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Nonsyndromic recessive deafness *DFNB18* and Usher syndrome type IC are allelic mutations of *USHIC*

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Abstract Human chromosome 11 harbors two Usher type I loci, *USHIB* and *USHIC*, which encode myosin VIIA and harmonin, respectively. The *USHIC* locus overlaps the reported critical interval for nonsyndromic deafness locus *DFNB18*. We found an IVS12+5G→C mutation in the *USHIC* gene, which is associated with nonsyndromic recessive deafness (*DFNB18*) segregating in the original family, S-11/12. No other disease-associated mutation was found in the other 27 exons or in the intron-exon boundaries, and the IVS12+5G→C mutation was not present in 200 representative unaffected individuals ascertained from the same area of India. An exon-trapping

assay with a construct harboring IVS12+5G→C generated wildtype spliced mRNA having exons 11 and 12 and mRNA that skipped exon 12. We conclude that mutations of *USHIC* can cause both Usher syndrome type IC and nonsyndromic recessive deafness *DFNB18*.

Introduction

Usher syndrome type I is genetically heterogeneous and characterized by bilateral sensorineural deafness, vestibular dysfunction, and visual impairment caused by retinitis pigmentosa (Petit 2001; Smith et al. 1994). Genes for six Usher loci (*USHIB*, *USHIC*, *USHID*, *USHIF*, *USH2A*, *USH3*) have been identified, two of which have mutant alleles associated with non-syndromic recessive deafness (Ahmed et al. 2001; Alagramam et al. 2001; Bitner-Glindzicz et al. 2000; Bolz et al. 2001; Bork et al. 2001; Verpy et al. 2000; Weil et al. 1995). *DFNB2* and Usher type IB (*USHIB*) are caused by mutant alleles of *MYO7A* (Weil et al. 1997), and we have recently reported that truncating mutations of *CDH23* encoding cadherin 23 cause Usher syndrome type ID, whereas less disabling mutant alleles result in nonsyndromic deafness *DFNB12* (Bork et al. 2001).

We have previously reported linkage of nonsyndromic recessive deafness (*DFNB18*) on chromosome 11p15.1 (lod score 4.4 at 0=0 for *DIIS1888*) in family S-11/12 from India; this co-localizes with *USHIC*. We have also postulated that these two hereditary disorders are associated with allelic variants of the same gene (Jain et al. 1998). In an Acadian population living in southwestern Louisiana, Usher syndrome type IC (*USHIC*) has been mapped to chromosome 11p15.2-p14 (Smith et al. 1992). Mutations of the gene encoding harmonin have been identified as the primary defect in *USHIC* patients (Bitner-Glindzicz et al. 2000; Verpy et al. 2000). *USHIC* has 20 primary and 8 alternatively spliced exons encoding several isoforms (Verpy et al. 2000). Depending upon the harmonin splice isoform, there are either two or three PDZ domains and one or two coiled coil regions in the en-

