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RESEARCH ARTICLE

STUDY OF GJB2 GENE MUTATIONS, REGULATING CONNEXIN 26(CX26) PROTEIN, IN PATIENTS WITH CONGENITAL NON-SYNDROMIC HEARING LOSS

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ABSTRACT

Background: Hearing loss is a common sensory disorder, which affects 1 in 1000 live births. Genetic causes are thought to be responsible for more than 50% of the cases with the majority of non-syndromic hearing loss being inherited in an autosomal recessive pattern. The most common form of Autosomal Recessive Non-syndromic Hearing Loss (ARNSHL) is caused by mutations in the gene GJB2 encoding the protein Connexin 26 (Cx26). Cx26 plays a key role in potassium homeostasis, which is essential for sound transduction. **Objectives:** The aim of this study was to determine the GJB2 gene mutations namely, p.W24X, p.W77X, c.35delG, IVS 1+1G→A and c.235delC in patients with Congenital Non-Syndromic Hearing loss. **Methodology:** This is a cross-sectional study, in which 50 patients were screened for five mutations in GJB2 gene, by ARMS PCR and/or RFLP. **Results:** This study revealed high prevalence of p.W24X mutation (8%) and low prevalence of p.W77X (2%). Other mutations associated with Cx26 gene like c.35delG, c.235delC and IVS 1+1G→A were not observed in our patients. **Conclusion:** We conclude that there is a significant contribution of GJB2 mutations to ARNSHL in this population. Screening for GJB2 mutations particularly, p.W24X and p.W77X should be offered to ARNSHL patients to confirm diagnosis of their congenital deafness, to deliver proper genetic counseling.

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INTRODUCTION

The major causes of hearing loss and ear diseases in India have been listed by WHO survey. Among these ear wax (15.9%) was the most common cause of reversible hearing loss. Non-infectious causes such as aging and presbyacusis (10.3%) and middle ear infections such as CSOM (chronic suppurative otitis media) (5.2%), Acute serous otitis media (3%), dry perforation of tympanic membrane are also responsible for hearing loss. Approximately 50% of all cases of congenital hearing loss are attributable to environmental factors, such as congenital hyperbilirubinemia, ototoxic medication exposure, neonatal hypoxia, viral infections (cytomegalo virus) and meningitis.(1)(2)(3) The other 50% of cases are thought to be inherited, i.e. of genetic causes.

Hereditary causes are classified as syndromic and non-syndromic. Among this 70% of hereditary cases are non-syndromic and remaining 30% cases are syndromic.(3)(4). 70% of hereditary cases are classified as Non-syndromic hearing impairment. This group is not associated with visible abnormalities of the external ear or any related medical problems. As a general rule, cases with autosomal recessive inheritance are typically born with bilateral, profound deafness to normal hearing parents. Therefore it is more difficult to determine whether the etiology is hereditary or acquired.(10)(11)(12) The different gene loci for nonsyndromic deafness are designated as DFN (for DeaFNess). Loci are named on the basis of mode of inheritance as (5) DFNA: Autosomal dominant(22%), DFNB: Autosomal recessive(75%), DFNX: X-linked (2%), Mitochondrial inheritance (<1%).

Approximately 50% of autosomal recessive nonsyndromic hearing loss can be attributed to the disorder DFNB1, caused by mutations in GJB2 (which encodes the protein connexin 26). The carrier rate in the general population for a recessive deafness-causing GJB2 pathogenic variant is approximately one in 33.Due to the high rate of consanguineous marriages, NSHL is a prevalent genetic disorder in India. Screening for mutations in Cx26 gene will be useful to identify the cause of deafness, develop suitable diagnosis and deliver appropriate genetic counseling to NSARD families. Moreover, through seeking pre-implantation genetic diagnosis (PGD), families with known mutations may have the possibility of having children free from those mutations. In India, very few studies have been conducted to find out genetic aetiologies of hereditary hearing impairment. Indian studies have shown that among different mutations of connexin 26, p.W24X is the most prevalent mutation.(6)(7)(9)(11). Whereas two frame-shift mutations, c.35delG and 167delT are the culprit mutations in more than 50% cases of Congenital Non-Syndromic Hearing Loss in the world. However, the incidence of these and other mutations varies significantly in different parts of the world. Another mutations i.e. p.W77X, c.235delG and IVS (1+1)G→A are also reported in India.(6)(8)(9).

