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A Pilot Study on Human CYP1B1 Gene Mutations in Three Cases with Primary Congenital Glaucoma in Calabar: Benefits for Disease Management

Roseline Duke[1], Mary E. Kooffreh[2], Anthony John Umoyen[2*], Ekerette Emmanuel Ekerette[2], Owoidihe Monday Etukudo[2], Ernest Eze[1], Essien Okon[3], Nkoyo Ephriam[2] and Michael Ozoje[2,4]

[1]Ophthalmology and Strabismus Unit, Department of Ophthalmology, University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria.
[2]Department of Genetics and Biotechnology, Faculty of Biological Sciences, University of Calabar, Calabar, Cross River State, Nigeria.
[3]Department of Biological Science, Cross River State University of Science and Technology, Calabar, Nigeria.
[4]Department of Animal Sciences and Genetics, Federal University of Agriculture, Abeokuta, Nigeria.

Authors' contributions

This research was carried out in collaboration between all authors. Authors RD and EE clinically characterized participants and collected samples. Authors MEK and AJU designed the study, performed laboratory work, wrote the protocol and the first draft of the manuscript. Authors EEE, OME and MO managed the statistical and bio-informatics analysis of the study. Author NE managed the literature searches and assisted in laboratory work. All authors critically read and approved the final manuscript.

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ABSTRACT

Background: Primary congenital glaucoma (PCG) is an inherited ocular congenital anomaly of the trabecular meshwork and anterior chamber angle, which results in optic nerve damage due to increase intraocular pressure if not properly managed. We explore CYPIBI gene mutations in three

**Corresponding author: E-mail: anthonyumoyen2015@yahoo.com;*

cases of primary congenital glaucoma, their family members in Calabar, helping to discuss future options for the disease management.

Methods: Clinical and molecular screenings were conducted on three cases of PCG, six parent and six siblings of the PCG patients recruited for this study. 51 unrelated, age-matched controls comprising 30 children control and 21 adult control were selected from individuals attending the eye clinic for general eye examinations that has no glaucoma or history of glaucoma. 2-3 ml of blood was collected from each participant, DNA extracted from blood, and PCR amplifications were carried out on exon 3. 25 µl of amplicon was utilized for bidirectional sequencing. The nucleotide sequences of the CYPIBI gene were edited from chromatograms using Bioedit software. Multiple sequence alignment and pairwise comparison of CYPIBI gene was carried out in the MEGA 6 software. Statistical analyses were performed using SPSS version 20 software. Significant level was set at $P<0.05$.

Results: Two missense mutations of CYPIBI gene namely: g.291G>C (p.Q97H) and 344C>T (P.T115M) were observed in this pilot study after the *in-silico* analysis. The g.291G>C mutation that resulted in an amino acid substitution of glutamine by histidine at position 97(p.Q97H) was observed in all the 3 cases of PCG, their parent (six) and siblings. The g.344C>T was detected in only one PCG patient. Two deletions of CYPIB1 gene namely: g.378-380delATG and g.535delG were also observed in this study. The g.378-380delATG was detected in all the PCG cases, their parents while g.535delG was seen in only one PCG patient that was 28 months old boy. These CYPIBI gene mutations were absents in all the controls.

Conclusion: This pilot study identified some CYPIBI gene mutations, which were peculiar to PCG cases, their parents and other family members. It suggests hereditary form of the disease although further studies are needed. This forms a baseline research for further molecular studies among PCG patients.

Keywords: Primary congenital glaucoma; children; CYP1B1 gene; mutation; Calabar.

1. INTRODUCTION

Primary congenital glaucoma (PCG) is one of the commonest causes of paediatric blindness and it has varying prevalence in Nigeria [1,2]. Glaucoma in infancy and childhood cases are potentially blinding conditions characterized by angle anomalies, presence or absence of mild congenital iris anomalies, ocular enlargement and increase intraocular pressure (IOP). Clinical presentation of primary congenital glaucoma (PCG) is that of the classic triad of symptoms including photophobia, blepharospasm, and epiphora [3]. Clinical studies stipulate that prognosis is mostly dependent on early, accurate diagnosis and treatment to control intraocular pressure [4,5] and glaucoma is attributed to 5% of blindness in children globally [5]. Childhood glaucoma is classified as primary when an isolated idiopathic development abnormality of the anterior chamber angle exists and secondary when aqueous out flow pathway is reduced due to either a congenital, an acquired ocular disease or systemic disorder [6].

