RESEARCH ARTICLE

HONG KONG JOURNAL OF PAEDIATRICS RESEARCH
Hong Kong J Pediatr Res 2022; 5(2):28-35

ISSN(e): 2663-5887
ISSN(p): 2663-7987

Mutational Analysis of GJB2 (Exon 2) Identified Four Novel Variants in Children with Nonsyndromic Congenital Hearing Impairment from South India

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Abstract

Background: GJB2 encodes gap junction proteins connexin 26 (Cx26) which are expressed in the cochlea where they colocalize, form heteromeric gap junctions and play an important role in cochlear homeostasis. GJB2 gene mutations on chromosome 13q11-q12 at the DFNB1 locus are responsible for up to 50% of autosomal recessive (AR) nonsyndromic sensorineural hearing loss (NSSNHL).

Aims and Objectives: The objective of the present study is to identify GJB2 (Exon 2) mutations in children with nonsyndromic congenital hearing impairment from South India.

Study Design: GJB2 mutational analysis was carried out in 175 children with congenital neurosensory nonsyndromic hearing impairment in the age group of 3-14 years.

Setting: Children with nonsyndromic congenital hearing impairment were enrolled from Government ENT hospital to identify the GJB2 mutations.

Materials and Methods: The entire exon 2 of GJB2 gene was amplified by polymerase chain reaction in 175 children with congenital non-syndromic hearing impairment. The sequence of each amplicon was confirmed by sequencing in both directions. The raw sequence data was analyzed and carefully edited against the standard Cx26 sequence using the AutoAssembler software.

Statistics: All the Cx26 variants were checked further in the world population from the connexin 26 gene databases (http://davinci.crg.es/deafness/). The secondary structural alteration due to the missense mutations of Cx26 gene was investigated using the GOR4 protein tool of the Biology Workbench of the San Diego Supercomputer Centre (http://workbench.sdsc.edu). The functional implications due to the variants were checked using the expasy tools PMut, SIFT and Polyphen.

Results: The results showed 13 variants in the coding region of Cx26 gene of which 4 variants were novel and were never reported in previous studies. R127H mutation was observed in majority of the probands followed by W24X, A40A, V27I, V153I and Q124X mutations. W77X and E147K mutations were observed in 5 patients each. While 4 probands showed I128I variant and 3 probands showed L28L mutation. M151R mutation was observed in only one proband.

Conclusion: The results of the present study shows that GJB2 gene mutations plays an important role in the genetic etiology of nonsyndromic hearing loss.

Keywords: Nonsyndromic hearing loss, Connexin, Mutation analysis, Autosomal recessive.

INTRODUCTION

Hearing impairment (HI) in humans is a genetically heterogeneous disorder with an incidence of about 1 in 1000 children worldwide, of which 50% of the cases can be attributed to a genetic cause [1]. Nonsyndromic hearing impairment (NSHI) accounts for nearly 70% of inherited hearing impairment, and is associated with more than 100 different genes with autosomal dominant (20–25%), autosomal recessive (75–80%), X-linked (1–2%), and maternal inheritance (1%) patterns [2]. GJB2 gene mutations on chromosome 13q11-q12 at the DFNB1 locus is responsible for up to 50% of autosomal recessive (AR) NSSNHL [3]. GJB2 encode gap junction proteins connexin 26 (Cx26) which are expressed in the cochlea where they colocalize, form heteromeric gap junctions [4], and play an important role in cochlear homeostasis [5]. Hearing loss associated with mutations in the GJB2 gene are assumed to be sensorineural in nature and fall into the moderate to profound range. In the present study, entire exon 2 of GJB2 (connexin 26) gene was amplified by polymerase chain reaction in 175 children with congenital non-syndromic hearing impairment.

