significance was set at P<0.005.

RESULTS

The sample population consists of nine clinically diagnosed retinoblastoma patients, 30 unrelated children. The mean age of the retinoblastoma cases and controls was 26.4±6.9 months and 10.8±1.9 years respectively. The patient population consists of six male retinoblastoma cases and three female cases, the control population consist of 16 males and 14 females (Table-1). After amplification, the 485-bp product was sent for sequencing (Plate 1). *In silico* analysis after sequencing revealed g.98A>G substitution in a male bilateral patient, where tyrosine was replaced by cysteine on the amino acid sequence (Fig-1). Another missense mutation: g.154A>G (p.I152V) was detected in a female bilateral patient. Nucleotide deletions observed include: g.1delC also found in the male patient with the missense mutation, g.1-12delCAGGAAAAGCCA, g.14-21delTTATTAAA, g.45-46delAA, and g.1-55delCAGGAA………..GTTTTC having the same frequency of 11.1% and observed in males bilateral cases. The RB1gene mutations where absent in all controls. The frequency of missense mutations and deletions was 22.2% and 33.3% respectively (Table-2).

Table-1: Socio-demographic of subjects

Variables	Retinoblastoma (n=9) Patients	Controls (n=30)	X^2	df	P-value
Gender	Males 6(66.7%)	16(53.3%)			
	Females 3(33.3%)	14(46.6%)	0.34	1	0.61
Ethnicity	Boki 1(11.1%)	3(10.0%)			
	Efiks 2(22.2%)	9(30.0%)			
	Ibibios 2(22.2%)	10(33.3%)			
	Ibos 3(33.3%)	6(20.0%)			
	Ijaws 1(11.1%)	2(6.6%)	0.19	4	0.29
Mean age (months/year)	26.4 $\pm$ 6.9 (months)	10.8$\pm$1.92 (years)			
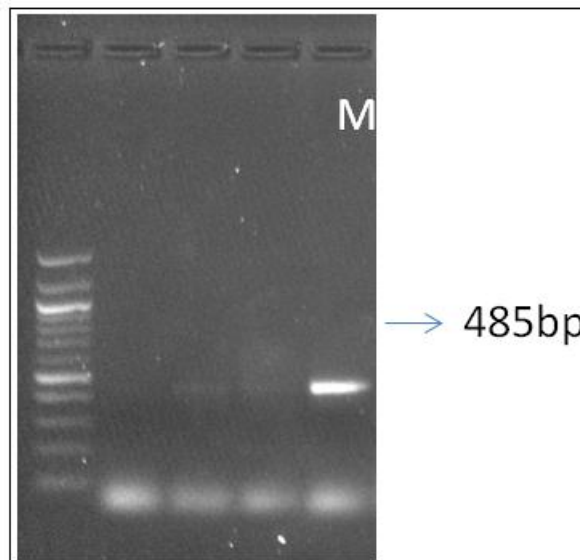
Plate-1: Gel showing PCR amplification of the RB1gene

Legend: M represents the DNA ladder of 100bp
Lane 1-3 did not amplify, the PCR was repeated to obtain products
Lane 4 is the 485bp product

Table-2: Prevalence of RB1 gene mutations in retinoblastoma cases based on gender

Type of mutation	RB1gene	Male		Female		Total	
Variant		N	%	N	%	N	%
Missense mutations	with mutation	1	11.1	1	11.1	2	22.2
	Without mutation	5	55.5	2	22.2	7	77.8
Deletions mutations	with mutation	3	33.3	0	0.0	3	33.3
	Without mutation	3	33.3	3	33.3	6	66.6

Summary of Mutations

g.98A>G (p.Y33C), g.1delC was found in one male bilateral retinoblastoma displaying both missense and deletion of RBI gene.

g.154A>G (p.I52V) found in only one bilateral female patient.

