Detection of Connexion 26 GENE (GJB2) Mutations in Cases of Congenital Non Syndromic Deafness

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Abstract Hearing loss is most common form of genetic hearing disorder. Non-syndromic sensory neural autosomal recessive deafness (NSRD) is the most common form of genetic hearing loss. Mutations in GJB2 gene, which encodes the connexin 26 protein, are major cause of NSRD. The aim of this study is directed towards the mutations caused along the connexin 26 gene using blood samples from nonsyndromic deaf children. The study was conducted on 36 congenitally hearing impaired children who visited to our department with complains of hearing loss and reduced speech and whose age was <10 years with no other congenital anomaly. After a thorough history, clinical examination and all audiological and radiological assessment, blood samples are collected and DNA extraction, PCR and sequencing were done for further genetic analysis. Annotated and documented autosomal recessive (pathogenic) mutations were observed in 57 % of NSRD cases. The frequency of pathogenic mutation was commonest for Ins G between nucleotide 30–35 (40 % of cases) followed by Del T at nucleotide 59(20 % of cases). These two common mutations (singly or doubly) were present in 51.4 % of cases. Present study helps to screen the families with hearing impaired children, which will facilitate the development of strategies for diagnosis and treatment of these common genetic disorders.

Keywords Deafness · Genetic · Connexin nonsyndromic

Introduction

All hereditary information is transmitted from parent to offspring through the inheritance of nucleic acid i.e. DNA & RNA. DNA (Deoxyribonucleic acid) is a linear polymer composed of purine and pyrimidine bases whose sequence ultimately determines the sequence of amino acid in every protein made by the body. The four types of bases in DNA are arranged in groups of three, each triplet forming a code word, or codon that signifies a particular amino acid. A gene represents the total sequence of bases in DNA that specifies the amino acid sequence of a single polypeptide chain of a protein molecule. The genes are arranged in a linear sequence of DNA that together with certain histone proteins forms rod shaped bodies called chromosomes. A mutation is a stable, heritable alteration in DNA. Mutation can involve a visible alteration in the structure of a chromosome, such as a deletion or translocation of a protein of a chromosome or they can involve a minute change in one of the purine or pyrimidine bases of a single gene. Most commonly, such “point” mutations consists of the substitution of one base for another, changing the meaning of the codon containing that base, hence their designation as “missense” mutation.

In most parts of the world, approximately one in 1000 newborn suffer from profound hearing loss and >50 % of these are caused by genetic factors [1]. Non syndromic neurosensory autosomal recessive deafness (NSRD) is the most common form of genetic hearing loss, accounting for 50 % of childhood prelingual deafness (1/2500) [2]. Various genes identified for NSRD include CDH23, CLDN14, COCH, COL11A2, DFNA5, D1APH1, DSPP, EVA4, GJA1, GJB2, GJB3, GJB6, KCNQ4, MYH9, MYO3A, MYO6, MYO7A,

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MYO15, OTOA, OTOF, PDS/SLC26A4, POU4F3, STRC, TECTA, TMC1, TMPRSS3, WFS1, WHRN etc. Mutation in ‘Gap junction beta 2 protein’ (GJB2) also known as ‘Connexin 26’, is a protein that in human encoded by the GJB2 gene, account for nearly 50 % of recessive non-syndromic hearing loss (NSHL) in populations all over the world as reported in several studies. Up to now, more than 90 variants of connexin 26 gene have been reported [3]. Connexin 26 is a member of a large family of protein involved in formation of gap junctions which allow direct transfer of small molecules and ions between neighboring cells [4]. Connexin 26 gene is expressed in many tissues, but in the cochlea of the inner ear, this gene product plays an important role in the normal hearing by controlling the potassium recycling pathway [4]. GJB2 formed by Connexin 26 gene is used for returning potassium ions to the endolymph, which plays a role in sensorineural hearing functions [4]. Mutations in the *GJB2* gene are responsible for 50 % of all cases of childhood prelingual ARNSHL the most common of which is 35delG, also referred to as 30delG [4–7]. It has been determined that more than 50 % of autosomal recessive non-syndromic hearing impairments result from the 35delG mutation in Northern and Southern European and American Caucasian populations [5]. Several types of Connexin 26 mutations cause recessive deafness according to ethnic background, as in the 167delT in Ashkenazi Jews [8] the R143 W in Africans [9] and the 235delC in the Japanese population [10]. World-wide 35delG mutation is the most common mutation of Connexin 26 gene accounting for up to 85 % of all GJB2 mutant alleles detected in Mediterranean populations [11–14]. Other mutations e.g. 235delC in Asian populations, R143 W in Africa were also shown to cause hearing loss [21].

