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RESEARCH ARTICLE

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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genetic counseling. Several international guidelines state that one of the fundamental aspects of the comprehensive management of patients with hearing loss is to define the genetic profile, which can vary in each population (or even within the family) and have individual mutations determined by the characteristics of the ethnic group and the patient in particular.[4]

In a third of patients with congenital deafness, it is possible to identify an environmental etiology such as infections, adverse perinatal factors, etc. The remainder have a genetic etiology[5] (Figure 1) with a large heterogeneity. Up to 70% are nonsyndromic congenital deafness and 30% are attributed to syndromic deafness.[6] Among these, some of the main ones, according to frequency or to high clinical impact are Waardenburg syndromes (OMIM 193500), Usher (OMIM 276900), Pendred (OMIM 274600), branchio-oto-renal (OMIM 113650), Jervell and Lange-Nielsen (OMIM 220400), Alport (203780) and mitochondriopathies.

Nonsyndromic congenital deafness (those in which deafness is the only manifestation) has a great genetic heterogeneity and >100 loci have been identified related with its etiology. The inheritance pattern identified, mostly present in >70% of cases, is autosomal recessive.[7] For the designation of congenital deafness cases and to highlight their etiologies, the internationally used nomenclature uses the abbreviation DFN (for deafness) followed by a letter. Thus, the letter A is used to define the autosomal dominant inheritance pattern (DFNA), B stands for autosomal recessive (DNFB) and X for X-linked (DFNX). Finally, the following number which each is designated corresponds to the order in which the gene responsible was mapped. Additionally, deafness may be due to a mutation in the mitochondrial genome, so it can be considered as a mitochondropathy.

Several studies in Europe, Asia, U.S. and South America show that 50-80% of cases of nonsyndromic congenital deafness are autosomal recessive and arise as a result of mutations in the DFNB1 locus (OMIM: 220290) at 13q11-q12.[8] There are two genes found in this region: GJB2, which codes for the protein connexin 26 and is mutated in 98% of cases of DFNB1 and the GJB6 gene, which encodes the protein connexin 30 and causes the remaining 2% of the DFNB1 deafness. It has been observed that there are other mutations in the same gene that cause autosomal dominant deafness (DFNA3)[9] and others that cause syndromic deafness with accompanying dermatological conditions.[10]

We have identified three autosomal recessive mutations in *GJB2* as the most frequent; c.35delG in Caucasian population, c.167delT in the Ashkenazi Jewish populations and c.235delC in Asians.[6-12] These three mutations produce a shift in the reading frame and a high premature codon. The most frequently reported mutation is the c.35delG, which could correspond to up to 70% of the mutant alleles of the *GJB2* gene related with deafness.[13]

To date there are no published studies on Mexican patients with congenital deafness. Although there are some preliminary works that have been presented at national conferences,[14] the most common mutation in our population is not currently known. Thus the "Multidisciplinary Study Group of Congenital Deafness" of the Hospital Infantil México Federico Gomez (HIMFG) proposed the research protocol to determine, in patients with nonsyndromic congenital deafness treated in the hospital, the three most common mutations in *GJB2*.

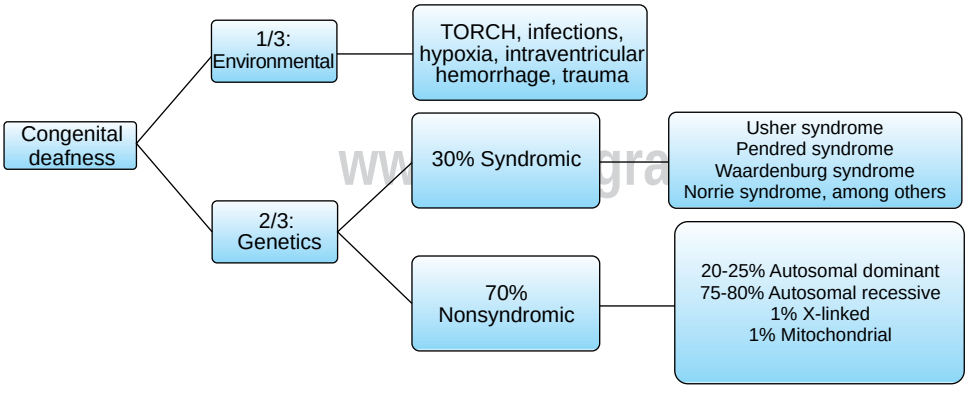

Figure 1.

