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MAIN ARTICLE

Connexin 26 and 30 mutations in paediatric patients with congenital, non-syndromic hearing loss treated with cochlear implantation in Mediterranean Turkey

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Abstract

Objective: Mutations in the genes for connexin 26 (*GJB2*) and connexin 30 (*GJB6*) play an important role in autosomal recessive, non-syndromic hearing loss. This study aimed to detect the 35delG and 167delT mutations of the *GJB2* gene and the del(GJB6-D13S1830) mutation of the *GJB6* gene in paediatric patients diagnosed with congenital, non-syndromic hearing loss and treated with cochlear implantation in Mediterranean Turkey.

Materials and method: We included 94 children diagnosed with congenital, non-syndromic hearing loss and treated with cochlear implantation. Blood samples were collected, DNA extracted and an enzyme-linked immunosorbent assay performed to enable molecular diagnosis of mutations.

Results: Of the 94 children analysed, the 35delG mutation was detected in 12 (12.7 per cent): 10 (83.3 per cent) were homozygous and 2 (16.7 per cent) heterozygous mutant. The 167delT and del(GJB6-D13S1830) mutations were not detected.

Conclusion: The GJB2-35delG mutation is a major cause of congenital, non-syndromic hearing loss in this study population.

Key words: Connexins; Hearing Loss; Cochlear Implants; Humans; GJB2 Protein, Human; GJB6 Protein, Human

Introduction

Sensorineural hearing loss is the most common congenital sensory impairment, affecting 1 in every 1000 newborns.[1] Genetic deafness is divided into syndromic and non-syndromic forms. Over 20 genes for non-syndromic autosomal recessive deafness (i.e. without other manifestations such as blindness) have been well characterised. In syndromic form, hearing loss is associated with a variety of anomalies. In non-syndromic genetic deafness of prelingual onset, autosomal recessive inheritance predominates (accounting for 80 per cent of cases); autosomal dominant (20 per cent), X-linked (1 per cent) and mitochondrial (less than 1 per cent) forms have also been described.[2] The 35delG mutation in the connexin 26 gene (*GJB2*), at the DFNB1 locus, is the most common mutation in patients with autosomal recessive sensorineural deafness.[3]

The *GJB2* gene is located on chromosome 13q12 at the DFNB1 locus. It encodes the connexin 26 protein and therefore is also termed the connexin 26 gene. Similarly, the *GJB6* gene, which encodes the connexin 30 protein, is also termed the connexin 30 gene.

Mutations in the connexin 26 gene are presumed to alter cochlear potassium recirculation via an effect on the gap junctions in cochlear cells, leading to the accumulation of potassium ions in the cochlear endolymph, and resulting hair cell dysfunction and deafness.[4] The phenotype profile of *GJB2*-related hearing loss depends on the severity of the *GJB2* mutation and the resulting effect on proteins.[5] More than 100 different mutations of the connexin 26 gene have been reported, with the 35delG, 167delT and 235delC mutations being the most common.[6]

Mutations in the connexin 30 gene have also been reported to cause autosomal recessive and autosomal dominant, non-syndromic hearing loss.[7] The connexin 30 protein functions as a component of the gap junction channels of cochlea cells. The del(GJB6-D13S1830) mutation of the connexin 30 gene causes genetic hearing loss by damaging the structure of this protein.

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This study aimed to detect the 35delG, 167delT and del(GJB6-D13S1830) mutations of the connexin 26 and connexin 30 genes, in 94 paediatric patients diagnosed with congenital, non-syndromic sensorineural hearing loss and treated with cochlear implantation in Mediterranean Turkey.

Materials and method

We enrolled in this study 94 children who had been diagnosed with congenital, non-syndromic, bilateral sensorineural hearing loss and treated with cochlear implantation between 2008 and 2010, within a cochlear implantation programme in Mediterranean Turkey. The patients comprised 53 (56.4 per cent) girls and 41 (43.6 per cent) boys, with a mean age of 4.6 years (range, 16 months to 13 years).

Blood samples were collected and stored at 4°C prior to DNA extraction. The presence of the 35delG and 167delT mutations (connexin 26 gene) and the del(GJB6-D13S1830) mutation (connexin 30 gene) was detected using the Pronto Connexin kit (Pronto Diagnostics, Rehovot, Israel), which utilises an enzyme-linked immunosorbent assay technique. This work was performed at the Department of Medical Biology and Genetics, Faculty of Medicine, Cukurova University, Adana, Turkey.

Assay procedure

Deoxyribonucleic acid was purified from peripheral blood using a proteinase K based extraction method. Two mutation detection methods were compared on a selected set of 48 DNA samples. Both were gel-based detection techniques which differentiated mutant and wild-type alleles according to their size; differences in size were due either to allele-specific priming or to enzymatic digestion.