Primary congenital glaucoma is usually bilateral and are more common in males [7,8]. Majority of PCG cases are sporadic but the disorder is familial in 10%-40% of cases. An autosomal recessive pattern of inheritance has been documented in population with high rate of consanguineous marriages [7,8,9]. Genetic ancestry markers may be useful in assessing risk factors in primary congenital glaucoma patients [10] and most mutations of CYP1B1 gene have been discovered among glaucomatous patients in ethnically homogenous population [10,11,12].

Primary congenital glaucoma is a sight-threatening disorder and treatment is fundamentally surgical [10,13,14]. The presence of CYP1B1 gene mutations was associated with severe disease manifestation, rapid progression and requiring more surgeries to control or reduce intraocular pressures [15]. In different populations, several molecular screening researches have been conducted concerning the associations of CYP1B1 gene mutation among primary congenital glaucoma patients [7,12,15,16,17,18]. There is paucity of reported study on genetic molecular screening of human CYP1B1 gene mutations among Nigerian children with primary congenital glaucoma especially in a tertiary referral centre for ophthalmic disorder in Calabar.

Establishing DNA diagnostic services is imperative for these patients and their family and

as such, will help ophthalmologist to decide whether the next child or a close relative should have constant ophthalmic surveillance. This will save considerable money, time, reduce psychological stress for these families [19]. Thus we looked at CYP1B1 gene mutation for three PCG patients, their parent and siblings to deduce further reproductive options for the disease management. This pilot study will form baseline information for larger molecular-genetic screening programme in this hospital and others populations.

2. MATERIALS AND METHODS

2.1 Study Population

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. Three congenital glaucoma patients, their parent (6), 6 siblings of the PCG patients and 51 unrelated age-matched controls were used for this pilot study. The control participants were of two groups namely: 30 children control and 21 adult control group. They were controls for primary congenital glaucoma cases and adult glaucoma cases (parent that have primary open-angle glaucoma), respectively. All participants recruited as controls were individuals who attend the UCTH eye clinic for general eye examinations who did not have glaucoma or other eye disorder and their consent were obtained accordingly. The parent of an affected child provided informed consent. All affected cases recruited for this research had disease onset from less than one year to 3 years old. Each participant underwent complete eye examinations by more than one ophthalmologist and the diagnosis was reviewed independently by the glaucoma consultant. Demographic details and clinical variables of patients, parent and relatives were collected as interview initiated and secondary data from case files. Parents diagnosed with glaucoma, glaucoma suspects and without glaucoma were utilized for the study.

2.2 DNA Extraction and PCR

Venous blood samples of 2-3mls were collected from each primary congenital glaucoma patient, parent, sibling and control. The samples were stored in separate EDTA bottles neatly labeled and kept at a temperature of about- 20°C. The blood samples were transported to International Institute for Tropical Agriculture (IITA), Ibadan for analyses. DNA extractions, PCRs were carried out at the Department of Virology and Molecular Diagnosis Units. IITA, Nigeria. DNA sequencing was performed at the DNA Facility Laboratory, Iowa State University, Ames, USA. DNA was extracted in the same fashion as previously reported [20], but 1% monothioglycerol was added instead of mecarpto-ethanol and the tubes were kept at -20°C for 20-30 minutes after the addition of cold isopropanol. PCR amplification of the targeted CYP1B1 gene on exon 3 and intron-exon boundary was undertaken using previously described primers [21] as follows: GL3-F1-CTCACTTGCTTTTCTCTCTCC and GL3-R1-CATCACTCTGCTGGTCAGGT. The PCR were performed in 50 µl cocktail containing 4 µl of genomic DNA, 10 µl of PCR buffer, 3 µl of $MgCl_2$, 1.0 µl od dNTPs, 1.0 µl of each primer (forward and reverse primer), 29.76 µl of nuclease free water, and 0.24 µl of taq DNA polymerase.

2.3 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 checked on agarose gel electrophoresis for PCR amplification of forward and reverse primers in Exon 3. The method for purifying the amplicons was carried out according to Bejjani [7] protocol. Seventy-five micro liters of 95% ethanol was added to eppendorf tubes containing 30 µl of PCR amplicons and inverted 3-5 times. The tubes were then transferred into -20°C for one hour. Then the tubes were centrifuged at 12000 rcf for 10 minuetes. The supernatant was decanted gently and 500µl of cold 70% ethanol was added, centrifuged again at 12000 rcf for 5 minutes. Then the alcohol was decanted and tubes air dried at room temperature until no traces of alcohol was seen. 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.

