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Molecular Genetics of Gucy2d Gene among Patients with Leber Congenital Amaurosis Attending National Eye Center Kaduna

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Leber congenital amaurosis (LCA) is a clinically and genetically heterogeneous disease that presents with the autosomal recessive pattern of inheritance. There are about 19 genes responsible for the pathogenic cause of the disease, with mutations in a few of the genes recorded. The study aims to identify the GUCy2d gene in patients with Leber Congenital Amaurosis attending the National Eye Center Kaduna, Nigeria. In this purposive sampling method made up of patients of all ages and sexes diagnosed with Leber congenital amaurosis or retinal dystrophy, DNA was extracted from the whole blood according to the manufacturer's guide (Bioneer USA). DNA was PCR-multiplexed by using primers designed as microsatellite markers to identify the GUCY2D gene, gel electrophoresed and sequenced to identify mutations. Multiple sequence alignment was carried out on the DNA sequence which was translated to protein sequence using MegaX software. The PCR products' band sizes correlated with the amplicon size of the genetic marker. After sequencing the Gucy2d gene, it was aligned with the reference gene obtained from the gene bank and mutations were identified in some positions. Gucy2d gene harbouring mutations were identified in the diseased patients, which resulted in the change in the translation of some of the Amino acid sequences.

Keywords: Heterogenous disease; autosomal recessive pattern of inheritance; Gucy2d gene; mutations.

1. INTRODUCTION

"Leber congenital amaurosis is the family of congenital retinal dystrophy that results in severe vision loss at an early age" [1]. "Retinitis pigmentosa (RP) is the generic name for a group of hereditary disorders characterized by progressive loss of photoreceptors and retinal pigmentary epithelium function [2]. "The estimated birth prevalence of LCA is two to three per 100,000 births" [3] "The condition is the most common cause of inherited blindness in childhood and constitutes more than 5% of all retinal dystrophies, LCA accounts for blindness in more than 20% of children attending schools for the blind[4]. "In addition, blindness does come with its share of challenges, considering most of the world is designed for sighted people. As a result, living with limited vision means that blind people have to overcome things that most people take for granted. Leber congenital amaurosis accounts for 3.5% of childhood blindness in the developed world" [5] "LCA is more common in cultures with frequent consanguineous marriages" [6]. "Leber congenital amaurosis represents the most severe end of the spectrum of early-onset retinal dystrophies. Some genes have been described as causing an early onset retinal dystrophy with a severely reduced or absent electroretinogram measured early with a clinical phenotype of milder nystagmus, normal pupil reactions and better vision" [7]. "Early severe retinal dystrophies presented in the first three months of infancy or before 5 years old are being described as early-onset severe retinal dystrophy (EOSRD or EORD). The genes responsible for the pathogenic cause of LCA are concerned with different divers' functions of the retina The pathophysiology of LCA is related to the inability of the eye to undergo photo transduction due to a disruption of the visual cycle. A dysfunctional mutation of any of the genes encoding for proteins that catalyze any of the series of enzymatic reactions to generate 11-cis retinal can block the visual cycle and lead to symptoms of LCA" [8]. "GUCY2D is the first gene identified as causative in LCA" [3]. "The prevalence of mutations of GUCY2D in LCA is 6-20.3%" [9-10]. "The GUCY2D gene is involved in phototransduction by coding for a protein that directly catalysis the synthesis of cyclic guanosine monophosphate (cGMP), retinal guanylate cyclase-2D or RetGC1, this recycling of cGMP allows recovery of the dark state after phototransduction cascade in rod and cone outer segments initiated when a photon (hv) strikes the visual pigment, rhodopsin in rods, and promotes it into an active state" [11]. "Guanylate cyclise 2D (GUCY2D) encodes 1103 amino acid (120kD) membrane guanylate cyclase, RetGC-1, an enzyme involved in the re-synthesis of cyclic guanosine monophosphate (cGMP), from guanosine triphosphate (GTP), Mutations in the gene lead to permanent closure of the cGMP gated cation channels with resultant hyperpolarisation of the plasma membrane" [9]. The study aimed to identify the molecular basis of Leber Congenital Amaurosis. The objective of the study was to carry out the genetic and sequencing analysis of some of the LCA genes.

2. MATERIALS AND METHODS

This Retrospective study was carried out on patients diagnosed with Leber congenital amaurosis of National eye center Kaduna. A total of 71(both males and females) subjects were interviewed and 15(both males and females) were subjected to molecular analysis.

