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HUMAN CYTOCHROME P450$_1$B$_1$ (CYP$_1$B$_1$) AND MYOCILIN (MYOC) GENES MUTATIONS POTENTIAL DIAGNOSTIC TOOL FOR GLAUCOMA DISEASE IN NIGERIAN POPULATIONS: A MOLECULAR EPIDEMIOLOGY APPROACH

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ABSTRACT

Background: Glaucoma is one of the world's leading causes of irreversible blindness. A complex, multifactorial disease, underlying pathogenesis and reasons for disease progression are not fully understood. Glaucoma bring about elevated intraocular pressure; leading to optic nerve damage and functional vision loss. In different populations, CYP$_1$B$_1$ and MYOC genes mutations had been associated with glaucoma presentations. There is paucity of updated information on genetic polymorphisms of CYP$_1$B$_1$ and MYOC genes mutations and their association with glaucoma in different Nigerian populations. Thus, this research seeks to investigate on human cytochrome P450$_1$B$_1$ (CYP$_1$B$_1$) and Myocilin (MYOC) genes mutations among glaucoma cases in Nigerian populations.

Methods: Cases and controls were recruited from Abuja (North Central); where patients from Jalingo (North East Nigeria) and Calabar (South-south). Genomic DNA extraction, PCR amplifications and sequencing of CYP$_1$B$_1$ and MYOC genes were carried out. Chromatograms were decoded into nucleotide sequences using ChromaPro software. Multiple sequence and pairwise alignments were performed using MEGA X software and Codon-Code Aligner software for SNPs analysis.

Results: Male and female glaucoma case was 19 (59.4%) and 13 (40.6%) respectively. The overall prevalence was 46.9% (15/32) and 43.8% (14/32) for CYP$_1$B$_1$ and MYOC genes mutations respectively. CYP$_1$B$_1$ gene mutation variants detected included g.291G>C (p. His97Glu), g.344C>T (p. Thro115Met), g.317delT and g.378-380delATG. MYOC gene mutation variants detected included g.299T>G (p. Gly102Arg), g.595A>C (p. Gln202His), g.513G>T (p. Gln171Pro) and g.341-347delTATACTG.

Conclusion: Glaucoma in Nigerian populations across different ethnic groups harbors CYP$_1$B$_1$ and MYOC genes mutations; and some were novel missense mutations. This study will help to establish the contribution of CYP$_1$B$_1$ and

MYOC genes mutations in molecular etiology of glaucoma in Nigerian populations. The information can serve as referral for future study.

Keywords: Glaucoma, CYP_1B_1 Gene, MYOC Gene, Mutations, Prognostic Biomarker

1 INTRODUCTION

Collaborative molecular genetics researches conducted by scientists had revealed molecular etiology, pathogenesis, clinical and genetic presentations of glaucoma in various populations [1-4]. Primary congenital glaucoma (PCG) presents as a developmental abnormality in the trabecular meshwork (TM), resulting in raised intraocular pressure (IOP); leading to blindness, if not treated medically [2,4,5]. Juvenile open-angle glaucoma (JOAG) shares several characteristics with adult-onset primary open-angle glaucoma (POAG), the commonest form of glaucoma [1]. The defining characteristics of both JOAG and POAG are cupping and corresponding patterns of visual field loss. Furthermore, JOAG has an earlier onset of disease than POAG and may sometimes be associated with high IOP that are less commonly observed with POAG [1,3,6]. Both JOAG and POAG are heritable and most cases of POAG have a complex genetic basis; caused by the combined actions of many genetic risk factors [1,6].

Primary congenital glaucoma (PCG) has an autosomal recessive mode of transmission. Cytochrome P450 family 1 subfamily B member 1 (CYP_1B_1, OMIM 601771) [7] is the most common identifiable cause of PCG globally [2-4,7]. Other forms of genes implicated in molecular etiology of glaucoma are myocilin gene (MYOC) [1,4,6,8] and latent transforming growth factor-beta binding protein [9]. Researchers documented that the clinical spectrum of glaucoma due to myocilin (MYOC) gene mutations can range from juvenile glaucoma to typical late-onset primary open-angle glaucoma (POAG) [4,6,8]. The implication of genes in molecular etiology of human eye diseases has made human genetics one of the most rapidly growing field in modern medicine; geared toward molecular diagnosis of diseases [2-6]. Glaucoma is eye disorder causing numerous visual function loss and blindness globally and its prevalence its prevalence is on the increase [1-4]. In different populations, several molecular genetics studies had been conducted concerning the association of CYP_1B_1 and MYOC genes polymorphism with glaucoma presentations [1-6]. Results in some studied populations highlighted the association of CYP_1B_1 and MYOC genes mutations in the pathogenesis of glaucoma, but others observed no association [1-4,7,8]. There is paucity of updated information on genetic polymorphism of CYP_1B_1 and MYOC genes mutations and their association with glaucoma in different Nigerian populations. Thus, this research seeks to investigate on human cytochrome $P450_1B_1$ (CYP_1B_1) and Myocilin (MYOC) genes mutations among glaucoma cases in Nigerian populations; a molecular diagnostic approach.

