RESEARCH NOTE

Two novel mutations in *ILDR1* gene cause autosomal recessive nonsyndromic hearing loss in consanguineous Iranian families

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Introduction

In a recent screening programme on hearing loss (HL), we examined 17 common autosomal recessive nonsyndromic hearing loss (ARNSHL) genes in every consanguineous Iranian family with ARNSHL that was referred to our centre. We first screened *GJB2* mutations and then utilized a panel of three to four short tandem repeats to analyse rest of the loci. Once a homozygous by descent (HBD) pattern was observed for a given locus, direct sequencing was performed to identify the possible mutation. Families that did not show HBD pattern were screened through otologic sequence capture of pathogenic exons (OtoSCOPE) targeted sequencing panel (http://medicine.uiowa.edu/morl/otoscope/). Using this strategy, we identified two novel mutations in the immunoglobuline-like domain-containing receptor 1 gene (*ILDR1*, MIM 609739): a 2-bp deletion, c.1217-18delTC and a substitution, c.305T>A, in consanguineous Iranian deaf families.

HL, which is the most common sensorineural impairment in humans, is caused by genetic defects in about half of all cases (Babanejad *et al.* 2012). In Iran, the frequency of HL is one in every 166 individuals and it ranks as the second most common disabling genetic impairment next to intellectual disability (Mahdieh *et al.* 2010; Babanejad *et al.* 2012).

The *ILDR1* gene in the DFNB42 locus (MIM 609646), which is located on chromosome 3q13.33, codes type-I transmembrane protein with a crucial role in epithelial barrier function in the ear (Borck *et al.* 2011; Higashi *et al.* 2013). Mutations in the *ILDR1* gene are common in the Iranian deaf population and have been reported in five unrelated families (Borck *et al.* 2011; Babanejad *et al.* 2012; Diaz-Horta *et al.* 2012). Additionally, one deaf family from Saudi Arabia and nine from Pakistan were also identified with *ILDR1* mutations (Aslam *et al.* 2005; Borck *et al.* 2011; Ramzan *et al.* 2014). In this study, we identified two other Iranian consanguineous deaf families with novel mutations in the *ILDR1* gene.

Materials and methods

Subjects

Recently, two consanguineous Iranian deaf families were referred to the Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, for genetic diagnosis. According to the guidelines of the Ethic Committee of Iran's Ministry of Health and Medical Education, written consent forms were obtained from participants. Physical examination of the affected individuals suggested a nonsyndromic HL pattern; no thyroid, ocular or cardiac symptoms were observed. Pure tone audiometry with air and bone conduction was completed at frequencies ranging from 250 to 8000 Hz. Tandem gait and Romberg testing were performed for vestibular function evaluation.

Mutation analysis

Blood samples were taken from affected individuals, normal siblings and parents. DNA was extracted from blood samples using the salting out method (Miller *et al.* 1988). Families were screened for *GJB2* mutations. Three pairs of primers were used to directly sequence the first and second exons of the gene by means of Big Dye Terminators (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, Foster City, USA) (table 1) (Najmabadi *et al.* 2002).

Families negative for *GJB2* mutations underwent screening for 16 known ARNSHL loci (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) (Babanejad *et al.* 2012). At each locus, allele segregation analysis was performed using three to four STR microsatellite markers (table 1 in electronic supplementary material). The markers were selected from the Genome Database (http://www.gdb.org/), and had heterozygosity in Iranian population.

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Table 1. Primers used for sequencing *GJB2* gene.