MATERIALS AND METHODS

Study Subjects

The present study was carried out in 175 children in the age group of 3-14 years with congenital neurosensory nonsyndromic hearing impairment attending Government ENT hospital to identify the GJB2 mutations. Children with post lingual or acquired hearing loss, and who had undergone cochlear implantation, were excluded from the study. The children were clinically examined by an ENT specialist and important characteristic features were recorded. An audiological evaluation was carried out by an audiologist. The severity of hearing impairment was assessed by Pure Tone Audiometry (ELKON 3 in3, Pure tone audiometer, ISO 1964-Standard, with TDH 39 headphones and OTICON-SR 80 bone conduction receiver), Impedance Audiometry (AN22T with built in perimeters having ipsilateral and contralateral reflex testing facility), Brain Stem Evoked Response Audiometry (BERA) and Otoacoustic Emissions (OAE) (Madsen Capedla make with facility from TEOAEs and DPOAEs with CPU band software). Audiometric test was applied to each ear at frequencies of 0.5, 1.0, 2.0, 4.0 and 8.0 kHz. An audiological scientist conducted the threshold testing based on which the deafness was categorized into various types. Detailed information on epidemiological, clinical and genetic aspects was collected using a standard questionnaire. The study was carried out in accordance with the ethical standards and the same was approved by the Ethical Committee for Biomedical Research of Institute of Genetics and Hospital for Genetic Diseases, Ameerpet, Osmania University, Hyderabad. Written, informed and educated consent was obtained from the parents or guardians of all the children who have participated in the study.

Mutational analysis of GJB2 coding exon (exon 2)

The entire exon 2 of GJB2 (connexin 26) gene was amplified by polymerase chain reaction in 175 children with congenital non-syndromic hearing impairment using the following oligonucleotide primers described by Scott et al [6] F-5'-TCT TTT CCA GAG CAA ACC GC-3' and R-5'-GGG CAA TGC GTT AAA CTG GC-3'. The coding region of Cx26 (exon 2) gene was sequenced using ABI 3100 genetic analyzer (Applied Biosystems). The sequence of each amplicon was confirmed by sequencing in both directions. The raw sequence data was analyzed and carefully edited against the standard Cx26 sequence using the AutoAssembler software.

Insilco analysis

All the Cx26 variants were checked further in the world population from the connexin 26 gene databases (http://davinci.crg.es/deafness/). The secondary structural alteration due to the missense mutations of Cx26 gene was investigated using the GOR4 protein tool of the Biology Workbench of the San Diego Supercomputer Centre (http://workbench.sdsc.edu). The functional implications due to the variants were checked using the expasy tools PMut, SIFT and Polyphen. The amino acid conservation across species of the protein subunits was checked by comparing the protein sequences of various species.

RESULTS

Molecular screening of Gap Junction Beta 2 or Connexin 26 (GJB2) gene:

Analysis of the sequences showed 13 variants in the coding region of Cx26 gene of which 4 variants were novel and were never reported in previous studies. The chromatograms of all the mutations are shown in figure 1. The mutation frequency of each variant is shown in Table 1. R127H mutation was observed in majority of the probands (34/19.42%), followed by W24X mutation (6/9.14%), A40A (15/8.57%), V27I (12/6.85%), V153I (10/5.71%) and Q124X (6/3.42%) mutations. W77X and E147K mutations were observed in 5 (2.85%) patients each. 2.28% (4) of probands showed I128I variant and 1.71% (3) showed L28L mutation. M151R mutation was observed in only one proband.

Distribution of hearing impaired by the genotypes of Cx26 gene mutation

Out of 175 probands studied, 91 (52%) showed mutations in the coding region of the Cx26 gene. 26 genotypes were observed in 91 patients with Cx26 gene mutation (Table 2). R127H/+ is the most frequent genotype, accounting for 9.14% (16) of the hearing impaired (n=175) or about 17.58% of the patients who have a Cx26 gene mutation (n=91). This mutation is due to G > A transition at the nucleotide 380 in cytoplasmic loop (CL or IC2) of the Cx26 protein. The R127H mutation occurred in homozygous state in only 6 patients (3.42%).

Apart from this, R127H mutation was observed in compound heterozygous state in 12 (6.85%) patients. The most frequently associated mutation was V27I. This mutation was found in 5 of the 12 patients who have R127H mutation. The V27I mutation is

due to G > A transition at 79th nucleotide in transmembrane (TM1) domain of the protein. In three of the 5 patients, the genotype was observed to be R127H/V27I. Further, two of the 5 patients who have R127H and V27I mutations, also had additional Cx26 gene mutations: One patient showed A40A mutation, which is a A > C transversion at 120th nucleotide in EC1 domain of the protein and another patient showed V153I mutation, which is a G > A transition at 457th nucleotide in TM3 domain of the protein.