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) *Pst l* 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) *Apa I* 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-

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

Variant	*Sense*	*Antisense*	*Tm (ºC)*	*Expected product (bp)*
c.35delG	AGCATGCTTGCTTACCCAGACTCA	TCCTTTGCAGCCACAACGAGGA	58	234
c.167delT	AGCATTGGAAAGATCTCGCTCACC	GGGAGATGGGGAAGTAGTGATCGT	57	163
c.235delC	GCTGCAAGAACGTGTGCTACGA	CGATGCGGACCTTCTGGGTTTT	58	210

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

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

ence in the type of population studied. The majority of patients who underwent molecular studies came from the State of Mexico (six patients) and from Michoacan (three patients). This is explained, in the first case, by the geographic proximity to the institution and, second, by the agreement of management and treatment of these patients that the institution has with the state. This may also represent a possible population bias.

Of the 11 patients who had molecular studies performed, in only three patients was the diagnosis established with neonatal hearing/screening tests. The remaining patients were diagnosed at a later time (age range between 2 and 5 years of age). Of the latter group, six patients had not developed language ability. Comparatively, some of the patients diagnosed in a timely manner and who used hearing aids and were admitted to a program for cochlear implant evaluation and began their rehabilitation and treatment from the time of clinical suspicion had already developed language ability. This highlights the great importance of early detection of hearing loss through the neonatal hearing screening program, which confers a better prognosis for the integration and quality of life of these patients.

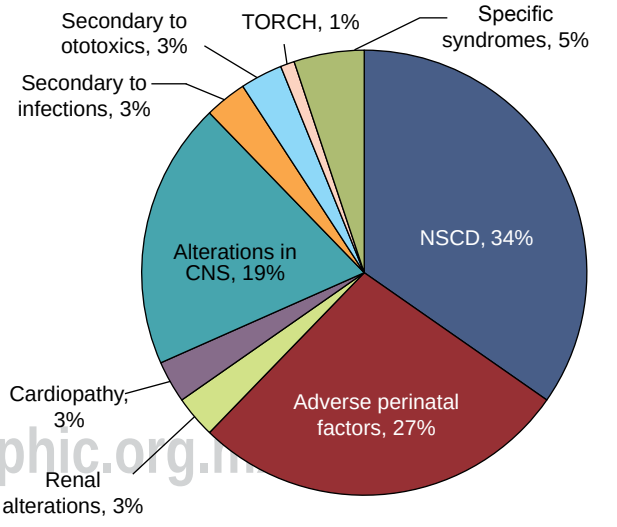

Figure 2. Causes of deafness identified in the 96 patients studied. The main causes were for nonisolated deafness and adverse perinatal factors. We identified only 33 (34%) cases of nonsyndromic congenital deafness (NSCD).

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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 2012[14-16] 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