2 MATERIALS AND METHODS

2.1 Study Design and Location

The study was hospital-based case-control study. Cases and controls were recruited from the Department of Ophthalmology, University of Calabar Teaching Hospital (UCTH), College of Heath Technology (CHT) Eye Clinic; both in Calabar, Cross River State. Also, subjects were recruited from the Ophthalmology Department, National Hospital Abuja; where patients from Jalingo (North East Nigeria) and other states visit the eye clinic for eye examinations and treatments. Therefore, subjects selected for this study were those residing in Taraba State (North East), Abuja (North Central) and Cross River State (South-south), Nigeria.

2.2 Ethical Approval

Ethical approval was obtained from the Health Research Ethics Committee (HREC), University of Calabar Teaching Hospital (UCTH), Calabar, Cross River State and the Institute Review Board (IRB) Committee National Hospital, Abuja. The rudiments of the research were explained to participants and consent were obtained before data and blood sample collections based on the Declaration of Helsinki guidelines.

2.3 The Study Population

A total of seventy-seven (77) blood samples were collected; comprising 32 glaucoma patients and 45 apparently healthy volunteers (control) subjects. Primary congenital glaucoma (PCG) cases were 14; while JOAG and POAG was 10 and 8 cases respectively; giving a total of 18 for open angle glaucoma (OAG) group. The control participants were selected in three groups namely: 15 children control group, 15 adolescent control group and 15 adult control group to mimic the age brackets for primary congenital glaucoma (PCG), Juvenile open-angle glaucoma (JOAG) and POAG cases. Then, the total controls were pulled together to be 45 apparently healthy control subjects. Also, apparently healthy controls were matched for sex, age and ethnicity. All participant underwent complete eye examinations by ophthalmologists and the diagnosis was reviewed independently by the glaucoma consultant. Demographic and clinical information of patient,

parent and relatives were collected as interviews were initiated. Also, secondary data were retrieved from case files. Parents diagnosed with glaucoma, glaucoma suspects and without glaucoma were used for this research.

2.4 Collection of Blood Samples

Five ml of venous whole blood were obtained from subjects through venipuncture under standard universal precautions. Blood samples were put into EDTA anti-coagulated tubes; label properly and stored in deep freezer.

2.5 Genomic DNA Extraction and PCR

DNA extraction was carried out according to Dellaporta *et al* protocol [10] with some few modifications as reported by Kooffreh *et al* [11]. The extracted DNA was stored at -20^{o}C until further use. PCR amplification of the targeted CYP_1B_1 gene on exon 3 was undertaken using previously described primers by Alfadhli et. al. [12]: GL3-F1-CTCACTTGCTTTTCTCTCTCC and GL3-R1-CATCACTCTGCTGGTCAGGT. The PCR were performed in 50µl cocktail containing 4µl of genomic DNA, 10µl of PCR buffer, 3µl of MgCl2, 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 [12]. Also, MYOC gene on exon 1 was amplified on previously described primers and cycling conditions by Whigham et. al. [13]: MYOC-F1-ATCTTGCTGGCAGCGTGAA and MYOC-R1- TCTCTGGTTTGGGTTTCC. Amplicons were purified using alcohol-based method as reported [14]. Twenty-five micro litres (25µl) of amplicons was put into sterile PCR tubes and 62.5µl of 95% ethanol (25µl x 2.5µl) to wash the amplicons. It was then centrifuged at 12,000 rcf for 10 mins and gently decanted; remaining the pellets attached to the wall of the tube. Five hundred micro litres (500µl) of 70% alcohol was added and then centrifuged at 12,000 rcf for five mins. It was carefully decanted and the pellets (containing purified amplicons) were air dry at room temperature (25^{0}C- 28^{0}C) [14]. Finally, 22µl of nuclease free water was added to each PCR tube containing pellets of purified amplicons and stored in deep freezer (-20^{o}C) for further usage. The suspended purified amplicons tubes were wrapped in foils and packaged in ice pack containers for onward shipment to Inqaba Biotec Laboratory, South Africa for sequencing.