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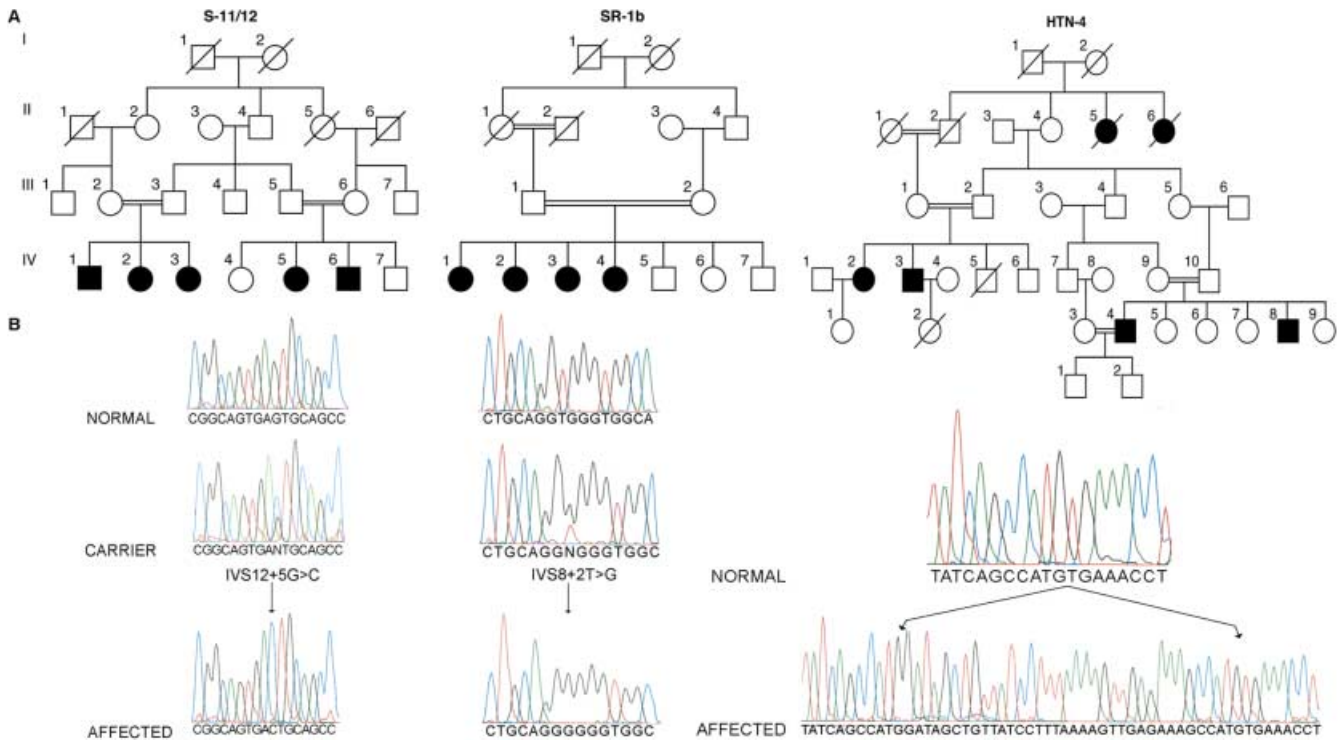


Fig. 1 **A** Pedigrees of family S-11/12 (*DFNB18*), SR-1b (*USH1C*), and HTN-4 (*USH1C*). Electroretinogram and electroneurogram results of affected individuals IV:1 and IV:6 revealed normal retinal and vestibular function in family S-11/12. Fundoscopic examination revealed pigmentary changes in the retina of affected individuals of Usher syndrome type I families SR-1b and HTN-4. **B** Sequence chromatograms for the splice sites of the homozygous wildtype, heterozygous, and homozygous mutant alleles found in families S-11/12 and SR-1b, and wildtype and homozygous mutant alleles found in family HTN-4. Arrows IVS12+5G→C and IVS8+2T→G replacements and the 36-base insertion found in these families

coded protein (Verpy et al. 2000). Here, we report a leaky splice site mutation of the *USH1C* gene associated with nonsyndromic deafness in the original Indian family (S-11/12) linked to *DFNB18* and two novel and two previously reported *USH1C* mutations in five consanguineous families segregating Usher syndrome type I (Fig. 1A).

Materials and methods

Families and genomic DNA isolation

Institutional review board approval (OH93-N-016) and written informed consent were obtained for all subjects in this study. Peripheral blood samples were obtained from participating subjects. DNA was extracted from peripheral blood or buccal swabs (BucalAmp DNA extraction kit, Epicentre Technologies). Pure-tone audiometry, funduscopy, and electroretinogram examinations were performed on selected affected individuals over the age of 13 years from each family.

Genotyping and sequencing

Linkage analyses were performed by using markers in the *USH1C* critical interval (Keats et al. 1994). After confirming linkage to