A40A and V153I mutations occurred individually in the compound heterozygote state with the R127H mutation in 2 patients each. One patient with A40A and R127H mutation also harboured an additional missense mutation, I121S, which is a T > G transversion at the 362-nucleotide position in IC2 domain of the Cx26 protein. This mutation was not described earlier and is considered to be a novel finding. Another patient with R127H mutation was found in compound heterozygote state with a nonsense mutation, C60X, which was also not reported previously. This mutation was also found in heterozygous state in one proband. The C60X mutation is due to C>A transversion at the 180-nucleotide position in the EC1 domain of the protein.

One more patient with R127H mutation also showed a homozygous E147K mutation. This mutation is due to G to A transition at 439th nucleotide in TM3 domain of the Cx26 protein. This indicates that 34 (19.42%) of the 175 probands have R127H mutation. Therefore, 37.36% of the patients who had a mutation in Cx26 gene also had R127H mutation and that most of them were heterozygous with the wild type allele.

The second most predominant mutant genotype of Cx26 gene was homozygous W24X/W24X. This mutation arises due to a G > A transition at 71st nucleotide in TM1 domain of the protein resulting in stop codon. This genotype was observed in 16 of the 175-hearing impaired. Two of these 16 patients also had an additional connexin heterozygous mutation, A40A. This indicates that W24X mutation was observed in 9.14% of all the hearing impaired and 17.57% of the probands having connexin 26 gene mutations. The W24X mutation is considered to be pathogenic.

The third most frequent mutant genotype was A40A/+. The A40A mutation is a silent mutation and is considered to be a polymorphic variant. This genotype was observed in 6 individuals accounting for 3.42% of the hearing impaired. The A40A mutation also occurred in compound heterozygote state with other Cx26 gene mutations. A total of 9 probands were found to be compound heterozygous for this mutation. Apart from the above-described genotypes, A40A was found additionally with three different variants, V27I, W77X and V153I in 3 patients each.

The fourth most common mutant genotype was V27I/+ (6.85%). This point mutation results in the replacement of Valine by Isoleucine. The V27I mutation was observed in 12 (6.85%) probands of all 175-hearing impaired. Four (2.28%) of the twelve probands were heterozygous for this mutation and the remaining 8 were compound heterozygotes with additional Cx26 gene mutations. Seven genotypes involving heterozygous V27I mutation in compound heterozygous state have been described above and one proband was found having E147K mutation along with V27I mutation in a compound heterozygous state.

The next most observed mutant genotype was V153I/+ in 6 (3.42%) probands. The V153I mutation was also observed in compound heterozygous state in 4 probands: (two (1.14%) probands with R127H mutation, one (0.57%) with A40A mutation and another proband with V27I and R127H mutations). The V153I mutation was observed in 10 (5.71%) probands out of 175 hearing impaired. The V153I mutation is considered to be pathogenic.

The Q124X mutation is a nonsense mutation that was observed in heterozygous state among 6 (3.42%) probands out of 175 hearing impaired and in 6.59% of all probands showing Cx26 gene mutations (n=91). This mutation is due to C to T transition at 370th nucleotide in IC2 domain of the Cx26 protein resulting in stop codon.

The known pathogenic mutation, W77X occurred in heterozygous state in 4 (2.28%) probands and in compound heterozygous state with A40A mutation in one proband (0.57%). This mutation was not found in homozygous state in the present study. The W77X mutation is due to a G to A transition at 231st nucleotide corresponding to the TM2 domain of the Cx26 protein.

The E147K mutation was found in homozygous state in 3 probands. Among one of these three probands, an additional connexin 26 mutations, R127H was also present. The E147K mutation was found in compound heterozygote state with V27I mutation in two patients. This mutation results due to G to A transition at nucleotide position 439 corresponding to the TM3 domain of the Cx26 protein. It causes the replacement of glutamic acid by lysine.