This study was designed to perform screening for 5 mutationsp.W24X, c.35delG p.W77X, IVS (1+1)G>A and c.235delC in Cx26, in non-syndromic autosomal recessive deafness cases who were referred from the ENT OPD.

METHODS

This Cross Sectional study was conducted in a tertiary care teaching hospital. Institutional Ethical Committee permission was obtained prior to commencement of the study. Sampling unit for the study is an individual with congenital non-syndromic hearing loss. Prevalence of Hearing Loss (in India) is 6.3%. Of which Non-syndromic hearing loss is 70%.(1) Hence, prevalence of Non-syndromic Hearing Loss in Indian population is 4.41% .The sample size for the practical purpose was taken as 50. Inclusion criteria:– Individuals with congenital bilateral, severe to profound sensorineural deafness without any systemic abnormalities and without any known etiology for deafness were included in the study. The degree deafness was confirmed by Pure Tone Audiometry (PTA) and/or Brain Stem Evoked Response Audiometry (BERA). Exclusion criteria – Individuals who suffered from hearing loss but had history and clinical findings suggestive of conductive deafness, otitis media, trauma or any other associated anomalies and systemic diseases.

Blood Sample Collection: Study details were explained to the subjects. 3 to 5 ml of blood in EDTA bulb was withdrawn from ante-cubital vein of each subject who visited ENT OPD. At the time of blood collection, a detailed history of hearing loss was obtained. A detailed local examination of both the ears was done. Reports of PTA, i.e. Pure Tone Audiometry and BERA, i.e. Brainstem Evoked Response Audiometry were noted. Reports of other important tests were also noted. The nature of hearing loss was confirmed. Before conducting the examination and eliciting history, the individuals were explained in vernacular about the purpose of the study and with their written consent, details were recorded.

Analytical Techniques: To extract DNA from whole blood, we followed 'salting out method' as described by Helms C.(10) PCR amplifies the sequence of DNA in vitro, using the basic elements of the natural DNA synthesis and replication processes. Post-PCR analysis analysis of p.W77X and c.del35G mutations were done simply by agarose gel electrophoresis, whereas the analysis of p.W24X, c.235delC and IVS(1+1)G>A mutations required Restriction Fragment Length Polymorphism (RFLP) of post-PCR product by restriction digestion enzyme, *Alu 1*, *Apa 1* and *Hph 1* respectively. (provided by Thermo SCIENTIFICS).

RESULTS

In the present study, five mutations p.W24X, c.35delG p.W77X, IVS (1+1)G>A and c.235delC of the GJB2 gene were screened in 50 cases of Non-syndromic Hearing Loss. Screening for ofmutations in Cx26 was performed by ARMS-PCR and RFLP. Among the 50 NSHL patients, male to female ratio was 1:1.Average age of study subjects in case group was 6.24 years and the youngest subject being 2 years old while oldest subject was 16 years old. Depending upon the presence or absence of mutations and the type of mutation, a characteristic electrophoresis pattern was observed. Based on these electrophoretic patterns, the frequencies of p.W24X, c.35delG, p.W77X, IVS(1+1)G>A and c.235delC mutations were analyzed. In this study, p.W24X mutation was observed in four cases and p.W77X mutation was identified in only one case.

