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# Construction and characterization of a vestibular-specific cDNA library using T7-based RNA amplification

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**Abstract** Using a very small amount of inner-ear tissue, we constructed a human vestibular cDNA library by means of T7-based amplification of RNA. This library should allow us to identify genes likely to be involved in auditory and vestibular functions. Here we first report the characterization of the human vestibular cDNA library. Among 506 cDNA clones randomly selected from the vestibular cDNA library, DNA sequences of 301 cDNA clones were identical to those of genes of known function. Twenty-two cDNA clones were considered to be novel because they did not match any cDNA sequences in the public database. The information in our study will provide a valuable resource for identifying several novel genes underlying deafness disorders and vestibular dysfunction.

**Key words** Human vestibule · cDNA library · T7-based RNA amplification · Hearing loss · Vestibular dysfunction

## Introduction

Analysis of the human genome at the molecular level has made it possible to identify genes responsible for hereditary deafness; to date investigators have cloned 28 genes associated with nonsyndromic deafness and dozens involved in deafness syndromes (multiple phenotypic disease).

Among several different approaches for identifying aberrant genes responsible for hearing disorders, the most common strategy has been the positional-candidate approach coupled with gene-mapping information and genetic linkage analysis, using selected groups of affected and unaffected individuals. Many genes, including *GJB2*, *TECTA*, and *OTOF*, have been identified in this way (Kelsell et al. 1997; Verhoeven et al. 1999; Yasunaga et al. 1999). Although this method is very effective for discovery of novel deafness-related genes, narrowing a candidate locus to a very small interval requires participation of relatively large families and comprehensive clinicopathological data. Another approach is based on specific expression patterns or functions in target organs. Inner-ear cDNA libraries have been constructed from multiple samples of fetal cochlear tissue to isolate genes associated with hearing (Robertson et al. 1994; Jacob et al. 1997). This approach has identified three genes responsible for deafness: *ATQ1*, *COCH*, and *OTOR* (Robertson et al. 1994, 1997, 2000; Skvorak et al. 1997). A large series of cochlea organ analysis has been performed, including more than 4000 human expressed sequence tags (ESTs) from the fetal cochlear library deposited in the public database (see http://hearing.bwh.harvard.edu/cochlearcdnalibrary.htm). However, to date, no attempt has been made to characterize the expressed genes of the human vestibule representing a functionally essential compartment in the inner ear. Vestibular symptoms are clinically either sole complaints or sometimes associated with deafness. Among the cochleo-vestibular dysfunctions, Meniere's disease, which is defined as a clinically representative inner ear disorder accompanied by sensorineural hearing loss and vertigo, was dominantly inherited in some families (Fung et al. 2002). The importance of genetic factors in Meniere's disease has been suggested, but very few genes associated with vestibular dysfunction have been cloned. The *COCH* gene, which is predominantly expressed in the inner ear, is demonstrated to be responsible for one type of nonsyndromic hearing loss with progressive hearing loss and vestibular dysfunction (Robertson et al. 1998). In contrast to the recent progress in the discovery of genes responsible for hearing loss, genes related to vestibular symptoms are not yet fully understood. Although many similarities are observed in the general features of cochlea and vestibular end organs, there are consid-

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erable differences in these homologous organs, including their evolution, structure, and function.

Therefore, as an additional resource for investigating genes essential for hearing and equilibrium, we constructed a human vestibular cDNA library by means of T7-based amplification of RNA from a very small amount of adult inner-ear tissue (Luo et al. 1999), and here describe the detailed analytical results obtained from reverse transcriptase-polymerase chain reaction (RT-PCR) experiments. To our knowledge, this is the first study of the gene expression profiles of human vestibular tissue.

## Materials and methods

### RNA extraction and T7-based amplification

Tissue samples of an adult vestibule were obtained from a single patient during labyrinthectomy with prior written informed consent, and immediately stored at −80°C. Total RNA was extracted from each tissue with an RNeasy mini kit (Qiagen, Valencia, CA, USA). Because of the extremely small amount of vestibular total RNA available at nanogram-level quantities, we applied one cycle of T7-based RNA amplification after DNase I treatment of total RNA and subsequent cDNA synthesis, as described previously (Luo et al. 1999), with some modifications.