2.6 Sequencing of Amplified CYP_1B_1 and MYOC Genes

Bidirectional sequencing of the purified amplicons was carried out at Inqaba Biotec Laboratory, South Africa using the same set of primers utilized during PCR amplification of CYP_1B_1 and MYOC genes.

2.7 Statistics and Bioinformatics Analysis

The socio-demographic variables, clinical data were computed and analysed using Statistical Package for Social Sciences (SPSS) version 20.0. Simple percentage was used to determined frequency of gene mutations. Sociodemographic data was compared using student *t*- test and chi-square (X^2) test. Significant was set at 5%. Nucleotide sequences of CYP_1B_1 and MYOC targeted on exon 3 and exon 1respectively were decodes from chromatograms into nucleotides sequences using ChromasPro software (www.technelysium.com.au). Multiple sequences and pairwise alignments were computed using Clustal W in MEGA X software according to Tamura *et al* [15] and excluding all gaps. Codon-Code Aligner version 6.06 was used to analysis SNPs in the aligned sequences.

3 RESULTS

Subject demographics are summarized in Table 1. Briefly, male and female glaucoma case was 19 (59.4%) and 13 (40.6%) respectively. Primary congenital glaucoma was 14 (43.8%) while JOAG and POAG was 10 (31.3%) and 8 (25%) respectively; giving a total of 18 (56.3%) for open angle glaucoma (OAG). The mean age was 19.71±1.12 years and 21.10±1.01 years for patients and controls respectively. Figure 1 displayed the CYP_1B_1 gene amplicons on exon 3 labelled from lane 1-5 and lane M is the DNA ladder of 100 base pairs. The MYOC gene amplicons on exon 1 are labelled from lane 1-5 and lane N is the DNA ladder of 200 base pairs (Figure 2). The overall prevalence of 46.9% (15/32) was recorded for CYP_1B_1 gene mutation among glaucoma cases and eight (8) of PCG cases had the gene mutation (Table 2). Table 3 displayed the prevalence of MYOC gene mutation among glaucoma patients with a total of 43.8% (14/32) and six (6) cases of JOAG had MYOC gene mutation. Additionally, some of these gene mutations variants were novel SNPs and were absent in the 45 healthy volunteers. Mutation variants of CYP_1B_1 and MYOC genes sequences in glaucoma cases (PCG, JOAG and POAG) are shown on Table 4. A total of 11 CYP_1B_1 gene mutation variants (missense mutations and deletions) were detected in this current study; including g.291G>C (p. His97Glu), g.344C>T (p. Thro115Met), g.317delT and g.378-380delATG. Also, a total of seven (7) MYOC gene mutation variants (missense mutations and deletions) were detected in this current research; including g.299T>G (p. Gly102Arg), g.595A>C (p. Gln202His), g.513G>T (p. Gln171Pro) and g.341-347delTATACTG.

4 DISCUSSIONS

Genetic variation of human CYP_1B_1 and MYOC genes mutations in different ethnics and geographical locations have been associated with susceptibility to PCG, JOAG and POAG (adult glaucoma) [2-4,6-8].; making it a strong candidate gene for investigation. This current study revealed the overall frequency of 46.9% (15/32) for CYP_1B_1 gene mutations among glaucoma patients (PCG, JOAG and POAG). The frequency of 70.6% was reported among PCG in Kuwait [12], 60.6% (43/71) in Northern Portugal [16], 50% (16/32) in Beijing, China [2], 42.8% among neonatal-onset and infantile-onset PCG in India [4], 6.7%-41.2% among glaucoma patients in Calabar [3,17], 36.4% in Nigeria among primary childhood glaucoma

Table 1: Socio-demographic characteristics of subjects

Variables	Cases (%) N=32	Controls (%) N=45	X^2cal	X^2tab
Sex				
Male	19(59.4)	28(62.2)	2.16	0.11
Female	13(40.6)	17(37.8)		
Age (years)				
Mean age	19.71±1.12	21.10±1.01		
Type of glaucoma				
PCG	14 (43.8)	0	0.50	0.10
JOAG	10(31.3)	0		
POAG	8(25.0)			
Educational status				
Tertiary	7(21.9)	7(15.6)	0.13	0.77
Secondary	12(37.5)	13(28.9)		
Primary	4(12.5)	11(24.4)		
Informal	9(28.1)	14(31.1)		
Marital Status				
Married	19(59.4)	18(40.0)		
Single	9(28.1)	22(48.8)	0.23	0.11
Widowed	4(12.5)	5(11.1)		

