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Non-Syndromic autosomal recessive deafness in Gaza strip: A study of five *GJB2* Gene mutations

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Abstract: Hearing loss is a common, pan-ethnic and highly heterogeneous sensory disorder with an incidence of around 1 in 1000 infants. Genetic causes are thought to be responsible for more than 60% of the cases with the majority of non-syndromic hearing impairment being inherited in an autosomal recessive pattern. The gene that is most frequently mutated in autosomal recessive non-syndromic hearing loss (ARNSHL) is gap junction protein beta-2 (*GJB2*) which codes for connexin 26 (Cx26). Cx26 plays a key role in potassium homeostasis, which is essential for sound transduction. The aim of this study was to determine the common *GJB2* gene mutations in 70 patients suffering from ARNSHL in Gaza strip. The patients were screened for five *GJB2* gene mutations namely, c.35delG, c.167delT, c.-23+1G>A, c.229T>C (p.Trp77Arg) and c.235delC. Study results revealed that *GJB2* mutations account for at least 35.7% of the ARNSHL with mutant allele frequency of 0.4%. The most frequently encountered mutation was c.35delG which accounted for 35.7% of the ARNSHL cases in either homozygous (34.3%) or heterozygous (1.4%) form and represented about 80.5% of all the detected mutations. The second most frequent mutation was c.235delC which was found only in heterozygous form. The third mutation was c.-23+1G>A which was identified in only one subject (1.4%) in a compound heterozygous form along with c.35delG. The c.167delT and p.Trp77Arg mutations were not observed in our patients. We conclude that there is a significant contribution of *GJB2* mutations to congenital ARNSHL in the Palestinian population of Gaza strip. Screening for *GJB2* mutations particularly, c.35delG, c.235delC and c.-23+1G>A should be offered to ARNSHL patients to confirm diagnosis of their congenital deafness, to deliver proper genetic counseling for the affected individuals and their families and to help them benefit from prenatal and pre-implantation genetic diagnosis.

Keywords: ARNSHL, Cx26, GJB2, Mutation, PCR-RFLP, Gaza Strip

1. Introduction

Hearing loss is the most prevalent form of sensory impairment in humans, with approximately 1 in 1000 infants being born with a serious hearing deficit (Shahin et al., 2002). Hereditary hearing loss can be subdivided into two types: syndromic or non-syndromic. The syndromic type is associated with other distinctive clinical features beyond deafness and accounts for about 30% of hereditary congenital deafness. The non-syndromic type, in which deafness is the only clinical manifestation, represents the other 70% (Matsunaga, 2009). Regarding non-syndromic hearing impairment, autosomal recessive form is the most frequent, accounting for 75-85% of the cases [Snoeckx et al., 2005; Ibrahim et al., 2011). Eighty (80) loci are already described for non-syndromic autosomal recessive deafness (http://hereditaryhearingloss.org).

The different loci for the numerous forms of deafness have been called DFN (for deafness) and are numbered in chronological order of discovery. The first ARNSHL locus, DFNB1 accounts for at least 50% of this type of hearing loss (Mukherjee et al., 2003). At chromosome 13q11-q12, DFNB1 *locus* comprises *Gap Junction β-2* (*GJB2*) and *Gap Junction β-6* (*GJB6*) genes that code for connexin 26 and connexin 30 (Cx30), respectively (Rodriguez et al., 2011). On the other hand, *DFNA* refers to autosomal dominant deafness loci.

Connexin 26 is one member of a family of related gap-junction channel forming proteins (Mukherjee et al., 2003). These gap junction proteins oligomerize as hexamers to form transmembrane channels called connexons. Connexons from the cell membranes interdigitate to form direct intercellular communications pathways, the gap junction channels (RamShankar et al., 2003).

Human connexins are classified either by molecular mass (26-59 kDa) or by sequence similarities into 5 subgroups: gap junction α (GJA), gap junction β (GJB), gap junction γ (GJC), gap junction δ (GJD) and gap junction ε (GJE). Sensorineural hearing loss is caused by mutations in the gap junction genes encoding the β connexins (Mukherjee et al., 2003).

GJB2, the gene encoding human connexin 26 (Cx26), was the first gene to be linked to an autosomal recessive form of deafness, DFNB1 (Kelsell et al., 1997). *GJB2* gene has 5500 bp and a single coding exon (exon 2), in a total of two exons. More than 200 different pathogenic mutations were identified in this gene that account for 10-40% of congenital deafness (Tang et al., 2006; Falah et al., 2011). *GJB2* mutation spectrum differs among various populations or even within a particular country. Therefore, it is necessary to establish the frequency of *GJB2* mutations in any population (Bonyadi et al., 2011).