### Construction and analysis of a vestibular cDNA library

For second-strand cDNA synthesis, a linker-primer that included an internal *XhoI* site (5′-AGTCTCGAGTCTAG TCGACGGCCAGTGAATTGTAATACGACTCACTATAGG $GCGT_{21}$-3′) was used. After double-strand cDNA fragments of less than 200 bp were removed by the use of CHROMA spin column-400 (CLONTECH, Palo Alto, CA, USA), a cDNA library was constructed using the cDNA Synthesis kit (Stratagene, LaJolla, CA, USA) according to the supplier's instructions. From recombinant clones, we randomly selected 506 clones and determined their DNA sequences. The nucleotide sequence of each clone was compared with those of cDNA sequences in the GenBank database of the National Center for Biotechnology Information as of October 2002. The clones were classified according to the entries in GenBank (Table 1).

**Table 1.** Classification of cDNA sequences from the vestibular tissue

| Database match | *n* (%) |
|---|---|
| Number of clones sequenced | 506 |
| Known function genes | 301 (59.5) |
| Repetitive sequences | 139 (27.5) |
| Clones identical to reported ESTs | 29 (5.7) |
| Novel ESTs | 22 (4.3) |
| Mitochodrial DNA sequences | 15 (3.0) |

EST, Expressed sequence tag

### RT-PCR analysis

From among the 506 EST sequences obtained from the vestibular cDNA library, we selected clones that showed no match to any cDNA sequences in the database. Twenty-two novel EST sequences were submitted to the Center for Information Biology and DNA Data Bank of Japan (DDBJ); their GenBank accession numbers are AB0728187, AB072818, AB077323, AB074042, AB076975, AB074038, AB074039, AB072821–AB072825, AB074046, AB07673, AB074133, AB074135, AB076955, AB076958, AB076959, AB076961, AB076962, and AB076966. To determine the tissue expression patterns of the selected ESTs, we examined them by semiquantitative RT-PCR analysis using one round of T7-based amplified cDNAs from one vestibular organ and eight organs (brain, heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas). Human glceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a quantity control. Sets of primers corresponding to each gene were designed from nucleotide sequences of the vestibular cDNA clones obtained, and these sets were first tested on genomic DNA to test the PCR. Amplification was performed at an annealing temperature of 95°C for 2 min, followed by 30–35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The annealing temperature was adjusted according to the Tm of the primers used. PCR products were electrophoresed on a 2%–3% agarose/Ethidium bromide gel.

## Results

### Evaluation and functional categorization of the vestibular cDNA library

The cDNA library was constructed from membranous labyrinths (vestibule) from adult inner ear. Very careful microscopic dissection was performed to avoid contamination by surrounding bony, cartilaginous, and fibrous elements. We obtained approximately $2.7 \times 10^6$ plaques and performed a partial characterization using 506 randomly selected clones. The insert sizes of these cDNA clones ranged from 0.2 to 0.5 kbp. Their DNA sequences were compared with sequences in the public database and classified as shown in Table 1. Of the 506 clones sequenced, 301 (222 independent clones), representing 59.5% of the total, were identical to sequences of genes whose functions were known; 29 (5.7%) were identical to archived EST sequences; 15 (3.0%) were identical to mitochondrial sequences; and 22 (4.3%) did not match any cDNA sequences in the public database. DNA sequences of all or part of the remaining 139 clones (27.5%) were repetitive elements. Genes corresponding to ESTs are summarized in Table 2. Comparison of our 222 independent vestibular genes of known functions with DNA sequences from a fetal cochlear library reported previously (Skvorak et al. 1999) revealed that 53 of these cDNAs were common to both studies (Table 2). The vestibular cDNA sequences that were identical to genes of known function were classified into 17 categories according to their functional

