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A NEW GJB2 GENE MUTATION THAT CAUSES NON-SYNDROMIC SENSORINEURAL HEARING LOSS DETECTED IN A NIGERIAN POPULATION.

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ABSTRACT
Mutations in the GJB2 gene at the DFNB1 locus on chromosome 13q12 are associated with autosomal recessive non-syndromic sensorineural hearing loss (SNHL). R143W mutation which is the common mutation in Ghana is widely reported in some journals as the commonest mutation among black Africans. This study examined mutation in GJB2 gene known to be responsible for non-syndromic sensorineural hearing loss and its pattern in our environment using molecular techniques. Data on the age, sex, age at onset of hearing loss, number of affected ears and family history of the patients were obtained through a structured questionnaire. The frequency and severity of hearing loss was obtained from the pure tone audiometry. Deoxyribonucleic acid (DNA) was isolated from the blood of patients using standard procedures and molecularly evaluated for the presence of mutation in the GJB2 gene. Sequencing was performed in samples whose single strand conformation polymorphism (SSCP) analysis showed a different banding pattern. A novel mutation, P 32 L which caused non-syndromic SNHL, was discovered from this study. A total of 4 probands out of 86 had mutations (4.7%) All the mutations were congenital probands which had severe to profound non-syndromic SNHL.The results of the study demonstrate that mutations in the GJB2 gene are a major cause of non-syndromic SNHL in the studied population. The importance of molecular tests for genetic counselling is re-enforced by this study.
Keywords: GJB2, Connexin 26, Autosomal recessive non syndromic hearing loss, Deafness, Mutation

1 INTRODUCTION.
Sensorineural hearing loss (SNHL) is one of the commonest congenital sensory impairments in humans (Dalzell et al., 2000). About one child in a thousand is born with hearing impairment significant enough to compromise the development of normal speech and language skills (Kenna *et al.,* 2010). Hearing loss can be classified as conductive,
sensorineural or mixed. Conductive hearing loss is caused by abnormalities of the external ear and the middle ear. SNHL occurs when there is damage to the inner ear (cochlea) or the nerve pathways from the inner ear to the brain and the auditory cortex of the brain (Lang *et al.,* 2007). Mixed hearing loss involves a combination of conductive

and sensorineural factors. Hair cells are the most vulnerable elements in the cochlea, and damage to them is the most common cause of SNHL (Hawkins and Lovett, 2004). The diagnosis of SNHL is based on the demonstration of reduced hearing acuity by auditory testing. Hearing is measured in decibels (dB) with the threshold of 0 dB for each frequency denoting the value at which normal young adults perceive a tone burst of a given intensity and frequency, 50% of the time (Kemperman *et al.,* 2002). A person's hearing acuity is classified as normal if it is less than or equal to 20 dB. Severity of hearing loss is graded as mild (21–40 dB), moderate (41–55 dB), moderately severe (56–70 dB), severe (71–90 dB), or profound (>90 dB).

SNHL can be congenital or acquired (Lim *et al.*, 2003). The congenital causes can be genetic or non genetic. It is estimated that 50% to 75% of all childhood hearing loss is due to hereditary causes. There are two main forms of genetic SNHL (hereditary hearing loss), namely, syndromic and non-syndromic (Noben-Trauth *et al*., 2003). Patients with syndromic SNHL have other clinical features in addition to the hearing loss. About 15-30% of genetic hearing loss is syndromic, while the majority (70%) is non-syndromic.

Genetic SNHL, syndromic or non-syndromic, can be transmitted in several inheritance patterns, including autosomal dominant, autosomal recessive, X-linked recessive and mitochondrial inheritance (Bitner-Glindzicz, 2002).

Undiagnosed SNHL and diagnostic delay have a profound impact on linguistic and communicative competence, as well as cognitive and psychosocial development of the individual. The overall aim of this study was to identify the kind of mutations that lead to non-syndromic SNHL in the studied population. SNHL affects language and speech development especially in neonates. Genetic counselling therefore re-enforces the need to identify the kind of mutation responsible for non-syndromic SNHL in Nigerians. Knowing the genetic cause of a person's hearing loss can lead to improved decision about its management. Genetic information can help predict whether the hearing loss will remain permanent or whether it will worsen over time. Knowledge of the genetic cause is also helpful in determining what kind of damage to the hearing system has led to the deafness. R 143 W mutation which is a common mutation in Ghana (Brobby *et al*., 1998) is widely reported in some journals as the commonest mutation among black Africans. This study therefore, would either confirm this assertion or dispute it.

