Molecular Investigation of the A1555G Mitochondrial Mutation in Brazilian Patients

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Abstract: *Introduction*: Among the alterations caused by the use of aminoglycosides, the A1555G mitochondrial mutation is one of the most important ones.

Objective: To investigate the prevalence of the A1555G mutation in patients with sensorineural hearing loss related with or without the use of aminoglycoside antibiotics.

Materials and Methods: Study of control-case in a sample of 27 patients with hearing loss, as the cases, and in 100 neonates, with normal hearing, as the control group. DNA was extracted from leukocyte blood samples and specific primers were used to amplify the Cytochrome b gene and the region that encloses the A1555G mutation of the mitochondrial DNA, using the polymerase chain reaction and the restriction fragment length polymorphism techniques.

Results: The presence of mitochondrial DNA was confirmed in all of the 127 study samples, due to the amplification of the Cytochrome b gene region in all such samples. The A1555G has not been identified in any of the 27 patients with hearing loss, nor in the 100 neonates of the control group.

Conclusions: Results are in accordance with studies which state that the A1555G mutation is not prevalent in the Americas. There is some interest in determining the real prevalence of this mutation and in the investigation of other mutations which may cause hearing loss associated to the use of aminoglycosides, or not, in the Brazilian population.

Keywords: Aminoglycosides, A1555G mutation, hearing loss.

INTRODUCTION

The relation between mitochondrial disease and hearing loss was stablished in 1986, by the study of a patient with mitochondrial myopathy and hearing loss [1]. Mitochondrial mutations, specially in *12S rRNA* and *tRNA^Ser(UCN)* genes, are important causes of non-syndromic sensorineural hearing loss, in some populations [2]. Among all these mutations, a replacement of the nitrogen bases A->G, in the position 1555 in the mitochondrial DNA *12S rRNA* gene, is of special interest as the main cause of hearing loss linked to the use of aminoglycoside antibiotics. It was identified in 1993 in a study which described it as a mutation in the mitochondrial rRNA which caused non-syndromic hearing loss, being this the first molecular genetic analysis related to aminoglycoside-induced ototoxicity [3]. Several studies have suggested that the A1555G mutation generates a new C->G pair of bases, making the secondary structure of the human *12S rRNA* strictly similar to the corresponding region of the *E. coli 16S rRNA* gene. This region is referred as the decoding region of the bacterial rRNA and an important action site for aminoglycoside antibiotics [3,4]. The aminoglycosides binding to the decoding region results in protein translation errors and ensuing bacterial death. When there is the mutation in the human *12S rRNA* gene its structure turns into similar to that of the bacterial rRNA. Then, when the aminoglycosides bind to the cochlear cells

mitochondrial DNA, there is an exacerbation of its toxic effect in the inner ear causing hearing loss. Therefore, these studies demonstrate the highly specific recognition way among the RNA molecules and aminoglycosides [4].

Contrary to the syndromic mitochondrial mutations, which usually affect only a fraction the molecules of the mitochondrial DNA (heteroplasmy), the mutations related to the non-syndromic sensorineural hearing loss are, frequently, homoplasmic and the phenotype differs considerably among the members of a same family, ranging from profound degree to a completely normal hearing leavel. Several factors such as nuclear genes, mitochondrial DNA haplotypes, environmental factors or tissue-specific effects act independently or in association, being able to influence the clinical expression, but studies have not identified yet modifying nuclear genes or correlation between mutation and mitochondrial haplotypes. The only environmental factor known in affecting the A1555G mutation is the group of aminoglycoside antibiotics [5].

Although mitochondrial mutations are of great importance in the sensorineural hearing loss etiology, there are few studies in developing countries, mainly in Brazil. Thus, the present study aimed at investigating the prevalence of the A1555G mitochondrial mutation, in sample of patients with non-syndromic sensorineural hearing loss with and without exposure to aminoglycosides.

MATERIALS AND METHODOLOGY

From September to October 2006, a transversal cut study was carried out, in which 20 index-cases with non-

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syndromic sensorineural hearing loss were studied (12 male and 8 female), age range from 1 to 37 years, who had already gone through molecular analysis of the *GJB2* gene and the Δ(GJB6-D13S1830) mutation and had no molecular alterations [6]. The studied index-cases consisted of 16 sporadic cases (only case in the family) and 4 familial ones (familial cases). From the familial cases the relatives with hearing loss were also evaluated (2 male and 5 female), age ranging from 5 to 45 years. Therefore, a total of 27 patients with hearing loss were studied.