2.4 Mutational Analysis of Sequenced CYP1B1 Gene

The amplicons were screened for CYP1B1 gene mutation on exon 3 and intron-exon boundary using ABI 3730XL sequence (Applied

Biosystems, USA). The nucleotide sequences of the CYP1B1 gene were edited from the chromatograms using Bioedit software [22]. Multiple sequence alignment was performed using CLUSTAL W in MEGA 6.06 software [23]. The nucleotide sequence of the targeted gene from patients, parents with glaucoma, siblings were compared with the published CYP1B1 sequence by blasting NCBI gene bank to query for similarity on the database.

2.5 Statistical Analysis of Socio-Demographic and Clinical Variables

The statistical analyses of socio-demographic data were carried out using Statistical Package for Social Sciences, (SPSS) version 20.0. Quantitative and Clinical variable were compare using chi-square (X^2) and simple percentage.

3. RESULTS

3.1 Demographic and Clinical Analysis

The sample population consists of three clinically diagnosed primary congenital glaucoma (PCG) patients, 6 parent and 6 siblings (brother and sister) by ophthalmologists at UCTH, Calabar, Cross River State. The mean age of the primary congenital glaucoma cases was 22.6±1.86 months and all had bilateral PCG. The patients were two males and one female. The mean IOP for right/left eye and CD ratio for right/left eye were 25/27 and 0.8/0.9 respectively in the PCG patients. The mean age of parent was 45.7±3.1 years, comprising 3 males and 3 females. The children controls were 16 males and 14 females with the mean age of 24.9±1.92 months, while the adult controls were 12 males and 9 females with the means age of 48.1±2.6 years as shown in Table 1.

3.2 Mutational Analysis of CYP1B1 Gene

The PCR amplicons (Fig. 1) was sent for sequencing. After sequencing, *in silico* analysis revealed g.291G>C substitution (Fig. 2) and g.378-380delATG in all PCG cases and their parent. The g.291G>C missense mutation of CYP1B1 observed lead to an amino acid substitution of glutamine by histidine at position 97 (p.Q97 H). The g.344C>T, and g.535delG were observed in one PCG cases, each from different patients. These CYP1B1 gene mutations were detected in some parents and siblings (Table 2), but were absent in all controls (Both children and adult controls).

4. DISCUSSION

Primary congenital glaucoma is common cause of pediatric blindness globally [7,13]. Intraocular pressure (IOP) [3], Optic nerve damage is imperative indicators of the disease progression and the course of disease after surgical operation. Angle surgery must be performed on patients as soon as the diagnosis is well established in order to save the sight of the patient, therefore preventing blindness. This rapid treatment is important to prevent the disease progression, that has impacts on visual acuity and visual field [7,23,24] and genetics is useful in identifying, counseling, managing and prognostication of disease [2,4].

Table 1. Clinical and demographic variables of participants

Variables	PCG (n=3)	Parent (n=6)	Controls (n=51)		X^2	df	p-value
			Children (n=30)	Adult (n=21)			
Gender	Males 2 (66.7) Females 1(33.3)	3 (50%) 3(50%)	16 (53.3%) 14(46.6%)	12(57.1%) 9(42.9%)	0.45	1	0.63
Mean age	22.6±1.86 (months)	45.7±3.1 (years)	24.9±1.92 (months)	48.1±3.6 (years)			
Mean IOP (mmHg) RE/LE	25/27	-	-	-			
C/D Ratio (mm) RE/LE)	0.8/0.9	-	-	-			

PCG = Primary congenital glaucoma, IOP= Intraocular pressure, RE= Right eye, LE= Left eye, C/D = Cup disc ratio

Table 2. Clinical and molecular analysis of the three patient with PCG

Variables		Patient 1	Patient II	Patent III
Clinical examination				
Sex		Male	Female	Male
Age of onset (months)		28	22	18
Laterality		Bilateral	Bilateral	Bilateral
IOP(mmHg) RE/LE		26/25	22/29	28/27
C/D ratio (mm) RE/LE		0.7/0.8	0.8/0.9	0.8/0.9
CYP1B1 mutation				
Missense mutation		g.291G>C	g.291G>C	g.291G>C
Deletion(s)		g.378-380delATG g.535delG	g.378-380delATG	g.378-380delATG
Family history		Mother: glaucoma patient (POAG)	Glaucoma suspect	Glaucoma patient (POAG)
	Parent:	Father : glaucoma patient (POAG)	Glaucoma patient (POAG)	Glaucoma patient (POAG)
	Siblings of patient:	Brother: JOAG	NE	Glaucoma suspect
		Sister: JOAG	ND	Glaucoma suspect