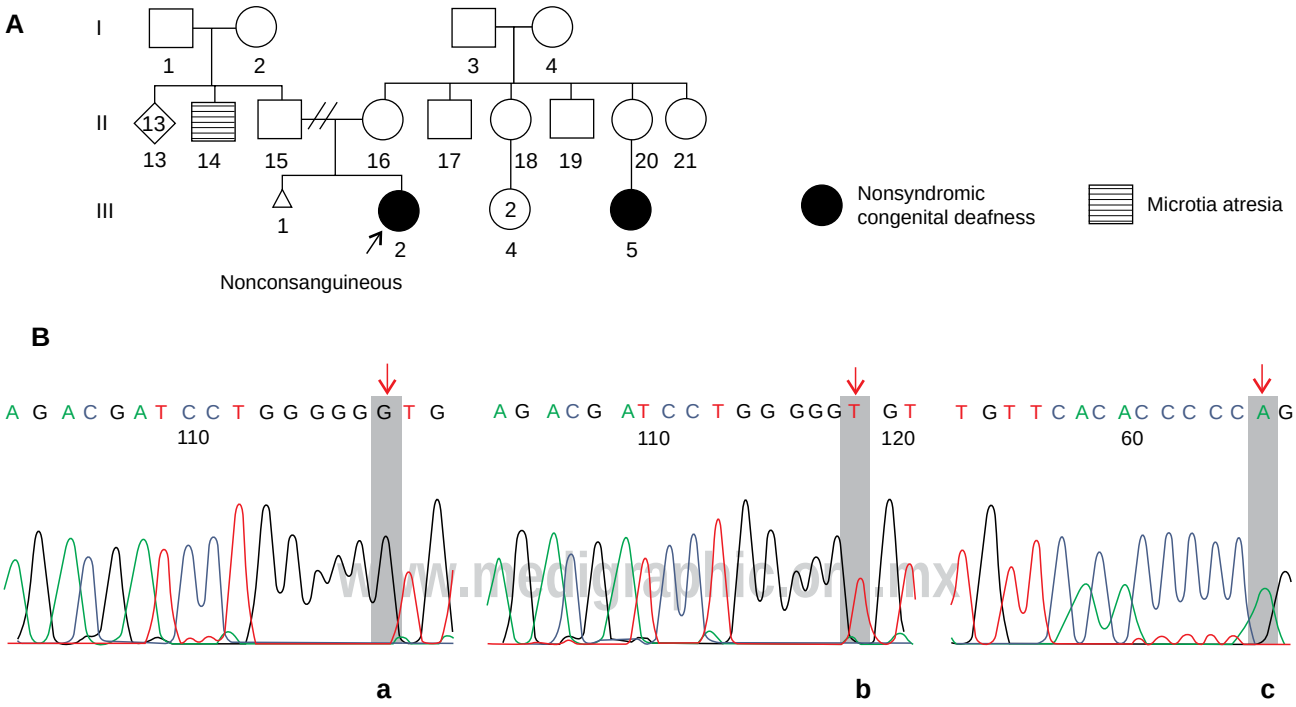
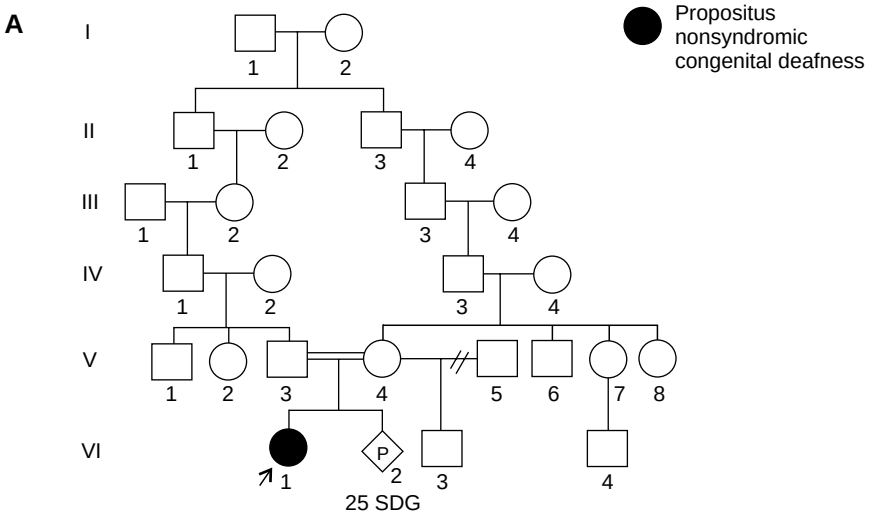


