Original Communication

Study of families of nonsyndromic hearing impairment segregating with mutations in Cx26 gene

Ramchander P. V., Nandur V. U.*, Dwarakanath K*, Vishnupriya S, Padma T
Department of Genetics, Osmania University, Hyderabad; *Government Ear, Nose and Throat Hospital, Koti, Hyderabad, India.

Autosomal recessive nonsyndromic hearing impairment (ARNSHI) is the most common form with profound hereditary hearing impairment linked to DFNB1 locus (connexin26 gene) at 13q12. Mutations in connexin26 (Cx26) gene are known to be frequently associated with ARNSHI. Here, we report results on 13 families with NSHI screened for entire coding region of Cx26 using ARMS-PCR, restriction digestion analysis, SSCP and sequencing. Cx26 mutations were found in seven of the 13 families with inheritance of W24X (G to A at 71bp) in six and R127H (G to A at 380bp) in one of them. The observations imply that the G to A transition at position 71 in exon2 of Cx26 gene could play a major role in the phenotypic expression of recessive hearing impairment while R127H could be an associated polymorphism in Indian population.

Key Words: Connexin26, W24X mutation, NSHI, India.

Introduction

Hearing impairment is one of the most frequent sensory defects occurring 1 in 500-650 new born with 60% of them being genetic in origin. Significant genetic heterogeneity has been observed with families of NSHI showing autosomal dominant, autosomal recessive, X-linked and mitochondrial inheritance. About 85% of the cases follow autosomal recessive pattern of inheritance. Recently, role of modifier gene(s) influencing the expression of hearing impairment has been implicated which is receiving greater attention. Based on the complexity of the inner ear, it is speculated that there may be more than ~1% of 30,000 human genes orchestrating the biological process of hearing. To date around 99 loci are mapped for autosomal dominant (DFNA1-51), recessive (DFNB1-39) and X-linked (DFN1-8) genes apart from modifier locus (DFNM1) (http://www.uia.ac.be/dnalab/hhh). Several studies indicate that mutations in Cx26 gene are responsible for ARNSHI at DFNB1 locus present on chromosome 13q12 (MIM # 220290; http://www.org.es/deafness/). Mutations in mitochondrial genome are also known to cause NSHI as found in patients with A1555G mutation in the 12SmRNA gene and an insertion of ‘C’ at position 961 in the 12SrRNA gene.

Cx26 is a member of a family of gap junction proteins which constitute a major system of intercellular communication involved in the exchange of electrolytes, secondary messengers and metabolites. Pathological mutations in Cx26 may disrupt gap junctions interfering with recycling of potassium ions in the inner ear or in gap junctions that are required for the differentiation of the cochlear neuroepithelium resulting in deafness. Though mutations in the Cx26 gene in various populations are known to be associated with NSHI, reports from the population of India specially from Andhra Pradesh are scarce. About 25000 children are born with hearing impairment every year in India, emphasizing the need to identify the underlying cause.
to improvise proper measures of their management. Further in geographic regions with increased rates of consanguinity, genetic hearing impairment is found to be high because majority are recessive in inheritance.\textsuperscript{12-16} South Indian populations are ideal for the study of ARNSHI in view of the high rate of consanguineous marriages practiced.\textsuperscript{17-19}

Materials and Methods

In an attempt to study the genetic basis of NSHI, 13 families were identified to screen for the segregation of mutations in the coding region of GJB2 gene. All the families were ascertained through the probands referred at the Government Ear, Nose and Throat (ENT) hospital, Hyderabad. All the probands were tested through audiometric analysis and they were diagnosed as cases of congenital profound hearing impairment. None of the cases were secondary to any other abnormalities or syndromes. The pedigrees were drawn based on interviews with multiple family members. Blood samples were collected in EDTA vacutainers from all the members of the families including the proband, their sibs, parents and available relatives after obtaining their informed consent. Genomic DNA was extracted by using a rapid, nonenzymatic method\textsuperscript{20} with a little modification at the SDS step. SDS treatment was given for 1 hour or sometimes more depending upon dissolution of the pellet.

All the members of the families were screened for mutations in the entire coding region contained in a single large exon\textsuperscript{2} of the Cx26 gene using protocols of Amplified Refractory Mutation System (ARMS) technique for detecting mutations 35delG, W24X, W77X and Q124X and restriction digestion analysis for 167delT, 235delC and W24X.\textsuperscript{19,21-23} Mutations detected by ARMS-PCR and Restriction digestion analysis were confirmed by direct sequencing (ABI 3100 DNA sequence analyzer).