Gene	Exon	Forward primer	Reverse primer
GJB2	Exon 1	5′TGTGGGGTGCGGTAAAAGGCGCCACGG	5′GCAACCGCTCTGGGTCTCGCGGTCCCT
	Exon 2	5′ACACAAGCAGCATCTTCTTCC	5′TTCGATGCGGACCTTCTGG
		5′GCATGCTTGCTTACCCAGAC	5′TGTGGCATCTGGAGTTTCAC

STR-markers were analysed in six family members including parents, affected individuals and normal siblings using polymerase chain reaction (PCR) amplification followed by resolving the PCR products on 8% polyacrylamide gel and then silver nitrate staining (Bassam *et al.* 1991). The patterns of STR-markers' bands on the gel were evaluated to figure out the HBD at each ARNSHL locus. The following pattern of STR markers is similar to the pattern of HL inheritance in the families: homozygous for affected individuals, heterozygous for parents and heterozygous or homozygous with different pattern for the healthy sibling. In case this pattern was observed, the related ARNSHL locus was considered HBD and a potential cause of HL in the family. Once HBD at a locus was noticed, the entire coding region, exon–intron boundaries and UTRs were sequenced using Big Dye Terminators (Applied Biosystems 3130 Genetic Analyzer), to find the causative mutation. Sequencing data were analysed with CodonCode aligner software, ver. 4.0.4 (CodonCode Corp., Dedham, USA).

A family with no HBD pattern at any of the 16 ARNSHL loci, underwent OtoSCOPE test for the proband's sample (http://medicine.uiowa.edu/morl/otoscope/) (Shearer *et al.* 2010). OtoSCOPE uses custom-designed Agilent SureSelect kit (Agilent Technologies, Santa Clara, USA) to capture 90 known HL genes implicating in NSHL, Alstrom syndrome, Branchiootorenal syndrome, Jervell and Lange Nielsen syndrome, Pendred syndrome, Usher syndrome and Wolfram syndrome. The captured regions are then subjected to massively parallel DNA sequencing using Illumina Hiseq (Illumina, San Diego, USA).

The consequent phenotypes of the observed variants were predicted using following web servers: PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), PROVEAN (http://provean.jcvi.org/index.php), Mutation Taster (http://www.mutationtaster.org/) and combined annotation dependent depletion CADD (http://cadd.gs.washington.edu/score) (Kumar *et al.* 2009; Adzhubei *et al.* 2010; Choi *et al.* 2012; Kircher *et al.* 2014; Schwarz *et al.* 2014). Once we identified a deleterious homozygous mutation in the proband, its cosegregation with HL was checked by direct sequencing of the mutated region in the other members of the family.

Results

In this study, as a part of a comprehensive study in investigating the genetic causes of Iranian deaf families, we identified *ILDR1* mutations in two Iranian deaf families: L-8600475 and L-1195.

Family L-8600475

Family L-8600475 had five affected individuals in its fourth generation; four of them were in the core family along with their two unaffected siblings and the mother (figure 1a). Audiometry revealed bilateral moderately severe to profound prelingual HL with downward sloping audiogram (figure 1b). The allele segregation analysis of four *ILDR1*-linked STR markers (table 1 in electronic supplementary material) in this family showed cosegregation of deafness with a homozygous pattern at the DFNB42 locus (figure 1a). Direct sequencing of the *ILDR1* coding regions, exon/intron boundaries and UTRs in the proband, using 12 pairs of primers (table 2), revealed a novel homozygous 2-bp deletion: c.1217-18delTC, in exon 7 of the *ILDR1* gene (figure 1c; table 3). Phenotype analysis of this mutation predicted that a premature stop codon (p.S406X) emerges at the deleted site which was deleterious (Mutation Taster, disease causing; scaled CADD score, 19.89) (figure 1c).

Family L-1195

Family L-1195 included three affected individuals, three normal siblings and the parents (figure 1a). Audiometry revealed bilateral severe to profound HL with a downward sloping audiogram (figure 1b). Allele segregation analysis in this family did not support HBD pattern similar to HL inheritance at any of the loci; however, the OtoSCOPE test disclosed a novel homozygous missense mutation: c.305T>A, in exon 3 of the *ILDR* gene in proband (figure 1c; table 3). This mutation was predicted to cause the deleterious valine to glutamic acid substitution at position 102 (PolyPhen2, damaging; SIFT, damaging; PROVEAN, deleterious; Mutation Taster, disease causing; scaled CADD score, 23.1) (figure 1c).

Both mutations showed cosegregation with deafness in the related families. We did not find these mutations in 285 Iranians with normal hearing ability and they were not reported in the exome variant server (http://evs.gs.washington.edu/EVS/).

