Original Article

Genetic and molecular analysis of the *CLDN14* gene in Moroccan family with non-syndromic hearing loss

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BACKGROUND: Hearing loss is the most prevalent human genetic sensorineural defect. Mutations in the *CLDN14* gene, encoding the tight junction claudin 14 protein expressed in the inner ear, have been shown to cause non-syndromic recessive hearing loss DFNB29.
AIM: We describe a Moroccan SF7 family with non-syndromic hearing loss. We performed linkage analysis in this family and sequencing to identify the mutation causing deafness.
MATERIALS AND METHODS: Genetic linkage analysis, suggested the involvement of *CLDN14* and *KCNE1* gene in deafness in this family. Mutation screening was performed using direct sequencing of the *CLDN14* and *KCNE1* coding exon gene.
RESULTS: Our results show the presence of c.11C>T mutation in the *CLDN14* gene. Transmission analysis of this mutation in the family showed that the three affected individuals are homozygous, whereas parents and three healthy individuals are heterozygous. This mutation induces a substitution of threonine to methionine at position 4.
CONCLUSION: These data show that *CLDN14* gene can be i mplicated in the development of hearing loss in SF7 family; however, the pathogenicity of c.11C>T mutation remains to be determined.

Key words: *CLDN14* gene, hearing loss, Moroccan family, mutation

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Introduction

Hearing loss is the most prevalent human genetic sensorineural defect. It occurs in 1 in 500 births and affects 278 million people world-wide.[1,2] The majority of the genes responsible for this disease have not yet been cloned and little is known about the corresponding gene products and their function in the cochlea. Nevertheless, several genes responsible for neurosensory deafness are involved in the regulation of the crucial inner ear ion homeostasis. The most studied include genes coding for the connexins (*GJB2*,[3] *GJB3*[4] and *GJB6*[5]), for the ion channels (*KCNQ4*,[6] *SLC26A4*[7] and *SLC26A5*[8]) and the tight junction proteins (*TRIC*[9] and *CLDN14*[10]). Claudin 14 is one of the members of the claudin family, which is expressed in the different tissues as liver and kidney, also in the cochlea the hair cells, the supporting cells and the sensory epithelium of the vestibular system. Claudin 14 participates in the formation of tight junctions in different epithelial cells including those of the cochlear sensory epithelia. The *CLDN14* (*NM_012130*) gene is composed of three exons, coding for a protein of 239 amino acid residues (10). In human *CLDN14* mutations cause profound, congenital, recessive deafness DFNB29, possibly related to failure of maintain the electrochemical gradient between the endo-lymphe and its surrounding tissues in the Corti inner ear organ.[10]

World-wide studies of the large varieties deafness genes are emerging rapidly. Identifying genes underlying

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hearing loss represents a powerful tool for discovering the molecular mechanisms that control the development, function and maintenance of the auditory system. In Moroccan population, we described the contribution of the *GJB2*,[11-13] *GJB6*,[14] *GJB3*,[14] *12sRNA*,[15] *ESPS*,[16] *TPRN*[17] and *TMPRSS3*[18] genes in inherited deafness. In order to add to the knowledge of genes involved in deafness in our population, we performed a genetic analysis of the *CLDN14* gene in Moroccan family with non-syndromic hearing loss (NSHL) with unknown etiology.

Materials and Methods

Subjects

Three affected females with NSHL of the Moroccan SF7 family were born to consanguineous parents. Three additional healthy siblings and the parents were also recruited. An informed consent was signed by each family member participating or by their parents. Clinical information was collected with a questionnaire and the study was performed in accordance with the Declaration of Helsinki protocols. Patients were previously tested negative for the most common connexin (*GJB2*, *GJB6* and *GJB3*) and mitochondrial (12sRNA) mutations. The control group consisted of 60 healthy Moroccan normally hearing from different geographic areas of Morocco with no familial history of hearing problems.

Genomic DNA was extracted from peripheral blood of affected individuals and their family members by standard phenol chloroform method.[19]

Genotyping and linkage analysis

In order to determine the genetic cause involved in deafness, we performed genetic linkage analysis in SF7 family. We genotyped DNAs from the available SF7 family members, using STR markers that have previously been linked to deafness in families of the various ethnic backgrounds (Hereditary Hearing Loss Homepage. URL: http://hereditaryhearingloss.org).