Table 1. Frequency of mutations W24X, W77X, IVS1+1G>A, 235delC and 35delG in Cx26 in patients with NSHL. (N=50)

	Mutations	No. of cases (out of 50)	Frequency in %
1	W24X	4	8%
2	W77X	1	2.17%
3	IVS1+1G>A	0	0
4	235delC	0	0
5	35delG	0	0

The observed frequency of p.W24X and p.W77X mutations among NSHL cases was 8% and 2% respectively. Other mutations i.e. c.35delG, IVS1+1G>A and c.235delC were not detected in the present study. p.W24X was the most common mutant allele in our study. After restriction analysis for p.W24X mutation, 4 out of 50 cases (8%) were found to be either heterozygous (2%) or homozygous (2%) for this mutation. Heterozygosity indicates a carrier frequency. Carrier frequency of p.W24X mutation in case group was 4%. Alu1 digestion ofDNA derived from unaffected subjects produced a single fragment of 286 bp, p. W24X homozygotes produced two fragments of 182 bp and 104 bp, and p. W24X heterozygotes produced three fragments of286 bp, 182 bp, and 104 bp. The second most prevalent GJB2 mutation p.W77X was detected only in one patient that too in heterozygous form. Heterozygosity indicates a carrier frequency. Carrier frequency of p.W77X mutation in case group was 2%, and unaffected subjects produced a single fragment of 648 bp, W77X heterozygotes produced two fragments of648 bp and 234 bp.

DISCUSSION

GJB2 gene codes for Cx26 which belongs to a family of transmembrane proteins with about 20 members in human(14).

Hexamers of connexins (connexons) are displayed in the plasma membrane. Several different connexins, including Cx26, have been shown to participate in the intercellular gap junction networks of the cochlea. These networks play a key role in potassium homeostasis, which is essential for the sound transduction mechanism(13). Most of the studies conducted in India and other countries of Asia have shown that mutations in GJB2 gene have a significant frequency in congenitally deaf populations. p.W24X was the common mutations identified in Asian countries. Whereas a significant ethnic and geographic variation in Cx 26 mutations across the world has been reported earlier, for example, 35delG mutation is predominant in Caucasian, 167delT in Ashkenazi Jews and c235delC in Japanese(6)(20). In this study, we screened 50 cases of NSHL for five mutations i.e. p.W24X, c.35delG p.W77X, IVS (1+1)G>A and c.235delC of the GJB2 gene. The p.W24X mutation was observed in four cases and p.W77X mutation was identified in only one case. The observed frequency of p.W24X and p.W77X mutations among NSHL cases was 8% and 2% respectively. Other mutations i.e. c.35delG, IVS1+1G>A and c.235delC were not detected in the present study. A G→A transition at nucleotide 71 forms a nonsense mutation at tryptophan 24 (p.W24X). p.W24X is present in the first transmembrane (TM1) domain of Cx26. Incorporation of a stop codon at this position results in the formation of a protein. This mutation was first described in a Pakistani family(14) and later in several Asian families.(12). This mutation was also found to be a common allele among the mutations causing autosomal recessive non-syndromic Hearing Impairment (ARNSHI) in previous studies from Indian population and haplotype analysis of markers flanking the GJB2 gene suggested a possible founder effect for this mutation in Indian population.(6)(8)(11)