Key: PCG: Primary congenital glaucoma, Juvenile open-angle glaucoma: Open angle glaucoma, POAG: Adult-onset primary open-angle glaucoma, X^2: Chi square

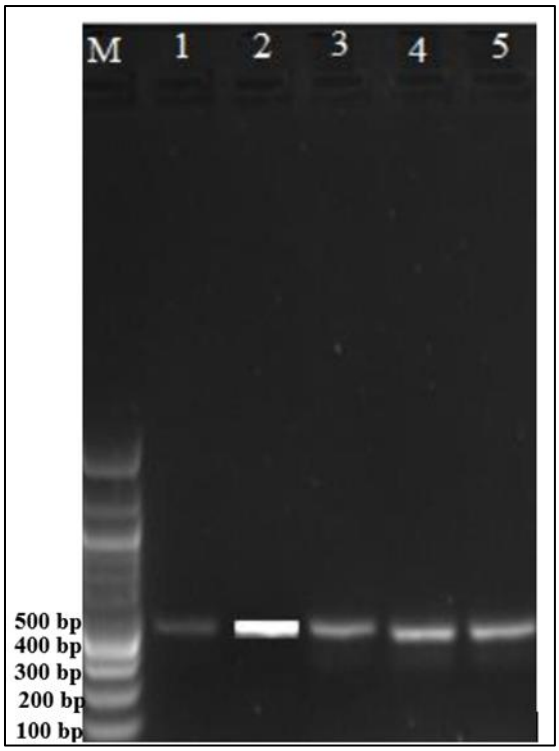

Figure 1: CYP1B1 gene amplicons using 100 base pair DNA ladder

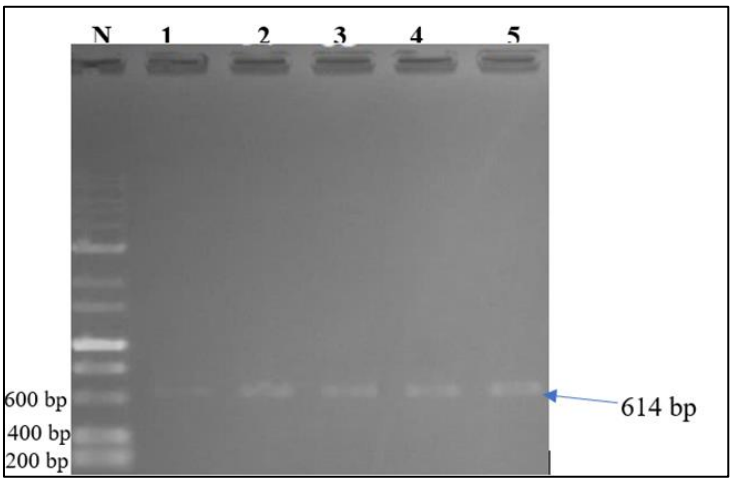

Figure 2: Electropherogram showing MYOC gene amplicons of 614 base pair using 200 base pair DNA ladder

Table 2: Prevalence of CYP_1B_1 gene mutation among glaucoma cases

Cases	Mutations in CYP_1B_1 gene	No mutation in CYP_1B_1 gene	Total	Prevalence (%)
PCG (N= 14)	8	6	14	57.1
JOAG (N= 10)	4	6	10	40.00
POAG (N= 8)	3	5	8	37.5
Total	15	17	32	46.9

Table 3: Prevalence of MYOC gene mutation among glaucoma patients

Cases	Mutations in MYOC gene	No mutation in MYOC gene	Total	Prevalence (%)
PCG (N= 14)	3	11	14	21.4
JOAG (N= 10)	6	4	10	60.0
POAG (N= 8)	5	3	8	62.5
Total	14	18	32	43.8

Table 4: Mutation variants of CYP_1B_1 and MYOC genes sequences in glaucoma cases