In this study, we used the genotyping approach with fluorescently labeled primers. Polymerase chain reaction (PCR) amplification was carried out in a total volume of 15 μl containing 30 ng of genomic DNA, 1 × PCR buffer, 200 μM of deoxyribonucleotide triphosphates (dNTPs), 0.75 U of Taq polymerase, 2 pmol of each primer and 0.2 pmol of the adapter, which is marked by the dye 6-FAM or HEX dye. Amplification conditions were: 7 min denaturing step at 94°C, followed-by 35 cycles consisting in 94°C for 35 s, 56°C for 35 s and 72°C for 35 s, with a final extension at 72°C for 10 min. After denaturation in 100% formamide (9 μl) at 95°C for 3 min, the PCR products (1 μl) and the internal size standard (0.5 μl) (GeneScan-500 LIZ; Applied Biosystems) were separated by capillary electrophoresis on ABI 3130 genetic analyzer. Allele sizes of STR markers, in base pair, were determined using ABI GenMapper software v. 2.4 by comparison with those of the internal size standard.

Genotyping showed linkage of the marker D21S1252 (known locus DFNB29-related gene containing *CLDN14*) to deafness in SF7 family. To confirm this linkage, we used the Sequence Tagged sites markers located on the region (21q22.13) [Table 1]. Allele sizes of the STR markers, in base pair, were determined using a ABI GenMapper software v. 2.4 by comparison with those of the internal size standard.

Molecular analysis of CLDN14 and KCNE1 gene

Mutation screening was performed using direct DNA sequence analysis of the *CLDN14* and *KCNE1* coding exon gene. PCR was carried out in a total volume of 15 μl containing 30 ng of genomic DNA, 1.5 mM MgCl2, 200 μM dNTP, 1 × PCR buffer, 0.75 U of TaqDNA polymerase and 7 pmol for each primer. We used two pairs of primers covering the entire *CLDN14* coding region: Cldn14F: 5'-CCCATTTCCTTTCTCTCCCT-3' and Cldn14R1: 5'-GGGCAGCAGCGGGTTGTAGA-3'; CIdn14F1-5-'TCGGCGGCACCCTCTTCATC-3' and Cldn14R: 5'-TTTCCCCCTCTGTCCCTGTG-3', generating two fragments of 586 and 531pb, respectively, overlapping on 101 bp.[20] The *KCNE1* gene was amplified using primers Kcne1F: 5'-TTTTGATTTGGGGTTGCA T-3' and Kcne1R: 5'-GCTAGCTGCAAGGGAGTCT-3'. PCR conditions were as follows: 94°C for 5 min followed-by denaturation at 94°C for 35 s, annealing at 58°C for 35 s and extension at 72°C for 1 min for 35 cycles, with a final extension at 72°C for 7 min.

Table 1: Microsatellite markers tested to confirm the association of the chromosomal region of chromosome 21 in family SF7

Marker	Chromosome	UniSTSid	Size in bp	Primers (5'→3')
D21S263	21q22.11	80288	175-201	TTGGCTTTGGAACCAG CATCAGCAAGGGTCCTC
D21S1910	21q22.11	78914	194-266	TTCTCTGGAATAAACGTGG CACGGCAAAGTAGTATTTAATG
D21S65	21q22.12	32298	184-192	CCGAAAACTTACTGGAGAAC GATCATCCAGGAATCACCAA
D21S1221	21q22.12	148244	226	CCCTATCTGTTCTTGCCAGC TTGCTTAAAAGGGAGTTTCACC
D21S1252	21q22.13	68655	231-251	TCTGTCTTTGTCTCACTATCTG GCAATGCTCTGTGGCT
D21S1222	21q22.13	148245	229	TCAAAGTGAGAATTCTTAAAATTCC AGCTCCTGAGACTGCAAATG
D21S267	21q22.13	19785	175-203	ATGGATCTGGATTTCTATCTTC CCTCCAACCTGGGTGA
D21S270	21q22.13	41735	199-223	GAAATGTTTTAATAAATGGTGGTTA ACAAAGTTATGGTCAAGGGG
D21S1255	21q22.2	13925	112-126	AGCTCTTTATTTTGCCACATAG CTGCATGTTTGCCTGG

The PCR products were purified by incubation with Exonuclease I and shrimp alkaline phosphatase. Direct sequencing of PCR products was performed with the ABI prism Big Dye Terminator cycle sequencing Ready Reaction kit v. 3.1 (ABI Prism/Applied Biosystems, Foster City, CA) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystem). Sequence analysis was carried out with the ABI SeqScape v. 2.5 Software.

