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DFNB66 and *DFNB67* loci are non allelic and rarely contribute to autosomal recessive nonsyndromic hearing loss

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ABSTRACT

We previously mapped the *DFNB66* locus to an interval overlapping the *DFNB67* region. Mutations in the *LHFPL5* gene were identified as a cause of DFNB67 hearing loss (HL). However, screening of the coding exons of *LHFPL5* did not reveal any mutation in the DFNB66 family. The objective of this study was to check whether *DFNB66* and *DFNB67* are distinctive loci and determining their contribution to HL. In the DFNB66 family, sequencing showed absence of mutations in the untranslated regions and the predicted promoter sequence of *LHFPL5*. Analysis of five microsatellites in the 6p21.31–22.3 region and screening of the *LHFPL5* gene by DNA heteroduplex analysis in DHPLC revealed a novel mutation (c.89dup) in one out of 129 unrelated Tunisian families with autosomal recessive nonsyndromic (ARNS) HL. Our findings suggest that two distinct genes are responsible for DFNB66 and DFNB67 HL. These loci are likely to be a rare cause of ARNSHL.

1. Introduction

Sensorineural hearing loss (HL) is the most frequent sensory defect in childhood, one child in 1000 is born deaf [1]. Several studies suggested that more than 50% of childhood HL has a genetic origin of which approximately 70% are nonsyndromic. Most frequently (80%) the affection segregates as an autosomal recessive trait [1]. To date, 91 loci for autosomal recessive nonsyndromic HL (ARNSHL) have been mapped and 35 of the corresponding genes have been identified (http://hereditaryhearingloss.org). We previously mapped the *DFNB66* locus to a 16.5-Mb critical region flanked by D6S1602 and D6S1665 on human chromosome 6p21.2–22.3 [2]. Meanwhile, another locus, *DFNB67*, was mapped in an overlapping interval on human chromosome 6 [3]. Sequence analysis of *LHFPL5* (lipoma HMGIC fusion partner-like 5) gene, also named *TMHS* (tetraspan membrane protein of hair cell stereocilia), revealed mutations in affected individuals from six Pakistani, Turkish and Palestinian families [3–5]. *LHFPL5* has four exons (NM_182548), which encode a 2162 base pair mRNA. The coding region is distributed along the first three exons. In the Tunisian DFNB66 family, no mutation in the coding exons of the *LHFPL5* gene has been detected [2]. No mutation was also detected in two Pakistani families segregating deafness consistent with linkage to DFNB66/67 intervals [3]. Similarity in the audiograms of DFNB66 and DFNB67 families was described [2–5].

The aim of our study was to check whether *DFNB66* and *DFNB67* are different and to determine their contribution to HL in the Tunisian population. We then analysed the Tunisian *DFNB66* family for mutations in the 5′ and 3′ untranslated regions (UTRs) and the predicted promoter region of the *LHFPL5* gene. In the absence of any pathologic change, we suggest genetic heterogeneity within the *DFNB66/67* region. In addition, we detected compatibility with linkage to markers of the *DFNB66/67* region only in 1 out of 129 Tunisian families with ARNSHL. Further screening of the *LHFPL5* gene identified a novel frameshift mutation. Therefore, we conclude that DFNB66 and DFNB67 genes are most likely to be a rare cause of ARNSHL in the Tunisian population.

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2. Materials and methods

2.1. Families and clinical evaluation

In this study we investigated 129 Tunisian families segregating ARNSHL. Clinical history interviews and physical examinations of members of these families ruled out the implication of environmental factors for causing the HL and the presence of a syndrome. Pure-tone audiometry testing was performed to define the severity of the HL. An additional 50 unrelated Tunisian subjects were recruited as controls with normal hearing to determine whether any novel sequence change might be a common polymorphism. Informed consent was obtained from all participants and from parents of subjects younger than 18 years of age.

2.2. Linkage analysis

DNA extraction was performed following standard phenol–chloroform method. Samples were genotyped using four known (D6S422, D6S276, D6S464 and D6S1560) and one novel (MLHFPL5) microsatellite markers in the DFNB66/67 locus. The primers designed to amplify the MLHFPL5 marker are: 5′-GTCCATCTC-TAGGGGCCTTC-3′ (forward primer) and 5′-ACATGTCCAGCCA-CCTCTTC-3′ (reverse primer). Fluorescently labelled alleles were analysed on an ABI PRISM 3100-Avant automated DNA analyzer (Applied Biosystems, Foster City, CA).

2.3. Promoter prediction

To predict the promoter region of *LHFPL5* gene, we used four complementary programs based on different algorithms and mathematical architecture: (i) FirstEF program (http://rulai.cshl.edu/tools/FirstEF/) which recognizes structural and compositional features such as CpG islands, promoter regions and first splice-donor sites by using discriminant functions [6], (ii) Promoter-Inspector (http:genomatix.de/online_help/help_gems/PromoterInspector_help.html) which predicts eukaryotic polymerase II promoter region in large genomic sequences with a high degree of specificity [7], (iii) Core Promoter (http://rulai.cshl.org/tools/genefinder/CPROMOTER/index.htm) using positional dependent 5-tuple measures, a Quadratic Discriminant Analysis (QDA) method to locate the transcription start sites (TSS) [8], (iv) Promoter2.0 (http://www.cbs.dtu.dk/services/Promoter/) which uses a neural network-genetic algorithm to predict TSS of vertebrate polymerase II promoters in DNA sequences [9].