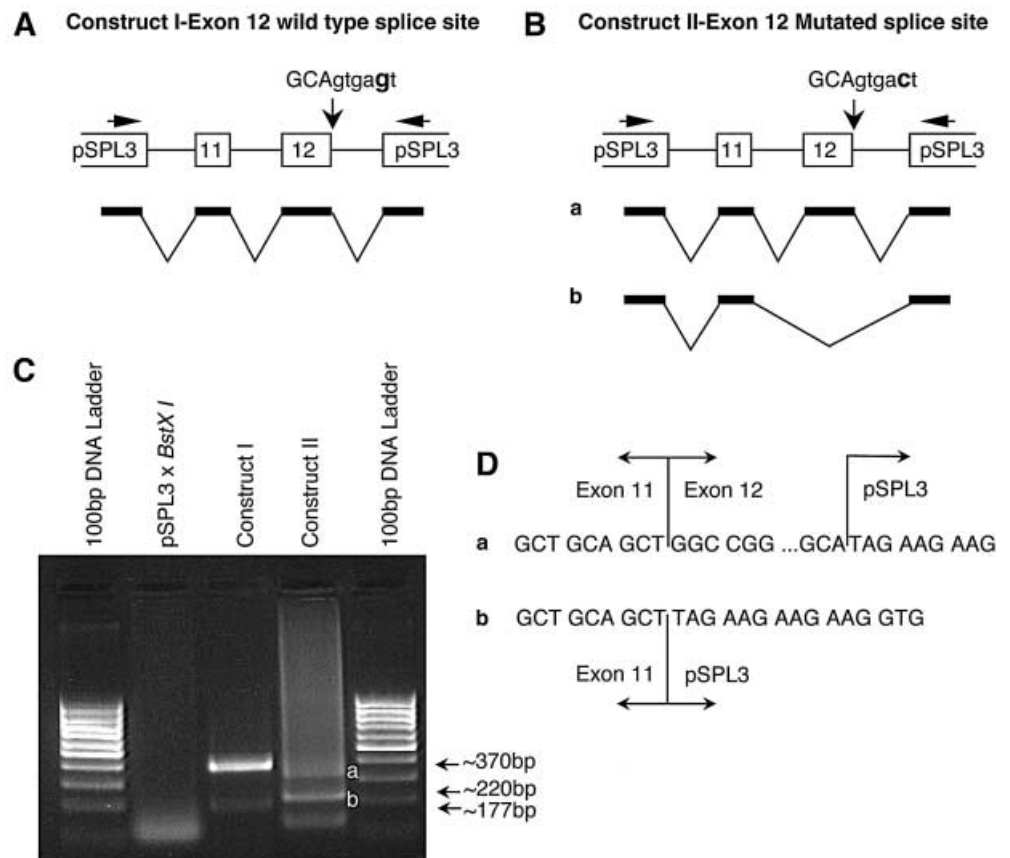
11p15.1 in nonsyndromic deafness (S-11/12) and Usher families (SR-1b, HTN-4, SR35a, DF080, DF086), we amplified the 28 exons of *USH1C* by polymerase chain reaction (PCR) and sequenced them on an ABI 377 with the reported primers (Verpy et al. 2000). Data was analyzed by using ABI Sequencing Analysis (v. 3.4.1) and LASERGENE-SeqMan software.

Exon trapping

To determine the effect of the splice site mutation found in family S-11/12, the wildtype and mutant alleles were PCR-amplified, cloned into pSPL3 (Invitrogen), and sequence verified (pSPL3 has an SV40 segment for replication and transcription in African green monkey kidney cells, COS-7). Purified cloned DNA of the experimental, wildtype, and empty-vector control constructs were separately transfected into COS-7 cells by using LipofectAMINE 2000 and PLUS reagent (Invitrogen). As a measure of the quality of transfection of COS-7 cells, GFP from pEGFP-N2 (Clontech 6081-1) was also transiently transfected. GFP was expressed in approximately 60%–70% of the cells.

Forty-eight hours after transfection of the pSPL3 constructs, RNA was extracted from the COS-7 cells by using TRIZOL (Invitrogen), and single-strand cDNA was synthesized (Invitrogen 18449-017). *Bst*XI endonuclease was used to digest vector-derived products as described by the manufacturer (Exon Trapping System, Invitrogen). Primary and nested secondary PCR amplifications of the cDNA were performed with vector primers. Aliquots of 10 μ l of each amplicon were analyzed on a 2% agarose gel (Fig. 2C). DNA bands were extracted (QIAquick 28706) and sequenced with the vector primers. The nested secondary PCR products were also cloned into pAMP10 (Invitrogen) and transformed into *Epicurian Coli* XL-10 Gold ultracompetent cells. Plasmid DNA was isolated (QIAprep 27106) and sequenced with the SD2 vector primer. We obtained the same sequence results from the subcloned amplicons and the agarose gel bands that were sequenced directly.