Results

Results of genotyping

Linkage analysis in SF7 family based on STRs markers genotyping showed the presence of homozygosity region by descent Marker D21S1252, located at the 21q22.13 region, in deaf individuals indicating the presence of a linkage between this region and deafness in this family. Confirmation of this linkage was tested by analysis of additional STRs markers in this region. The phasing of alleles identified in each individual SF7 family confirmed the linkage potential of deafness in this family to the region bounded by D21S263, D21S1910, D21S65, D21S1221 and D21S1252 markers. This connection is expressed by homozygosity of the markers of the region's potential susceptibility in affected individuals. Thus, two affected individuals shared the same haplotype covering the region tested. Another individual with this haplotype shared common part indicating the presence of possible recombination. However, parents and other individuals of the family are clinically normal in the heterozygous state of this haplotype [Figure 1].

These results indicate that the variation gene is likely located in this candidate region. Indeed, it has been reported in the literature that two genes in this region are involved in deafness, *CLDN14* and *KCNE1*, the latter was responsible for Jervell and Lange-Nielsen syndrome, it is also involved in non-syndromic autosomal recessive hearing loss.[21] So we considered that these two genes may be involved in deafness for SF7 family.

Results of sequencing

The sequence analysis of *KCNE1* gene showed no deleterious mutation or variations in deaf patients tested in SF7 family.

The analysis of the *CLDN14* gene in SF7 family revealed a c.11C>T mutation. Transmission analysis of this variant in the family showed that the three affected individuals are homozygous, whereas parents and three healthy individuals are heterozygous, suggesting that we are facing a recessive mutation. The c.11C>T mutation [Figure 2] leads to the substitution of threonine to methionine at position 4 of the protein (p.T4M).

In controls, we revealed the c. 11C >T mutation at heterozygous state in 4 individuals with a frequency of 3.33%.

Discussion

CLDN14 gene encodes a member of the claudin family of tight junction proteins. The importance of claudin 14 for normal hearing was demonstrated by the association between profound congenital deafness and mutations of *CLDN14*.[10] A recent study made in 30 consanguineous Pakistani families with multiple affected individuals and 57 sporadic cases with moderate to severe hearing loss, showed that individuals with mutations of *CLDN14* suffer from the different degrees of hearing loss with an increase of severity in high frequencies.[22]

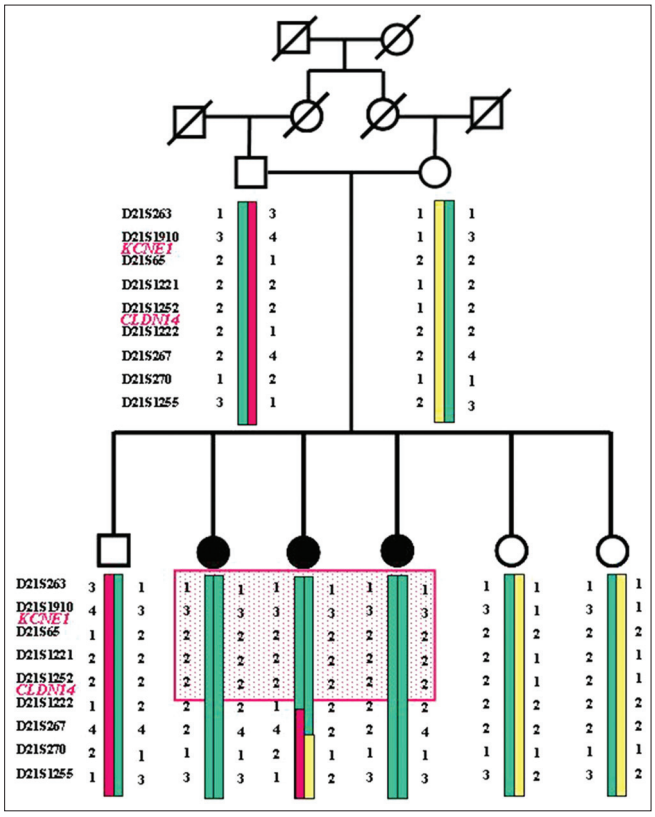

Figure 1: Pedigree and haplotypes of the SF7 family

In this study, linkage analysis in SF7 family with NSHL showed the presence of homozygosity region located at the 21q22.13 region, in deaf individuals indicating the presence of a linkage between this region and deafness in this family. Later, the sequencing of the *CLDN14* gene in this family showed a missense mutation (c.11C>T) leading to a change of p.T4M. It was found at homozygous state in three patients and at heterozygous in parents and three healthy brothers. We also found this mutation in Moroccan healthy individuals at heterozygous state in 4 controls.