To obtain alignments of similar regions in two DNA sequences, we used PipMaker (http://pipmaker.bx.psu.edu/pipmaker/). Regions of more than 5 kb upstream the coding sequence of *LHFPL5* gene in human and mouse were compared. The resulting alignments are summarized with a "percent identity plot". To check for the significance of the similar sequences and to find transcription factors binding sites (TFBSs), we used the Transfac database (http://www.gene-regulation.com).

2.4. Mutation analysis

For mutation detection, DNA heteroduplex analysis in Denaturing High Performance Liquid Chromatography (DHPLC) was performed. For a given sequence, WAVEMAKER™ software was used to predict temperature and gradient conditions that should resolve heteroduplexes on the WAVE system. Prior to DHPLC analysis, 5 μL of each PCR product of a patient sample was mixed with 3.5 μL of the wild type. The PCR products were denatured at 95 °C for 5 min and cooled to 25 °C using a temperature ramp of 1 °C/min to induce heteroduplex formation. DHPLC analysis was performed on the automated WAVE DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE). The mix was injected into the mobile phase (buffer A, 0.1 M TEAA; buffer B, 0.1 M TEAA/25% acetonitrile) using a flow rate of 0.9 mL/min. The PCR products were eluted from the solid phase by a linear gradient in 8.8 min sample run under partially denaturing conditions. The eluted products were detected by UV analysis at 260 nm.

Any variant identified by DHPLC in the 3 coding exons of *LHFPL5* was verified by sequencing. In addition, the predicted promoter sequence and the 5′ and 3′ UTRs of *LHFPL5* were amplified by PCR and sequenced using Big Dye Terminator Sequencing Kit and an ABI 3100-Avant automated DNA analyzer (Applied Biosystems, Foster City, CA). Sequences of primer pairs are listed in Table 1. All sequences were compared with the RefSeq Gene *LHFPL5* reference sequence (NM_182548).

3. Results

In this study, we screened the 5′ and 3′ UTRs of the *LHFPL5* gene in one affected individual from the DFNB66 family. However, mutations were not found in these regions. We analysed a region of 5 kb upstream the *LHFPL5* coding sequence with different software to predict the promoter region of this gene. A sequence of 686 bp between −192 and −877 positions (+1 being the A of the ATG translation start codon) was found using FirstEF, PromoterInspector and containing a TSS predicted by Core Promoter. Using Promoter2.0, a TSS was obtained at position −1978, 1100 bp upstream of the first promoter predicted region. The alignment of the 5 kb region between human and mouse using PipMaker software enables us to detect 37 similar regions with a percent identity ranging from 46% to 100%. In order to determine if one of these regions corresponds to a transcription factor binding site, we used the TRANSFAC database. One region of 24 bp from −337 to −360 corresponding to NRSF's (Neuron-restrictive silencer factor) transcription factor binding site was found. Finally, a region of about 2 kb from −192 to −1998 was screened for mutation but no variation was detected.

To determine the contribution of *DFNB66* and *DFNB67* loci to childhood HL, we genotyped 129 unrelated consanguineous Tunisian families affected with ARNSHL using five fluorescent microsatellite markers bordering the *DFNB66/67* loci and then performed

Table 1
Primer pairs used to sequence promoter and non coding regions of *LHFPL5* gene.

Region	Primer	Primer sequence (5′…3′)	PCR product size (bp)
5′UTR and Predicted Promoter	F	CTCTGCCCCTTCCCCGCCTCTG	656
	R	CGGAGGACAGCACGTTACCCAC	
	F1	GGCCTGTAGTGGGAGCTC	391
	R1	TGTGGATGGAGGCCTAGAAG	
	F2	GTGGTGGAGTTGGAGGTTCT	366
	R2	GCCCGTCTCCCTCTTC	
	F3	TCTGTTATCACACAATCAGTCT	373
	R3	CAATTCCAGCAGCAAAAA	
	F4	TCCTTAGCTCCGGACG	622
	R4	CCCCGTCCTCAAAGAC	
	F5	GTACCTTTGAGCACTTTAA	698
	R5	TGGCTAGTGTAAAGAACTAG	
	F6	AGGGGGAAACTGCAGAGATT	354
	R6	AAAGTGCTCAAAGGTACAT	
3′UTR	F1	TCTTGGAATCTTTTGTTCTTTT	389
	R1	ATGGACCCATTTAGCCCTCT	
	F2	GCAAATCGCTTCACCTTCTT	394
	R2	GTTCTGATGGCCCCTCCAT	
	F3	TTTTCTTATTGCTGCTCAGAG	495
	R3	CAACAAATACAAATTAAAAG	