g.1-12delCAGGAAAACCA and g.45-46delAA detected in one male bilateral patient

g.1-55delCAGGAA…..TTC detected in one male bilateral patient

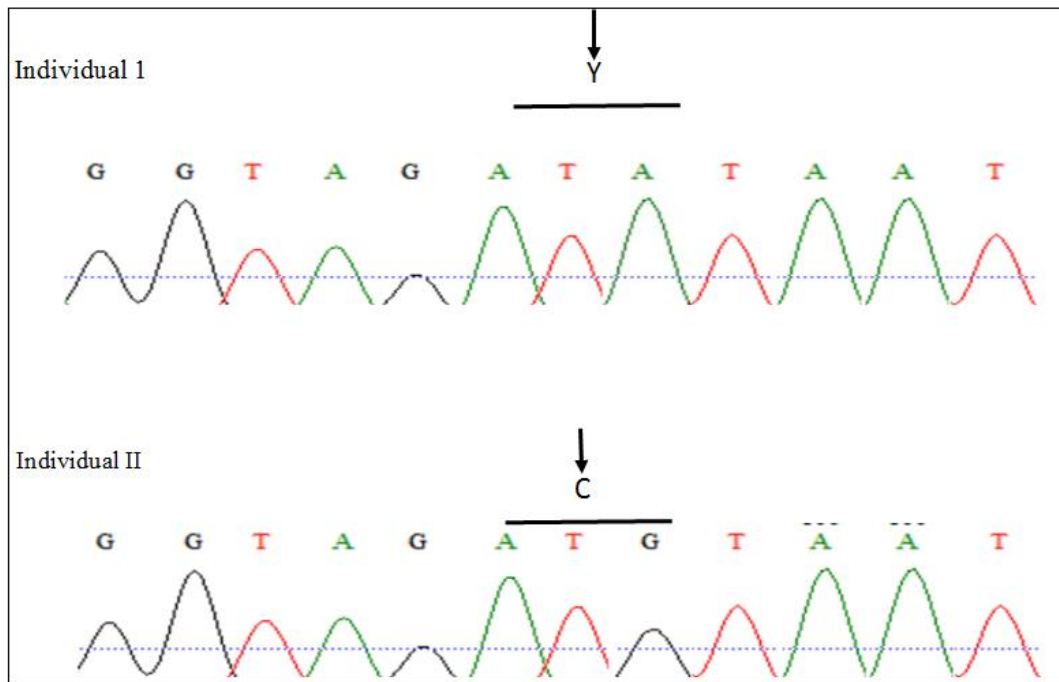g.14-21delTTATTAAA detected in one male patient.

Fig-1: Chromatogram showing missense mutation of RB1 gene on exon 19. Upper panel displayed the normal case (individual I) and lower panel showed the disease case (individual II) with the mutation. The arrow indicates the A>G transition. The encoded amino acid at codon 98 (underline) is indicated, TAT encodes tyrosine (Y), TGT encodes cysteine (c)

DISCUSSION

Genetic variations of RB_1 gene in different ethnic group, race and geographical locations have been associated with susceptibility to retinoblastoma, making it one of the strongest candidate gene for investigation. In this pilot study, the prevalence of RB_1 gene missense mutations and deletions were 22.2% and 33.3% respectively. A missense mutation prevalence of 11.1%, was documented by Kalsoom et al., [17]. The RB_1 gene mutations detected in this study were only observed among bilateral cases of retinoblastoma patients. The findings of Abidi et al., [18] revealed mutations in bilateral cases of the disease which were absent in all unilateral cases of retinoblastoma in Moroccan patients. Two missense mutations namely g.98A>G (p.Y33C) and g.154A>G (p.I52V) was detected on exon 19 in our study. In Taiwanese family, c.1960G>T (p.V654L) missense mutation of RB1 gene was observed among retinoblastoma patients on the same exon 19 [19]. In Brazil, g.39408A>G, g.39598A>G, g.76737A>G, g.5978A>G, g.59789A>G and g.156616A>G missense mutations were detected among retinoblastoma patients for RB_1 gene mutations [20] but we observed the same A>G missense mutations on different nucleotide sites on exon 19 and its intronic flanking sequences. Kadam-pai et al., [14] also reported an A>G mutation at position 153,104 in Southeast Asian populations. The g.73788-73789delAA was reported in Lausanne among retinoblastoma patients [21], but we detected g.45-46 delAA of RB1 gene on different nucleotide sites. Kumaramanickavel et al., [22] identified g.2-9delTTATTAAA (eight base-pair deletions) on exon 4

from the tumour DNA of a 21-month old boy in India. We also identified (the same eight-base-pairs deletions of TTATTAAA) but on exon 19 in one male bilateral case with the mutation located between nucleotide sites 14-21 (g.14-21delTTATTAAA). More male cases were observed than females but the females to male ratio varied for different studies [23-25]. However Al Hasan et al., [26] and Manjandavida and Chahar [27], reported a male preponderance. A limiting factor of this study is the small population of patients. The prevalence of retinoblastoma among children in Calabar was reported by Duke et al., [24] to be 4.4%. Despite this limitation, the information obtained from this research will provide baseline for more robust research in the future to identify the actual mutations that are responsible for retinoblastoma in our population.

CONCLUSION

The exon 19 of the RB_1 gene mutations was investigated in 9 retinoblastoma children and 30 control children. Two missense mutations and five deletions were observed in four males and a female patient and were not observed in the controls. The low frequency suggests that the causal allele may be present on other exons and genes.

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