



## Prospects for genetic hearing loss screening: 35delG mutation tracking in a newborn population

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### Abstract

**Objectives:** To investigate the prevalence of the 35delG mutation in a newborn population, with specific molecular testing, and to evaluate the prospects for genetic neonatal screening for hearing impairment.

**Population and method:** 233 newborn were evaluated at the *Hospital de Base de São José do Rio Preto, SP*, for molecular analysis of the 35delG mutation in the connexin 26 gene, with the reaction technique in allele-specific polymerase chain reaction, after genomic DNA extraction from umbilical cord blood.

**Results:** Five heterozygotes were identified, obtaining a prevalence of 2.24% of 35delG mutation carriers in the study population.

**Conclusion:** Using the molecular test allowed for the identification of the 35delG mutation in the study population with the possibility of being used as a complement to neonatal audiometric screening as being simple, fast, and easily to perform with low costs.

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### Introduction

Due to its high frequency and to its clinical impact, the early identification of hearing loss has become an important public health goal. Neonatal screening was recommended by the National Institute of Health and Joint Committee on Infant Hearing Screening for the implementation of electrophysiological tests such as evoked otoacoustic emissions and brainstem auditory evoked response.<sup>1,2</sup> Along with the technological improvements in the diagnosis of hearing loss, recent and continuous advances in the field of molecular genetics have increasingly allowed for the

identification of the genes responsible for the inherited form of this disorder and, consequently, for its early detection.<sup>3,4</sup>

Sensorineural hearing loss affects one in every 1,000 newborns, and 60% of the cases may be attributed to genetic factors, whereas the remaining 40% has the widest variety of etiologies in industrialized countries. Among genetic causes, syndromic hereditary forms account for 30% of hearing loss in children, while the non-syndromic forms affect around 70% of the population.<sup>5</sup>

In 1994, the first autosomal recessive non-syndromic deafness locus (DFNB1) was identified on the long arm of chromosome 13 (13q)<sup>6</sup> and, in 1997, connexin 26 gene (Cx26) or gap junction beta-2 protein (GJB2) was detected on chromosome 13q11-12.<sup>7</sup> In the inner ear, Cx26 plays a crucial role in the regulation of cochlear homeostasis and in the production of the endocochlear potential.<sup>8</sup> Therefore, mutations in the Cx26 gene cause a structural and functional defects in these gap junctions, leading to persistently high intracellular potassium concentration; this damages the mechanism that allows the quick response of ciliated cells to new auditory stimuli, consequently resulting in hearing loss.<sup>9</sup>

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Among the mutations in the Cx26 gene described so far, which cause DFNB1, a particular mutation and the first one described, the 35delG mutation accounts for most of the mutant alleles (60-85%) in the Mediterranean European population.<sup>10</sup> However, around 10 to 42% of patients with hearing loss have mutations in the Cx26 gene in only one allele, despite the fact that most mutations have a recessive trait.<sup>11</sup> In several

European countries, the prevalence of the 35delG mutation, in normal-hearing individuals, has been estimated to range from 2 to 4%.<sup>10</sup>

The 35delG mutation arises from the deletion of a guanine (G) from a six-guanine stretch, at nucleotide positions 30 to 35, in the Cx26 coding region, resulting in premature stop codon mutations, at nucleotide position 38, with a consequent frameshift and a stop at codon 13, triggering the synthesis of an incomplete polypeptide, with 12 instead of 226 amino acids.<sup>12</sup>

In developing countries, few studies have been carried out to assess the prevalence of hereditary hearing loss. In Brazil, a prevalence of 0.9% was described in carriers of the 35delG mutation, that is, 1:103 heterozygotes in a neonatal screening.<sup>13</sup> In another study, now with hearing impaired patients, the 35delG mutation was identified in 84.2% of the alleles.<sup>14</sup>

The aim of the present study was to investigate the prevalence of the 35delG mutation, using an allele-specific polymerase chain reaction (AS-PCR) in a sample of newborns, and to draw attention to the necessity of neonatal screening for hearing loss.