I128I/+ mutant genotype was observed in four probands. The I128I is a silent mutation resulting due to C -> T transition at the 384th nucleotide position corresponding to IC2 domain. Out of 4, one proband was compound heterozygote to I128I mutation with an additional novel mutation, M151R. The M151R mutation is due to T to G transversion at the 452nd nucleotide position corresponding to the TM3 domain of the protein. The clinical significance of this mutation is unknown as it was not reported previously. It was observed only in one patient in the current study.

L28L, a silent mutation arising due to C to G transversion at 84th nucleotide corresponding to TM1 domain of connexin 26 protein was observed in heterozygous state in 3 (1.71%) probands of all 175-hearing impaired. It was not found in homozygous or compound heterozygous condition in any of the patients.

Novel Mutations

In the present study, 4 out of 13 variants of Cx26 gene were found to be novel. All these novel mutations occurred either as heterozygotes or compound heterozygotes with other connexin 26 mutations. Table 3 lists the novel mutations along with the

genotypes in which they occurred.

Predicting the changes in the secondary structure and function using In-silico analysis

The In-silico analysis of the mutations using GOR4 tool revealed a change in secondary structure in the V27I, I121S, R127H and E147K mutant alleles (Table 4). The PSIC score difference of the Polyphen software indicated that the E147K (2.339) and M151R (2.695) mutant alleles were probably damaging. However, the NN output predicted all the variants to be pathological except the V27I and V153I mutations. The median sequence conservation values and scores of the SIFT bioinformatics tool indicated that the two missense mutations, E147K (3.00) and M151R (3.00) might probably affect the protein function of the connexin gene. The highly conserved amino acid sequences across the mammalian species were found to be altered by all missense mutations of Cx26 gene.

Table 1: Mutation Frequency of the Cx26 gene (exon2) variants

S.No	Mutation	N	% Among probands (175)
1	W24X	16	9.14
2	V271	12	6.85
3	L28L	3	1.71
4	A40A	15	8.57
5	C60X	2	1.14
6	W77X	5	2.85
7	1121S	1	0.57
8	Q124X	6	3.42
9	R127H	34	17.42
10	11281	4	2.28
11	E147K	5	2.85
12	M151R	1	0.57
13	V1531	10	5.71

Table 2: Genotype Frequencies of Cx26 gene mutations among 175 children with congenital hearing impairment

S. No	Genotype	N	% Among probands (175)	% Among mutants (91)
1	W24X/W24X	14	8	15.38
2	W24X.W2X:A40A/+	2	1.14	2.19
3	1.281/+	3	1.71	3.29
4	V28L/+	4	2.28	4.39
5	V271/+; R127H/+	3	1.71	3.29
6	V271/+; R127H/+; V1531/+	1	0.57	1.09
7	V271/+;A40A/+	1	0.57	1.09
8	V271/+;E147K/+	2	1.14	2.19
9	V271/+;A40A/+; R127H/+	1	0.57	1.09
10	A40A/+;1121S/+;R127H/+	1	0.57	1.09
11	A40A/+;R127H/+	2	1.14	2.19
12	A40A/+V1531/+	1	0.57	1.09
13	A40A/+	6	3.42	6.59
14	A40A/+;W77X/+	1	0.57	1.09
15	W77C/+	4	2.28	4.39
16	C60X/+	1	0.57	1.09
17	C60X/+;R127H/+	1	0.57	1.09
18	Q124X/+	6	3.42	6.59
19	R127H/R127H	6	3.42	6.59
20	R127H/+	16	9.14	17.58

21	R127H/+; V1531/+	2	1.14	2.19
22	E147K/E147K/R127H/+	1	0.57	1.09
23	E147K/E147K	2	1.14	2.19
24	11281/+; M151R/+	1	0.57	1.09
25	11281/+	3	1.71	3.29
26	V1531/+	6	3.42	6.59
Total		91	52	100

N= Number of affected individuals; -/+= Heterozygous condition.