Table 2. The functional categories of known genes found in the vestibular cDNA library

| Gene description | Occurrence | Accession no. | Identical to cochlear ESTs |
|---|---|---|---|
| ***Cell cycle/cell proliferation*** | | | |
| Apoptosis inhibitor survivin | 1 | HSU75285 | |
| BBP-like protein 1 (BLP1) | 1 | AF353991 | |
| bcl-1 | 2 | HUMBCL1 | |
| Cell cycle regulator protein p95 | 1 | AF058696 | |
| Chondroitin sulfate proteoglycan 2 (versican) (CSPG2) | 1 | NM004385 | |
| WD repeat domain 7 (WDR7), transcript variant 1 (= TGF-beta) resistance associated gene) | 1 | NM_015285 | |
| Programmed cell death-2/RP8 homolog (PDCD2) | 2 | AL031259 | |
| ***Cell surface markers/cell adhesion*** | | | |
| Integral transmembrane protein | 1 | ITM1 | |
| Junctional adhesion molecule 3 (JAM3) | 1 | NM_032801 | |
| Ligatin | 2 | AF159586 | |
| Lysosomal-associated protein transmembrane 4 alpha (LAPTM4A) | 1 | NM_014713 | |
| Progesterone membrane binding protein (PMBP) | 1 | NM_006320 | |
| Pro-oncosis receptor inducing membrane injury gene | 1 | HSM800854 | |
| Putative type 1a integral membrane protein | 1 | HSS171 | |
| Retinoic acid receptor responder (tazarotene induced) 1 (RARRES1) | 2 | RARES1 | |
| Similar to integral membrane protein 3 | 1 | BC002424 | |
| Tight junction protein 1 | 2 | TJP1 | |
| Transmembrane protein 1 (TMEM1) | 1 | XM009794 | |
| Transmembrane protein BRI | 1 | AF152432 | |
| ***Extracellular matrix*** | | | |
| Alpha-3 type IV collagen (COL4A3) | 1 | HUMCOL4A3X | |
| Collagen type IV alpha 5 (COL4A5) | 1 | U04520 | + |
| Endoplasmic reticulum glycoprotein | 1 | NM_006816 | |
| ***Cellular metabolism*** | | | |
| Aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase | 1 | AF151838 | |
| Beta1-4 galactosyl transferase | 1 | AF038662 | |
| Carbonic anhydrase II (CA2) | 1 | NM000067 | + |
| CDP-diacylglycerol synthase (CDS) | 1 | HSU6008 | |
| Cisplatin resistance associated (CRA) | 1 | AW00589 | + |
| Cytochrome C oxidase subunit IV precursor (COX4) gene, encoding mitochondrial protein | 1 | AF017115 | + |
| Cytosolic selenium-dependent glutathione peroxidase | 1 | HUMGLPEX | + |
| Diaphorase (NADH) cytochrome b-5 reductase, nuclear gen encoding mt protein, | 1 | DIA1B | |
| Dioxin inducible cytochrome 450 | 2 | HSU56438 | + |
| Ectonucleotide pyrophosphatase/phosphodiesterase 4 | 2 | NM_014936 | |
| Filamin (FLN) gene to glucose-6-phosphate dehydrogenase (G6PD) | 1 | HUMFLNG6PD | |
| FRA3B common fragile region diadenosine triphosphate hydrolase (FHIT) | 1 | AF020503 | |
| Glutathione peroxidase 4 (CPX4) | 1 | GPX4 | |
| Inducible 6-phosphofructo-2-kinase/fructose 2, 6-bisphos | 1 | AF056320 | + |
| Lactate dehydrogenase B (LDHB) | 2 | LDHB | |
| p6=cytochrome c oxidase subunit VIc homolog/COSVIc/prostatic carcinoma upregulated | 1 | S82616 | + |
| Phenylalkylamine binding protein | 3 | AF196969 | |
| Prostaglandin D2 synthase gene, exon 7 | 1 | HUMDS03 | |
| Putative DNA dependent ATPase and helicase (ATAX) | 1 | HSU72938 | |
| Pyruvate dehydrogenase E1-alpha subunit | 1 | HUMPDHE1B | + |
| Retinal short-chain dehydrogenase/reductase | 1 | AF126782 | + |
| Rhodanese (=thiosulfate sulfurtransferase) | 1 | D87292 | |
| Serine palmitoyl transferase (SPTL1) | 1 | AF286717 | |
| Sphingomyelin phosphodiesterase | 1 | X52678 | + |
| Stannin | 5 | AF286717 | + |
| Thiopurine methyl transferase (TPMT) | 3 | HSTHSMT1 | |
| TPI1 gene for thiosephosphate isomerase | 1 | HSTPI1G | |
| Unspliced mRNA for glutathione peroxidase | 2 | HSPEROXP | |
| ***Cytoskeletal proteins*** | | | |
| Actin-gamma 1 | 1 | ACTG1 | + |
| Alpha-actin | 2 | HSAPACT | |
| Beta-2-microglobulin | 1 | B2M | + |
| C3 (complement component) (C3) gene 3'-end | 1 | M63422 | |
| Cytokelatin exon, delta-aminolevulinate synthase (erythroid) | 1 | HS884M20 | |
| Lamin A/C | 1 | BC003162 | |
| WAS protein family, member 2 | 1 | AK025566 | |