Study design: Retrospective study.

Study Location: The National Eye Center (NEC) is located in Kaduna state in the north-central part of Nigeria. The state is geographically located at 10˚20'N and 7˚45'E covering a land mass of about 17,781 square miles [12]. The mandated of NEC Kaduna aims to provide eye health and prevent blindness through programs utilizing methodologies of public health, community medicine and ophthalmology.

Study Duration: May 2019- December 2020.

Study population: The Sample population was made up of patients of all ages and sexes diagnosed with Leber Congenital Amaurosis and or Retinal Dystrophy at the National Eye Centre Kaduna between (2016-and 2019).

Data collection: Medical records of patients diagnosed between 2016 and 2019 were examined and their contact information was extracted with the assistance of medical record officers. Families of patients were contacted through extracted contact information in their medical records.

Inclusive and Exclusive criterion: The inclusive criterion for this study was all the patients diagnosed with LCA and RD, willing to participate in the study but only patients diagnosed with LCA were exclusively included in the molecular analysis study due to study specific variables and most importantly the ability and willingness to participate in the study, The retrospective part of the study include both RD and LCA Patients because congenital RD is the family name of LCA and are often misdiagnosed. And some exclusion criterion was that Some case folders had incomplete contact information for the diagnosed subjects with the disease; hence they were excluded from the study, and some uncontrolled variables like some patients fitness level.

Collection of Blood Samples: Five millliters (5mL) of venous blood were collected separately from both normal (parents with no symptoms) and patients diagnosed with retinal dystrophies and or Leber congenital amaurosis and optic nerve damage using an EDTA vacutainer set. Blood samples collected were stored in a thermo flask and immediately transported to Biotechnology laboratory Nigerian Defence Academy (NDA) and stored at -4˚C until analysed.

DNA Extraction. Extraction of DNA was carried out according to manufacturer's guide [13]. The collection and binding column tubes were well labeled with a marker from 1-11 with blood of patients diagnosed with LCA for the affected individuals and 11-15 of the binding column tubes for non-diagnosed patients (Parents) before starting the reaction mixture.

Multiplex polymerase chain- reaction: The method described by Abid and Kamal (2015) was adopted for the M-PCR [14]. The M-PCR was carried out by mixing 2µL of DNA sample, and 2µL of each of the forward and reverse 0primer (2×5 Fw and Rv primer of the five genes (Guanylate Cyclase 2D gnee, Retinal Pigmentary Epithelium 65, Centrosomal protein 290, Aryl hydrocarbon interacting like protein 1, Crumbs homologue 1 gene, making 10µl each), and 10µL of Negative control which does not contain all the FW and RV (Table 1.0) primers and Amplicon sizes.

Agarose Gel-Electrophoresis of PCR Products: The agarose gel was prepared by dissolving 1.5% agarose powder in 750ml of deionized water and acetic acid and microwaved for 2 minutes before adjusting to 1L mark with deionized water.
The molten agarose was allowed to cool before staining with 8µL of ethidium bromide, and a comb was inserted into the slots of the casting tray. The molten agarose was poured into the tray and allowed to solidify for 20 minutes, a buffer was poured into the barely submerged gel tank and 2µL of 10x bromophenol blue gel loading dyes, and then 10µL of each PCR product were loaded into the wells using a micro-pipette. 2000bp DNA ladder [13].The Gel was electrophoresed at 120V for 1 hour 30 minutes and bands on the gels were visualized using ultraviolet trans-illumination and photographed using gel documentation system.

Sequencing of gel electrophoresed product

***Dye terminator cycle sequencing with quick start kit*:** This method was carried out with a DTCS kit according to the manufacturer's guide [13].

***Ethanol precipitation for the sequencing reaction*:** All the sterile sample tubes were labeled and the stop solution glycogen mixtures were prepared. The suspended sample was loaded into the appropriate wells of the sample plate. Each of the suspended samples was overlaid with a drop of mineral oil from the kit. Loaded into the sequencing instrument and sequenced

Mutation analysis: The resultant sequence where aligned with MegaX software to determine the point of mutation by comparing the Sequence analysis with reference sequence from Gen-bank to determine the point of mutation. And the translated the protein sequence to determine the effect on the amino acid sequence**.**

3. RESULTS

3.1 Result of Sequence Analysis

Result of Sequence analysis: Chromatograms from the affected individuals were compared with the corresponding control gene sequence from Ensemble Genome Browser database [15]. Sequence variants were identified via BIOEDIT sequence alignment editor version 6.0.7.