Table 3: Novel Cx26 gene mutations observed in children with CHI in the present study

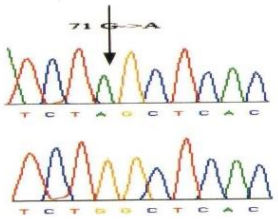
S. No.	Name of the Mutation	Base pair change	Structural domain	N	Genotype
1	L28L	CTC -> CTG	TM1	3	L28L/+
2	C60X	TGC -> TGA	EC1	2	C60X/+, C60X/R127H
3	1121S	ATC ->AGC	CL	1	A40A/1121S/R127H/+
4	M151R	ATG -> AGG	TM3	1	11281/M151R

N= number of affected individuals

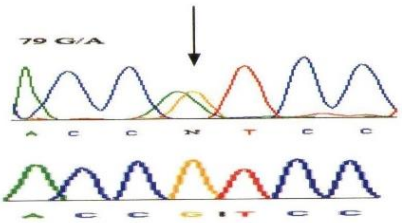
Table 4: Prediction of changes in secondary structure and function due to Cx26 gene missense variants

S.No	Name of the Mutation	GOR4	Polyphen			Pmut			SIFT		
			PSC1 Score difference	Prediction	Substitutional difference	NN output	Reliability	Prediction	Score difference	Prediction	MSC
1	V271	Random coil to Alpha Helix	0.334	Benign	NA	0.1402	7	Neutral	0.21	Tolerated	3
2	1121S	Alpha Helix to Beta sheet and Alpha helix to Random coil	0.572	Benign	NA	0.5303	0	Pathological	0.22	Tolerated	3.02
3	R127H	Alpha helix to Beta sheet	0.519	Benign	NA	0.5112	0	Pathological	0.14	Tolerated	3.01
4	E147K	Alpha helix to Beta sheet and Alpha helix to Random coil	2.339	Possibly damaging	Improper substitution in transmembrance region	0.6716	3	Pathological	0	Affect protein function	3
5	M151R	No change	2.695	Possibly damaging	Improper substitution in transmembrance region	0.9338	8	Pathological	0	Affect protein function	3
6	V1531	No change	0.235	Benign	NA	0.0508	8	Neutral	1	Tolerated	3.01

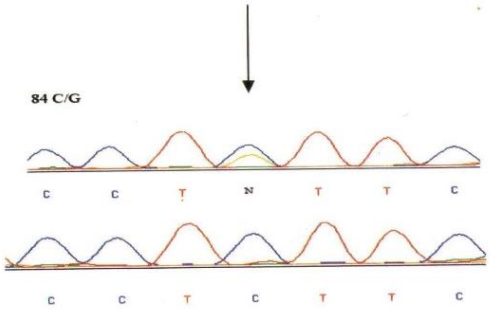
W24X mutation (homozygous)



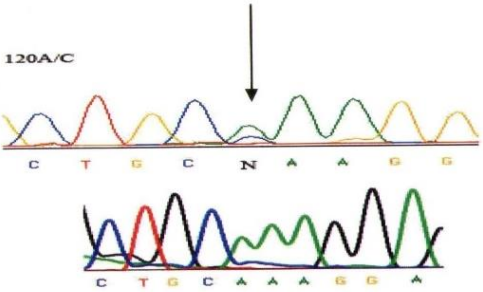
V27I mutation (heterozygous)



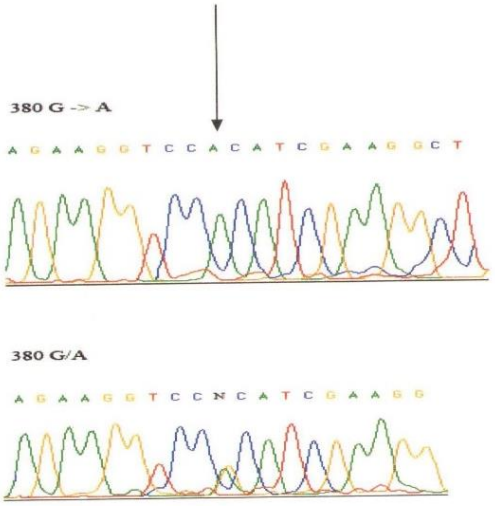
L28L mutation (heterozygous)



