Study of six mutations in the gjb2 *gene in Cuban patients with nonsyndromic sensorineural deafness*

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RESEARCH

ABSTRACT

Deafness is a partial or total hearing loss that can appear at any ages and with different degrees of severity. About 50% of hearing disorders have a genetic origin, and among them, the nonsyndromic sensorineural deafness represents 70% of the cases. Out of these, the 80% correspond to autosomal recessive inheritance deafness. Autosomal recessive deafness has not been characterized enough at molecular level in Cuba. The purpose of this work was to determine the frequency of six *gjb2* mutations in the nonsyndromic sensorineural deafness. In order to detect W24X, M34T, E47X, V95M, W77R, and 35delG mutations in the *gjb2* gene, we employed the PCR-RFLP and the heteroduplex techniques. Four out of the six tested mutations were detected only by using the PCR-RFLP technique, being the most frequent mutations 35delG (70%) and M34T (20%). The other two mutations detected employing this technique (W77R and E47X) represented 5% each one. The heteroduplex analysis was very informative because we detected the formation of seven heteroduplexes in the analyzed fragments, corresponding to six different patients. The formation of heteroduplexes was obtained in two amplified fragments corresponding to one of these patients. In two of these patients, the heteroduplex complemented one mutation previously detected by the PCR-RFLP technique. This work allowed us to identify five families in which the carrier diagnosis of the autosomal recessive inheritance deafness led to the definition of the genotype responsible for the illness.

Keywords: *gjb2*, Connexin 26, mutation, *PCR-RFLP*, heteroduplex

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RESUMEN

Estudio de seis mutaciones en el gen *gjb2* en pacientes cubanos con sorderas neurosensoriales no sindrómicas. La sordera es una pérdida parcial o total de la audición, que puede manifestarse a cualquier edad y con diferente grado de severidad. El 50% de las afectaciones de la audición presentan un origen genético, y entre ellas, las sorderas neurosensoriales no sindrómicas representan el 70%. Las más frecuentes son las de herencia autosómica recesiva, que constituyen más del 80% de los casos. En Cuba, las sorderas autosómicas recesivas a nivel molecular no se han caracterizado suficientemente. El objetivo de este estudio fue determinar la frecuencia de seis mutaciones en el gen *gjb2*, en pacientes cubanos con sorderas neurosensoriales no sindrómicas. Para la pesquisa de las mutaciones W24X, M34T, E47X, V95M, W77R y 35delG en este gen, se empleó la técnica PCR-RFLP y la técnica de heteroduplex. De las seis mutaciones, solamente cuatro se detectaron mediante la técnica PCR-RFLP, y la 35delG (70%) y M34T (20%) resultaron más frecuentes. Cada una de las otras dos mutaciones (W77R y E47X) representó el 5%. El análisis de heteroduplex resultó ser muy informativo al detectarse la formación de siete heteroduplex en los fragmentos analizados, correspondientes a seis pacientes, y en un paciente se observó la formación de heteroduplex en dos fragmentos amplificados. En dos de los pacientes, el heteroduplex complementó una mutación anteriormente detectada por la técnica PCR-RFLP. Se identificaron cinco familias en las que el diagnóstico de los portadores de la sordera hereditaria autosómica recesiva llevó a definir el genotipo responsable de la enfermedad.

Palabras clave: *gjb2*, conexina 26, mutación, *PCR-RFLP*, heteroduplex

Introduction

Deafness is the partial or complete loss of the hearing capacity, affecting 6 to 8% of the developed countries' population at any age and with a varying degree of severity [1]. Its consequences to the individual and the society are highly influenced by the age of appearance and its severity. When occurring early in childhood and comprising a severe defect, it causes profound limitations for speak learning and subsequent cognitive and psico-social disorders. In the elderly, it affects the quality of life due to the isolation of the individual affected.

The fifty percent of hearing affections are genetically determined [2], with the neurosensory non-syndrome deafness without any other accompanying symptoms but isolated hypoacusia accounting for the 70% of the cases [3].