2Materials and Methods

2.1Preliminary screening

Outpatients attending clinic at Ear, Nose and Throat (ENT) unit of Lagos University Teaching Hospital were evaluated for non-syndromic SNHL using tuning fork. The air and bone conduction threshold were evaluated. Questionnaire was administered on the patients. On the basis of this examination and other physical examinations, patients were either recruited or excluded. Patients were further referred to laboratories for full basic audiological evaluation. The work was reviewed by the Research Grants and Experimentation Ethics Committee of College of Medicine, University of Lagos.

2.2 DNA extraction.

DNA extraction was carried out using the protocol of Iranpur and Esmailizadeh, (2010). For the analysis of GJB2 gene, the following primers which had been used in literature (Heinz *et al*., 2001), were used to amplify the coding region of GJB2 gene.

Forward primer 5^{1}-TTC TGTCTTCACCTGTTTTG-3^{1}

Reverse primer 5^{1}-GGTCAG AATCTT TGTGTTGG-3^{1}

PCR reaction was performed in a final volume of 25 µl reaction mixture containing 2 µl genomic DNA, 2.0 µl $MgCl_2$, 0.5 µl dNTPs, 0.2 µl of each primer, 0.125 µl of *Taq* polymerase (Promega- USA), 2.5 µl of buffer and 17.47 µl of water. The thermal cycler was programmed according to the following steps to undergo the amplification reaction for GJB2 gene coding region. A complete cycle was achieved by denaturation at 94 °C for 15 seconds followed by annealing at 55.5°C for 30 seconds and extension was done at 72 °C for 1 minute, followed by 5 minutes of post extension. This was repeated 40 times.

2.3 Detection and Visualization of Amplified PCR Products.

PCR products were loaded to a 1.5 % agarose gel. The desired band of the coding region of GJB2 gene was visualized using Ethidium Bromide florescence under ultraviolet light. Band size was determined by loading DNA marker Qx 174 DNA/*Hae* III to one lane with PCR products.

2.4 Single strand conformation polymorphism (SSCP) analysis

PCR product was first digested with DraII restriction enzyme. PCR product was diluted fourfold according to manufacturer's recommendation and master mix was prepared as follows: using sterile Eppendorf tubes 10 µl of PCR product, 18 µl of deionized water, 2 µl of buffer, and 2 µl of restriction enzyme were digested for 8 hours at 37^0 C. Enzyme activity was inactivated at 65^oC for 20 minutes.

Ten microlitre of digested PCR product and 10 µl of gel loading dye were put into sterile eppendorf tubes and denatured at 94^0C for 4 minutes. Samples were snap cooled on ice to prevent renaturing and kept on ice until use. Forty percent polyacrylamide was prepared by dissolving acrylamide and bis acrylamide in ratio 39:1 in 100 ml of deionized water. Ten percent casting polyacrylamide gel (10 ml 40% acrylamide/bis, 4 ml 10X TBE, 26 ml H_2O, 40 µl TEMED and 400 µl 10% ammoniumpersulphate), was prepared and immediately poured into the glass plates before polymerization starts and combs were inserted to create wells. Denatured samples were loaded into the wells and electrophoresis was carried out in a Bio Rad Protean II xi vertical electrophoresis unit using 1x TBE buffer at 60 Watts constant power for about 8 hours. Staining of gel was done using silver stain kit according to manufacturer's guide line. Samples that showed different SSCP banding pattern were selected for sequencing.

3 Results.

A total of 216 subjects (150 patients and 66 controls) were enrolled in this study. Control individuals were people free from non-syndromic SNHL. However, some probands were dropped because the individuals did not carry out the prescribed tests to confirm that they had sensorineural hearing loss. Parental consanguinity was absent in all studied families.

Hearing loss was without any other accompanying clinical features. Age of the patients varied from two to fifty years (Table 1). Eighty nine patients (59.3 %) were males while 61 patients (40.7 %) were females (Table 2) The level of formal education in the studied population differed greatly; sixty two patients (41.3 %) had no education at all, 43 (28.7 %) had secondary education, 24 (16 %) had tertiary education while 21 (14 %) had primary education (Table 3). Fifty four patients (53 %) had high frequency SNHL, 30 (29.4 %) had middle frequency SNHL while 18 (17.6 %) had low frequency SNHL (Table 4). Among the 102 patients that did audiological evaluations, 43 patients (42.1 %) had moderately severe SNHL, 28 (27.4 %) had severe SNHL, 24 (23.5 %) had profound SNHL, 7 (6.8 %) had moderate SNHL while none had mild SNHL (Table5).