## Patients and methods

The study was carried out between September 22 and October 10, in 2003, at the Obstetric Unit of Hospital de Base de São José do Rio Preto, state of São Paulo, Brazil. A total of 227 births occurred at the unit, of which four corresponded to monozygotic twins; therefore, the final sample included 223 newborns. After cord clamping, 4 ml of blood was collected from the umbilical cord, and stored in a Vacutainer® tube containing EDTA. This procedure was performed after the mother or couple signed an informed consent form. The study protocol was approved by the local Research Ethics Committee.

The genomic DNA was extracted from the cord blood samples using GFX™ Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech Inc. Limited, 2000), according to the manufacturer's instructions; the procedure was performed at the Molecular Biology Laboratory of the School of Medicine of São José do Rio Preto (FAMERP).

For detection of the 35delG mutation, we used an allele-specific polymerase chain reaction (AS-PCR),<sup>15</sup> in Applied Biosystems thermal cycler – GeneAmp PCR System 9700,™ performed at the Center for Molecular Biology and Genetic Engineering of UNICAMP (CBMEG). Three ARMS (amplification-refractory mutation system) primers were synthesized for mutation screening: normal primer (NOR),

used to amplify the allele without the 35delG mutation; mutant primer (MUT), to amplify the allele with the 35delG mutation; and the common primer (COM), used as reverse primer, along with NOR or MUT primers used as forward primers.<sup>16</sup> The oligonucleotide primer sequences and the conditions for the AS-PCR were carried out as described in the literature.<sup>15</sup> These two reactions (NOR/COM and MUT/COM) allow identifying each sample as normal homozygote (without the 35delG mutation in both alleles), heterozygote, or mutant homozygote (with the 35delG mutation in one or in both alleles, respectively). Control primers A (forward) and B (reverse) were also synthesized for Cx26 gene co-amplification of a portion of the XY homologous amelogenin gene. These primers were, therefore, used as internal amplification controls.<sup>17</sup>

The AS-PCR products were analyzed by 1.5% agarose gel electrophoresis, in Tris-Borate-EDTA or TBE 1X buffer, containing ethidium bromide at 0.5 µg/ml, exposed to ultraviolet light in order to confirm that the reaction was successful, and submitted to gel photodocumentation.

## Statistical analysis

One hundred newborns were evaluated in a pilot study with the aim of estimating the proportion (p) of hearing loss in the initial sample. After determining this proportion, we defined the final sample size (n) necessary to statistically represent the total population (Np) of newborns during the study period (Np = 223 newborns). The following parameters were used to calculate the size of the final sample (n): p = 0.01 (estimated by the pilot sample); q = 0.99; zc = 3.00 (99.74% of reliability); e = 0.03 (3% of estimation error); Np = 223 (population size).

$$n = zc^2 \times p \times q \times Np / e^2 \times (Np - 1) + zc^2 \times p \times q$$

The results of the study were expressed as percentage values.

## Results

Among 223 newborns, five were heterozygotes, with a prevalence rate of 2.24% (1:44.6) of carriers of the 35delG mutation in the study population. These heterozygotic newborns have undergone regular audiometric testing.

## Discussion

The population of 223 newborns evaluated at the Hospital de Base de São José do Rio Preto, in São Paulo, by molecular analysis of the Cx26 gene, and by the AS-PCR technique, showed a prevalence rate of 2.24% (1:44.6) of 35delG mutation. This result and the methodology used are in agreement with previous studies and with those studies described in the literature, including several populations, in which the prevalence of carriers in samples of 53 to 560 normal-hearing newborns, children or adults, ranged from 0% (USA, Afro-Americans, England, France and Egypt) to 4.4% (Estonia).<sup>18</sup> In the State of New York (USA), a

screening performed in a population of 2,089 newborns revealed a prevalence of 1.29% of 35delG mutation.<sup>19</sup>