The present study is directed toward the mutations caused along the Connexin 26 gene using blood samples from nonsyndromic deaf children. Children <10 years are selected because firstly testing for GJB2/GJB6 mutation in pediatric age has considerable value in establishing an etiologic diagnosis for SNHL hearing loss where as adult is of very low yield except in cases of early onset SNHL and strong family history secondly genetic hearing loss diagnosed in young children and infants is congenital hearing loss that was present but missed during the neonatal period. Mild congenital hearing loss was undetectable by available screening methods but has become more serious and detectable in later age and early detection and early intervention is very important in children and infants. The Connexin 26 gene analysis provides a better understanding

of the biology of normal and abnormal hearing and helps to form the basis for diagnosis.

Materials and Methods

The study was performed in 36 prelingual deaf children who attended ENT department of Dr. Bhim Rao Ambedkar Hospital associated with Pt. J. N. M. Medical College, Raipur. Only those hearing impaired patients were selected whose age was less than 10 years and with no other congenital anomaly. A detail history was taken from mother and relatives to get sufficient particulars of cases and significant history, if present. Beside present history, past history, detailed birth history (antenatal history, natal history and postnatal history) a thorough family history of both maternal and paternal side relatives has been taken to know the same problem in family.

Clinical examination of children were done including general examination, systemic examination to rule out presence of any other congenital anomaly and thorough local examination of ear, nose and throat has been done to rule out any congenital anomaly or any pathology. Children were also examined for mental retardation. IQ assessment was done in each case which would indicate that deafness was part a syndrome. All suitable investigations including Pure Tone Audiometry, Otoacoustic Emission, Brainstem Evoked Response Audiometry, HRCT bilateral temporal bones, 3D-MRI were done to find out membranous labyrinth and neural bundle involvement.

DNA Extraction

DNA was isolated from venous blood samples following a standard protocol and stored at -20°C . Genomic DNA was diluted to 5 ng/ μl and optimized under standard polymerase chain reaction (PCR) conditions with forward and reverse primers. The coding exon (Exon2) and flanking intronic regions of GJB2 gene were PCR amplified with forward primer 5'TTG GTG TTT GCT CAG GAAGA 3' and reverse primer 5'GGCCTACAGGGGTTTCAAAT3' using the Genbank Accession Number: M86849. The PCR products were then separated by electrophoresis to check proper amplification with genomic DNA.

The PCR products were purified on spin columns and the second exon of GJB2 was sequenced using either the forward or the reverse primer and the BigDye Terminator Cycle Sequencing kit and automated DNA sequencers with Sequence Analysis Software (Chromas Version 2.33). The

Table 1 Frequency of common mutations (>30 %) along with observed mutations at respective positions

S. No.	Position	No. of mutations	Frequency (%)	Mutations	Predominant mutations
1.	60	15	42.8	InsG, Mut T → G, DelT, Mut G → A, InsA	~46 % InsG ~40 % Mut G → A
2.	30–35	15	42.8	InsG, Mut G → C	~93 % InsG
3.	122	12	34.2	DelA, InsG	~91 % InsG
4.	732	12	34.2	Mut G → A, Mut G → T	~58 % Mut G → T ~41 % Mut G → A
5.	43	11	31.4	DelA, Mut A → C	~81 % DelA
6.	731	11	31.4	Mut G → A, Mut G → T, DelG	54 % Mut G → A 36 % Mut G → T

PCR products were sent for sequencing to Biotechdesk Pvt Ltd, Hyderabad, India. The statistical analysis was performed using *SPSS 17.0 software*.

DNA sequence variations were identified by comparison of subject DNA sequence to GJB2 reference sequences, Genbank Accession Number NM_004004. The sequence of PCR product of samples was matched with exon-2 of gene corresponding to Homo sapiens gap junction protein, beta 2, 26 kDa (connexin 26) (GJB2) sequence: URL: <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=42558282> Nucleotide NM_004004 using The European Bioinformatics Institute (EBI) bioinformatics tool: URL: <http://www.ebi.ac.uk/Information/> following application of pairwise sequence alignment.

The translation product of this gene is a 26 kDa protein (connexin 26) (GJB2) with amino acid sequence matched with nucleotide sequence with numbering begin with nucleotide A of ATG start codon in Exon2 as cDNA position number 1.