Each patient went through a complete anamnesis to investigate the beginning age of the hearing loss, the presence or not of other cases in the family, the use or not of ototoxic drugs (aminoglycosides) and to exclude the possibility of other environmental causes: materno-fetal infections, perinatal complications, meningitis, accustic trauma, and blood-marriage. The physical, otorhinolaryngo-logic and systemic exams and complementary exams were carried out in order to exclude suggestive signals of hearing loss syndromic forms (specially cranio-facial dismorphism, tegumental alterations, anomalies of branchial, cardiac, tireoideal origin, sight disorder, etc). Moreover, patients went through ophthalmologic evaluation (including fundo-scopy), vestibular test and computerized tomography of the temporal bone. Therefore, all the clinical evaluation was carried out to exclude patients with hearing loss caused by environmental factors, except the use of aminoglycosides, by consanguinity, congenital malformation of inner ear or by genetic syndromes. The patients were audiologically tested by pure tune audiometry and included those with non-syndromic sensorineural hearing loss classified as slight (25-40 dB), moderate (41-60 dB), severe (61-80 dB) or profound (>81 dB) [7].

The included patients were identified as Hearing Loss Group (HLG) being divided in 2 subgroups:

- HLG subgroup 1: patients who used aminoglycoside antibiotics (n=13)

- HLG subgroup 2: patients who did not use aminoglycoside antibiotics (n = 14)

A hundred neonates were included in the study as Control Group (CG), with normal test of otoacustic emissions carried out up to 3 days after being born and index of Apgar ≥7 in the 1st minute.

A total of 4.0mL of blood was collected (from peripheral vein in the HLG group and from the umbilical cord, after its tying, in the Control Group) in a Vacutainer® tube containing anticoagulant (EDTA), with previous obtaining of free and clarified consent term of patients or responsibles. The protocol for this study was approved by the Institution Research Ethics Committee (Protocol #2784/2006, Resolution #138/2006). The genomic DNA was extracted from blood samples of both Groups using the GFX^{TM} Genomic Blood DNA Purification Group (Amersham Pharmacia Biotech Inc.), according to the manufacture´s protocol.

In order to detect the A1555G mutation [8], fragments of the mitochondrial DNA, which include the mutation region, were amplified by the PCR technique, in the Applied Biosystems – Gene Amp PCR System 9700® termociclator.

For this reaction a pair of primers was synthetized. From these, the deoxinucleotide triphosphates (dNTPs) are incorporated, beginning the DNA amplification, respecting the complementarity of the bases (A-T/C-G), finally obtaining the amplification of the mitochondrial DNA. The sequence of the primers oligonucleotides and the conditions for the PCR reaction were according to those described in the literature [8].

As a product of the PCR reaction, a fragment of 643 bp was amplified, which was, afterwards, submitted to the restriction analysis by the RFLP technique, using the *BsmAI* enzyme [8] (New England Biolabs)®, overnight at 55ºC. The enzymatic digestion of the normal samples fragment for the A1555G produce two fragments of 413 bp and 230 bp due to the recognition of the restriction site of the *BsmA*I enzyme. The samples with the mutation only produce the fragment of 643 bp, because there is not the recognition of the enzyme site due to the substitution of the nitrogen bases A->G in the position 1555 in the mitochondrial DNA.

Another pair of primers was also synthetized for the amplification of a determined Cytochrome b region [9]. This specific region, a highly conserved area of the mitochondrial genome, serves as an inner control of amplification in order to verify the presence of the mitochondrial DNA in the samples. The expected size of the amplified fragment by PCR using the CitbF and CitbR primers was 161 bp. The sequences of the primers oligonucleotides and the conditions for the PCR reaction were according to those described in the literature [9].

Both pairs of primers used for the PCR include regions of the Human Mitochondrial DNA Revised Cambridge Reference Sequence [10].

The products from both reactions, PCR and RFLP, were analysed by agarose gel electrophoresis 2% in TBE 1x buffer, containing ethidium bromide, in 0.5µg/mL concentration. The agarose gel was submitted to ultraviolet lighting to confirm the success of the reaction and the gel was photodocumented.