The relative contribution of the 35delG mutation to non-syndromic hearing loss, in different populations, ranged from 0% (Oman, Korea, Japan) to 7% (Italy, Spain, Greece), showing the genetic heterogeneity across countries, although some of these studies included a small number of patients, in addition to some differences regarding the screening methods for detection of the mutation.<sup>20-23</sup>

In Brazil, the prevalence of carriers of the 35delG mutation amounted to 0.97%, approximately 1:103 heterozygotes, in a screening carried out in 620 newborns in the region of Campinas, state of São Paulo.<sup>13</sup> The methodology used in the current study was similar to the study carried out in Campinas, so as to maintain the methodological consistency, but some variation was observed in the frequency of alleles containing the 35delG mutation. This fact can be explained by the difference in the sample or maybe because the ethnic composition of the Brazilian population is highly heterogeneous. Several ethnic groups, especially Caucasians and Africans, miscegenated, which results in different prevalence rates across Brazilian regions or even between cities/towns within the same state.<sup>24</sup> Therefore, a multicenter study is necessary to determine the actual prevalence of the 35delG mutation in the Brazilian population.

According to the literature, the analyses of the Cx26 gene in patients with hearing loss often show heterozygosis in approximately 10 to 42% of the cases, despite the fact that most mutations, especially the 35delG mutation, have a recessive trait.<sup>11,25</sup> The results of the study carried out in patients with non-syndromic neurosensory hearing loss at the Hospital de Base de São José do Rio Preto are in agreement with these data, since the prevalence of 35delG heterozygotes corresponded to 12.12%.<sup>26</sup> Other two Brazilian studies found a prevalence of heterozygotic individuals with hearing loss of 6.45<sup>14</sup> and 2.66%.<sup>27</sup> The prevalence in both studies was smaller than in the literature, perhaps because of the differences in the sample or due to ethnic and regional characteristics of the Brazilian population,<sup>14</sup> as previously mentioned.

However, since hearing loss caused by the 35delG mutation occurs when there is mutation in both alleles (homozygosis), characterizing autosomal recessive inheritance in most cases, a more comprehensive analysis of the Cx26 gene is necessary in order to distinguish heterozygotic patients with hearing loss (prevalence in 10-42% of the cases)<sup>11,25</sup> from normal heterozygotes (healthy carriers, that is, individuals with mutation in just one allele, but with normal hearing, whose prevalence is estimated to vary from 2 to 4% of the population with hearing problems).<sup>10</sup> These more comprehensive analyses and studies (including the screening of the entire coding and noncoding regions or the screening of D(GJB6-D13S1830) mutation in the Cx30 gene) should investigate the audiometric and clinical characteristics of these individuals.<sup>26</sup> In case of heterozygotic patients with hearing loss, recent studies have suggested the existence of another mutation in the Cx26 gene, or the

co-occurrence of D(GJB6-D13S1830) mutation in the Cx30 gene, which may contribute to the digenic inheritance of hearing loss.<sup>26</sup>

These data support the future use of genetic screening tests, such as AS-PCR, for the detection of the 35delG mutation, as occurred in this study, since they allow the identification of healthy carriers, homozygotes or carriers of the 35delG mutation with hearing loss in a large number of cases. These tests can add to the existing neonatal audiometric screening, as they are simple, quick, easily applied and inexpensive.<sup>28-30</sup> Newborns whose genetic test shows heterozygosis must be submitted to serial follow-up, because they may carry the 35delG mutation and have hearing loss, or may only carry the mutation and have normal hearing, as occurred in this study.

Our conclusion is that this test allowed for the identification of the 35delG mutation, and that if it is performed immediately after birth, it may help the early differential diagnosis between healthy carriers and children with hearing loss (35delG homozygotes or heterozygotes). In addition, it will provide parents with valuable information about the treatment and prognosis, combined with genetic counseling.<sup>30</sup> Therefore, it is imperative that there be interest in establishing the prevalence and the types of mutations that cause non-syndromic hearing loss in Brazil, with continuous population-based studies, so that a program of neonatal genetic screening can be implemented throughout Brazil. This program may bring significant reductions in hospital and medical expenses and improvement to public health, providing patients with a better quality of life.

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