The gene alignment search tool started comparison from position 9674 and ended at 9925. About 35.3% were all transversional mutation and in turn changed cytosine to guanine and transitional mutation of about 17.6 % of all the mutated sequence which resulted in substitution of cytosine to thymine and thymine to cytosine respectively. And there was also deletion of Guanine, Cytosine and thymine making about 47.1% of all the mutated sequence.

Table 1. Primer sequence for the genetic analysis

Gene amplicon		Primer sequence 5'-3'	Amplicon size
GUCY2D	GUCY2DFw	Cctagagcctctctgggc	703bp
	GUCY2DRv	Ggggtagaaatcaggcttcc	703bp

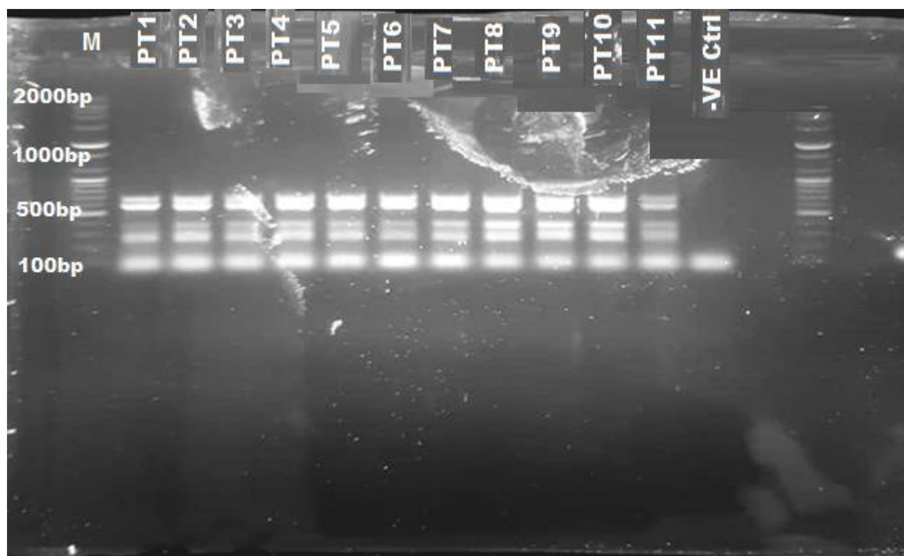

Plate 1. Gel electrophoresis documented result

Key; PT1-PT11 Electrophoresed result of patients diagnosed with LCA, M-Molecular ladder, -VE Ctrl= negative control
GUCY2D gene between 500bp and 1000bp about 703bp as band size

Table 2. Sequencing result for GUCY2D Gene

Position	Type of mutation	Wild type gene	Mutated gene sequence	Type of substitution
9674	Substitution	C	G	Transversion
9686	Substitution	C	G	Transversion
9688	Substitution	C	G	Transversion
9695	Substitution	C	G	Transversion
9699	Substitution	C	G	Transversion
9709	Deletion	C	-	-
9710	Deletion	T	-	-
9744	Deletion	C	-	-
9764	Substitution	C	G	Transversion
9765	Deletion	T	-	-
9854	Deletion	C	-	-
9869	Deletion	G	-	-
9890	Deletion	G	-	-
9891	Deletion	T	-	-
9892	Substitution	C	T	Transition
9893	Substitution	T	C	Transition
9895	Substitution	-	T	Transition

Key: C=cytosine, G=Guanine T=Thymine

3.2 Homosapiens Guanylate Cyclase Retinal Protein 2D (GUCY2D)

703 GUCY2D Homosapiens guanylate cyclase retinal
GGCCCAGGGAAGGAACCAAATTTACGGAAAA
AGAAACTGGCTCCTGCCTCT
CTGCTCCCCCACCTTTCAGAGGTGCCCCCAT
GTGGGTGGAGAAAGGTGATG
TCGTCCACGGTCAGGATGATCTTGTTGGGGC
CGGAGACCATTTGCATGTGA
AGTCCCGGTGCCTGGTGGAGAAAAGGTCAC
CTCCACCAAGGGCACCAAGTT
CCGTCAAGAGACCACCAGCGGATGCCGCGG
AGGGATAAC