Table 2. Continued

| Gene description | Occurrence | Accession no. | Identical to cochlear ESTs |
|---|---|---|---|
| ***DNA replication and repair*** | | | |
| Topoisomerase II beta | 1 | U65315 | + |
| X-ray repair complementing defective repair in Chinese hamster cells 3 (XRCC3) | 1 | XM_050297 | |
| ***Serum/blood cell proteins*** | | | |
| Apoliprotein cluster | 1 | HSALINT | + |
| B-cells—hypothetical protein | 1 | AA362439 | |
| Beta-globin | 2 | AF007546 | |
| hHa2 gene from circulating lymphocyte | 1 | X90761 | |
| Ig gamma heavy chain, membrane-bound type, and secret type | 1 | D78345 | |
| Immunoglobulin heavy chain variable region | 1 | HSA7320 | |
| Immunogobulin lamda | 1 | D88268 | + |
| ***Neuronal*** | | | |
| Ceroid-lipofuscinosis, neural 3, juvenile (CLNS3) | 1 | NM000086 | |
| Glioblastoma amplified secreted protein (GASP) | 1 | AF395824 | |
| Highly similar to SPECTRIN beta chain, brain | 1 | AK023762 | |
| NDRG family member 4 (NDRG4) | 1 | NM_022910 | |
| Neuroxin III-alpha | 3 | AF099810 | |
| Purkinje cell protein 2 | 1 | S40022 | |
| Sine oculis homeobox homolog 3 (*Drosophila*) (SIX3) | 2 | NM_005413 | |
| ***Translation/protein synthesis*** | | | |
| 28S ribosomal RNA | 7 | AF152922 | |
| 40S ribosomal protein S18 | 2 | AL031228 | + |
| 60S ribosomal protein L13a | 1 | X56932 | |
| 60S ribosomal protein L15 | 1 | AF279903 | + |
| 60S ribosomal protein L37A | 1 | HS495010 | |
| Heterogeneous nuclear ribosomal protein11 | 1 | HNRPA1 | |
| Ribosomal proteon S6 | 1 | HSRPS6G | + |
| Ribosomal protein L3 | 3 | HUMRPL3A | + |
| Ribosomal protein L9 | 3 | RPL9 | + |
| Ribosomal protein L15 | 1 | HS90L6 | |
| Ribosomal protein L17 | 1 | RPL17 | + |
| Ribosomal protein L27 | 2 | HUMRIBPROE | |
| Ribosomal protein L35a | 1 | RPL35A | + |
| Ribosomal protein L37 | 1 | L11567 | + |
| Ribosomal protein L41 | 1 | AF026844 | + |
| Ribosomal protein L44 | 1 | RPL44 | |
| Ribosomal protein S11 | 1 | RPS11 | + |
| Ribosomal protein S12 | 2 | NM001016.1 | + |
| Ribosomal protein S19 | 1 | RP19 | |
| Ribosomal protein S24 | 3 | HSU12202 | + |
| Ribosomal protein S25 | 1 | RPS25 | + |
| Ribosomal protein S27A (ubiquitin carboxyl extension protein) | 1 | RPS27A | + |
| Ribosomal protein S7 | 1 | RPS7 | + |
| Ribosomal protein S23 | 1 | RPS23 | + |
| Surf 3 gene for ribosomal protein L7A | 2 | HSSURF3 | |
| TATA binding protein (TBP)-associated factor | 1 | TAF2G | |
| The gene for U2 small nuclear RNA-associated beta antigen | 1 | HS705D16 | + |
| ***RNA processing*** | | | |
| Nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs) (NOLA2) | 1 | NM_017838 | |
| The alternatively spliced gene for the human orthologs of mouse QKI-7 and QKI-7B | 1 | HS51J12 | |
| ***Transcription/nuclear proteins*** | | | |
| HIV Tat-SF1 | 2 | U76992 | |
| Homeobox protein NKX3.1 | 1 | AF247704 | + |
| Max | 1 | HSMAXG | |
| Nuclear protein family 3 similar to integral membrane | 1 | BC002424 | |
| Nuclear transport factor 2 | 1 | BC002348 | |
| Transcription factor ETR103 | 1 | HUMETR103 | |
| Zinc-finger protein 147 (ZNF 147) (estrogen-responsive finger protein) | 1 | XM012593 | |
| ***Kinase and phosphatase*** | | | |
| Eph-like receptor tyrosine kinase hEphB1b (EphB1) | 2 | AF037332 | |
| PI-3 kinase | 2 | HSpI3KINK | + |
| PNP1 mRNA (thyrosine phosphatase) | 1 | HSPNP1 | |
| Protein kinase inhibitor gamma (PKIG) | 2 | AF182032 | |
| Protein phosphatase 1, gamma | 1 | L07395 | |
| Protein phosphatase 1, catalytic subunit, alpha isoform | 2 | BC004482 | + |