Statistical Analysis

A hundred neonates were evaluated in a piloting study, for estimating the proportion (p) of probable carriers of the A1555G mutations in the initial sample. After having been determined, this proportion (p) had the "known population sample dimensioning" statistical formula applied to obtaining the size of the final sample (n) needed to statiscally represent the total population (Np) of neonates, born in the right period for the carrying out of the research (Np=352 neonates). For the final sample (n) calculation, the referred statistical formula was used with the following parameters: p=0.00 (estimated by the pilot sample); q=1.00; zc=3.00 (99.74% of trust); e=0.03 (3% of estimate error); Np=352 (population size during the study period).

$$\text{Formulae: n} = \frac{zc^2 \text{ x p x q x Np}}{e^2 \text{ x (Np} - 1) + zc^2 \text{ x p x q}}$$

The study results were expressed in percentage.

Table 1. Clinical and Audiometric Data of the 20 Index Cases and of the 7 Affected Familial Cases (n=27) which were Submitted to Molecular Analysis for Detecting A1555G Mitochondrial Mutation

Index Cases (IC)	Gender	Age (Years)	Beginning of the HL	Degree of HL	Use of AMG	Familial Cases	Age (Years)	Degree of HL	Use of AMG
1	F	12	Pre-lingual	Profound	No				
2	M	9	Pre-lingual	Severe	Yes				
3	F	28	Post-lingual	Profound	No				
4	F	3	Pre-lingual	Profound	Yes				
5	M	8	Pre-lingual	Severe	Yes				
6	M	4	Pre-lingual	Profound	Yes				
7	F	10	Post-lingual	Severe	Yes				
8	M	7	Pre-lingual	Profound	Yes				
9	M	3	Pre-lingual	Severe	Yes				
10	F	3	Pre-lingual	Profound	Yes				
11	M	9	Pre-lingual	Profound	Yes				
12	M	5	Pre-lingual	Profound	Yes				
13	F	33	Pre-lingual	Severe	No	Sister - 14 Brother - 15 Brother - 16	28 37 19	Moderate Profound Profound	No No No
17	M	37	Post-lingual	Profound	No				
18	F	5	Pre-lingual	Profound	Yes				
19	M	10	Pre-lingual	Moderate	No	Mother - 20	38	Moderate	No
21	M	8	Pre-lingual	Severe	Yes				
22	M	7	Pre-lingual	Profound	No	Mother - 23	45	Moderate	No
24	M	32	Pre-lingual	Profound	Yes				
25	F	10	Pre-lingual	Profound	No	Sister - 26 Sister - 27	8 5	Severe Severe	No No

(IC) Index cases; (M) Male; (F) Female; (HL) Hearing Loss; (AMG) Aminoglycosides.

RESULTS

The amplification of the Cytochrome b gene region was obtained after the PCR reaction demonstrating the fragment corresponding to the 161 bp confirming, thus, the presence of the mitochondrial DNA in all of the 127 study samples 100% (HLG Groups I and II: n = 27 and Control Group: n = 100).

The PCR reaction allowed the amplification of the mitochondrial DNA fragment of 643 bp, which involves the mutation region, in all of the samples (HLG Groups I and II: n=27 and Control Group: n=100). These samples (100%) presented the 413 bp and 230 bp DNA fragments after enzymatic digestion confirming absence of A1555G mitochondrial mutation

The clinical and audiometric data obtained from the index-cases such as gender, age, beginning time and degree of hearing loss, use or not of aminoglycosides and familial cases are described on Table **1**.

DISCUSSION

Complementing the research, which identified that mutations in the conexine 26 gene (*Cx 26*) cause hearing loss in a significant proportion of cases in populations of several European countries, the finding of other mutations made possible the molecular etiological identification in many patients, enabling not only early rehabilitation, but also genetic counseling. However, it has been interesting to observe that the frequency of genes and mutations that cause hearing loss have varied considerably, among populations. The 35delG mutation, in the *Cx26* gene, for instance, has a high prevalence among deaf people of European origin [11], while it is virtually absent in the Japanese, Koreans or Mongolians [12,13].

Likewise, the frequency of the mitochondrial mutations also differs among several populations, being the A1555G mutation found mainly in patients with maternal inheritance hearing loss although it has been sporadically identified in patients with recessive autossomic heritage. In both modes of inheritance, differences are mainly associated to ethnic origins. According to recent studies, this is a frequent cause of non-syndromic hereditary hearing loss, associated or not to the use of aminoglycoside antibiotics in populations of the Asian continent [8,14-18], Arabic countries [19] and South Africa [20], being it rare in most part of the European and American populations [21,22].