Detection of 35delG mutation using polymerase chain reaction 'Bandeiraea simplicifolia' lectin assay. Genomic DNA was amplified using the primers 5′-GGTGAG GTTGTGTAAGAGTTGG-3′ and 5′-CTGGTGGAG TGTTTGTTCCCAC-3′ (where G = guanine, T = thymine, A = adenine and C = cytosine) and cut with the BsiY1 restriction enzyme.[8] The wild-type sequence yielded a single, uncut, 207 base pair (bp) fragment, while the 35delG mutant allele yielded two fragments of 184 and 23 bp.

Detection of 167delT mutation using polymerase chain reaction restriction fragment length polymorphism assay. Deoxyribonucleic acid was amplified by polymerase chain reaction, using the primers 5′-GGTG AGGTTGTGTAAGAGTTGG-3′ and 5′-AAGACAG TCTTCTCCGTGGG-3′.[9] A PstI restriction enzyme digest of the 729 bp, amplified, wild-type DNA produced four fragments of 339, 170, 150 and 70 bp size; digestion of DNA from homozygotes produced 220- and 170-bp fragments.

Detection of del(GJB6-D13S1830) mutation using allele-specific priming. Deoxyribonucleic acid was amplified using the following primers: Cx30-F-CTGTGGTGG ACGTACACCAG, Cx30-R-GCGTCTGTGCTCTCT TTGATC and BKR-1CACCATGCGTAGCCTT AACC.[10] Two different polymerase chain reaction products were obtained using these three primers together: Cx30-F-Cx30-R (289 bp) and BKR-1-Cx30-R (430 bp). This enabled discrimination between wild-type subjects (289-bp product), homozygotes for the deletion (430-bp product) and heterozygotes (both products). The polymerase chain reaction conditions for all three mutations were as follows: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds then 55°C for 30 seconds then more than 72°C for 30 seconds; and finally 72°C for 5 minutes. The Pronto Connexin kit was used; this is a post-amplification, single-nucleotide, primer extension assay utilising the enzyme-linked immunosorbent assay technique, and intended for the identification of the most common mutations at the DFNB1 locus on chromosome 13q12 (associated with autosomal recessive deafness), that is, the 35delG and 167delT mutations in the *GJB2* gene and the del(GJB6-D13S1830) mutation in the *GJB6* gene

Amplification. The *GJB2* and *GJB6* gene fragments (580 and 372 bp, respectively) were amplified using a multiplex amplification mix. An additional 242-bp fragment was amplified in samples carrying the del(GJB6-D13S1830) mutation.

Post-amplification treatment. The remaining free nucleotides were inactivated using the post-amplification mixture supplied with the Pronto Connexin kit.

Primer extension reaction. This high throughput reaction tests for the presence of wild-type alleles and also the 35delG, 167delT and del(GJB6-D13S1830) mutant alleles, simultaneously. Each screened allele was tested in a single well of a 96-well microtitre plate. This format detects the complete genotype of the 35delG mutation, which accounts for more than 75 per cent of all *GJB2* deafness alleles.[11] Complete genotyping of samples carrying the less frequent 167delT and del(GJB6-D13S1830) mutations was provided by the Pronto Connexin Strip (Pronto Diagnostics), which tested for each mutant and its wild-type allele, utilising two wells of an eight-well strip.

Enzyme-linked immunosorbent assay detection. Reaction products were transferred to a streptavidin-coated enzyme-linked immunosorbent assay plate and treated according to a standard enzyme-linked immunosorbent assay protocol. Results were determined both visually (noting the appearance of a blue colour) and colorimetrically, using a standard enzyme-linked immunosorbent assay reader (optical density 450 nm).

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Results

A total of 94 paediatric patients with hearing loss was analysed.

The 35delG mutation was detected in 12 of these children (12.7 per cent). Of these mutations, 10 (83.3 per cent) were homozygous mutant and two (16.7 per cent) were heterozygous mutant. The 167delT and del(GJB6-D13S1830) mutations were not found in our study group. All children except one had been diagnosed during their first year of life (mean age at diagnosis, 6.33 months). The mean age at cochlear implantation was 19.9 months. All the children routinely used and benefited from their device. Details of those children with mutations are given in Table I.

Discussion

More than 50 per cent of cases of pre-lingual deafness in developed countries is attributed to monogenic defects. Non-genetic causes include neonatal infection, ototoxic medication and trauma. Syndromic forms of hearing loss account for 30 per cent of pre-lingual genetic deafness, and include several hundred different deafness syndromes, whereas non-syndromic hearing loss accounts for 70 per cent. In patients with non-syndromic genetic deafness of pre-lingual onset, autosomal recessive inheritance predominates (80 per cent). Autosomal recessive hearing loss is usually more severe than other forms, and is almost exclusively due to cochlear defects (i.e. sensorineural deafness).[12]

The 35delG mutation is the commonest connexin 26 mutation. Its prevalence varies widely in different countries. In the USA, Kenna *et al.* found the 35delG homozygous mutation in 2 per cent of patients with non-syndromic hearing loss, whereas Prasad *et al.* found it in 14.8 per cent.[13,14] In a study from China, none of 118 deaf patients had homozygous 35delG mutation.[15] In studies of non-syndromic sensorineural hearing loss patients from Japan, Ghana, India, South Korea, Taiwan and Thailand, no homozygous 35delG mutation was reported.[16–21] The present study assessed Turkish children with congenital, non-syndromic hearing loss who had been treated with cochlear implantation, and found a high incidence of 35delG mutation compared with other studies.