Mutations at the DFNB1 locus can be classified into two groups: (i) those that affect the coding sequence of *GJB2*, and (ii) those that lie outside the coding sequence of *GJB2* and affect the expression and/or regulation of this gene (Gandia et al., 2013).

This study was designed in order to determine for the first time the common mutations in connexin 26 gene in non syndromic autosomal recessive deafness cases in Gaza strip.

2. Materials and Methods

2.1. Subjects and Selection Criteria

The population of this descriptive analytical study consisted of 70 deaf patients**.** The subjects were recruited from three Gaza Strip hearing loss centers: Mustafa Sadiq Rafii Secondary School for the Deaf, Atfaluna Society for the Deaf Children and Basma Center for Audiology and Speech Therapy**.** The participants were from both genders and their age ranged from 2-27 years and they all suffered from prelingual severe to profound bilateral hearing loss. Apart from their deafness the subjects did not have any other remarkable health problems. The study protocol was presented for approval to the Ministry of Education and to the Administrations of the three centers. Approval and written consent of patients and/or their parents was also taken. In order to ensure that hearing loss in the study subjects is most probably of the autosomal recessive non-syndromic type, an interview was conducted with each patient and/or his/her family to help exclude other hearing loss causes.

2.2. Mutation Analysis

Anticoagulated venous blood (~2 ml) was collected from each patient. Genomic DNA was prepared from peripheral blood lymphocytes using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol. Extracted DNA was used in screening for the selected mutations. PCR-Restriction fragment length polymorphism (PCR-RFLP) analyses were performed for detecting all five mutations as previously described by other investigators and as indicated in Table 1. Restriction enzymes and PCR primers were purchased from Promega (USA). Digested PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide, and the results were documented using a gel documentation system.

Table 1. *Primer sequences, restriction enzymes and allele designation used for identifying the five GJB2 mutations*

Restriction enzyme	Primer sequence (5'-3')	Mutation	PCR Product (bp)	Allele size (bp)	Reference
BstI	F:TCTTTTCCAGAGCAAACCGC R: GCTGGTGGAGTGTTTGTTCACACCCGC	c.35delG	89	Normal: 69+20 Hetero: 89+69 Homo: 89	(Freitas et al., 2010)
MwoI	F: GATTGGGGCACGCTGCA R: CCCTTGATGAACTTCCTCTTCTTC	c.167delT	322	Normal: 322 Hetero: 322+161 Homo:161	(Shahin et al., 2002)
ApaI	F: TGTGTGCATTCGTCTTTTCCAG R:GGTTGCCTCATCCCTCTCAT	c.235delC	410	Normal: 262+148 Hetero: 410+262+148 Homo: 410	(Padma et al., 2009; Scott et al., 1998)
HphI	F: TCCGTAACTTTCCCAGTCTCCGAGGGAAGAG R: CCCAAGGACGTGTGTTGGTCCAGCCCC	c.-23+1G>A	360	Normal: 242+118 Hetero: 360+242+118 Homo: 360	(Padma et al., 2009)
MspI	F: CCATCTCCCACATCCGGCTC R: GCCTTCGATGCGGACCTTCT	p.Trp77Arg	182	Normal: 100+82 Hetero: 182+100+82 Homo: 182	(Al-Achkar et al, 2011)

DNA samples were first screened for c.35delG and c.167delT mutations and when no c.35delG or c.167delT mutation was detected, screening was continued for the c.235delC, IVS1+1 G>A and p.Trp77Arg mutations. The rationale behind selecting those mutations is based on their common presence in the West Bank part of Palestine (Shahin et al., 2002) and in neighboring Arab countries e.g., Lebanon, Syria and Jordan (Mahayri and Monem, 2012; Medlej-

Hashim et al., 2002; Mustafa et al., 2001) which are believed to be historically related to our population.

3. Results

This study investigated five *GJB2* gene mutations associated with non-syndromic autosomal recessive deafness in 70 deaf patients in Gaza strip. Among the deaf patients, 37 (53%) were males and 33 (47%) were females. The age of patients ranged from 2 to 27 years.

The results of the interview revealed that consanguineous marriage is common between the parents of the patients (76%), and that there is a relevant family history in the family of the father (34%) or the mother (33%). Additionally, 63/70 (90%) of the patients' parents had normal hearing. Available data for 6 of the remaining 7 patients showed that their maternal and paternal grandparents had normal hearing. Therefore, interview outcomes allowed us to consider almost all the patients as having non-syndromic autosomal recessive hearing loss.