Figure 3. Family pedigree of the patient and electropherograms of the five patients with c.35delG. **(A)** The proband (III.2) and a cousin (III.4) present diagnosis of NSCD. **(B)** Electropherograms: **a)** normal sequence, **b)** sequence sense in which the deletion in the homozygous state is observed,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



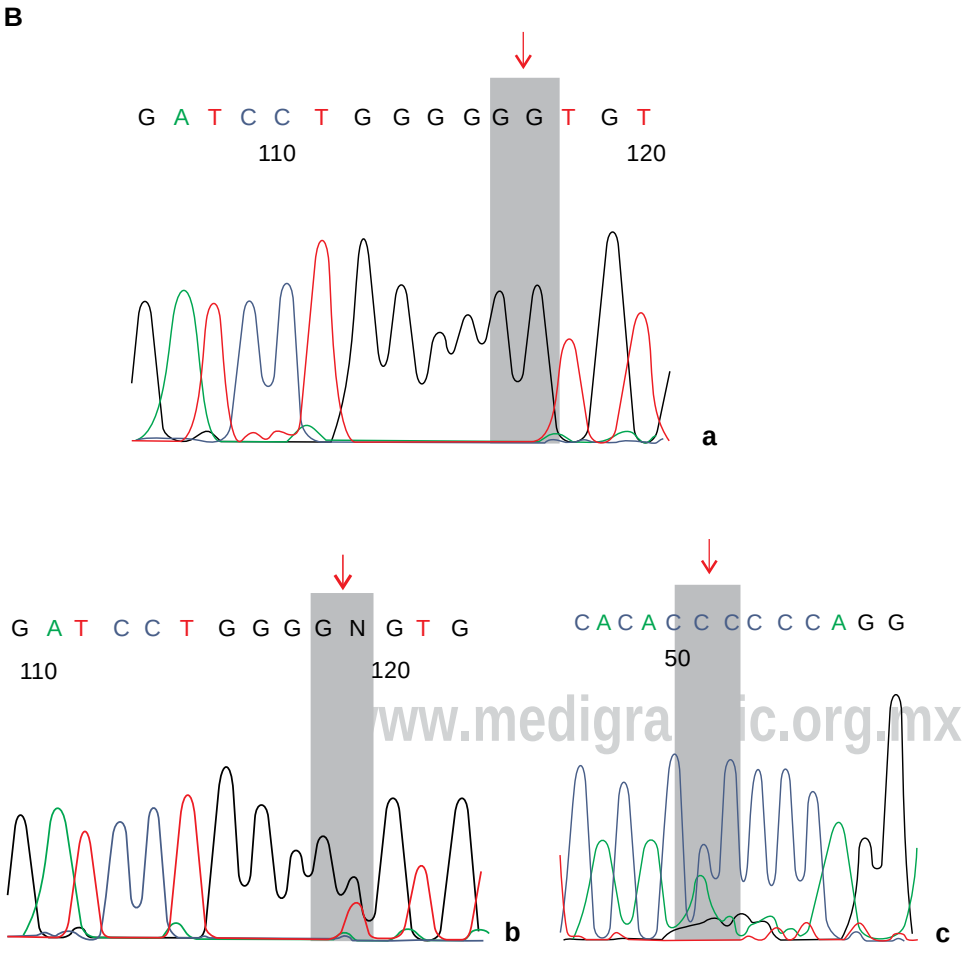


Figure 4.

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.