Mutations that are not detected by ARMS and restriction digestion analysis were screened by SSCP for the novel mutations with primers suggested by Scott et al\textsuperscript{21} followed by electrophoresis on a fan cooled 12% mini slab gel (49:1) for ~14 hr at 100V after staining the gels by silver staining. Sequencing was done to confirm the mutations detected, using primers suggested by Kelsell et al.\textsuperscript{14}

Results

The pedigree analysis of 356 NSHI probands (comprising 83.1\% Hindus and 15.9\% Muslims) revealed 36.2\% of the cases with positive family history. Majority of the cases showed autosomal recessive (87.5\%) inheritance and in the remaining families dominant (8.5\%) and X-linked (3.8\%) pattern of inheritance were indicated. In 86.4\% of these cases the parents were first cousins, in 8.2\% cases uncle niece and in 5.4\% cases second cousins. The incidence of consanguinity among the parents of the probands with positive family history (67.4\%) was higher than that is found in sporadic cases (49.3\%). DNA was isolated from 200 probands (males-56.0\%; females- 44.0\%) and were screened for mutations in the coding region of Cx26 in comparison to 200 normal hearing controls (males- 53.5\%; females- 46.5\%) without family history of NSHI. The analysis revealed prevalence of W24X mutation in 6.5\% cases, all showing homozygosity. In addition, in 0.5\% cases mutation W77X (G to A at 231bp) and in 0.5\% cases 235delC were detected. None of the controls carried alleles for the mutations screened. Apart from W24X mutation, prevalence of R127H was also found to be high where frequency of heterozygotes was 28.0\% among 200 probands as compared to 36.5\% of 52 controls screened for this mutation. Only in two (1.0\%) of the probands the mutation occurred in homozygous state (Table 1).

Out of all the cases recorded only in 13, members of the families responded for further studies. Of these, seven families showed positive family history of NSHI while six were sporadic. In six of them W24X mutation segregated following autosomal recessive pattern. In these six families all the affected members were homozygous for W24X and had profound hearing impairment (>90dB). Two of the probands showed the possibility of being compound heterozygotes when the entire families were studied. In one family cousin of the proband (Figure 1C) was affected with NSHI and showed heterozygosity for the W24X mutation. His parents were phenotypically normal but only father was the carrier for
W24X mutation and mother carried wild type alleles at this site. The child is evidently a carrier for another mutation inherited through the mother in all possibility which has to be explored further. In another family the mutation for R127H co-segregated with profound deafness but in heterozygous condition. The parents in this case were phenotypically normal and the mutation is inherited through carrier mother. This family is further being followed for the identification of other gene (s) responsible for hearing impairment found in the proband.

**Family Studies, Familial Cases**

Among the familial cases in the family DF-148, the proband and his affected brother were homozygous for the W24X mutation. Out of 3 unaffected sibs, 2 carried homozygous wild type alleles and one sib was a heterozygote. Both the parents of the proband were carriers for the mutation and contributed one allele each to the proband and to his affected brother. It is interesting to observe that a cousin of the proband who was the son of his maternal uncle was affected but showed carrier status when analyzed for W24X mutation. This was confirmed by the analysis by ARMS-PCR (Figure 1B) and also restriction digestion analysis (Figure 1A). Heterozygosity in this person is considered as a compound heterozygosity because both his parents were phenotypically normal but only father showed heterozygosity for W24X mutation while mother was homozygous for the normal allele for the mutation. Apparently the mother may be carrier for a recessive mutation for NSHI occurring either at a different site of the CX26 gene or elsewhere in the genome which may be the second mutant gene in the individual III-6 in the pedigree DF-148. We ruled out in this case the presence of other mutations like 35delG, W77X, Q124X, 167delT and 235delC for which ARMS and restriction digestion methods are available. Further, sequence analysis for the coding region of Cx26 in the individual III-6 (Figure 1C) and his parents did not reveal any variation that can be accounted for the presence of second mutant allele in Cx26 gene. The location of second mutant gene in this individual has to be explored still.

In family DF-161 (Figure 1D), the homozygous proband has phenotypically normal father and a male sib who were carriers for W24X mutation. The mother and maternal aunt of the proband are affected and homozygous for the mutation. The pedigree shows apparently a dominant mode of inheritance for NSHI without skipping of generation but the mutation analysis supports the recessive nature of the allele and hence considered as a case of pseudo-dominance.

Family DF-217 (Figure 1E) similarly appears to be a case of pseudo-dominance. The proband and his affected father were found to be homozygous for W24X mutation while the phenotypically normal mother was a carrier for the mutation. The pedigree though appeared to show dominant inheritance with NSHI in successive generations, mutation analysis revealed the recessive nature of the gene.

**Sporadic Cases**

In the 3 sporadic families DF-168, DF-230, DF-320 (Figure 2) the parents were phenotypically normal and they were identified as carriers for W24X mutations. In pedigree DF-168, the proband had phenotypically normal sib with both normal alleles and out of the 2 paternal uncles studied one was a carrier and other was homozygous normal for the mutation. In family DF-230, the proband had 3 phenotypically normal sibs of whom 2 were carriers and 1 was homozygous normal for the

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Proband Homozygotes</th>
<th>Proband Heterozygotes</th>
<th>Control Homozygotes</th>
<th>Control Heterozygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>35delG</td>
<td>0/200</td>
<td>0.0</td>
<td>0/200</td>
<td>0.0</td>
</tr>
<tr>
<td>167delT</td>
<td>0/200</td>
<td>0.0</td>
<td>0/200</td>
<td>0.0</td>
</tr>
<tr>
<td>G to A 71bp</td>
<td>13/200</td>
<td>6.5</td>
<td>1/200</td>
<td>0.5</td>
</tr>
<tr>
<td>G to A 231bp</td>
<td>1/200</td>
<td>0.5</td>
<td>0/200</td>
<td>0.0</td>
</tr>
<tr>
<td>235delC</td>
<td>1/200</td>
<td>0.5</td>
<td>0/200</td>
<td>0.0</td>
</tr>
<tr>
<td>G to A 380 bp</td>
<td>2/200</td>
<td>1.0</td>
<td>56/200</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Nonsyndromic hearing impairment segregating with mutations in Cx26 gene
mutation. In pedigree DF-320 the proband who was the only child (female) was homozygous for the mutation while her parents were carriers.

