A Novel Mutation in the GJB2 (Connexin 26) Gene in Egyptian Children with Non-syndromic Sensorineural Hearing Loss

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Abstract
This study aimed to investigate the frequency of any novel gene mutations, in human GJB2 gene among Egyptians with familial sensorineural non-syndromic hearing impairment. PCR amplifying the entire coding region of GJB2 gene and direct DNA sequencing to analyze mutations in this gene among 78 cases with autosomal recessive congenital non syndromic hearing loss was used.

We describe for the first time a novel mutation in the coding region of the GJB2 gene. A deletion mutation of T at position 59, in the intracellular domain of connexin 26, resulting in a frameshift at the 20th amino acid leading to a premature termination of the protein was detected in 9% of the studied cases.

These data provide a novel molecular explanation for the role of GJB2 mutation in hearing loss to be taken into consideration in the genetic diagnosis and counseling of non-syndromic sensorineural hearing loss in Egyptians.

Introduction
Hearing loss is the most common sensory deficit in humans and most auditory system dysfunctions resulting in hearing loss are genetically inherited [1]. It affects approximately 10 per cent of the world population, which is significant enough to compromise the development of normal language skills and social development. It can appear at any age with varying degrees of severity [2].

Over 100 loci, both nuclear and mitochondrial, are already implicated in the development of this most common sensory disorder of humans [3].

GJB2 (Gap Junction protein β Type 2), encodes Connexin 26 (Cx26), a member of a highly conserved protein family found throughout the animal kingdom [4]. Six connexins oligomerize to form pore-like plasma membrane hemichannels called connexons, they form gap junction channels which facilitate electrical and biochemical coupling between cells. Ultrastructural studies have identified two gap junction networks, including that of the epithelial tissue and that of the connective tissue, in the mammalian cochlear duct and vestibular system [5]. The gap junction system is the most likely pathway for cochlear K+ recirculation following hair cell depolarization maintaining cochlear homeostasis [6].

The GJ channels have diverse functions, including electrical signal propagation, metabolic cooperation, growth control, spatial buffering of ions, and cellular differentiation [7]. Mutations in one gene,
Patients and Methods

Studied Population

Seventy eight Egyptian children (38 males and 40 females) with prelingual non-syndromic hearing loss were enrolled in this study. Their ages ranged between 1 to 15 years. Parental consanguinity was present in all studied families (34 families). All patients were recruited from the genetic counseling service for deaf people at the Clinic of Children with Special Needs, National Research Center, and the outpatient clinic of the Hearing and Speech Institute, Cairo, Egypt.

The study was approved by the Ethics Committee of The National Research Center, Cairo, Egypt, and written informed consent was obtained from all parents or caregivers. All cases were subjected to the following:

1. Detailed history was taken for each patient that included: Parental consanguinity, family history of similar condition, pregnancy history, perinatal history, history of drug intake, recurrent ear infections, ear trauma, and history of any chronic illness. Onset, course and duration of the hearing loss were obtained.

2. Three generations pedigree was constructed for each case.

3. Careful clinical examination had been done to exclude syndromic deafness, and other associated anomalies.

4. Ear, nose and throat (ENT) examination had been carried out to check for any ear malformations, signs of trauma or scars of previous operations.

5. Full audiological assessment: Auditory brain stem response (ABR) for cases ≤ 4 years old and pure-tone audiometry (PTA) for cases > 4 years old to determine the degree of hearing for both ears.

Molecular Analysis

Genomic DNA Extraction: Five ml venous blood was obtained from each patient in a sterile tube containing EDTA as an anticoagulant for genomic DNA extraction. Extraction was carried out by using a ready-made QIAGEN kit (QIAamp DNA Mini kit and, QIAGEN; Germany). Genomic DNA was diluted 1:100 in TE buffer and the A260 absorbance was read and the concentration was calculated.

DNA amplification using polymerase Chain Reaction (PCR): Amplification of the connexin 26 (Cx 26) coding region was achieved by using the following primers:

Forward Primer: 5’ tct tt cca gag cca acc gc 3’
Reverse Primer: 3’act cgt gcc caa cgg agt ag 5’

PCR reaction was performed in a final volume of 50 μl, containing 200 ng genomic DNA, 10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton® X-100, 1.5 mM MgCl₂, 200 μM dNTPs, 10 μM of each primer and 2.5 units of Taq polymerase (Promega-USA). Then the thermal cycler was programmed according to the following steps to undergo the amplification reaction for Cx26 gene coding region. First; samples were denatured at 94 °C for 5 minutes. Subsequently; 40 cycles of denaturation were achieved at 94 °C for 15 seconds, annealing was carried out at 55 °C for 30 seconds and extension was done 72 °C for 1 minute, followed by 5 minutes of post extension.