Of the 70 deaf patients, 31 (44.3%) were found to harbor *GJB2* mutation in homozygous, compound heterozygous or heterozygous form. However, 39 (55.7%) of the study subjects failed to show any of the tested mutations.

GJB2 c.35delG was the most frequently encountered mutation where 25 of the 70 probands (35.7%) were found to be either homozygous (34.3%) or heterozygous (1.4%) for this mutation. Heterozygosity was recorded in only one proband who was compound heterozygous for c.35delG/ c.-23+1G>A. The second prevalent *GJB2* mutation c.235delC, was detected only in heterozygous form (one mutant copy) in six probands (8.6%). None of the 70 samples showed the c.167delT or the p.Trp77Arg mutation. Frequency of the detected mutations is summarized in Table 2.

Table 2. *Frequency of mutations in Cx26 in ARNSHL patients in Gaza strip*

Mutation	Genotype	Frequency No. (%)	Allele frequency
c.35delG	c.35delG/c.35delG c.35delG/ C.-23+1G>A	24 (34.3%) 1 (1.4%)	0.35
c.-23+1G>A	c.-23+1G>A /c.35delG	1 (1.4%)	0.007
c.235delC	c.235delC/unknown	6 (8.6%)	0.043
c.167delT	Not detected	-	-
p.Trp77Arg	Not detected	-	-
Total		31(44.3%)	0.4

4. Discussion

This work investigated five *GJB2* mutations in Gaza strip Palestinian families with presumable ARNSHL. The results revealed that the *GJB2* mutations were evident in 31 (44.3%) probands. When compared to its frequency in other populations, this result is among the highest *GJB2* mutation rates reported so far.

ARSNHL patients from different ethnic groups also show variation in terms of the frequency of individual mutations. Our results demonstrated that c.35delG accounts for a significant proportion (~80.5%) of the pathogenic *GJB2* alleles. This mutation is the commonly encountered *GJB2* mutation in Caucasians however, it is very rare in Asian patients (Cifuentes et al., 2013).

On the other hand, the second most common mutation observed in this study c.235delC represents the most frequent mutation in some Asian populations, Japan in particular (Abe et al., 2000), whereas it is rare in Africans (Chan and Chang, 2013) and Indonesians (Gaffar et al., 2009).

Likewise, c.167delT which was not detected in our patients is predominant in Ashkenazi Jews (Morell et al., 1998). Interestingly, Shahin et al., (2002) detected this mutation in a group of Palestinians from Bethlehem (Shahin et al., 2002) indicating that variation in *GJB2* mutant allele frequency may also be evident in groups of the same population.

The third detected mutation was the splicing mutation c.-23+1G>A which was found only in one patient in compound heterozygous form with c.35delG. This result is similar to that reported in many Arab populations (Shahin et al., 2002; Snoeckx et al., 2005; Riahi et al., 2013). This mutation is frequent in ARNSHL cases of Iranian Azeri Turkish population, Eastern Europe, Turkey and Kurdish population of Iran (Bonyadi et al., 2011; Mahdieh et al., 2004; Seeman and Sakmaryova, 2006; Sirmaci et al., 2006; Toth et al., 2007).

The trend is not different for mutation p.Trp77Arg which also was not detected in our study. This mutation is commonly encountered in certain populations e.g., Spanish and Cubans (Perea et al., 2007), and is detected in low frequency in Arabs (Al-Qahtani et al., 2010).

Variation between different studies in terms of the frequency and type of *GJB2* mutations associated with ARNSHL 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* mutant alleles.

In more than half of the examined patients none of the five screened *GJB2* mutations could be detected and in 6 patients only one mutant allele (c.235delC) could be identified. This finding is comparable to those found by other investigators where they showed that their ARNSHL patients harbored only one mutated allele in the *GJB2* gene (Kenneson et al., 2002). This could be due to three possible causes: (i) presence of other mutations in the coding or regulatory regions of *GJB2*; (ii) mutations (particularly deletions) in the contiguous *GJB6* gene which has been shown to co-occur in *GJB2* heterozygotes and, (iii) mutation in other hearing loss

loci. Work is ongoing to find other pathogenic alleles by sequence analysis of the *GJB2* gene and screening for *GJB6* gene mutations.

5. Conclusion

There is a significant contribution of *GJB2* mutations to congenital ARNSHL in the Palestinian population of Gaza strip. By screening *GJB2* c.35delG, c.235delC and C.-23+1G>A, the molecular cause in as high as 35.7% of ARNSHL patients of Gaza strip can be identified. Moreover, defining the mutation responsible for ARNSHL is crucial for confirming diagnosis and delivering proper genetic counseling for the affected individuals and their families.

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Author Disclosure Statement

The authors hereby declare that no competing interests exist.

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