Table 2. Continued

| Gene description | Occurrence | Accession no. | Identical to cochlear ESTs |
|---|---|---|---|
| PTPL mRNA for protein tyrosine phosphatase | 2 | HSPTPL1 | + |
| RYK = related to receptor tyrosine kinase from human hepatoma | 1 | S59184 | |
| Similar to serine threonine kinase pim3 | 1 | BC017083 | |
| YSK1 | 1 | D63780 | |
| ***Proteolysis*** | | | |
| F-box only protein 7 | 3 | XM_00994 | |
| Polyubiquitin UbC | 3 | AB009010 | |
| Proteosome (prosome, maropain) subunit, beta type 1 | 1 | NM_002793 | |
| Sorting nexin1A (SNX1) | 2 | AF065484 | |
| ***Signaling molecules/growth factors/receptors*** | | | |
| Amyloid beta (A4) precursor protein (Alzheimer disease) | 1 | XM009710 | + |
| Calmodulin 1 | 1 | JO4046 | + |
| Cytoplasmic interactor of beta 4 integrin P27BBP protein (ITBG4BP) gene, exon 4, 5, 6, 7 | 1 | AF289541 | |
| Diaphorase (NADH) (cytochrome b-5 reductase) (DIA1), nuclear gene encoding mt protein | 1 | NM007326 | |
| Elongation factor 2 | 2 | HSEF2MR | + |
| Glutamate receptor, metabotropic 1 (GRM1) | 1 | XM_054004 | |
| Insulin-like growth factor 2 receptor (IGF2R) | 1 | XM604237 | |
| Insulin-like growth factor binding protein 6 (IGFBP6) | 1 | HSA6952 | |
| Metabotrophic glutamate receptor 6 (mGluR6) | 2 | HSU82083 | |
| Okadaic acid-inducible and cAMP-regulated phosphoprotein 19 | 1 | AF084555 | + |
| p0071 protein | 1 | HSP0071 | |
| Solute carrier family 1 (glial high-affinity glutamate transporter), member3 (SLC1A3) | 1 | SLC1A3 | |
| Testosterone-repressed prostate message 2 (TRPM-2) protein gene | 1 | M63379 | |
| Thrombin receptor gene, exon1 | 1 | AF054633 | |
| Transmembrane receptor protein | 1 | HSTRECP | + |
| Uterin bom besin receptor | 1 | HSUBR | |
| Zona pellucida glycoprotein 2 (sperm receptor) | 1 | ZP2 | |
| ***Channel protein*** | | | |
| Apamin-sensitive small-conductive calcium-activated potassium channel (hSK2) (KCNN2) | 1 | AF239613 | |
| ATPase, $H^+$ transporting, lysosomal mRNA | 1 | ATP6C | |
| ATPase, $Na^+/K^+$ transporting, alpha2 (+) polypeptide | 2 | ATP1A2 | + |
| Potassium channel, subfamily K, member 6 (TWIK-2)(KCNK6) | 1 | KCNK6 | |
| Vestibule-1 protein (mechanosensitive ion channel domain containing protein) | 2 | AB056652 | |
| Sodium/myo-inositol cotransporter (SLC5A3) | 1 | AF027153 | |
| Voltage-dependent anion channel 3 (VDAC3) | 1 | BC002456 | |
| Voltage-dependent calcium channel beta2 subunit (CACNB2) | 1 | AF465485 | |
| ***Miscellaneous*** | | | |
| 40-kDa keratin intermediate filament precursor | 1 | HUMIFP | |
| 6.2-kDa protein | 1 | NM_019059 | |
