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*Cover Letter

A novel p.Leu213X mutation in *GJB2* gene in a Portuguese family

Dear Sirs,

Herewith we are submitting an Article, focused on a new mutation found in the *GJB2* gene. This mutation, p.Leu213X, introduces a premature STOP codon avoiding the formation of the functional connexin 26 protein and in the present study was found in compound heterozygosity with another *GJB2* mutation already identified (c.333-334delAA), being this genotype responsible for the hearing impairment observed in the two patients.

All the co-authors have been involved in the study and have other publications in the field of Hereditary Deafness published in different journals as Genetics Research International, Am J Med Genet, Hear Research., Int J Ped Otorhinolaryngology, Eur J Hum Genet, J Med Genet.

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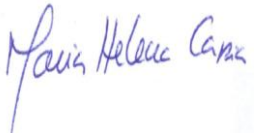
We would like to inform that there is no conflict of interest regarding this study.

Thanking you in advance for your prompt attention to our submission.

Best regards,



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A Novel p.Leu213X Mutation in *GJB2* Gene in a Portuguese Family

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Conflict of Interest:

There is no conflict of interest

Abstract

Introduction: Hearing loss is the most common sensory disability and is present in about 1.9 per 1000 infants at birth. The DFNB1 *locus* (13q11-q12) includes the genes *GJB2*, coding for connexin 26, and *GJB6*, encoding connexin 30. More than 100 mutations have been identified associated with autosomal dominant and recessive hearing loss in the *GJB2* gene.

Objectives: The aim of the present study was to identify the genetic etiology of deafness in two Portuguese individuals, presenting nonsyndromic sensorineural moderate and severe hearing loss, respectively.

Patients and Methods: The individuals were evaluated in both ears by pure tone audiometry and blood samples were collected after written informed consent was signed. DNA extraction and PCR amplification of *GJB2* coding region followed standard methodologies. PCR products were automatically sequenced in both directions.

Results:We identified a novel mutation, c.638T>A (p.Leu213X), in *GJB2* gene. This nonsense mutation was found in both siblings, and was inherited from their hearing father. Molecular analysis showed that the two siblings were also heterozygous for c.333-334delAA, a previously described *GJB2* deletion. This novel mutation was not found in a random control sample of 480 individuals that were screened for coding region of *GJB2* gene. p.Leu213X mutation identified in this study for the first time changes the codon 213, coding for a highly conserved and slowly evolving residue of connexin 26, localised to the C-terminus domain of the protein, to a stop codon, leading to the deletion of the last 14 amino acids of the protein.

Conclusion: We can conclude that the etiology of deafness in these individuals is due to the *GJB2* genotype involving the c.333-334delAA deletion and the novel p.Leu213X mutation in compound heterozygosity.

Key-words – novel mutation, *GJB2* gene, connexin 26, deafness, sensorineural, hearing impairment

Introduction

Clinically significant hearing loss (HL) is present in at least 1.9 per 1000 infants at birth and the prevalence rises to at least 2.7 per 1000 by the age of four [1]. HL may be due to genetic and/or non-genetic (environmental) factors. Most hereditary HL is inherited as a simple Mendelian trait and is nonsyndromic (70%), being classified as sensorineural when it is caused by problems in the inner ear, cochlear nerve, and/or central auditory pathway [2]. Nonsyndromic sensorineural hearing loss (NSSHL) is predominantly inherited in an autosomal recessive pattern (DFNB *loci*). The DFNB1 *locus*, at chromosome 13q11-q12, includes the *GJB2* and *GJB6* genes, which encode connexin 26 (Cx26) and connexin 30 (Cx30), respectively. These connexin proteins are co-expressed and co-localised in the cochlea, where they are constituents of the cochlear gap junction systems, which are thought to have a role in the recirculation of K^+ contributing to cochlear homeostasis [3,4,5], and might also be important for Ca^{2+} signalling [6] and the intracellular diffusion of large cationic molecules [7].

GJB2 gene is about 5500 bp length with 2 exons, of which only one contains the coding region [8]. More than 100 different mutations have been identified in association with autosomal dominant and recessive HL in this gene [8]. Three nonsyndromic recessive mutations, c.35delG, c.167delT and c.235delC have been found at high frequency in Caucasian, Ashkenazi Jewish, and Asian populations, respectively [9].