A study carried out with 32 Asian people with hearing loss and 100 American controls (63 deaf and 37 with normal hearing) found the mutation in 87.5% in the deaf Asian group, but have not found it in any of the individuals from the control group, concluding that the mutation is not prevalent in populations of non-Asian origin [22]. This result has been confirmed in a study in Greece, in which the mutation has not been found in the 106 cases [23], and also in another study involving 202 patients with early-onset non-syndromic hearing loss in the United Kingdom [2]. Likewise, in the present study, the A1555G mitochondrial mutation has not been found in HLG groups (Group I: 13 index-cases who used aminoglycosides and Group II: 14 index-cases who did not use aminoglycosides) and Control Group (100 neonates with normal otoacustic emissions test). This result demonstrates that the mutation is not a common cause of hearing loss in the present studied sample.

In the USA, two mutation screenings were carried out in neonates. In the first one using blood from the umbilical cord of 1773 neonates, only one neonate was carrying the mutation [24]. In the second screening of 25 neonates, with alterations in the otoacustic emissions test, the mutation has not been found [25]. Screening carried out in 300 neonates and in 712 normal hearing subjects, in Argentina, did not detect the A1555G mutation [26]. These studies demonstrate the low prevalence in the American populations, but do not rule out the need for molecular investigation in neonates' screening tests, mainly in countries in which the use of aminoglycosides is quite common.

The present study methodology has been similar to that described in the literature [8], including the amplification of a specific region of the Cytochrome b gene, a highly conserved area in the mitochondrial genome, which served as an amplification control in order to verify the presence of the mitochondrial DNA in all of the analysed samples [9]. Although the number of cases in both groups was different from the number in some published studies, and although the mutation was not found in both groups (HLG and control groups), this study was able to confirm the results of studies that also didn't find the mutation in non-Asian or Arab ethnic groups [21-26].

Two studies in families with mitochondrial inheritance have been carried out in Brazil. The first one has been carried out in 5 families with hearing loss, in which the mutation prevalence of 2% (4 cases) was determined. From these positive cases, only 1 was associated with a supposed exposure to aminoglycosides, and the mutation has not been found in the control group made of Afro-Brazilians, "whites" and from Asian origin (Japanese or Chinese) [27]. The second study has been carried out in one family only with 9 cases with deafness. The A1555G mutation has been found in all of them, but the association between deafness and use of aminoglycosides was not stablished [28]. Such results, different from the present study, may be explained by the highly heterogeneous ethnic composition of the Brazilian population, due to the miscegenation among several ethnic groups; resulting, thus, in different prevalence in the various Brazilian regions.

CONCLUSIONS

The PCR/RFLP molecular techniques, with the protocol used in the study, are easy methods for tracking the A1555G mutation, helping the molecular investigation of the hearing loss. Many mutations may contribute to hearing loss and the A1555G mitochondrial mutation, related to the use of aminoglycoside antibiotics, may be only one of them [14,17]. The identification of underlying causes of hearing loss caused by exposure to aminoglycosides in essential to help the therapeutics, mainly in neonatal intensive care unit, besides promoting the genetical counseling and early rehabilitation, allowing the inclusion or re-inclusion of these patients in their social or professional activities.

There is a considerable interest in determining the real prevalence of the A1555G mitochondrial mutation in the Brazilian population, and in the investigation of other mutations which may cause hearing loss associated or not to the use of aminoglycosides, by means of molecular tests, according to those used in the present study, which may be a valuable complement to neonatal audiometric tracking.

ABBREVIATIONS

Cx26	=	Conexina 26
DNA	=	Deoxyribonucleic Acid
dNTPs	=	deoxinucleotide triphosphates
EDTA	=	Ethylene Diamine Tetraacetic Acid
PCR	=	Polymerase Chain Reaction
RFLP	=	Restriction Fragment Length Polymorphism
rRNA	=	ribosomal Ribonucleic Acid
TBE	=	Tris-Borate-Edta Buffer
tRNA	=	transfer Ribonucleic Acid

CONFLICT OF INTEREST

There are no financial bounds or agreements between the authors and companies that may be interested in the material addressed in this article.

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