S/N	SNPs mutations	Amino acid	Non Syn/Syn
	CYP_1B_1 gene		
1	g.291G>C	p. His97Glu	Non synonymous
2	g.219G>C	p. His73Glu	Non synonymous
3	g.344C>T	p. Thro115Met	Non synonymous
4	g.344G>C	p. Ala115Thro	Non synonymous
5	g.182G>A	p. Gly61Glu	Non synonymous
6	g.130G>A	p. Ala389Thro	Non synonymous
7	g.46T>C	p. Glu15Pro	Non synonymous
8	g.317delT	-	Non synonymous
9	g.535delG	-	Non synonymous
10	g.378-380delATG	-	Non synonymous
11	g.370-380delGCAGAGTGATG	-	Non synonymous
	MYOC gene		
1	g.299T>G	p. Gly102Arg	Non synonymous
2	g.595A>C	p. Gln202His	Non synonymous
3	g.595A>C	p. Gln202Pro	Non synonymous
4	g.595A>C	p. Gln202Asp	Non synonymous
5	g.595A>C	p. Gln202Val	Non synonymous
6	g.513G>T	p. Gln171Pro	Non synonymous
7	g.341-347delTATACTG	-	Non synonymous

Key: Syn - Synonymous; Non Syn - None Synonymous; SNPs – Single nucleotide polymorphism

[18], 19% [19], 48.2% in Cairo [20]; both in Egyptian PCG patients and 0.42% - 28.13% in reviewed studies across different populations using primary glaucoma cases [21]. Also, documented frequency of CYP_1B_1 gene mutation was 44% in Pakistani PCG family [22], 78% among glaucomatous individuals in Iraq [23] and 80% and100% in Saudi Arabians and Slovakian Rom populations, respectively [24]. Patients with CYP1B1 pathogenic variants had a poorer outcome than those without the gene mutation [4] and similar findings were observed among glaucoma patients in this study. Reasons for differences in the prevalence of gene mutations among glaucoma patients can be attributed to differences in sample size, variations in risk factors (family history, genes, environmental factors, anatomical defects, age and gender of subjects enrolled in various study. These reasons were documented in previous researches [2-4, 8, 16-17-19, 24]; and been in tandem with this current study.

The human CYP_1B_1 gene encodes a cytochrome p450 monooxygenase enzyme, and several variants have been associated with primary congenital glaucoma, juvenile open-angle glaucoma and primary open-angle glaucoma and

other eye diseases [2, 16, 20, 25]; and in this study gene variants where detected among PCG, JOAG and POAG cases. The CYP_1B_1 gene mutation can result to rise in intraocular pressure (IOP), which can cause anatomical damage to the optic nerve if not treated medically and functional damage to the visual field [1, 8, 16, 19]. In this study, CYP_1B_1 gene mutation variants (missense mutations) were detected; like g.182G>A (p. Gly61Glu) and g.130G>A (p. Ala389Thro). In Beijing, China [2], Egyptian population [19] and in meta-analysis involving different countries (like Iran, Portugal, Saudi Arabia, Brazil and Vietnam) [26], the G61E (p. Gly61Glu) variant of CYP_1B_1 gene mutations were detected among PCG patients and the same missense mutation [p. Gly61Glu (g.182G>A)] were also found among glaucoma patients (PCG, JOAG and POAG) in our current study. Also, G>A missense mutations were observed among PCG patients in India at a different nucleotide position (g. 4793G>A: p. Ala330Thro) [27] but in our study; the CYP_1B_1 mutation was g.130G>A (p. Ala 389 Thro). Other G>A missense mutations of CYP_1B_1 gene were documented in Iraq (c.182G>A, c.685G>A, g.6813G>A, and g.6705G>A) [23], consanguineous Pakistani families (c.1314G>A, c.732G>A: p. Met244Ile) [22], India (c.1169G>A, [p. R390H]) [4], among PCG cases. In this current study, other CYP_1B_1 gene mutation variants were g.291G>C (p. His97Glu), g.219G>C (p. His73Glu), g.344G>C (p. Ala115Thro), g.344C>T (p. Ala115Val) and g.46T>C (p. Glu 15 Pro). In this study, we observed CYP_1B_1 gene mutation variant of g.291G>C (p. His97Glu); a non-synonymous missense mutation among glaucoma cases (PCG, JOAG and POAG). The same nonsynonymous missense mutation was reported among open-angle glaucoma patient [3], PCG cases [17] and in primary childhood glaucoma cases [18]; all in Nigeria. In Spanish POAG, the QI44H of CYP_1B_1 gene mutation variant was detected [28] but, in our study the Q73H (p. His73Glu: g.219G>C) was observed among glaucoma patients.