The non-syndrome hypoacusia is manifested as a clinically and genetically heterogeneous group, with the inherited recessive autosomy representing up to 80% [3].

Inherited deafness is genetically heterogeneous, with more than 100 genes identified as responsible, disseminated throughout the human genome [4]. The *gjb2* gene is one of them, located in the 13q12 chromosome and coding for the Connexin 26 protein [5, 6]. Defects in this gene account for both dominant

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and recessive autosomic inherited patterns of deafness [1, 7].

The molecular diagnosis of inherited deafness allows detecting the people carrying the genetic imprints which predisposing to suffer it and to assist the for their reproduction. The genetic consultation defines the risk for a couple of having a child affected by the disease and offers the possibility of pre-born diagnosis. Additionally, the molecular diagnosis characterizes the possible disease based on the genetic defect detected, very relevant when considering the new options for personalized treatment of genetic diseases.

The high genetic heterogeneity of deafness makes it difficult to be addressed by molecular diagnosis, with all the genes demonstrated to be involved in the inherited pattern detected having to be identified. This is also troublesome with the recessive autosomic pattern due to inaccuracy while detecting it and when considered as sporadic for the patient, based on insufficient information provided about familiar deafness or environmental conditions triggering the disease, also having relatives carrying some of those mutations in an heterocygotic pattern.

The high frequency of affections in the *gjb2* gene accounting for recessive autosomic deafness is the basis to molecularly characterize the disease by an early diagnosis. However, this is a difficult task due to more than 90 mutations reported in this gene [8].

For this reason, the goal is the search for those highly frequent mutations in the entire gene *gjb2* in the population under study. Otherwise, a very convenient strategy involves the use of analytical methods to restrict the mutation to a segment of the gene, facilitating its identification.

The recessive autosomic deafness has been insufficiently characterized in Cuba at the molecular level. The aim of this work was to determine the frequency of six mutations in the *gjb2* gene in Cuban patients suffering from non-syndrome neurosensitive deafness.

Materials and methods

Sampling

Peripheral blood samples were collected from 55 patients with non-syndrome neurosensitive deafness and without familiar relationship. Of them, 23 referred familiar precedents of the disease, considered as recessive autosomic outcomes based on family tree analyses. The rest 32 accounted for sporadic cases, without any record of familiar affection.

All the patients were remitted to our laboratory by the Ear-nose-throat services of the Clinical-Surgical "Hermanos Ameijeiras" and Pediatric "William Soler" hospitals. All the patients signed an informed consent agreeing to provide a blood sample for research. Ten milliliters were collected in sample tubes containing 150 mL of 5.6% EDTA and stored at 4 °C for 24-48 hrs. Besides, a survey was carried out to structure familiar trees.

Peripheral blood DNA extraction and purification

The DNA was extracted by the Bunce and Welsh's method [9], based on salt precipitation of proteins present in the sample, and precipitated by adding two volumes of absolute ethanol. The pelleted DNA was washed with 70% ethanol, vacuum-dried and resuspended in 150 µL of sterile water heated at 56 °C for 30 min.

Integrity test of DNA purified by electrophoresis

In order to verify the integrity of the DNA obtained and estimate its concentration, submarine electrophoresis in 0.8% agarose gels was carried out in 0.5 X Tris-Borate (TBE) running buffer [10]. A loading buffer (LB-BB) containing Ficoll 400 and a dye (bromphenol blue) was added to the sample prior to electrophoresis. The DNA molecules were visualized in the gels by etidium bromide staining (at 0.5 µg/mL) and exposure to ultraviolet light (252 nm). A volume of 10 µL from each sample was applied, composed of 2 µL of the sample and 2 µL of the LB-BB 6X and 6 µL of sterile water.