Discussion

ILDR1 is an evolutionary conserved protein among vertebrates which is expressed in various epithelial tissues. It has a major role in the maintenance of full barrier function in the inner ear epithelium by localizing at tricellular contacts and recruiting tricellulin (Borck *et al.* 2011; Higashi *et al.* 2013). ILDR1 is dominantly expressed around hair cells, but different mutations in *ILDR1* gene affect ILDR1 localization at

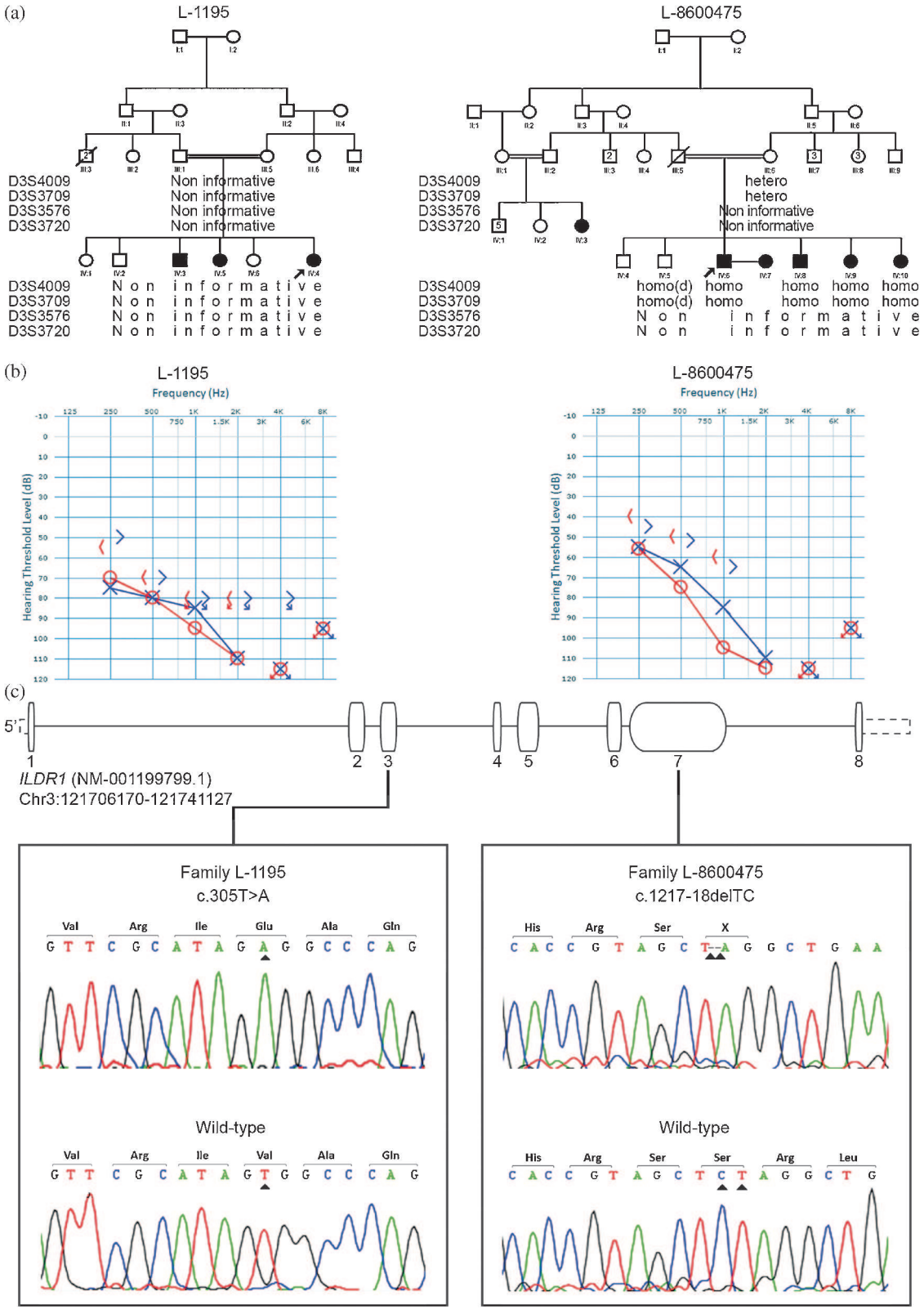

Figure 1. Families' data: (a) Pedigrees of families and allele segregation analysis results: for *ILDR1*-linked STR markers. Filled symbols for males (squares) and females (circles) represent affected individuals and empty symbols represent unaffected individuals. Arrow denotes the proband. Hetero, homo and homo(d) indicates heterozygous, homozygous and homozygous with different pattern statuses, respectively, for each STR marker in each analysed member of the family. Noninformative shows that the STR markers' patterns were inconclusive. (b) Pure tone air and bone conduction audiograms from left (blue) and right (red) ears of an affected individual in each family. (c) Sequence chromatographs of the mutated part of the *ILDR1* gene in proband of each family as compared to wild type. Arrows denote the mutations.

Table 2. Primers used for sequencing *ILDR1* gene.

Gene	Exon/intron	Forward primer	Reverse primer
ILDR1	Exon 1	5′GCAGCAGGTAGGGAAGGTG	5′GCTCCAGGTTTCTCAGTTGC
	Exon 2	5′TTCCTGGGAGTTTTCCTC	5′TGGGCTCCGTTTGTTTATCT
	Exon 3	5′CAAGAGTCCCAAGGGATG	5′CAACAGGCAGCAGAAAGAAA
	Exon 4	5′CACCTCGGTCTCCAGAGAAG	5′CCAAGCAGGGGTTGAACTAA
	Exon 5	5′TCAAGAGCTGTGTTTATGAGACC	5′TTCAGGGCTGAGGCTAATGT