DNA Purification: Amplified fragments on agarose gel were purified by using a ready-made extraction kit (Invisorb® Spin DNA,Invitek; Germany).

Sequencing: Direct DNA sequencing was carried out in both directions using the forward and reverse primers on the Automated Sequencer “ABI Prism 310 Genetic Analyzer”. The cycle–sequencing reaction was performed in a volume of 20 μl containing: 8 μl of the terminator ready reaction mixture 3.2 pmole of either the forward primer or the reverse primer and 30 ng of purified PCR product.
The thermal cycling protocol was followed on the “Perkin Elmer, Gene Amp PCR System 9700” ; 95°C for 5 minutes followed by 25 cycles of (96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes). Centri-Sep Columns (Princeton Separations, Philadelphia) were used for effective and reliable removal of excess Dye Deoxy™ terminators from completed DNA sequencing reactions. Data were compared and aligned with different sequences of different strains using the Clustal W software for multiple alignments http://www.genome.jp/tools/clustal.

Table 1: Different strains that showing alignment with the coding sequence of Cx26 gene in Egyptian patients.

<table>
<thead>
<tr>
<th>GI/GB accession number</th>
<th>Description</th>
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<td>gi/33391196/gb/AY28097.1</td>
<td>Homo sapiens connexin 26 (GJB2)</td>
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Results

We studied 78 Egyptian patients with prelingual non-syndromic hearing loss. Parental consanguinity was present in all studied families (34 families). Forty eight cases (61.5%) had severe hearing loss, 16 cases (20.5%) showed moderate hearing loss, 14 cases (17.9%) had mild hearing loss.

After direct sequencing of all samples using the primers in both directions, forward and reverse primers, the obtained sequence was aligned with human Cx26 gene on NCBI database revealing a novel GJB2 deletion frameshift mutation (Figure 1) in 3 out of 34 families.

A novel mutation was detected, which was the deletion of T at position 59 resulting in a frameshift at the 20th amino acid, leading to a premature termination of the protein (Table 2). The mutation was segregated in the family according to a recessive mode of inheritance. No other homoygous loss-of-function mutation segregating with affected status was identified in other family members. This variant was not present in more than 150 ethnically-matched, healthy, in our in-house dataset. The sequence is shown in the reverse direction. All patients with the novel mutation showed severe hearing loss.

Table 2: A novel mutation in Cx26 gene in Egyptian patients with non-syndromic sensorineural hearing loss.

<table>
<thead>
<tr>
<th>Nucleotide Change</th>
<th>Effect</th>
<th>Type of Mutation</th>
<th>Protein Domain</th>
<th>No. of Patients</th>
</tr>
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<tbody>
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<td>Del of T at position 59</td>
<td>Stop Codon (Term.)</td>
<td>Framshift</td>
<td>IC1</td>
<td>3 out of 34 families (7/78 cases, 9%)</td>
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</table>

Discussion

Deafness is a worldwide prevalent disease that seriously impairs human quality of life. The deafness-associated gene mutation spectrum as well as dominant gene profile varies greatly among regions and races [11]. 35de1G, 167de1T, and 235de1C are reported to be the most prevalent mutant genes among Caucasian, Jewish, and Asian populations, respectively [12-14]. Contemporary studies of the causes of congenital and early-onset deafness have incorporated the tools of genetic epidemiology, including molecular testing, to identify a growing number of potential genetic and environmental causes.

In most cases, inherited HL is monogenic. In 70% of neonates who fail newborn hearing screens and are presumed to have inherited HL, there are no other distinguishing physical findings and the HL is classified as nonsyndromic. In the remaining 30%, the HL is accompanied by other physical findings and is said to be syndromic [15]. Of the more than 400 syndromes in which HL is a recognized feature, Usher syndrome, Pendred syndrome and Jervell and Lange-Nielsen syndromes are the most frequent [16]. Monogenic hearing loss can be inherited in different ways. Autosomal recessive HL (ARNSHL) occurs in 80% of cases and is typically prelingual, while autosomal dominant HL (ADNSHL) accounts for about 20% of cases and is most often postlingual. In less than 1% of cases, the inheritance occurs through the X-chromosome or the mitochondria [17]. Monogenic hearing loss is an extremely heterogeneous trait, with

Figure 1: The sequence of a novel deletion frameshift mutation. An arrow indicates the mutation site (Deletion of T at bp 59).
over 100 mapped loci and 46 causally implicated genes (Hereditary Hearing Loss Homepage; http://webh01.ua.ac.be/hhh/).

The important role of intercellular communication via gap junctions has been confirmed by findings that certain CX gene mutations, particularly those of GJB2 and GJB6, cause hearing loss [18].