Methods

We studied one Portuguese family, composed of two hearing-impaired siblings with HL, aged 11 and 15 (proband) years and their normal hearing parents (fig. 1A). Each affected individual was clinically evaluated to ensure that the HL was not syndromic neither a result of infection, acoustic trauma, ototoxic drugs or premature birth. None of the patients had undergone cochlear implantation.

Written informed consent was obtained from siblings and parents prior to blood sampling. Hearing levels were determined by pure-tone audiometry with a diagnostic audiometer in a soundproof room. The level of HL was classified, following the European

Working Group on Genetics of Hearing Impairment [10], as mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB), or profound (>95 dB), from an average at 500, 1000, 2000 and 4000 Hz in the better ear. Total genomic DNA was extracted from peripheral blood using the JetQuick Blood and Cell Culture Kit (GENOMED), according to the manufacturer's instructions. PCR amplification of *GJB2* coding exon was made using primers 2AF and 2BR described previously [11]. After direct bidirectional sequencing electrophoretograms were evaluated by visual inspection and pairwise alignment to reference sequences using NCBI's BLAST (Basic Local Alignment Search Tool) [12]. A random control sample composed of 480 Portuguese individuals was also screened for the coding region of *GJB2* gene.

Results

Pure tone audiometry showed bilateral, sensorineural moderate and severe HL in the proband (individual II:1) and his sibling (individual II:2), respectively (fig. 1D,E). Both parents had normal hearing (fig. 1B,C). A novel variant, c.638T>A (p.Leu213X) was found through molecular genetic analysis in the two siblings in compound heterozygosity with the c.333-334delAA previously described deletion. The deletion c.333-334delAA (fig. 2A,B) was inherited from the mother, who is heterozygous for this mutation. The novel mutation p.Leu213X (fig. 2C,D) found in both siblings, was later identified in their father, in heterozygosity. This new mutation was not present in the random control sample of 480 Portuguese individuals that were screened for coding region of *GJB2* gene.

Discussion

Mutations in *GJB2* are the most common cause of nonsyndromic, autosomal recessive, hereditary HL, and may account for 10–40% of all congenital HL depending upon ethnicity [9]. The recessive deletion c.333-334delAA [13] causes a frameshift which results in chain termination after an additional novel amino acid, truncating about half of the protein. This is the first time that this mutation is reported in Portuguese HL patients.

The novel recessive mutation p.Leu213X, found in this study, creates a premature STOP codon by changing the codon 213 (TTG) which codes for a leucine, for a STOP codon (TAG). This mutation leads to the deletion of the last 14 amino acids of the protein. The Leu213 amino acid residue is localised to the C-terminus domain of the protein. The residues in the intracellular loop region and C-terminus are very different among different connexins and are hence thought to be responsible for regulation. These residues could thus impart unique properties to the various connexin molecules [14]. According to ConSeq [15] the mutation p.Leu213X affects a highly conserved residue that has evolved slowly (fig. 3).

Previously to this study, seven other missense/nonsense mutations were found in the C-terminus of Cx26 [16]. Interestingly, one of those mutations, identified in French patients [17], is also a nonsense mutation, p.Cys211X, occurring close to p.213LeuX and eliminating the conserved amino acid residues 212 and 213 as well as the remnant of the C-terminus of the protein.

Conclusions

Considering all this, we can conclude that the HL of the two Portuguese siblings is most probably due to the presence of the c.333-334delAA deletion and the p.Leu213X novel mutation, in compound heterozygosity. However, the functional characterization of the p.Leu213X nonsense mutation should be performed in order to investigate the effect of the mutation on the protein, and to provide insight on the role of the conserved 213Leu residue and subsequent amino acid residues for the normal Cx26 function.

Legends

Figure 1 – Pedigree of the family analysed in this study (A). Representative audiograms of individual I:2 (B), individual I:1 (C), individual II:1 (D) and individual II:2 (E) showing pure-tone audiometry results for air conduction bilaterally. Circles in blue represent the right ear; crosses in red represent the left ear.