IOP: Intraocular pressure, RE/LE: Right eye/left eye, ND = Not determine, NE: Not establish, C/D: cup/disc ratio, JOAG: Juvenile open angle glaucoma, POAG: Primary Open Angle Glaucoma

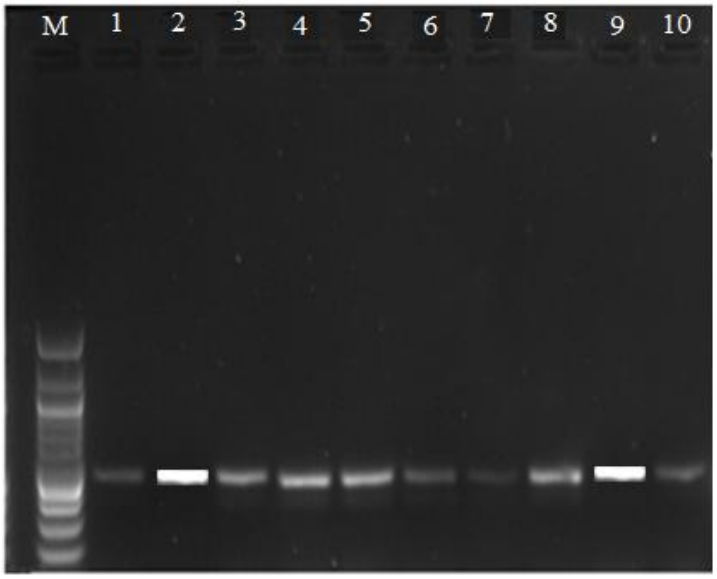

Fig. 1. Agarose gel electrophoresis showing PCR product after amplification of CYP1B1 gene. Lane M is the 1kb DNA Ladder and Lane 1-10 contains the amplified PCR products

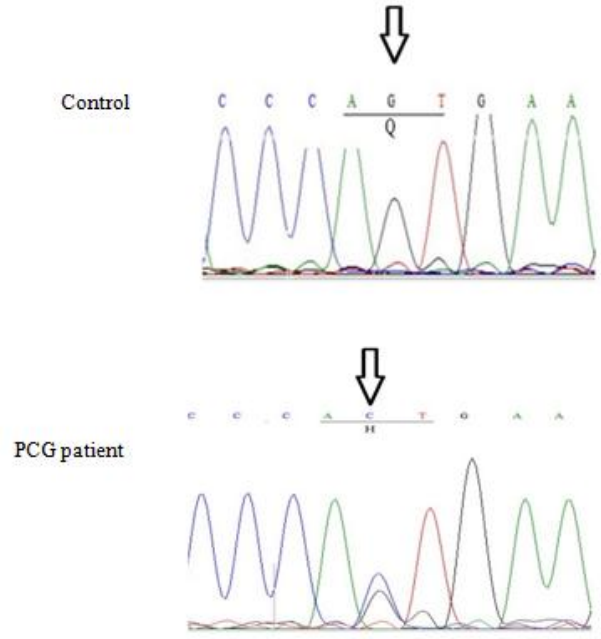

Fig. 2. Missense mutation (g.219G>C) on exon 3 of the CYP1B1 gene. The upper panel (control) has no mutation while the lower panel (PCG patient) has mutation. The arrows indicate the G>C substitution and the codons underlined: H represent Histidine while Q represent Glutamine

Sequence analysis in different populations have revealed many mutations in the CYP1B1 gene associated with primary congenital glaucoma cases [7,12,15-18,22-23]. We identified g.291G>C missense mutation among the three cases of PCG and their parents in Calabar resulting in a substitution of glutamine by histidine at position 97(p.Q97H). The human