The non-synonymous missense mutation of CYP_1B_1 gene mutation namely g.344C>T (p. T115M) was detected among glaucoma patient (PCG, JOAG and POAG) in this current study. The same non-synonymous missense mutation [g.344C>T (p. T115M)] was detected among open-angle glaucoma patient in Nigeria [3,18], among PCG cases in Calabar [17]. In France, g.4547C>T and g.8167C>T missense mutations were detected [29] and the g.55C>T (p. Q19X) non-synonymous missense mutations in Korea with respect to CYP_1B_1 gene mutations among primary congenital glaucoma [30] and were similar to our current findings in Nigerian populations but at different nucleotide site.

In this present study, the CYP_1B_1 gene deletion was detected namely: g.535delG. The same g.535delG mutation was reported among primary childhood glaucoma cases in Nigeria [18], Tunisian individuals [31], Brazilians [32], Moroccans [33], Portuguese children [34], among open-angle glaucoma patient [3], PCG cases [17] in Calabar; therefore, been in harmony with our current findings. The 4339delG of CYP_1B_1 gene was detected in PCG patients in Cairo, Egypt [20], Moroccans PCG cases [35] but in our study, the deletion of nucleotide T occurred at position 535. Additionally, complete nucleotides deletions of CYP_1B_1 gene mutant variants were detected among PCG patients in Brazil and United States of America [36], India [27], Pakistani families [22] and in Maghreb, Morocco [35]. The following CYP_1B_1 gene deletions: g.317delT, g.378-380delATG and g.370-380delGCAGAGTGATG were also detected among glaucoma patients in this study and similar mutations were previously reported among open-angle glaucoma patient [3,18], PCG cases [17] in Nigeria; therefore, been in tandem with our current findings.

The overall frequency of 43.8% (14/32) for MYOC gene mutations was recorded in this study. The prevalence of 87.5% was reported among glaucoma individuals in China [37] and 71.4% in six generations of a Chinese POAG family [38]; which are high when compared with 43.8%; not similar to our current research. The MYOC gene frequency of 36.4% was documented among Chinese family having glaucoma [38], 33% in a Japanese population [39], 30.23% (13/43) in Indian population among PCG [4], 20.2% in Portugal among POAG [40]. 19% among in Egyptian patients with PCG [19], 14.5% in Zambia [41]. A lower frequency of 8.4% was previously reported among POAG patients in Rivers State Nigeria [8], 7.5% in north Indians JOAG population [42], 6.7% of 45 JOAG probands in Ibadan, Nigeria [1] and 1.4% in African-Americans POAG patients [43].

The mutation of MYOC gene may lead to expression of a mutant MYOC protein; causing lost wild type function and may deploy a dominant negative regulation over the rest of wild type that regulate IOP in the eye [44]. The overlaps between glaucoma-causing variant and neutral polymorphisms are the residue positions where the mutated amino acids are different [44]. The alteration resulted to increased intraocular pressure at pathological rates, which after some time causes anatomical damage to the optic nerve and functional damage to the visual field [19, 40, 44]. In this study, g.299T>G (Glycine 102 Arginine) missense mutations were detected among subjects investigated and the same T>G missense mutations were also found among POAG patients in Zambia at a different nucleotide position; yielding Leucine 158 Arginine (g.39T>G) [41]. In Egyptian population, p. Gln48His was observed among PCG patients [19] but in our current study in Nigerian populations, the p. Gln48His (g.595A>C) mutant variants were detected among glaucoma patients (PCG, JOAG and POAG). The genetic diversity in African population is more than anywhere else on earth, making it the most likely environment for establishing novel MYOC mutations, especially in sub-Saharan Africa populations [13]. Studies conducted in Ghana [45], Zambia [41] and South Africa [13] documented novel glaucoma causing mutations different from those found in other populations [37-40]. In this study, novel missense mutations were observed in five glaucoma cases and were absent in all controls.

5 CONCLUSION

Glaucoma patients in Nigerian populations harbors CYP_1B_1 and MYOC genes mutations; and some were novel missense mutations. This study has established the contribution of CYP_1B_1 and MYOC genes mutations in the molecular etiology of PCG, JOAG and POAG in Nigerian populations; a Sub-Saharan population. The information can also serve as a basis upon which future works can be developed in genetic screening in Nigeria and identify novel mutations. The presence of genetically associated PCG, JOAG and POAG cases in the study population; suggest the presence of public health challenge in Nigeria. There is need to examine the possible occurrence of other new glaucoma genes and mutations as well as investigating environmental factors and gene-gene interactions that may contribute to the disease molecular etiology in Nigerian populations.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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