Table 1. Age of patients

age(years)				
0-9	10-19	20-29	30-39	40-above
54	16	13	29	38
36%	10.7%	8.7%	19.3%	25.3%

Table 2. Sex of patients

sex	
male	female
89	61
59.3%	40.7%

Table 3. Level of education

level of education			
none	primary edu	secondary edu	tertiary edu
62	21	43	24
41.3%	14%	28.7%	16%

Table 4. Frequency of hearing loss.

frequency of hearing loss		
low	middle	high
18	30	54
17.6%	29.4%	53%

Table 5. Severityof hearing loss.

severity of hearing loss				
mild	moderate	mod. severe	severe	profound
0	7	43	28	24
0%	6.8%	42.1%	27.4%	23.5%

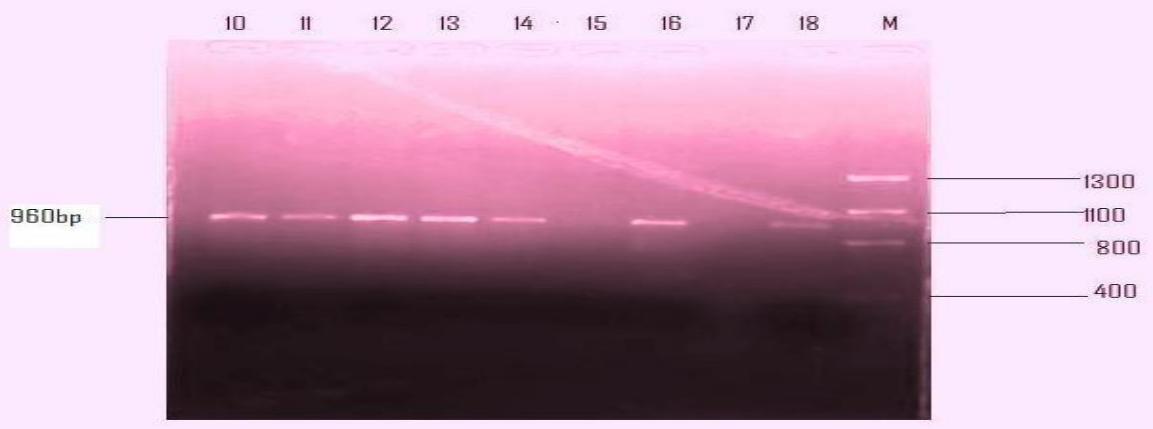

Figure 1. PCR from DNAsamples.

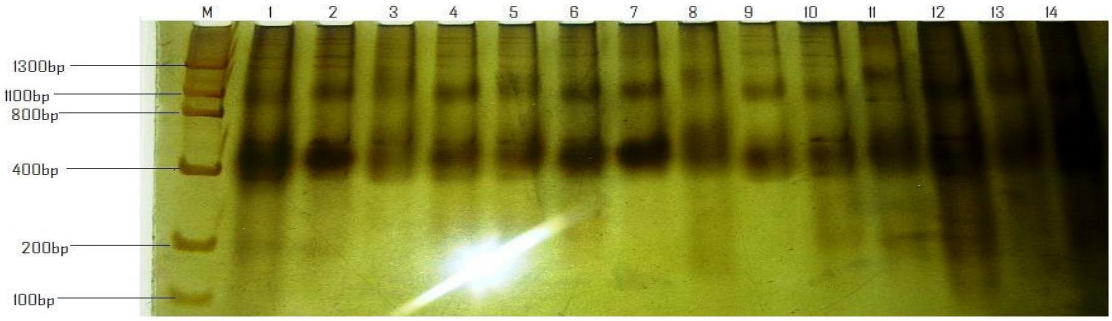

Figure 2. Single strand conformation polymorphism (SSCP) showing band variation in some probands. M is the marker.