Detection of mutations

Primers

The change caused by each independent mutation in the restriction pattern for the enzymes commercially available was analyzed with the PCGene software [11]. In those cases satisfactorily detected, a primer set was designed with the OLIGO software version 4.1 (National Biosciences Inc.) to amplify the fragment containing the mutation and easily differentiated by enzyme restriction and electrophoresis.Primers sets were designed for Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) systems to detect the following 5 mutations: W24X, M34T, E47X, V95M and W77R and with that previously described to detect the 35delG *gjb2* mutation [12], and introduced in the Laboratory for Genetic Molecular Diagnosis at the Clinical-Surgical "Hermanos Ameijeiras" hospital (Table 1).

PCR-RFLP technique

The mutations were detected by the PCR-RFLP technique [10], based on allele differentiation for each mutation by enzyme restriction of an amplified gene fragment carrying it. Six PCR-RFLP systems were used to detect the previously mentioned mutations (Table 1). Reaction mixes were prepared containing 1 mM $MgCl_2$, 0.16 mM of each deoxyribonucleotide, 0.7 µM of each primer, and 0.75 units of the Taq DNA polymerase (Promega, USA) and 1X reaction buffer supplied by the manufacturer to a final volume of 15 µL. The amplification was carried out in a thermocycler PTC 150 (MJ Research, USA). The parameters of the amplification programs used were: Mutations W24X and M34T-denaturing at 94 °C for 5 min, 10 cycles (94 °C, 15s; 62 °C, 15s; 72 °C, 30s), 30 cycles (89 °C, 15s; 62 °C, 15s; 72 °C, 30s), followed by a final extension at 72 °C for 10 min; mutations E47X and W77R -denaturing at 94°C for 5 min, 10 cycles (94 °C, 15s; 65 °C, 15s; 72 °C, 30s), 30 cycles (89 °C, 15s; 65 °C, 15s; 72 °C, 30s), followed by a final extension at 72 °C for 10 min; mutation V95M-denaturing at 94 °C for 5 min, 1 cycle (94 °C, 2 min; 58 °C, 1 min; 72 °C, 4 min), 30 cycles (94 °C, 40s;

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58 °C, 30s; 72 °C, 1 min), followed by a final extension at 72 °C for 5 min; mutation 35delG- denaturing at 94 °C for 5 min, 30 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min), followed by a final extension at 72 °C for 7 min.

Each reaction set also included a negative control reaction, devoid of template DNA, and a positive control reaction containing a DNA template carrying the specific mutation).

Amplification products were checked by submarine electrophoresis in 1.5% agarose gels with 0.5X TBE as running buffer and applying 5 µL of each reaction volume. A sample of a molecular weight marker (100 bp DNA ladder, Promega, USA) was included in each run. When amplified as expected, 5 µL of each reaction product were digested for 16 hrs with the buffer and at the temperature recommended by the manufacturer.

The sample was genotyped by electrophoresis in 6% polyacrylamide gels [10] under 500 V for 3 hrs, and the restriction fragments were visualized by staining with etidium bromide and exposure to ultraviolet light. The genotype resulting from each mutation for a given individual was established from the restriction pattern observed by electrophoresis.

RNA/DNA Heteroduplex Assay

The RNA/DNA heteroduplex assay was used, due to the low prevalence of the six mutations studied for the gene *gjb2* in the human population [13]. It is a simple methodology detecting mutations regardless of their type.

Four sets of primers were designed, amplifying four overlapped fragments of the *gjb2* gene and spanning the entire Connexin 26 coding region (Table 2) [14].

The respective locations of mutations tested in the four fragments are: Fragment I 35delG, W24X, E47 and M34T), Fragment II- W77R and V95M, Fragments III and IV (none of the mutations studied). All the mutations were verified for causing the heteroduplex formation in their respective fragments prior to running the samples.

The amplification reaction was carried out for the four fragments as mentioned above for the PCR-RFPL technique, using a Minicycler (MJ Research, USA).