Fig. 2A–D Exon trapping results. **A, B** Genomic constructs I and II with wildtype and mutant alleles, respectively, of exon 12 splice site transfected into COS 7 cells. **C** Representative results of three independent experiments performed in duplicate. Wildtype construct I shows only one band of ~370 bp, whereas construct II with the IVS12+5G→C allele gives rise to both an ~220-bp band and an ~370-bp band. **D** comparison of the nucleotide sequences at the boundaries of exons 11 and 12 and the vector in construct I (a) and in construct II (b). Only the 5' and 3' ends of exon 12 are shown. The remainder of the exon is indicated by the dotted line. Arrows Exon boundaries and exon-vector splicing



Results and discussion

Locus *DFNB18*

In family S-11/12, the oldest affected individuals at the time of ascertainment, IV-1 and IV-6, were 18 and 19 years old, respectively (Jain et al. 1998). Neither vestibular nor ocular phenotypes were detected in profoundly deaf individuals of this family. Linkage analysis of this family defined the *DFNB18* locus on chromosome 11p15.1. Sequence analysis of *USH1C* revealed a putative +5 splice site mutation in intron 12 (IVS12+5G→C; Fig. 1B). Homozygosity for the IVS12+5G→C mutation co-segregated with profound congenital deafness in family S-11/12 (data not shown). No other disease-associated mutation was found in the other 27 exons or in the intron-exon boundaries, and the IVS12+5G→C mutation was not present in 200 normal representative unaffected individuals ascertained from the same area of India.

In order to determine whether IVS12+5G→C alters normal splicing of *USH1C* mRNA, we made two constructs of genomic DNA for exon trapping. One construct was for the wildtype genomic sequence spanning intron 11 through intron 13, and the other had the splice site mutation IVS12+5G→C (Fig. 2A, B). The transfected empty pSPL3 vector produced the expected product of 177 bp, whereas the wildtype exon 12 splice site produced the expected single band of approximately 370 bp when ampli-

fied with vector primers (Fig. 2C). The construct with a mutated 5' splice site (IVS12+5G→C) when transfected in the exon trapping system produced a band of ~370 bp containing the wildtype sequence (exons 11 and 12) and a band of ~220 bp (Fig. 2C), which had skipped exon 12 (Fig. 2D). These results demonstrate that, in the exon trapping assay, IVS12+5G→C results in a mixture of wildtype spliced mRNA and mRNA that skips exon 12. If only exon 12 is skipped, then there is a shift in the reading frame and a predicted premature stop codon in exon 13 resulting in an mRNA that presumably undergoes nonsense-mediated mRNA decay (Lykke-Andersen et al. 2000). However, the effect, if any, of IVS12+5G→C on splicing of the downstream exons or on in vivo splicing of *USH1C* pre-mRNA is not known.

Locus *USH1C*

We also ascertained five families segregating Usher syndrome linked to markers on chromosome 11p15.1 (SR-1b, HTN-4, SR35a, DF080, and DF086). In family SR-1b from Pakistan, there were four offspring of a consanguineous mating all of whom had congenital profound deafness and retinitis pigmentosa. In this family, we found a novel splice donor site mutation, IVS8+2T→G, in *USH1C* (Fig. 1B). The IVS8+2T→G mutation was not found in 100 representative DNA samples from unaffected individuals from the same region of Pakistan. In