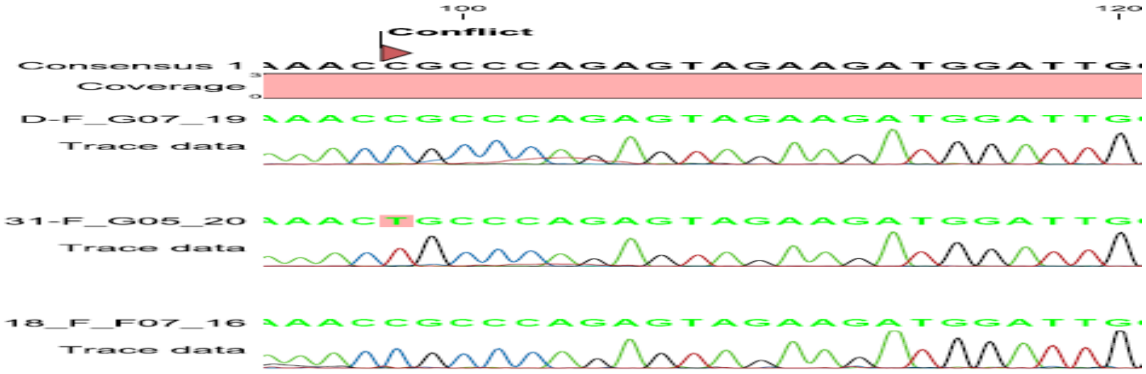

Figure 3. Electropherogram of alignment of probands 18 and 31 against control

Proband	Mutation	Nucleotide change	Age at onset	Degree of hearing loss
17	P32L	C to Tat 98	Congenital	Profound
31	P32L	C to T at 98	Congenital	Profound
54	P32L	C to T at 98	Congenital	Severe
59	P32L	C to T at 98	Congenital	Profound

Table 6. Summary of CX 26 mutations found in the study.

4 Discussion

The main objective of this study was to identify the kind of mutation that could lead to non-syndromic SNHL in a Nigerian population. This was done with a focus on gap junction beta 2 (GJB2) genes. There is dearth of information on the kind of mutation that is responsible for non-syndromic SNHL among black Africans. Most reported studies in Africa were only in North African countries such as Egypt, Tunisia, Morocco and Libya. Mutations in the GJB2 were implicated. However, most of the mutations were 35 del G. The only known published study of this sort was the work of Brobby *et al.,* (1998) in Ghana. This made so many authors to presume that the mutation identified in Ghana is the common mutation among black Africans.

For GJB2 analysis, Single strand conformation polymorphism (SSCP) was performed on the samples that were PCR-amplified. Sample that showed different banding pattern were marked for sequencing. The sequence of each amplicon was confirmed by sequencing in both directions. Alignments and analysis were performed using CLC Main Workbench version 6.7.1. Sequence analysis demonstrated that 4 probands had mutations. This gave a prevalence of 4.7 % (4 out of 86). The contribution of GJB2 gene mutation in this study is lower than western populations; 40 % in U.S.A (Kelley *et al.*, 1998), 49 % in Italy (Estivill *et al.*, 1998), 54 % in Russia (Posukh *et al.*, 2005), 36.6 % in Iran (Hamid *et al.*, 2009), 22 % in Germany (Heinz *et al.*, 2001), 17.7 % in India (Ramshanker *et al.*, 2003). The high frequency of mutation in GJB2 gene in white population possibly is the result of a founder effect rather than a mutational hot spot. However, Cordeiro-Silva *et al.*, (2010) reported a prevalence of 7.8 % in a Brazillian study and Chalestori *et al.*, (2006) had 7.8 % in an Iranian study. The low prevalence in this study could be that some of the hearing-impaired probands had non genetic origin.

Suprisingly the Arg 143 Trp mutation reported in most literature to be the most frequent among black Africans but was only identified in Ghana was not found in this study.

One variants of GJB2 gene sequence (P32L, a missence mutation) was identified in four probands in this study. This is the first time to the best of the knowledge of the author that this mutation was identified.

Given the extraordinary genetic heterogeneity of non-syndromic SNHL, it was believed that no single gene would play a significant role in its etiology. So it was surprising to discover that sequence variations at the GJB2 locus accounts for up to 50% of cases of non-syndromic SNHL in some populations. While more than 90 alleles have been described in the literature, three accounts for the majority of GJB2-related non-syndromic SNHL in studied populations. They include 35 del G commonly found among populations of northern European descents, 167 del T most common among Ashkenazi Jewish population and 235 del C common among Korean and Japanese populations.

5 CONCLUSION

Mutations detected in this study were only found among patients with severe to profound non-syndromic SNHL but not in mild to moderate cases. Additionally, all the mutations were homozygotes. Absence of heterozygosity in this study could be that these mutations were point mutations that only existed in the patient. It could also mean that the common founders were only recent thus it has not permeated the population. However this can only be substantiated when a similar study is done in other regions of the country.

The results of the study demonstrate that mutations in the GJB2 gene are a major cause of non-syndromic SNHL in the studied population. The importance of molecular tests for genetic counselling is re-enforced by this study.

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