Importance of the diagnosis of protein connexin 26 mutations in the integral management of nonsyndromic congenital deafness

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ABSTRACT

Background. Congenital deafness is a public health problem affecting 2-3/1000 newborns in Mexico. Neonatal audiologic screening allows early detection with important implications for the functional prognosis. About 70% of cases of congenital deafness are associated with a genetic etiology with an autosomal recessive pattern of inheritance. Most cases are caused by mutations in the GJB2 gene, which codifies connexin 26. The three most commonly reported mutations in this gene are c.35delG, c.167delT and c.235delC.

Methods. After obtaining informed consent, DNA was extracted from a blood sample, and the three previously mentioned mutations were searched for using PCR-RFLP or PCR followed by sequencing.

Results. Molecular analysis was carried out in 11 patients. In five of these patients, a change in sequence was observed. In none of the patients were c.167delT and c.235delC mutations found. One patient was homozygous for c.35delG and another patient was heterozygous for c.35insG, which is a mutation not previously reported. A third patient was heterozygous for c.34G>T. Two additional patients had the c.79G>A (p.V27I) polymorphism.

Conclusions. Frequency of the three mutations analyzed was lower compared to other populations. Five sequence changes were observed, two polymorphisms and three mutations, one of them novel. This study also demonstrates the relevance of early diagnosis and multidisciplinary management and the importance of determining the genetic basis of this disease in pediatric patients with congenital deafness.

Key words: congenital deafness, connexin 26.

INTRODUCTION

Congenital deafness is a global public health problem. Its incidence varies with different ethnic groups.1 Several studies indicate that in Mexico ~2-3/1000 children are born with hypoacusia.2,3 The diagnosis of hearing loss during the first months of life is of great importance because children who are identified in a timely manner and who are offered early treatment have a better cognitive, language and social development. For this reason, in Mexico the program of Neonatal Hearing Screening and Early Intervention 2007-2012 (TANIT) was implemented, which includes strategies for early detection of deafness in institutions affiliated with the Secretariat of Health.3

Part of the complexity of the study of congenital deafness is to determine its etiology, which will impact on the various management and treatment decisions for the patient including the possibility of cochlear implants and
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Congenital deafness

1/3: Environmental
TORCH, infections, hypoxia, intraventricular hemorrhage, trauma

2/3: Genetics
30% Syndromic
Usher syndrome
Pendred syndrome
Waardenburg syndrome
Norrie syndrome, among others

70% Nonsyndromic
20-25% Autosomal dominant
75-80% Autosomal recessive
1% X-linked
1% Mitochondrial

Figure 1.
Etiological classification of congenital deafness. Etiologic heterogeneity is observed in the cases of congenital deafness. The autosomal recessive nonsyndromic are the most frequent.
SUBJECTS AND METHODS

Following institutional approval by the Committee of Ethics and Biosafety of the Investigation Protocol (HIM/2010/011) the patients who came in for treatment during the last 5 years (2007-2012) to the institution with a diagnosis of NSCD, without apparent perinatal risk factors, were identified. After obtaining Informed consent, a 1-ml sample of peripheral blood was taken and DNA was extracted using the Puregene Kit (Qiagen) according to the manufacturer’s specifications. Subsequently, the oligonucleotides were designed to perform amplification with the PCR technique of the three fragments of the GJB2 gene where the three most frequently reported mutations are found (Table 1).

For identification of the c.35delG mutation, sequencing was carried out (in both chains) from a 210-bp fragment of the coding region. Identification of the mutations c.235delC and c.167delT was performed using the restriction fragment length polymorphism (RFLP) technique. The enzymes used were 1) PstI to identify the mutation c.167delT, which recognizes the sequence 5’-CTGCAG-3’ and breaks the normal sequence, producing a 116-bp fragment and another of 47 bp. The PCR product with the mutation c.167delT is not produced at the time of the break. 2) ApaI was used for the search of the c.235delC mutation. Its recognition sequence is 5’-GGGCCC-3’. In the normal sequence it produces 151- and 59-bp fragments. If the mutation c.235delC is found, the enzyme does not make the break. RFLPs were visualized on agarose gel using 2% ethidium bromide with a 50-bp molecular weight marker as a reference to confirm the expected size of the fragments generated.

RESULTS

There were 96 patients with probable diagnosis of nonsyndromic deafness identified. In 63 of the patients, there were events associated with adverse factors at birth, so they were excluded from the study. Of the 33 remaining cases of nonsyndromic congenital deafness (Table 2), 11 had a molecular analysis performed (Figure 2). In all cases, PCR-RFLP study allowed for ruling out the presence of mutations c.167delT and c.235delC. In five cases, changes in the nucleotide sequence of the 210-bp amplified fragment were identified. Three of the variants corresponded to mutations and two to polymorphisms. In patient 5, the c.35delG mutation was identified in the homozygous state (Figure 3). In patient 4, the c.34G>T mutation was heterozygous (Figure 4). In patient 8, the c.35insG mutation was heterozygous (Figure 5). The latter has not been reported previously in the literature. In patients 2 and 3, c.79G>A polymorphism was heterozygous (Table 2).