	Exon 6	5′TGGGGTACAAAAGTTGCAGTC	5′GAACAAATGGCCCAAAACAT
	Exon 7	5′AAGACCTCTGTGACCGAT	5′CTAGAGCTACGGTGCCTTCC
		5′ATCTGAGGGAGGGGAGAA	5′AGAGGCAGCCTGTGTTGG
	Exon 8	5′ACCTAAGCCTGGGGAGAGAG	5′TTGCACTCCTGGGCTCAA
		5′TGGGAGGCTAAGAATCACTTG	5′ATGGGCTGGCAGGATAAACT
		5′GCCTTGGTTCATGCTCTCTT	5′TGCCAGGAGAATGGTAGCTC
	Intron 4	5′TGTGTAGGAGCAGATCTC	5′CTGCTTGTCTGTCCCCTCTC

Table 3. *ILDR1* mutations found in Iranian, Pakistani and Saudi Arabian families.

	Mutation (c.DNA)*	Mutation classification	Position	Predicted protein*	Ethnicity	No. of reported families	Reference
1	c.59-5_88del	Splice Site-deletion	Intron 1/ Exon 2	–	Iran	1	Borck *et al.* (2011)
2	c.583 C>T	Nonsense	Exon 5	p.Q195X	Iran	1	Borck *et al.* (2011)
3	c.379+1G>A	Splice site	Intron 3	–	Iran	2	Babanejad *et al.* (2012)
4	c.820 C>T	Nonsense	Exon 7	p.Q274X	Iran	1	Diaz-Horta *et al.* (2012)
5	c.1217_18delCT	Deletion	Exon 7	p.S406X	Iran	1	Present study
6	c.305 T>A	Missense	Exon 3	p.V102E	Iran	1	Present study
7	c.1135G>T	Nonsense	Exon 7	p.Glu379X	Pakistan	1	Borck *et al.* (2011)
8	c.3G>A	Start codon	Exon1	p.Met1?	Pakistan	1	Borck *et al.* (2011)
9	c.290G>A	Missense	Exon 3	p.Arg97Gln	Pakistan	1	Borck *et al.* (2011)
10	c.411delG	Deletion	Exon 4	p.Trp137CysfsX25	Pakistan	1	Borck *et al.* (2011)
11	c.1387C>T	Missense	Exon 7	p.Arg463Cys	Pakistan	1	Borck *et al.* (2011)
12	c.499+1G>A	Splice site	Intron 4	–	Pakistan	1	Borck *et al.* (2011)
13	c.1032delG	Deletion	Exon 7	p.Thr345ProfsX20	Pakistan	2	Borck *et al.* (2011)
14	c.1180delG	Deletion	Exon 7	p.Glu394SerfsX15	Pakistan	1	Borck *et al.* (2011)
15	c.1358G>A	Missense	Exon 7	p.Arg453Gln	Pakistan	1	Borck *et al.* (2011)
16	c.325_333dupAATGAGCCC	Duplication	Exon 3	p.Asn109_Pro111dup	Saudi Arabia	1	Ramzan *et al.* (2014)