Results

Sequencing was successful in 35 cases. While with one case, case number 22, sequencing failed due to technical error. 34 Cases out of 35 sequenced cases revealed at least one mutation (variant/polymorphism/single point mutation/benign mutation/pathogenic mutation) except case number 28 which perfectly matched the exon-2 of gene corresponding to Homo sapiens gap junction protein, beta 2, 26 kDa (connexin 26) (GJB2) sequence with nucleotide Accession number NM_004004. One case (case no. 28) had no mutation (and hence encoding a native protein), nevertheless it resulted in NSHL due to factors unknown to the present study.

Most common mutations occur at position 60 and 30–35. Out of the two most common positions, commonest mutation is insertion of nucleotide G between 30 and 35 nucleotide of coding part (Exon2) of GJB2 gene (Tables 1, 2).

Frequency of documented pathogenic mutations observed in present study: 1 = 30–35 Ins G. 2 = 44 Mut A → T. 3 = 51 Mut C → A. 4 = 59 Del T. 5 = 71 Mut G → A

Table 2 Documented mutations observed in present study

Name of mutation	Description	Type of effect	Reference	Present in cases
R127H	G to A at 380	Polymorphism	Estivill et al. [11] and others	2,5 (Mut G → A)
K15T	A to C at 44	Lys at 15 into Thr	Bradshaw et al. [27] (PC)	12 (Mut A → T)
51del12insA	del of 12 nt and ins of A at 51	Frameshift	Sobe et al. [28] and others	36 (Mut C → A)
I20T	T to C at 59	Ile into Thr at 20	Loffler et al. [29] and others	10, 17, 23, 26, 29, 30, 32 (DelT)
W24X	G to A at 71	Trp at 24 into Stop	Kelsell et al. [7] and others	19, 31 (Mut G → A)
35insG	ins of G at 30–35	Frameshift	Estivill et al. [11] and others	1, 4, 5, 6, 7, 8, 18, 19, 20, 23, 29, 30, 32, 36 (all show insG) 3 (Mut G → A)

Discussion

We found monoallelic variations in most of our cases. Bhalla et al. [15] also observed that most of the patients (50/58) carried monoallelic variations. These monoallelic variants may not necessarily contribute the pathogenesis of disease. 94.2 % of cases have mutations that resulted in shifting of reading frames of coding part of Exon2 of GJB2 gene in such a manner that the product formed is a mutated protein with translation of mRNA stopped prematurely at first codon encountering a stop codon during translation of in coding part of Exon2 of GJB2 gene. This resulted in a dysfunctional protein. Dai et al. [16] also observed that Overall, 92.6 % (684/739) of the pathogenic mutations were frame-shift truncation or nonsense mutations. Whereas Case 26 had 3 deletions between 8 and 59 (8DelG, 27DelC, 59DelT). These mutations alters the reading frame between nucleotides 8 and 59 resulting in a mutant protein with one amino acid less than native proteins along with amino acid sequence alterations at 16 positions between 2nd and 17th amino acids in encoded protein. These mutations could be possibly deleterious causing formation of dysfunctional protein. In the present study one case (case no. 28) had no mutation (and hence encoding a native protein), nevertheless it resulted in NSHL due to unknown factors. Our observations regarding the formation of mutated proteins with altered sequence so as to render it dysfunctional is corroborated by Santos et al. [17] who observed that all putatively functional GJB2 variants occurred on the wild-type haplotype & were identified in 6.1 % of families. None of the putatively functional GJB2 variants were observed in the compound heterozygous state. The six putatively causative variants noted were 231G A(W77X), 71G > A(W24X), 167delT, 95G > A(R32H), 358–360delGAG(delE120), and 269T > C(L90P), with 231G > A(W77X) and 71G > A(W24X) being the most common, but for putatively benign variants there were a few cases in which individuals were observed to have two GJB2 polymorphisms. Two individuals were

identified to be compound heterozygous for the polymorphisms [380G > A (R127H)] + [457G > – > A(V153I)]. Thus it could be deduced that for putatively benign variants, almost all variants are essentially single point variants with few exceptions.