DISCUSSION

Of the group of patients in the HIMFG with deafness, we identified 96 patients with probable diagnosis of nonsyndromic congenital deafness. Of these, only 33 cases were candidates for molecular study because the etiology was considered to be exclusively genetic. It is interesting to note that of the original group of 96 candidates, in 63 subjects it was possible to identify a cause, environmental risk factor or syndrome; therefore, they were rejected as candidates for molecular genetic study of the connexin 26 gene. However, this high frequency of nongenetic causes of deafness is an important data of the profile of the population attending our hospital and differs from that reported worldwide where most cases correspond to nonsyndromic congenital deafness or are not associated with predisposing perinatal factors. This situation could be explained by the type of institution because patients treated at the HIMFG are referred for evaluation across the country, particularly cases requiring tertiary care due to their complexity or associated features, which indicates a significant differ-

Table 1. Oligonucleotides used to amplify regions of GJB2 containing variants of this gene most frequently associated with deafness

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sense</th>
<th>Antisense</th>
<th>Tm (°C)</th>
<th>Expected product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG</td>
<td>AGCATGCTTGCTTACCAGACTCA</td>
<td>TCTTTGCAAGCCACAACGAGGA</td>
<td>58</td>
<td>234</td>
</tr>
<tr>
<td>c.167delT</td>
<td>AGCATGAAAGATCATGCTGCTACCC</td>
<td>GGGAGATGGGAAATGATGATGT</td>
<td>57</td>
<td>163</td>
</tr>
<tr>
<td>c.235delC</td>
<td>GCTGGAAGACGTGGCTACGA</td>
<td>CGATGGACCTTCTGGGTTTT</td>
<td>58</td>
<td>210</td>
</tr>
</tbody>
</table>

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The main causes were for nonisolated deafness and adverse perinatal factors. We identified only 33 (34%) cases of nonsyndromic congenital deafness (NSCD).

Table 2. Clinical, demographic, and familiar characteristics and results of molecular testing in the 11 patients studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Origin</th>
<th>History of consanguinity</th>
<th>Family history of deafness</th>
<th>Grade and type of deafness</th>
<th>Mutation in GJB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>8 months</td>
<td>State of Mexico</td>
<td>No</td>
<td>Yes (younger sister)</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>1 year</td>
<td>State of Mexico</td>
<td>Yes</td>
<td>Yes (sister)</td>
<td>Sensorineural medium bilateral</td>
<td>Polymorphism c.79G&gt;A</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>16 years</td>
<td>State of Mexico</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>Polymorphism c.79G&gt;A</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>5 years 3 months</td>
<td>State of Mexico</td>
<td>Yes</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>c.34G&gt;T heterozygote</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>2 years</td>
<td>State of Mexico</td>
<td>No</td>
<td>Yes (cousin, sister)</td>
<td>Sensorineural profound bilateral</td>
<td>c.35delG homozygote</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>3 years</td>
<td>Michoacán</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>2 years</td>
<td>State of Mexico</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>3 years</td>
<td>Guanajuato</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>c.35insG heterozygote</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>2 years</td>
<td>Michoacán</td>
<td>Yes</td>
<td>Yes (cousin, brother)</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>9 years 3 months</td>
<td>D.F.</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>11 years</td>
<td>Michoacán</td>
<td>No</td>
<td>No</td>
<td>Sensorineural profound bilateral</td>
<td>---</td>
</tr>
</tbody>
</table>

Figure 2. Causes of deafness identified in the 96 patients studied. The main causes were for nonisolated deafness and adverse perinatal factors. We identified only 33 (34%) cases of nonsyndromic congenital deafness (NSCD).
In three cases the existence of consanguinity between the patient’s parents was confirmed and in four patients there was family history of hereditary deafness affecting the same generation, which suggests an autosomal recessive inheritance pattern. In most of our patients the deafness was of a profound bilateral sensorineural type. This is the most common type of deafness in the cases of nonsyndromic congenital deafness; therefore, the population analyzed reflects that which has been previously reported in the literature.4

Mutations in the GJB2 gene were identified in 3/11 patients analyzed, which corresponds to 27% of the cases. This is consistent with a proportion similar to what is expected according to what is reported in the literature. It is calculated that up to 20% of cases of nonsyndromic congenital deafness originate due to mutations of this gene.15 However, the sample analyzed for this study is too small to be able to establish frequencies. In addition, only the three most frequent mutations were searched for, including sequencing of a 210-bp fragment, so that the presence of mutations in the gene regions not analyzed cannot be ruled out.