The second most prevalent GJB2 mutation p.W77X was detected only in heterozygous form. Heterozygosity indicates a carrier frequency. Carrier frequency of p.W77X mutation in case group was 2.17%. p.W77X mutation was first reported in two families from Pakistan and one case from India(12)(14). A G→A transition at nucleotide 231 forms a nonsense mutation at tryptophan 77 (W77X). p.W77X is present in the second transmembrane (TM2) domain of Cx26. Incorporation of a stop codon at both these positions is predicted to result in a truncated protein with a complete loss of function (8). None of the 50 cases of NSHL, in our study carried 35delG mutation in contrast to a recent study by Bhalla et al., which reported a 10.9% prevalence of this mutation in North Indian patients . Presence of 35delG in NSHL patients has also been reported by Ghosh et al and Mani et al. but with low prevalence of 2.5% and 0.75% respectively (9).This mutation is highly prevalent in Caucasians and other Mediterranean populations but it has a very low frequency in the Indian subcontinent (8)(11). 35delG is also known as 30delG. The mutation involves deletion in a stretch of six G nucleotides, which lies in codon 10, resulting in a frameshift; a glycine is converted to a valine at codon 12 and a stop codon is formed at codon 13. (6)(16). The fourth mutation 235delC, which is a frameshift mutation due to deletion of a single cytosine at position 235 and was described for the first time in Japan (17). It represents the most frequent known mutation in Asian populations. In our study, this mutation was not detected. 235delC mutation was reported in Chinese (16.3%) (Dai et al., 2007)(21), Japanese (73%) (Abe et al., 2000)(17) and Taiwanese (5%) (Wang et al., 2002)(19). The mutation IVS (1+1)G>A was also known as –3170G>A. This splice site mutation is predicted to disrupt splicing, yielding no detectable mRNA (18). In our study, this mutation was not found. This mutation was previously reported in Palestine (15)(18). IVS (1+1)G>A is reported from India for the first time by RamShankar et al., 2003(8). He found that one proband was heterozygous for IVS(1+1)G>A. In 2009, Padma et al.(6)were reported, one homozygous and one heterozygous mutants for c.IVS1+1G>A mutation, the frequency of which works out to be 0.3% (1 of 303 each) for both. A heterozygous mutation of p.W24X and p.W77X in the NSHL subejcts indicates the simultaneous presence of another etiology, which needs to be further evaluated. There might be a presence of another mutated gene, making study subject a co-incidental carrier of p.W24X and p.W77X mutation. This another mutated gene could be related to connexin 26 or could be altogether a different gene on a distant locus. This observation demands for the further evaluation of the other areas of genetic spectrum related to congenital SNHL. A possibility of digenic SNHL as well as a non-genetic etiology cannot be ruled out in that subject. Discrepancies between different studies in terms of the frequency and type of GJB2 mutations associated with NSHL could be due to several reasons including: sample size (higher sample size increases the chance of detecting rare or exceptionally low mutations), precision of genotyping method employed, selection criteria of the patients investigated, rate of consanguineous marriage, and population genetic structure in terms of the type of circulating founder GJB2 mutations.

In a developing country like India, where patients cannot afford genetic analysis by sequencing, a restriction enzyme analysis along with electrophoresis could be very helpful for diagnosing NSHL. As concluded by Mukheree et al, ARMS PCR accompanied by restriction digestion and Agarose Gel Electrophoresis can be equally efficient in diagnosing NSHL, as compared to Sequencing (20). Detection of causative mutations beyond the 1st year of age is of minimal benefit as far as patient's treatment is considerd. A better rehabilitatioin can be offered to a patient, who is diagnosed during infancy. In the absence of significant auditary input during infancy, a marked delay in maturation of higher brainstem structures is observed.(22) (23). Cochlear implantation at an early age has been found to provide significant benefits to children with congenital NSHL. Statistically significant betterment was observed in Meaningful Auditory Integration Scale (++), categories of auditory performance (CAP), speech intelligibility rating (SIR) results in cochlear implant recipiants having GJB2 related mutations.(23) Another study supported the benefits of cochlear implants in GJB2 related profound NSHL (24). Currently available techniques like BERA are not capable enough to diagnose congenital hearing loss at the earliest age. Rather no other technique can offer a pre-natal diagnosis of hearing loss. To overcome these difficulties, mutational analysis at a peri-natal or even a pre-natal period is highly recommended. As of date, in India, there is hardly any awareness among a general population, about genetic testing for NSHL. The present study will help increasing awareness about importance of genetic analysis in congenital NSHL.

CONCLUSION

Our findings indicate that there is low prevalence of pW24X and p.W77X GJB2 mutations in NSHL cases in our population. In conclusion, there is a significant contribution of

GJB2 mutations regulating Connexin 26 protein to congenital NSHL (Autosomal recessive) in our study group. Screening for GJB2 mutations particularly, p.W24X and p.W77X should be offered to NSHL patients to confirm diagnosis of their congenital deafness, to deliver proper genetic counseling for the affected individuals and their families,to help them benefit from prenatal and pre- implantation genetic diagnosis. In a country like India where consanguineous marriages and marriages within the same communities is common, the incidence of recessive hearing loss could be proportionately higher. Early detection programs for NSHL are strongly recommended. Such an approach helps in early intervention by way of speech therapy and language development that would save the children from the dual disability of "deaf-mutism".

CONFLICTS OF INTEREST: Nil

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