The programs used to amplify the four fragments of the *gjb2* gene in the heteroduplex assay were: Fragment I- denaturing at 94 °C for 2 min, 30 cycles (94 °C, 45s; 60 °C, 30s; 72 °C, 30s, followed by a final extension at 72 °C for 2 min; fragment II- denaturing at 94 °C for 2 min, 1 cycle (94 °C, 2 min; 58 °C, 1 min; 72 °C, 1 min), 30 cycles (94 °C, 40s; 58 °C, 30s; 72 °C, 30s), followed by a final extension at 72 °C for 7 min; fragments III and IV- denaturing at 94 °C for 2 min, 10 cycles (94 °C, 15s; 62 °C, 15s; 72 °C, 30s), 30 cycles (94 °C, 15s; 62 °C, 15s; 72 °C, 30s), followed by a final extension at 72 °C for 10 min. The results of the reactions were checked as previously mentioned.

The analysis of the correspondent heteroduplex fragment was carried out by taking 3-8 µL of the amplification product and denaturing it at 95 °C for 5 min and renaturing it at room temperature down to 37 °C. Afterwards, it was placed in an ice bath, 2 µL of the LB-BB were added and the prepared sample was run in a 20% mutation detection enhancement (MDE; FMC, USA) gel in 0.5 X TBE buffer for 15-20 hrs. The heteroduplex formation was visualized by staining with etidium bromide and exposure to ultraviolet light.

Table 1. Information of the PCR-RFLP systems used to detect the point mutations studied

Mutation	Primers	Sequences (5'-3')	Size of the amplified fragment (bp)	Restriction enzyme	Allele size (bp)
35delG (**)	35delG -1 35delG -2	GGTGAGGTTGTGTAAGAGTTGG CTGGTGGAGTGTTTGTTCCCAC	207	Bse I (Promega)	Normal: 207 Mutated: 181 + 26
W24X	W24X Cx26 del ID	GAGGTATAATTGACAGATGAA CAAACCGCCCAGAGTAGAAG	114	Xba I (Promega)	Normal: 114 Mutated: 88 + 26
M34T	M34T Cx26 del ID	CCTTTGCAGCCACAACGAT* CAAACCGCCCAGAGTAGAAG	144	Bcl I (Promega)	Normal: 121 + 23 Mutated: 144
E47X	E47X-A E47X-B	GCAAAGGAGGTGTGGGGAGAC* GGATGTGGGAGATGGGGAAGTA	106	Bfa I (Promega)	Normal: 106 Mutated: 85 + 21
V95M	Cx26-2B Cx26-E	CCAGGCTGCAAGAACGTGTG TCGAAGATGACCCGGAAGAA	279	Pml I (Promega)	Normal: 279 Mutated: 190 + 89
W77R	W77R-A W77R-B	CCATCTCCCACATCCGGCTC* GCCTTCGATGCGGACCTTCT	182	MspI (Promega)	Normal: 100 + 82 Mutated: 182

(*) Bases that had to be altered to generate the restriction site for the enzyme selected.
(**) Developed by Storm K *et al*. (1999) [12].

Results and discussion

The study of six mutations of the *gjb*2 gene followed two strategies. First, we hypothesized that the Connexin 26 gene is relevant in the ethiopathogenesis of inherited hipoacusia with recessive autosomic pattern and that the six mutations should account for a high percent of the alterations present in this gene. In this sense, we established the following strategy:

1. All the individuals affected were screened for the six mutations mentioned, always beginning with the 35delG mutation. The screening for new mutations were stopped if detecting two mutations (in Composed heterocygotic individuals) or verifying the heterocygocity for one of them.

2. A heteroduplex analysis of the gene will be carried out when not detected the two mutations

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Table 2. Heteroduplex systems used to locate the mutations in the *gjb2* gene

Gene *gjb2* fragment	Nucleotide sequence (5'- 3')	Amplified fragment (bp)	Fragment location in the gene*
I	CAAACCGCCCAGAGTAGAAG GTGATCGTAGCACACGTTCTTG	221	179 to 399
II	CCAGGCTGCAAGAACGTGTG TCGAAGATGACCCGGAAGAA	269	370 to 639
III	TCGAGGAGATCAAAACCCAGAAG GCAAATTCCAGACACTGCAATCA	255	550 to 804
IV	GCCTTGTCCCAACACTGTGGACT TGAGCACGGGTTGCCTCATC	231	714 to 943