CHROMOSOME11p15.1

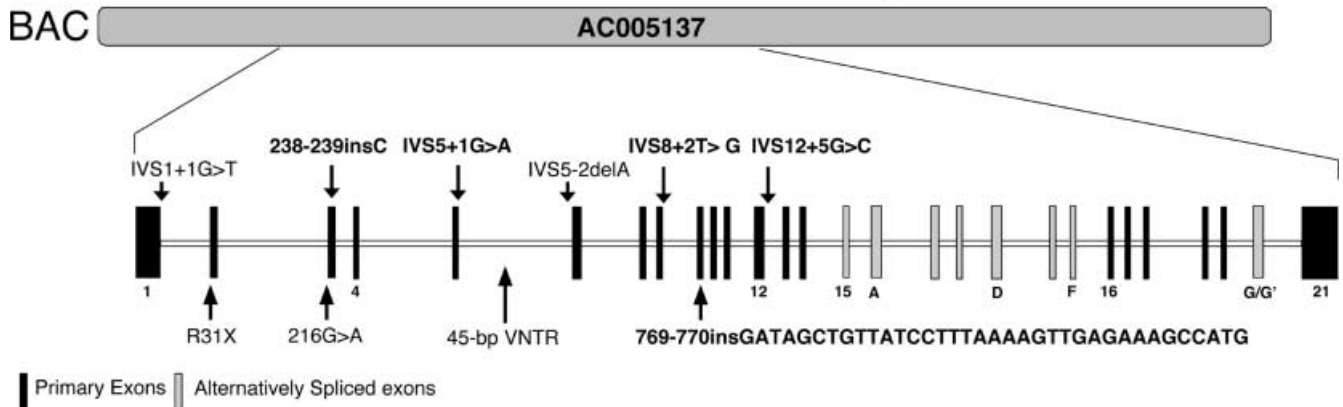


Fig. 3 Structure of the *USH1C* gene with reported mutant alleles. The 20 primary exons are shown in *black*, whereas the eight alternatively spliced exons are depicted in *light gray* (Verpy et al. 2000). Mutations found in this study are represented in *bold*. The 45-bp VNTR in intron 5 is also shown; it has nine repeats in the Acadian population and a maximum of eight in non-Acadian individuals. The expanded VNTR is in linkage disequilibrium with *USH1C* in Acadians (Savas et al. 2002)

family HTN-4 from India, we found an insertion of 36 bases in exon 9 at position 769–770, an insertion leading to a stop codon in exon 9. This mutation was not found in 100 representative DNA samples from India. In family SR35a, a mutational screen of *USH1C* revealed a homozygous IVS5+1G→A mutation, which was previously reported in a patient from Europe (Verpy et al. 2000). A 238–239insC mutation was found in two Usher type IC families, DF080 and DF086, a mutation that was also reported in a family from Pakistan and in many patients of European origin (Zwaenepoel et al. 2001). A summary of the *DFNB18* and Usher syndrome type IC mutations of *USH1C* is shown in Fig. 3.

In the mouse inner ear, harmonin is found in at least eight different alternatively spliced variants, differing in length from 420 to 910 amino acids (Verpy et al. 2000). Interestingly, isoforms of harmonin that include eight alternatively spliced exons (15, A, B, C, D, E, F, G/G; see Verpy et al. 2000) are not expressed in the retina. It has been hypothesized that mutations in these exons may cause nonsyndromic hearing loss *DFNB18* (Verpy et al. 2000). However, *in vivo*, the mutation IVS12+5G→C associated with nonsyndromic deafness in family S-11/12 may, in some of the transcripts, cause skipping of a primary exon of *USH1C* present in both eye and ear transcripts.

The correct splicing of pre-mRNA requires the presence and recognition of a number of sequences within introns, such as the highly conserved ag of 3' and gt of 5' splice sites (Padgett et al. 1986) and the less conserved nucleotides of the acceptor (y₁₁nyag|G) and donor sites (AG|guragu; Hawa et al. 1996; Maquat 1996). For example, mutations have been reported to occur at 5' splice site positions +2, +4, +5, +6, and +9 in several diseases (Bonadio et al. 1990; Svenson et al. 2001; Treisman et al.

1983), such as β -thalassemia, hereditary vitamin D resistance, protein C deficiency, and hereditary tryrosinemia type I (Divoky et al. 1992; Grompe and al-Dhalimy 1993; Hawa et al. 1996; Khan and Riazuddin 1998; Old et al. 2001; Reitsma et al. 1991). The β -globin genes expressed in HeLa cells with an IVS1+5G→C or IVS1+6T→C mutation produce wildtype mRNA and aberrantly spliced messages (Treisman et al. 1983). Similarly, for exon trapping experiments with the IVS12+5G→C mutation of *USH1C*, we report both normal and aberrantly spliced mRNA. Whereas normal retinal function is preserved in the presence of this leaky mutant allele, the cochlea is abnormal. Although many more mutations associated with Usher syndrome type IC or non-syndromic hearing loss *DFNB18* are required for a definitive answer, our data suggest that there may be a genotype-phenotype relationship in which recessive hypomorphic alleles of *USH1C* cause nonsyndromic hearing loss, whereas more severe mutations of this gene result in Usher syndrome.

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