Thus, in only one of our cases was the c.35delG mutation identified, the most frequently associated with autosomal recessive nonsyndromic deafness. This corresponds to 9% of the cases studied, which comprises a smaller percentage to that reported in the literature and corresponds to 28 to 63% of the GJB2 mutations depending on the population.6

To our knowledge, there are no publications that report on the type or frequency of mutations associated with cases of congenital deafness in the Mexican population. Recently, two groups of researchers in Mexico presented their results to the National Congress of Human Genetics 201214-16 in which only 3% of the cases were homozygous for c.24delG. This corresponds to an even smaller percentage to that found in this study. However, as has already been mentioned, our sample is even smaller for determining the frequencies. In our case it is notable that the mutation was found in the homozygous

Figure 3. Family pedigree of the patient and electropherograms of the five patients with c.35delG. (A) The proband (III.2) and a cousin (III.4) present diagnosis of NSCD. (B) Electropherograms: a) normal sequence, b) sequence sense in which the deletion in the homozygous state is observed, c) antisense sequence in which deletion is confirmed.
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Figure 4.

Family pedigree and electropherograms of the patient with c.34G>T.

(A) Family pedigree of six generations in which the proband (VI.1) is observed with diagnosis of NSCD. Consanguinity: 1/256, coefficient of kinship: 1/128.

(B) Electropherograms: 
   a) normal sequence, 
   b) the sense sequence in which transversion is observed in heterozygous state, 
   c) anti-sense sequence in which change is corroborated.
state and that there was no consanguinity between the patient’s parents or are from the same Mexican state, for which reason it would be important to determine the frequency of healthy carriers (heterozygous) of the mutation in the Mexican population.

In one of the patients the c.34G>T change was found in the heterozygous state. This had been previously reported, but its pathological effect had not been demonstrated. At present it is classified as a probable pathological change (rs104894408). This mutation of incorrect direction causes a glycine change for cysteine in position 12 within the aminoacid chain of the protein (p.G12C). Glycine is an aminoacid with aliphatic lateral chains and is hydrophilic and polar, whereas cysteine is also a hydrophilic aminoacid and polar but has lateral chains with sulfur atoms, giving it the capacity to produce disulfide bonds. It is probable that for this reason it generates disturbances in the protein structure. This change was reported in patients with nonsyndromic congenital deafness in a study performed by Putcha et al. In this study the GJB2 and GJB6 genes were analyzed in more than 7000 patients with nonsyndromic congenital deafness in

![Family pedigree and electropherograms of patient 8 with the c.35insG.](image-url)
the U.S. The p.G12C change corresponded, as the only alteration, to only 0.4% of all mutations detected in the two genes studied.

Another mutation was found that corresponds to an insertion of one guanine within the sequence of the six guanines in which occurs that of c.35delG. As has already been mentioned, this mutation has not been reported and causes a change in the reading frame with a high premature codon in codon 67. In this case the insertion was found in the heterozygous state. On the face of this alteration, there are various aspects to consider. It cannot be ruled out that this is a compound heterozygote because molecular analysis was not carried out of the complete gene (only of specific mutations by PCR-RFLP and of a fragment by sequencing). Neither can the presence of another mutation in the other gene copy be ruled out, a mutation which would be in a different location to those studied.6

Another possibility is that it is a double heterozygote because the connexins operate in groups of six proteins and can be linked to other connexins, particularly connexin 30, forming a heteromeric connexon.18 Cases of double heterozygous with a mutation in an allele of the GJB2 gene and another mutation in an allele of this gene family, mainly in the GJB6 gene, codifies for connexin 30 and is found in the same locus.19

Finally, we identified the polymorphism c.79G>A in two patients in a heterozygous state. It is interesting that this polymorphism has been identified in 41% of patients14 so it is probable that this change is common in the Mexican population and not associated with disease. However, the possibility has been proposed that this single nucleotide polymorphism with another change in the sequence of the GJB2 gene could cause deafness.20 Therefore, it would be important to determine the frequency of this change in Mexican controls.

In conclusion, this preliminary study represents the first report of the frequency in the mutations c.35delG, c.167delT and c.235delC of the GJB2 gene in Mexican patients with congenital deafness. In this group the presence of the mutations c.167delT and c.235delC was ruled out and in five patients (45% of the cases) there were changes found in its sequence: two polymorphisms and three mutations, one of them previously reported. This highlights the importance of knowing the genotypic profile of our population.

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