In other sporadic family DF-86 (Figure 2), the proband showed heterozygosity on SSCP analysis which was identified as due to mutation R127H on sequencing. Both his parents were phenotypically normal. Mutation R127H was inherited through his carrier mother. The father carried normal alleles for R127H indicating that the proband may be harbouring another gene responsible for his hearing impairment. We ruled out in this case the presence of other mutations like 35delG, W77X, Q124X, 167delT and 235delC for which ARMS and restriction digestion methods are available and also by sequencing.

Figure 1: Families showing segregation of NSHI and W24X mutation. A & D with restriction digestion analysis; B & E with ARMS analysis; C with Sequencing. ■ ● ➔ Affected with NSHI, □ ○ ➔ Phenotypically normal L-50bp ladder; N-Normal; C-Carrier; M-Mutant; UC-Uncut control; each lane correspond to individuals in the pedigree.
The second mutant allele in this proband has to be explored still.

In the remaining six families mutations 35delG, W24X, W77X, Q124X, R127H, 167delT and 235delC were not detected in the probands or in their relatives.

Discussion

Mutations in Cx26 are the most common cause of moderate to profound congenital inherited hearing impairment in many populations. The mutation 35delG is reported to be more prevalent in many of the Southern European descent populations resulting in a frameshift generating premature stop codon. In some populations as observed in the Ashkenazi Jews, frameshift mutation 167delT resulting in premature termination has been observed with a carrier frequency of 4.03%, compared to the carrier frequency of 35delG.
(0.73%). In Japanese, frame shift mutation 235delC resulting in truncation of protein is found to be prevalent with the carrier frequency of 7.8%. In these populations, screening of the newborn for early detection of relevant mutation is recommended and practiced.

Reports from India showed a new mutation W24X occurring with a frequency of 13.3% and 18.1%. This mutation is not reported from other populations. Several other mutations within Cx26 gene with low frequencies are reported among hearing impaired showing clearly the presence of ethnic diversity. 35delG mutation, a hotspot among Caucasian, 167delT in Ashkenazi Jews, 235delC in Japanese and W24X mutation in Indians are considered to be the result of founder effect. In the present study the prevalence of W24X mutation is consistent with that reported by Ramshanker et al and Maheswari et al.

From India, report of Ramshanker et al revealed high frequency of R127H among hearing impaired with homozygosity (4.6%) and heterozygosity (15.3%) among deaf cases and as well as controls (5.0% homozygous and 25.0% were heterozygotes). They suggested that the substitution of G to A at 380bp (R127H) could be a polymorphism and not causative for NSHI. Present results also showed the prevalence of heterozygotes for R127H in probands (28.0% heterozygotes) and as well as in controls (36.5% heterozygotes). Further two (1.0%) of the probands showed homozygosity raising doubt about the possibility of pathogenic nature of the mutation specially when viewed in the light of the finding of modifier genes influencing the expression of genes for NSHI.

Roux et al reported two unrelated families where 2 parents with normal hearing and children genotyped as R127H/M34T or R127H/W24X were found to be homozygous for R127H. In each of these families, a normal hearing sib also carried the genotype R127H/M34T or R127H/W24X. The authors are of the opinion that R127H may not be associated with deafness or combined genotypes with variants such as M34T, V37I, or R127H could have a phenotypic expression modulated by environmental factors or modifier genes.

In the present attempt mutation analysis of the families enabled confirmation of the recessive inheritance of W24X mutation and also identification of carriers who were phenotypically normal. This information further enabled in counseling the families regarding the possible risk of recurrence in their progeny, and also counseling them against assortative mating to reduce enhancement in recurrence of NSHI. This approach is needed specially in view of the high rate of consanguineous marriages occurring in the local population and the preference of the families to encourage the affected member to marry a blood relative for sake of convenience and social reasons.

**Conclusion**

In a country like India, with occurrence of high consanguineous marriages and high incidence of recessive hearing impairment, early detection programmes for NSHI 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”. In view of the diversity present in Indian population and reports on the ethnic association of mutations causing NSHI worldwide, it is also important to consider the ethnic background of the affected as it would help in identifying founder population. Further, screening for carriers for mutation like W24X is required for effective counseling.

**Acknowledgements**

We are thankful to the families, members for their participation in this study. Ramchander is thankful to CSIR, New Delhi, India for the award of a Senior Research Fellowship. This work was supported by Department of Atomic Energy, Mumbai, India.

**References**

Nonsyndromic hearing impairment segregating with mutations in Cx26 gene