This mutation was described in other studies such as Tunisian, Turkish, Greek and Spanish patients.[20,23,24] The c.11C>T mutation modifies a conserved residue between the claudins 9, 10, 11 and 18 genes.[20] p.T4M protein (O95500) showed a diffuse cytoplasmic localization. It was suggested that the cytoplasmic and/or transmembrane regions of claudins play a role in establishing fibril architecture.[25]

A study conducted in 102 Tunisian patients with autosomal recessive NSHL, showed the presence of six variants in the *CLDN14* gene, c.11C>T, c.58G>A, c.63G>A, c.372C>A, c.243C>T, c.687G>A, which were classified as non pathogenous. The c.11C>T mutation

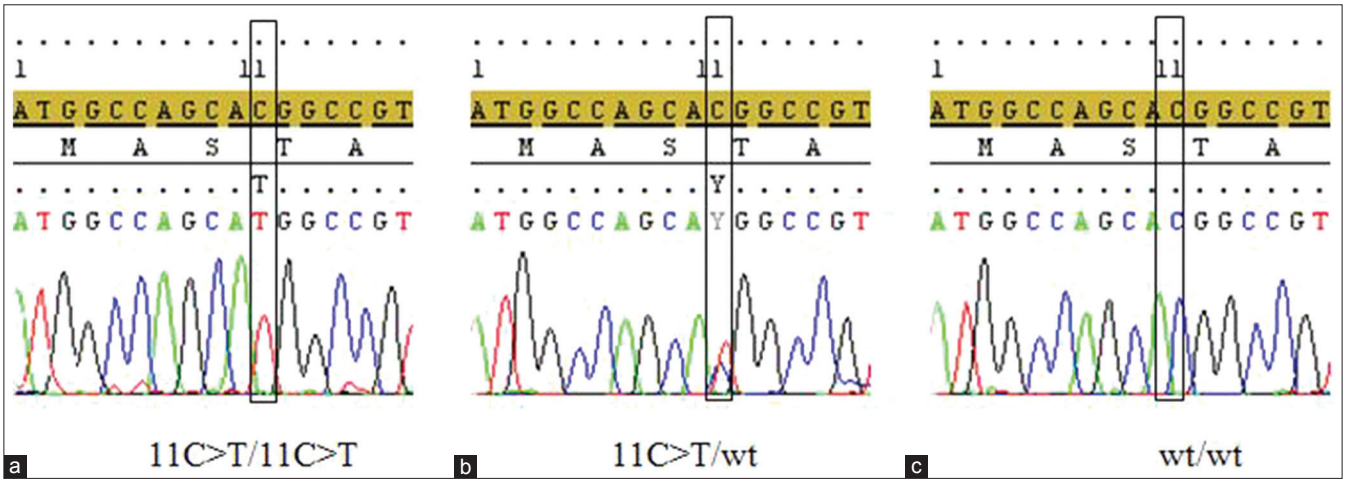

Figure 2: Sequence chromatograms showing the c.11C>T (threonine to methionine at position 4) variant in the *CLDN14* gene. (a) homozygous deaf patient (c.11C>T/c.11C>T), (b) Heterozygous deaf patient (c.11C>T/wt), (c) Homozygous individual with the wild type variant (wt/wt)

was found in 8 homozygous families presenting severe to profound NSHI and two heterozygous families.[23]

In addition, another study performed in 60 index patients from large Turkish families with autosomal-recessive NSHL, suggested that the c. 11C >sT mutation is frequent and non pathogenous.[24]

Unlike these studies, others have shown the presence of the pathogenic mutations in the *CLDN14* gene.[10,20] Recently, three new mutations (c.167G>A, c.242G>A and c.694G>A) responsible to cause deafness were identified in Pakistan, in addition to c.11C>T variant, which is not pathogenic according to bioinformatics analyzes.[26]

Pathogenicity of a mutation is an ongoing subject of discussion. The quandary becomes particularly acute when we are trying to determine if a missense alteration in a candidate gene is important (disease associated). In our study, the c.11C>T mutation was found at the homozygous state only in patients and in healthy individuals at the heterozygous state. The absence of a functional study of this mutation does not give an idea of its pathogenic effect. This homozygous state in our patients might be observed in many other loci in the SF7 family, but the *CLDN14* gene is the most distinguished gene.

In conclusion, our results show that *CLDN14* gene can be responsible for deafness in Moroccan SF7 family. While, the pathogenicity of c.11C>T mutation is still under discussion and functional studies are needed to further determine its pathogenicity.

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