*****_ILDR1_ transcript (NM_001199799.1) and encoded protein (NP_001186728.1) used for mutation nomenclature.

tricellular contacts and the recruitment of tricellulin (Higashi *et al.* 2013).

Earlier, 17 different *ILDR1* mutations—including missense, nonsense, deletion, duplication, splice site mutations and a start codon mutation—have been found in Pakistani, Iranian and Saudi Arabian families with ARNSHL (Aslam *et al.* 2005; Borck *et al.* 2011; Babanejad *et al.* 2012; Diaz-Horta *et al.* 2012; Ramzan *et al.* 2014).

ILDR1 mutations were first described by Borck *et al.* (2011). They reported 10 different homozygote mutations in nine Pakistani and two Iranian deaf families (table 3). The Iranian families which were recruited by our group were diagnosed with severe to profound HL and identified with a splice site deletion in intron 1 and exon 2 (c.59-5_88del) as well as a nonsense mutation in exon 5 (c.583C>T, p.Q195X) (Borck *et al.* 2011) (table 3). Later, three additional Iranian deaf families were identified with *ILDR1* mutations. The genetic cause of deafness in two unrelated families with profound HL was the same: a homozygote splicing site mutation, which leads to the omission of exon 3 in the transcript (c.379+1G>A) (Babanejad *et al.* 2012) (table 3). In the third family, a stop codon gaining mutation in exon 7 leads to a truncated protein with no function (c.820C>T, p.Q274X) (Diaz-Horta *et al.* 2012) (table 3). Recently, Ramzan *et al.* (2014) has identified mutated *ILDR1* in one out of 100 Saudi Arabian deaf families. Although rare in Saudi Arabian population, *ILDR1* mutations seem to be common in Pakistani and Iranian populations (Borck *et al.* 2011; Babanejad *et al.* 2012; Diaz-Horta *et al.* 2012; Ramzan *et al.* 2014).

In this study, we observed two mutations out of 17 in the *ILDR1* gene that have been reported worldwide: a 2-bp deletion (c.1217-18delCT) and a missense mutation (c.305T>A), which cosegregate with ARNSHL in two consanguineous Iranian families. Both families showed downward sloping audiograms. The reported Iranian, Pakistani and Saudi

Arabian deaf families have shown downward sloping or flat audiograms, suggesting either pattern could be observed in *ILDR1*-mutated deaf families (Aslam *et al.* 2005; Borck *et al.* 2011; Babanejad *et al.* 2012; Diaz-Horta *et al.* 2012; Ramzan *et al.* 2014).

The c.1217-18delCT mutation was predicted to introduce a premature stop codon that may lead to a truncated protein with no function, which can induce NSHL phenotype in the family. This novel mutation occurred in exon 7 of *ILDR1*, where 50% of mutations reported by Borck *et al.* (2011) took place. This finding is in concordance with Borck *et al*.'s suggestion that exon 7 of *ILDR1* is a critical region for *ILDR1* mutations (Borck *et al.* 2011). The *ILDR1* missense mutation has not been observed in Iranian deaf families earlier. In this study, we have identified the first one: c.305T>A, which leads to a valine-to-glutamic acid substitution in the 102 amino acid residue. This substitution is located in the immunoglobulin-like domain of ILDR1 protein and is predicted to be damaging by five *in silico* human variants evaluating programmes.

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