In the present study most common mutations occur at position 60 and 30–35. Out of the two most common positions, commonest mutation is insertion of nucleotide G between 30 and 35 nucleotide of coding part (Exon2) of GJB2 gene. Two most common mutations are Insertion of G at positions 30–35 (~93 %) and 122 (~91 %) respectively. Positions 30–35, 122, and 43 have one predominant mutation each as shown in table while positions 60, 732 and 731 have roughly equally two predominant mutations (Table 3). Three mutations, c.35delG, c.167delT, and c.235delC, are found to be the most frequent mutations in Caucasian, Ashkenazi Jewish, and Asian populations, respectively (Gabriel et al. [18], Morell et al. [19], Ohtsuka et al. [20], Park et al. [21], Rabionet et al. [4], Wilcox et al. [22], Abe et al. [24], Ballana et al. [25], Roux et al. [26]). In an Iranian study by Galehdari et al. [23] it was reported an absence of any mutation associated with deafness, including the commonly described mutations 35delG, 427C > T(R143 W), 167delT, and 235delC in the connexin-26 (GJB2) gene in an ethnic group of the Iranian Arab NSARD patients. In a report published in Lancet in year 1998, Estivill et al. [11], found insertion of G at position between 30 and 35 of exon 2 of GJB2 gene. In our study we could not find a single mutation describing a deletion of G at 30–35 instead we found insertion of G at position between 30 and 35 of exon 2 of GJB2 gene. Interestingly 235delC is considered to be commonest pathogenic mutation affecting Asian populations [16]. But we, again, did not witness a single case having this specific mutation.

Frequency of pathogenic mutation is commonest for InsG between nucleotides 30–35 (40 % NSHI cases) followed by Del T at nucleotide 59 (20 % NSHI cases). These two common mutations (singly or doubly) are present in 51.4 % of NSHI cases. Similar observations were reported from China in which Pu Dai et al. [16] found that the four

Table 3 Documented mutations and non documented common mutations (>30 %) in cases

Cases	Documented mutations at specified position					Non-documented predominant common mutation (>30 %)				
	30–35	44	51	59	71	60	122	43	731	732
1	InsG									
2										Mut G → A
3	Mut G → A									
4	InsG									
5	InsG									
6	InsG									
7	InsG									
8	InsG									
9						Mut T → G		Del A		
10				DelT						
11						Del T	Ins G	Mut A → C		
12		Mut A → T								
13						Ins G	Ins G	Del A		
14						Ins G	Ins G	Mut A → C		
15									Mut G → T	Mut G → T
16						Mut T → G	Ins G			
17				DelT						
18	InsG									
19	InsG				Mut G → A					
20	InsG					none	none	none	none	none
21										
22						x	x	x	x	x
23	InsG			DelT						
24						Ins G				Mut G → A
25						Mut T → G	Ins G			
26				DelT						
27						Del T	Ins G	Del A		
28						none	none	none	none	none
29	InsG			DelT						
30	InsG			DelT						
31					Mut G → A					
32	InsG			DelT						
33								Del A		
34						Ins G			Mut G → A	
35						Mut T → G			Mut G → A	Mut G → T
36	InsG		Mut C → A							

prevalent mutations; c.235delC, c.299_c.300delAT, c.176_c.191del16, and c.35delG, account for 88.0 % of all mutant alleles identified. Though the mutation spectrum in Chinese population may not match our Indian probands because of regional or racial differences.

Finally in the present study rest of 43 % cases in which documented pathogenic mutations were not present, commonest mutation was at position 60 (InsG, Mut T → G, DelT, Mut G → A, InsA) with a frequency of 66 % followed by mutation at position 43 (DelA, Mut A → C) with a frequency of (40 %) and at position 122 (DelA, InsG) with a frequency of (40 %). Together these three mutations are present in 100 % of ARNSHI cases.

Conclusion

- The frequency of pathogenic mutation is commonest for InsG between nucleotides 30–35 (40 % NSHL cases) followed by Del T at nucleotide 59 (20 % NSHL cases). These two common mutations (singly or doubly) are present in 51.4 % of NSHL cases.
- Sequencing analysis evidenced a total of six pathogenic mutation namely R127H, K15T, 51del12insA, I20T, W24X, 35insG. These six mutations have already been annotated and documented.
- Annotated and documented autosomal recessive (pathogenic) mutations are observed in 57 % of NSHL cases.

- In many regions globally, 30–35 DelG is the commonest mutation but a few regions, including the Central India, registers insertion instead of deletion i.e., 30–35 InsG.

Maximum no of mutations [13] were present position 665(5 %) and 11(4 %) mutations were found at position 660. A total of 10 mutations were present at position 627,625 and 643 each making 3.6 % of total mutations.

Compliance with ethical standards

Conflict of interest Author A- Dr. Hansa Banjara, Author B- Dr. Varsha Mungutwar, Author C- Dr. Neha Swamkar and Author D- Dr. Pradeep Patra declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standard of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all parents/legal guardians of children included in the study.

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