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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,[17] 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.[17] In this study the *GJB2* and *GJB6* genes were analyzed in more than 7000 patients with nonsyndromic congenital deafness in

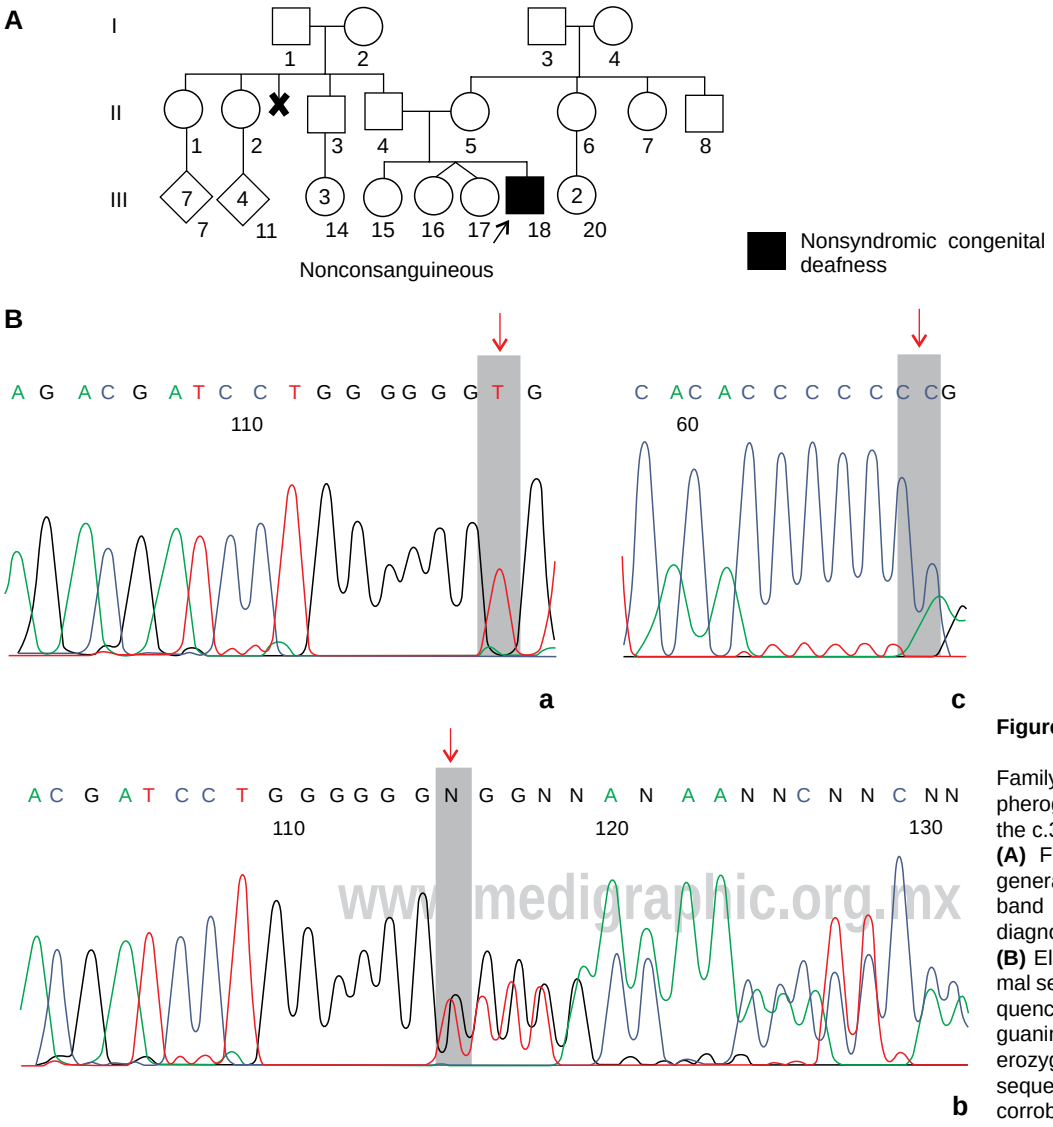

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Figure 5.

Family pedigree and electropherograms of patient 8 with the c.35insG.
(A) Family pedigree of three generations in which the proband (III.7) is observed with diagnosis of NSCD.
(B) Electropherograms: **a)** normal sequence, **b)** the sense sequence in which the insertion of guanine is observed in the heterozygous state, **c)** anti-sense sequence in which insertion is corroborated.

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 patients[14] 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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