(*) Positions in the messenger RNA nucleotide sequence reported by Lee *et al*. (1992) [14].

present in the individual, or will be limited to those fragments without mutations to avoid heteroduplex formation *per se.*

Results corresponding to the first phase indicated a prevalence of mutations lower than that previously assumed, determining a change in the strategy followed by a second phase of work:

1. Screening for the 35delG mutation (the only mutation showing a considerable frequency in the first phase)

2. A heteroduplex analysis of the gene will be carried out when not detected homocygocity for this mutation; excluding the fragment I when detecting heterocygocity for the 35delG mutation.

3. In case of heteroduplex formation for one of the fragments covering the other 5 mutations (fragments I and II), the respective mutations should be detected directly.

We verified in our laboratory the heteroduplex formation by these mutations, validating the second strategy.

Eighteen patients with familiar outcome and 17 sporadic ones were analyzed during the phase I of our study, and 4 and 12 (respectively) were analyzed during the second phase.

Other 4 patients (1 with familiar outcome and 3 sporadic), considered as occasional cases, were lately incorporated to the study, only being possible to be screened for the 35delG. They were taken into account to increase the number of individuals used to calculate the gene frequency for this mutation.

The heteroduplex analysis was carried out in 17 of all the patients studied in both phases of work.

Only four of the six mutations screened were detected by PCR-RFLP (Tables 3 and 4), with 20 chromosomes carrying any of them. The percent of each of these four mutations in respect to the alleles affected are represented in table 5. The most frequently detected mutations were the 35delG (70%) and M34T (20%), with the W77R and E47X accounting for the 5% each (Table 5).

The 70% prevalence of the 35delG mutation is very similar to that reported by Alvarez *et al.* (74%) when studying patients from Cuba and Spain [15], and by Morenoet *al.* (67.8%) in Spanish patients. The prevalence of the M34T mutation was below 1% in the study of Alvarezet *al.* [15], while being the second most frequent mutation in ours (with 20% of the chromosomes mutated). In the case of the other two mutations (W77R and E47X), the 5% values were very similar to the 4% values reported by Alvarezet *al.* [15], and those of 3.7% and 5.1% for E47X and W77R in Spanish and Cuban patients, respectively, reported by Morenoet *al.* [16].

The heteroduplex assay brought an important piece of information, with 7 heteroduplexes detected in the analized fragments, corresponding to five patients with one heteroduplex and another patient with heteroduplex formed in two of the amplified fragments. In two of these patients the heteroduplex complemented a previously detected mutation.

Five of the heteroduplexes formed corresponded to fragment III. The R184P mutation described for the Cuban-Spanish population by Alvarezet *al.* [15]

Table 3. Resultados del estudio molecular en pacientes con antecedentes familiares

Patient code	Direct detection of the mutation						Heteroduplex in the fragment				Genotype
	35delG	W77R	E47X	M34T	W24X	V95M	I	II	III	IV	
Phase I											
0203	+/+										35delG / 35delG
3401	+/+										35delG / 35delG
6306	+/+										35delG / 35delG
7901	+/+										35delG / 35delG
0201	+	+									35delG / W77R
0101	+	-	-	-	-	-			+		35delG / III
0301	+	-	-	-	-	-		-	-	-	35delG / ?
7103	+	-	-	-	-	-		-	-	-	35delG / ?
0801	-	-	+	-	-	-		-	+	-	E47X / III
6709	-	-	-	+	-	-		-	-	-	M34T / ?
1301	-	-	-	-	-	-	-	-	-	-	
1401	-	-	-	-	-	-	-	-	-	-	
6403	-	-	-	-	-	-	-	-	-	-	
6503	-	-	-	-	-	-	-	-	-	-	
6607	-	-	-	-	-	-	-	-	-	-	
6803	-	-	-	-	-	-	-	-	-	-	
6901	-	-	-	-	-	-	-	-	-	-	
7002	-	-	-	-	-	-	-	-	-	-	
Phase II											
0401	-						-	-	-	-	
0601	-						-	-	-	-	
4301	-						-	-	-	-	
4501	-						-	-	-	-	
Ocassional cases											
7301	-										

(+) Presence of the mutation or heteroduplex formation.
(-) Absence of the mutation.
(?) Second mutation undetected.
Ocasional cases: lately incorporated patients to the trial.