cytochrome $P450_1B_1$ (CYP1B1) gene is one of the major candidate gene participating in trabecular meshwork development leading to ocular abnormality [6] if there is alteration or mutations of the gene [7,18-22]. We observed g.291G>C missense mutation in our pilot study in Calabar, associated with rapid disease manifestation and progression, but G>A transition of CYP1B1 missense mutation on exon 3 displaying the substitution of met364val among primary congenital glaucoma cases was reported in Japan [25], Indonesians and Europeans [16]. In Nigeria, among primary childhood glaucoma's patients g.291G>C missense mutations was observed and not found among controls [26]. CYP1B1 gene analysis in primary congenital glaucoma cases in Brazil were associated with severe, rapid disease manifestation and required more surgical operations to control intraocular pressure [15]. Alfadhi [21] previously documented that some PCG patients in Kuwait responded poorly to treatment, and surgery which correlate to CYP1B1 gene mutations. These findings were similar to our present study in Calabar. In this present pilot study in Calabar, g.291G>C mutations were observed in the three patients (PCG), their parents and some siblings suggesting parent-of –origin effect in those three families, and Klutz [27] documented a patent-of-origin effect of ocular disease among two families in Germany. Molecular genetic diagnosis should be included in the management of patients with glaucoma especially when gene mutations are detected among family members as reported [28], so this molecular genetic study will be of benefits for PCG management and counseling of families.

The g.344C>T (T115M) missense mutation was observed in only one case of PCG in our study and this patient showed a rapid disease manifestation, progression and response poorly to treatment. These clinical presentations by the patient may indicate that this gene mutation acts in consort with other variants of CYPIBI gene mutations since the patient did not only show g.344C>T mutation. Authors [17] documented that g.4547C>T, g.8167C>T mutations of CYPIBI gene were known to cause primary congenital glaucoma in France. The damaging effects attributing to CYBIBI gene mutation was associated with primary congenital glaucoma cases in Tunisia [18]. Previous documented studies have shown that missense mutations of CYPIBI gene affect highly conserved and functionally important regions of the gene, resulting in significant structural changes, reduced CYP1B1 activity linked the disease etiology [29,30,31].

A single nucleotide deletion: g.535delG mutation was detected in one of the three cases of PCG recruited in this pilot study. This same deletion was previously documented among primary congenital glaucoma cases in Portuguese [16], Brazil [32] Tunisia in North Africa [18], Morocco [33] and in Nigeria among primary childhood glaucoma's patient [26]. Also *a* three base nucleotides deletion namely: g.378-380ATG was detected in all children, their parents and some siblings (a brother and sister of patient) that were diagnosed with PCG, juvenile open angle glaucoma (JOA) and POAG respectively. It was interesting to note that parents of "patient I" were clinically diagnosed with primary open angle glaucoma (POAG), having severe presentation of the disease with increase intraocular pressure and responded poorly to treatment. These similar disease presentation were observed in their offspring and the molecular screening results revealed CYPIBI mutation (Missense mutations and deletions), which corroborates the report of [3]. Therefore, we can suggest that the disease predisposition in this family may be familial, pointing to hereditary forms of glaucoma. To the best of our knowledge through literature search, g.378-380delATG deletion is novel and was detected in all cases of PCG, their parents and some family members but further studies are needed to confirm if it is a pathogenic variant of the gene. All primary congenital glaucoma patient recruited for this study were bilateral and more males were presented with this eye disorder, similar to previously reported research in Nigeria [1], Iran [34] and Brazil [11] where more male subjects had primary congenital glaucoma. Deletions mutations of CYPIBI gene caused abnormal development of the trabecular meshwork in humans [6] and mouse eye [35] and in this present pilot study in Calabar, deletions of CYPIBI gene mutations were observed in glaucoma patient (primary congenital glaucoma, juvenile open angle glaucoma and primary open-angle glaucoma) in the three families study. This information will benefit in the disease management and counseling of other family members. The human CYPIBI gene is known to be involved in trabecular meshwork development of the ocular structure, which function as a drainage pathways for aqueous humour [36,37] and mutations of this CYPIBI gene is associated with the molecular pathogenesis of primary congenital glaucoma in several populations [7,12,15-18,38-40]. In this pilot study, CYPIBI

gene mutations showed reasonable role in the molecular pathogenesis of primary congenital glaucoma and correlated to the clinical variables like increased IOP, cup/disc ratio considered as important risk factors [11,40]. Molecular genetic screening would help to determine the penetrance or expressivity and help decisively in clinical management and counseling of individuals, which is concomitant to previous findings in ocular diseases [41,42,43]. The CYPIBI gene mutations identified in our pilot study need further investigation in a large population for significant conclusions to be reached.

5. CONCLUSION

This pilot study identified g.291G>C, and g.378-380delATG in all the PCG cases, their parents and some siblings. This information will form baseline for understanding the molecular genetic basis of the disease.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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