Gap junctions (GJs) are the only known intercellular channels linking the cytoplasm of adjacent cells. They facilitate intercellular exchanges of ion, metabolite and signaling molecules. GJs in all vertebrates are assembled from connexins (Cxs). Connexins are small transmembrane proteins that belong to an extensive protein family found in most metazoans. Six connexins can oligomerize to form a hemichannel (connexon) in the cell membrane [4]. The Cx gene family has twenty-one members in human genome [19]. New information on the physiological roles of vertebrate connexins has emerged from genetic studies. Mutations in connexin genes underlie a variety of human diseases, including deafness, demyelinating neuropathies, and lens cataracts [20].

To date, more than 154 GJB2 mutations have been identified in the coding exon of GJB2 (http://davinci.crg.es/deafness), but a single chain-termination mutation, 35delG, accounts for up to 70% of pathologic alleles in many populations. Although this mutation is common in Western Europe and the Middle East [21], much lower frequencies have been observed in Asia [22]. The 35delG mutation exhibits linkage disequilibrium, and haplotype analysis suggested that it arose from a single individual in the Middle East approximately 10,000 years ago [23].

The GJB2 mutation spectrum diverges substantially among populations, as reflected by specific ethnic biases for common mutations like 235delC in the Japanese (carrier rate of 1%–2%) [24], 167delT in the Ashkenazi Jewish population (carrier rate of 7.5%) [25], V37I in Taiwan (carrier rate of 11.6%) [26], R143W in Ghana and W24X in Indians [9]. Despite this variability, the combined frequency of all GJB2 mutations is sufficiently high in most populations to make mutation analysis of this gene a clinically useful, and therefore widely available, genetic test. Among the Caucasians, populations of Slovak Romany, Italy and Northeastern Hungary show very high rate of Cx26 mutations. On the contrary, relatively low frequency rate was observed in a few African populations; Kenya (2%) and Sudan (7%) while high frequencies in Tunisia (17%) [2].

In Egypt, Meguid et al. (2008) [10] reported that the frequency of 35delG mutation in GJB2 gene among cases with recessive nonsyndromic congenital sensorineural hearing impairment was 10.17%. All cases were homozygous for the mutation. Compared to other populations, 35delG mutation comprised a lower frequency among Egyptians which confirms the genetic heterogeneity and ethnic variation of nonsyndromic sensorineural hearing impairment. They stated that audiological evaluation of cases with 35delG mutation revealed that severe hearing loss was detected in 66.7% of cases, and moderate hearing loss in 33.3%. In the present study, we aimed to characterize the mutation profiles, of 78 patients with autosomal recessive nonsyndromic hearing loss in Egypt to find out any other novel mutations. The entire coding region of the GJB2 gene was directly sequenced in all patients.

We describe for the first time a new mutations in GJB2 gene, which also has not been previously described, is a deletion of T at position 59 (c.59delT), in the intracellular domain of connexin 26 among 9% of the studied cases, resulting in a frameshift at the 20th amino acid, leading to a premature termination of the protein that would severely disrupt the protein structure.

The structure and function of human connexins have been extensively studied at the biochemical and physiological levels. Studies of mutant forms of connexin have preceded their connection to human disease. Site-directed mutagenesis and domain replacement forms of human Cx26 have aided in the definition of important and essential elements of connexin primary structure [27]. Connexins contain four transmembrane domains (TM1-TM4), two extracellular domains (EC1-EC2), one cytoplasmatic loop (CL), and N- and C-cytoplasmatic termini (NT-CT). The N-terminal domain is involved in the process of membrane integration and hexamer formation [28] and, together with the first transmembrane domain, determines voltage gating. The extracellular loops regulate the connexon-connexon interactions, including heterotypic channel formation. Each loop contains three cysteine residues, conserved across all connexins and that form essential intramolecular disulfide bonds [29]. The intracellular loop and C-terminal domains regulate pH gating [30]. The TM domains are important for protein folding.

Many HL-causing mutations of GJB2 gene have been reported in the connexin-deafness database (http://davinci.crg.es/deafness/). However, after reviewing the literature, our described novel mutation had not been previously identified for cases with autosomal recessive nonsyndromic hearing loss. One novel mutation at position 59 (c.59T>C) affecting a highly conserved residue within the N-terminal domain of GJB2, was previously described in an adult with apparently sporadic congenital HL in Austria [31]. These findings may indicate that this site is a mutational hot spot.

Summing up, in addition to the 35delG mutation which was previously reported to occur in almost 10.17% of Egyptian individuals suffering from HL [10], this study emphasises the importance of GJB2 gene mutations in inherited cases of nonsyndromic sensorineural hearing loss in Egypt. A
novel mutation was identified. The characterization of novel mutant alleles may contribute to a better understanding of the function of the connexin 26 domains. Moreover, these data show the importance of further investigations and future genetic counseling for GJB2 gene mutations.

References