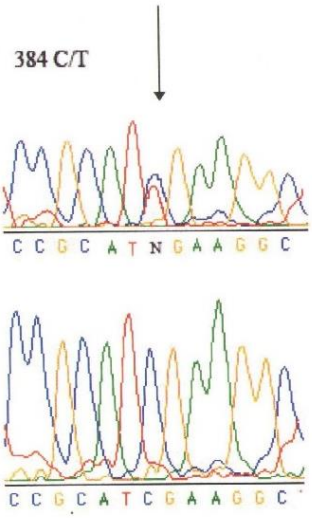
A40A mutation (heterozygous)



R127H mutation (Homozygous & Heterozygous)



I128I mutation (Heterozygous)



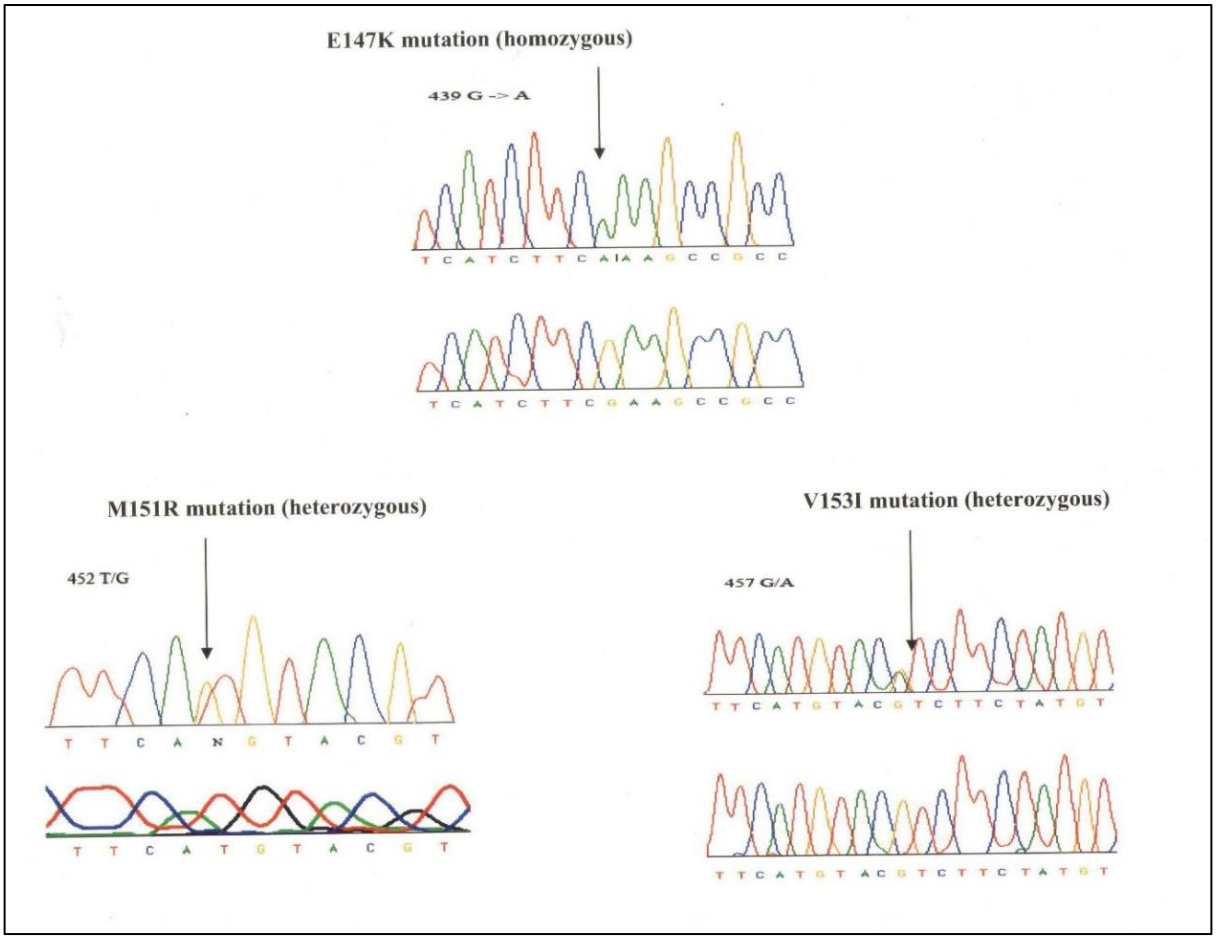


Figure 1: Chromatograms showing different nucleotide substitutions

DISCUSSION

Connexin 26 is expressed in the stria vascularis, spiral ligament, spiral limbus, in between the supporting cells of cochlea, and looks like functioning in the recycling of potassium that is required by the hair cells to create an action potential in response to sound waves. GJB2 gene consists of two exons, perhaps only exon 2 (681 bp) is coding. Cx26 product of GJB2 forms a gap junction protein with four transmembrane domains [7]. GJB2 (DFNB1) gene mutations are mainly responsible for pre-lingual hearing loss in various populations including India [8, 9]. c.35delG and c.235delC gene mutations were the main cause of autosomal recessive non-syndromic sensorineural hearing loss in the Iraqi deaf population [10]. Connexin 26 (GJB2) mutations are responsible for 19.4% of NSHL in Indian population. The c.71G > A(W24X) and c.35delG were the most prevalent GJB2 mutations accounting for 72.2% and 15.4% of NSHL [11], while low prevalence of GJB2 mutations in non-syndromic hearing loss was reported from Western India [12]. Although several mutations of GJB2 have been reported from India, p. Trp24Ter is the most common mutation, followed by p. Trp77Ter, c.35delG and p. Gln124Ter3 [8,13,14]. Pawan et al (2018) identified 17 different variants in the coding exon 2 of GJB2 gene, in children with non-syndromic hearing loss from northern (Delhi, UP) and western (Gujarat) regions of India. Among these variants, 16 variants (c.35delG, c.71G>A, c.148G>A, c.223C>T, c.231G>A, c.238C>T, c.283G>A, c.313_326del14, c.340G>A, c.341A>G, c.370C>T, c.407dupA, c.439G>A, c.79G>A, c.380G>A and c.457G>A) were known and one variant (c.616A>C) was novel [15]. GJB2 mutations frequency amongst hearing impaired individuals in India is considerably lower than European and North American population. Likewise, the frequency of GJB2 mutations also differs in south and north India [8]. In the present study