Another *GJB2* mutation, 167delT, has been found to account for 40 per cent of pathological alleles in a Jewish deaf population, and has a 2 to 4 per cent carrier frequency among Ashkenazi Jews.[22] However, Tekin and colleagues' 2001 study of a large Turkish population did not detect the 167delT mutation.[23] This group's 2003 study screened multiplex cases for other mutations of the *GJB2* gene, and did detect the c.167delT mutation (1 allele; 0.3 per cent).[24] The present study did not detect the 167delT mutation.

The second most common mutation causing autosomal recessive hearing loss, after the 35delG mutation of the connexin 26 gene, is the del(GJB6-D13S1830) mutation of the connexin 30 gene.[25] This mutation spans a 342-kb region and is particularly common in Spain. Del Castillo *et al.* conducted a multicentre study in nine countries and found that this deletion was present in most of the screened populations, with higher frequencies in France, Spain and Israel.[26] Studies of patients with non-syndromic hearing loss in Italy, Morocco and India did not detect the del(GJB6-D13S1830) mutation.[27–29] Furthermore, this mutation was detected neither in the present study nor in two earlier Turkish studies of patients with autosomal recessive, non-syndromic, prelingual hearing loss.[30,31] The failure to detect this mutation in the Turkish populations assessed by the present study and the above two studies suggests that the del(GJB6-D13S1830) mutation is specific to certain populations, in a similar manner to the 35delG mutation. However, Propst and colleagues' Canadian study did not detect the del(GJB6-D13S1830) mutation in paediatric cochlear implant users of various different ethnicities.[6]

TABLE I
PATIENT DEMOGRAPHIC INFORMATION

Sex	Age			Mutn type	Pre-op ABR (R:L; dBnHL)	HL sev	Family Hx
	At study (y)	At Dx (mth)	At CI (mth)				
M	6	9	25	Homo	90:90	Severe	RP
F	5	4	20	Hetero	90:90	Severe	RP
F	4.5	5	22	Homo	90:90	Severe	RP
F	5.5	N	18	Homo	90:90	Severe	RP
F	6	18	26	Homo	90:90	Severe	RP
F	6	12	24	Homo	90:90	Severe	Bro HL
M	4	N	16	Homo	90:90	Severe	RP
M	4	N	16	Homo	90:90	Severe	None
M	4.5	N	16	Homo	90:90	Severe	None
M	5	6	18	Homo	90:90	Severe	RP
F	6	10	17	Hetero	90:90	Severe	RP
F	4	8	21	Homo	90:90	Severe	RP

Mutn = mutation; N = newborn; Pre-op ABR = pre-operative auditory brainstem response test result; R = right; L = left; dBnHL = decibel normalised hearing level; HL sev = hearing loss severity; Hx = history; y = years; Dx = diagnosis; mth = months; CI = cochlear implantation; M = male; F = female; Homo = homozygous; Hetero = heterozygous; RP = related parents; Bro HL = brother with hearing loss

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Mutations of the *GJB2* (connexin 26) gene have been found to be associated with a significant proportion of patients with prelingual, non-syndromic, autosomal recessive deafness, in all the cochlear-implanted populations studied thus far. Wiley *et al.* studied 108 children from a cochlear implant database, and found *GJB2* mutations in 16 of the 46 children (34.7 per cent) who met the inclusion criteria for idiopathic, non-syndromic hearing loss.[32] Daneshi *et al.* found *GJB2* mutations in 19.9 per cent of Iranian children with cochlear implants.[33]

- **The GJB2-35delG mutation is a major cause of congenital, non-syndromic hearing loss in the Turkish paediatric cochlear implant programme population studied**
- **No patient had the 167delT or del(GJB6-D13S1830) mutation**

The present study was conducted in the Mediterranean region of Turkey, the population of which comprises various ethnicities and is more heterogeneous than that of any other region of Turkey. This was one of the strengths of the study.

Conclusion

This study assessed connexin 26 and 30 mutations in paediatric cochlear implant users in Mediterranean Turkey. The 35delG mutation of the connexin 26 gene was the most common mutation detected, similar to previous Turkish studies. The present study findings emphasise the importance of genetic diagnosis in the clinical setting. The possibility of prenatal or early postnatal assessment should be considered in all pregnancies for which both parents are known to be carriers of a connexin 26 gene mutation. It is hoped that such assessment will enable early diagnosis of affected newborns and will facilitate the early use of a hearing device, reducing subsequent hearing disability and improving intellectual development.

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