Figure 2 – Partial sequence of an electrophoretogram showing (A) wild-type sequence and (B) the c.333-334delAA heterozygous deletion (the arrow). Partial sequence of an electrophoretogram showing (C) the wild-type sequence and (D) the novel mutation identified in this study, p.Leu213X, in heterozygosity (arrow).

Figure 3 – Schematic representation of the Cx26 protein showing the two mutations identified in this study. The extent of amino acid conservation is colour-coded, with residues shown in shades of blue (1-2) not conserved and rapidly evolving. Residues in white (3-6) show an average degree of conservation and residues in shades of red (7-9) are highly conserved and are slowly evolving. The degree of conservation of the polymorphic residues was analysed using ConSeq [15], the sequence only variant of Rate4Site, an algorithmic tool for the identification of functional regions in proteins. Figure adapted from [14].

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Figure(s)

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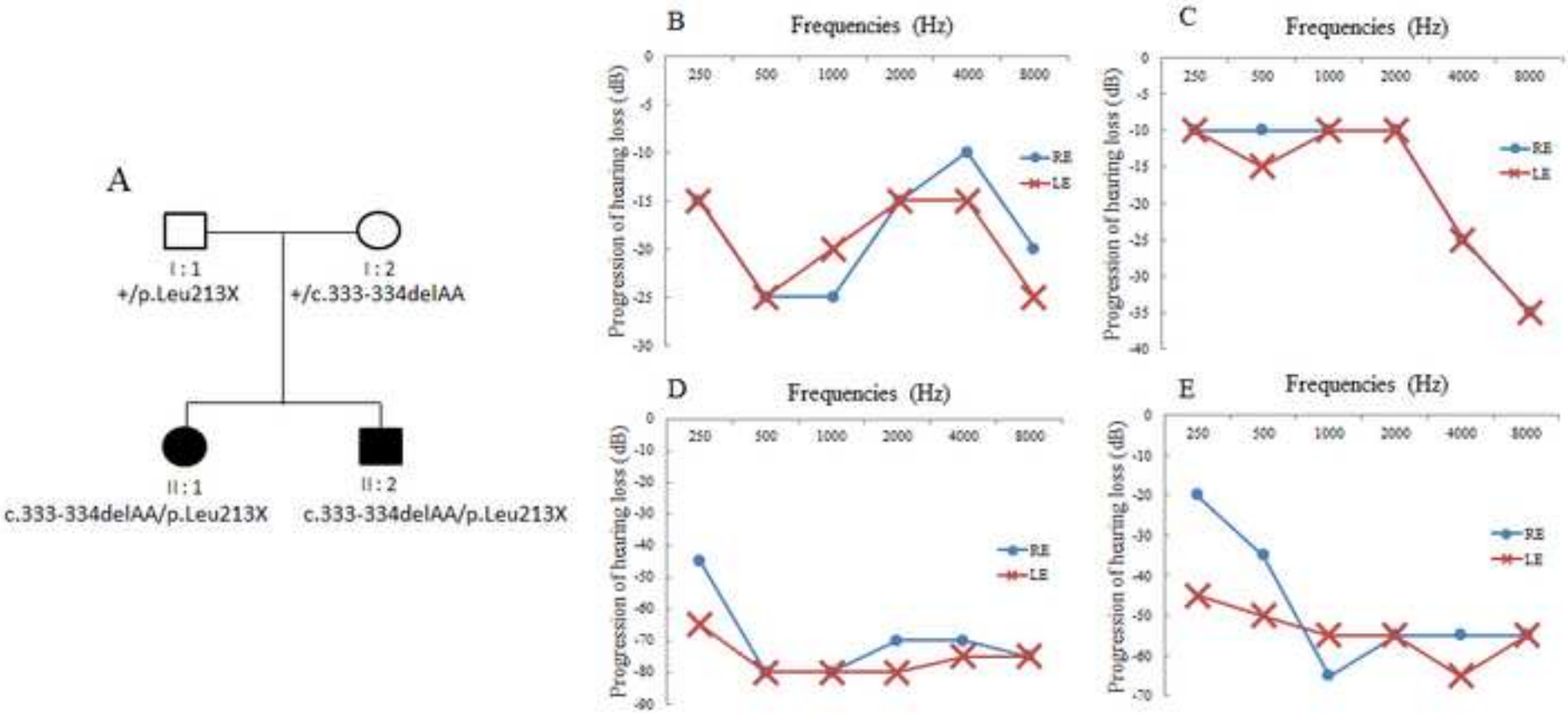