Table 3. Translated protein sequence of Gucy2d gene

cDNA	Translated protein position	Mutation on protein sequence
c9674C>G c9686C>G	3225	T>? P>R R>G L>W L>?
c9688C>G c9695C>G		L>?
c9699C>G c9709C>G	3229	
c9709delC c9710delT		Y>?
c9744delC c9764delC>G	3230	
c9765delT c9854delC		L>?
c9869delG	3233	
		R>?
	3237	
		L>?
	3249	S>? S>F P>?
	3256	
	3285	
	3290	
	3297	
	3298	
	3299	
	3209	

In comparison with reference sequence: NG_009092 Homo sapiens guanylate cyclase 2D, retinal (GUCY2D), RefseqGene on chromosome 17 1-2467 nucleotide). There was the substitution of cytosine(C) to guanine(G) at positions 9674,9686,9688,9695,9699,9764. And also transitional mutation of cytosine to thymine(T) at positions 9892 and 9895 with substitution at 9893 of thymine to cytosine. Deletions on positions 9709,9710,9744,9765, 9854,9869,9890 and 9891 deletions of C,T,C,T,C,G,G and T respectively.

The table shows the translated protein sequence of the Gucy2d gene and its codons. It starts at position 3225 and ends at position 3309 in relation to the codons from position 9674-9895. The translated protein are termed as follows: ?=Signifies unknown protein sequence P=Proline, R=Arginine, C=Cysteine, L=Leucine, Y=Tyrosine, S=Serine, F=Phenylealanine, W=T ryptophan, G=glycine. 80% were translated to an unknown sequence noted as ? on the mega x software.

4. DISCUSSION AND CONCLUSION

4.1 Discussion

In this research, we identified a LCA-1 substitution (Missense). A potentially pathogenic mutation in GUCY2D was detected in a patient child utilizing Agarose gel electrophoresis and Sanger sequencing technique. This is the first research to be mentioned on the GUCY2D mutation in NEC, Nigeria. GUCY2D is one of the most prevalent gene related to LCA/EOSRD pathogenesis [16] and is one of the most famous cases, accounting for about 10% – 20% of cases [17] Guanylate Cyclase 2D, mutations are liable for autosomal recessive LCA-1 retinal degeneration & autosomal dominant/recessive cone dystrophy - 6 disease [18]. The GC1 protein encoded by GUCY2D, is one of the fundamental enzymes in the phototransduction cascade and is situated in the disc membranes of either the outer segments of the rod and cone photoreceptors, that regulates GMP and Ca2+ level in these cells [19,20].

Rwandzy et al. extricated the Genomic DNA from peripheral blood lymphocytes using a standard protocol to detect and found mutations using single nucleotide polymorphisms (SNPs) in particular genes associated with LCA disease and has detected a homozygous substitution (Missense) mutation (NM_000180.3:c.2944G>A) of the GUCY2D gene, situated on the chromosome 17p13.1 cytogenetic band p.(Gly982Ser) which is located in exon number 16 which causes variation in amino acid from Glycine to Serine at position 982 [21]. Homosapiens Guanylate Cyclase 2D refseqgene on chromosome 17 with accession number NG_009092 and 1-24671 nucleotides, the sequence from our result was translated into protein sequence from position 3225-3309 and 80% of the sequence where unknown and at postion 3229 proline was changed to Argine (p3229r)of the regions where unkown this was as a result of missence mutation when the change of the single base pair caused substitution of the Amino acid and thus rendered the protein non-functional which relates to the findings of Perrault et al. [3] that found homozygosity for a G-to-T transversion at nucleotide 227 of GUC2D converting an alanine into a serine (A52S) and also found 2 missense mutations and 2 frameshift mutations associated with LCA1 and with the findings of Yzer et al. (2006) he identified homozygosity for a c.2302C-T transition in exon 12 of the GUCY2D gene [22].

4.2 Conclusion

There was both tranversional and transitional substution and deletion. After the translation of DNA sequence to protein it was found that there was a missense mutation that rendered the protein non-functional. We conclude that the Gucy2d genes is present and mutations in Gucy2d were found in patients diagnosed with leber congenital amaurosis, The molecular modeling of the mutated protein shows compositional alter in the protein that could influence the correct phosphoregulation of the enzyme and could therefore hinder its work. To conclude of the LCA may have been due to known potentially pathogenic mutations in GUCY2D. This research is one of the first to report molecular findings of LCA with patients with GUCY2D mutations. The method used in this study is very definitive and readily available.

ETHICAL APPROVAL

The ethical clearance for the study was obtained from the Ethical and Human Research Committee of the Kaduna State Ministry of Health and the Medical Advisory Committee of the National Eye Centre, Kaduna.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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