Table 4. Results of molecular studies on patients without familiar records (sporadic)

Patient code	Direct detection of the mutation						Heteroduplex (fragment)				Genotype
	35del G	W77R	E47X	M34T	W24X	V95M	I	II	III	IV	
Phase I											
0801	-	-	-	-	-	-	-	-	-	-	
1001	-	-	-	-	-	-	-	-	-	-	
1101	+	-	-	-	-	-		-	-	-	35delG / ?
1201	-	-	-	-	-	-	-	-	-	-	
1501	-	-	-	-	-	-	-	-	-	-	
1701	-	-	-	-	-	-	-	-	-	-	
1801	-	-	-	-	-	-	+		+		I / III
1901	-	-	-	-	-	-	-	-	-	-	
2201	-	-	-	+	-	-		-	-	-	M34T / ?
2301	-	-	-	-	-	-	-	-	-	-	
2401	-	-	-	-	-	-	-	-	-	-	
2501	-	-	-	-	-	-	-	-	-	-	
2701	-	-	-	+	-	-		-	-	-	M34T / ?
2901	-	-	-	-	-	-	-	-	-	-	
3001	-	-	-	+	-	-		-	-	-	M34T / ?
3501	-	-	-	-	-	-	-	-	-	-	
3701	-	-	-	-	-	-	-	-	-	-	
Phase II											
0501	-						-	-	-	+	? / IV
2001	-						-	-	-	-	
3201	-						-	-	+	-	? / III
3301	-						-	-	-	-	
3601	-						-	-	-	-	
7201	-						-	-	-	-	

is located in this fragment, arising as a possible candidate mutations causing deafness in the Cuban people.

In seven out of 17 familiar cases (41% of the patients) where the study was finished, the gene *gjb*2 was demonstrated as responsible for the disease, with two mutations detected in this gene either by the direct technique or by the heteroduplex assay (Table 3). In a similar study, Murgiaet *al*. reported a prevalence rate of 34-50% for the recessive autosomic cases [17].

In other 3 familiar cases are high probabilities for the *gjb*2 of being involved in the ethiopathogenesis of the disease, with one mutation detected with both techniques.

Therefore, the involvement of the *gjb*2 gene in the ethiopathogenesis was demonstrated in 10 out of 17 familiar cases (59% of the patients) that completed the study.

This study allowed the identification of five families, in which the genetic assistance and the diagnosis of carriers and pre-born diagnosis can be accurately carried out, because of defining the genotype predisposing for the disease. These results demonstrate the relevance of extending these studies to other families of the Cuban population affected, to unravel the epidemiological properties of the disease and to develop a prevention program of its inherited manifestations.

Table 4. Results of molecular studies on patients without familiar records (sporadic)

Patient code	Direct detection of the mutation						Heteroduplex (fragment)				Genotype
	35del G	W77R	E47X	M34T	W24X	V95M	I	II	III	IV	
Phase II											
7401	-						-	-	-	-	
7701	-						-	-	-	-	
7801	-						-	-	-	-	
8001	-						-	-	-	-	
8101	-						-	-	+	-	? / III
8201	-						-	-	-	-	
Ocasional cases											
6001	+										35delG / ?
7501	-							-			
7601	-							-			

(+) Presence of mutation or heteroduplex.
(-) Absence of heteroduplex.
(?) Second mutation undetected.
Ocasional cases: lately incorporated patients to the trial.

Table 5. Prevalence of the different mutations found in the alleles affected

Mutation	Affected alleles	%
35delG	14	70
M34T	4	20
W77R	1	5
E47X	1	5
Total	20	100

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