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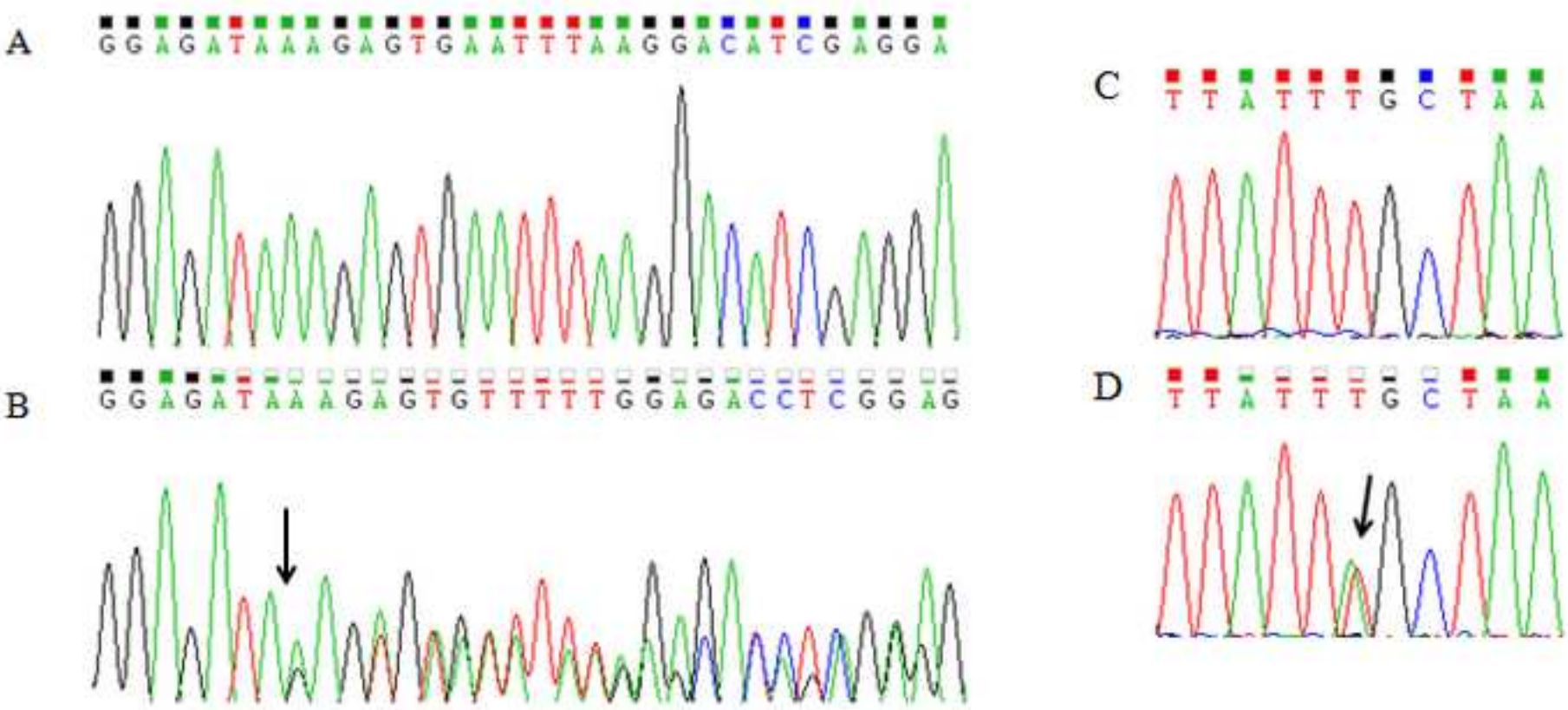

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