analysis of the sequences showed 13 variants in the coding region of Cx26 gene of which 4 variants were novel and were never reported in previous studies. R127H mutation was observed in majority of the probands followed by W24X mutation A40A, V27I, V153I and Q124X mutations. W77X and E147K mutations were observed in 5 patients each. While 4 probands showed I128I variant and 3 probands showed L28L mutation. M151R mutation was observed in only one proband. Incredible development has been made on the molecular basis of hearing and hearing loss. In current years the molecular genetic background of nonsyndromic hereditary hearing impairment is quickly increasing and used for diagnostic purposes, treatment, genetic counselling, and management of the disease. Early diagnosis and intervention are recommended to improve the cognitive, social, speech, and language development of children living with hearing impairment [16].

CONCLUSION

GJB2 gene mutations play an important role in the genetic

etiology of nonsyndromic hearing loss. Hence, identification of mutations in GJB2 helps in early diagnosis and genetic counselling.

Conflicts of interest: None to declare.

Authors' Contribution: Study design, execution, data analysis and manuscript draft are done by Hema Bindu L. Shehnaz Sultana helped in literature search and manuscript drafting. Penagaluru Pardhanandana Reddy approved the final version to be published.

Acknowledgements

The authors would like to thank Dr. K. Thangaraj, Director, Centre for DNA Finger printing and Diagnostics and former Senior Scientist and Group Leader Centre for Cellular Molecular Biology (CCMB) for encouragement, support, advice and for providing facilities to carry out study at CCMB and Dr. M.V. Vishnuvardhan Reddy ENT specialist for the diagnosis of cases. Authors also like to thank shri. Mahendra Ranka, chairman, Bhagwan Mahavir Memorial Trust and Mrs. Sunita Kumar, Chairman, Managing Director, MAA Research Foundation for their support. Dr. L. Hema Bindu would like to thank Indian Council of Medical Research (ICMR) for providing her Senior Research Fellowship (SRF).

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