| Arg/Abl-interacting protein ArgBP2 (ARGBP2), transcript variant 1 | 1 | NM003603 | |
| Arg/Abl-interacting protein ArgBP2 (ARGBP2), transcript variant 2 | 1 | NM021069 | |
| ATPase, class VI, type 11B (ATP11B) | 1 | XM_087254 | |
| CGI-56 protein | 1 | HSA011117 | |
| Chemokine-like factor super family 8 (CKLFSF8) | 1 | XM_087402 | |
| Coagulation factor C (limulus polyphemus) homology (Coch-5B2) | 4 | COCH | + |
| Cystein-rich protein (CROIP) gene, exon 5, 6 | 1 | HUMCRP04 | |
| deK (putative oncogen) | 1 | HS298J15 | |
| DLEC1 to DRCTL4 gene region section 1/2 | 2 | AB026898 | |
| Down syndrome critical region protein A (DCAA) | 1 | NM006052 | |
| Enhancer of rudimentary (*Drosophila*) homolog (ERH) | 2 | NM_004450 | + |
| Finkel-Biskis-Reilly murin sarfoma virus (FBR-MuSV) ubiquitously expressed (fox derived) | 2 | NM006522 | |
| Gene trap ankyrin repeat (GTAR) | 1 | AB014597 | + |
| HIV-1 TAR binding protein | 1 | HUMTAP2 | |
| HLA class III region containing NOTCH4 gene, homeobox | 1 | HSMHC3W5A | |
| Jun D proto-oncogene (JCND) | 1 | JUND | |
| KIAA0941 protein (= rab11-family interacting protein 2) | 1 | AB023158 | |
| Kiss-1 metastasis-suppressor | 5 | NM002256 | |
| Mu-crystallin gene, exon 8 | 1 | HSMVCRY6 | + |
| NY57-antigen | 4 | AF1155114 | |
| Org/ALI-interacting protein Arg BO2 & translated products (transcript varinat 2) | 1 | NM021069 | |
| PAR (Parader-Willi/Angleman region) 5 gene from brain, 15q11–q13 | 1 | AF019618 | |
| Prohibin | 1 | PHB | |
| Rearranged mRNA for glutamine synthase | 1 | X59834 | |
| Ring finger protein 11 | 1 | AB024703 | + |
| S-adenosyl homocysteine hydrolase-like | 1 | AHCYC1 | |
| Selenium binding protein 1 | 2 | NM_003944 | |

Table 2. Continued

| Gene description | Occurrence | Accession no. | Identical to cochlear ESTs |
|---|---|---|---|
| Selenoprotein P, plasma 1 (*SEPP1*) | 3 | SEPP1 | + |
| Serine (or cysteine) proteinase inhibitor, member 2 | 1 | BC015663 | |
| SET translation (myeloid leukemia-associated) | 1 | NM003011 | |
| Similar to selenium binding protein 1 | 1 | BC009084 | |
| STX16 (syntaxin16) | 1 | HSJ907D15 | |
| Testis-specific Y-encoded-like protein (TSPYL) | 1 | AF042181 | |
| The HMG2L 1 gene for high moleculity group protein 2-like 1 | 1 | HS510H16 | |
| The N-O oct5a POU domain proteins | 1 | HS273N12 | |
| Thyroglobulin gene, exon 32 | 1 | HSTHRG08 | |
| Thyroid autoantigen 70kD (ku antigen) (*G22P1*) | 1 | XM010020 | |
| Transposon-derived Buster1 transposase-like protein | 1 | AF205600 | |
| V-ski avian viral oncogene homolog | 1 | SKI | |
| Wbscr1 and replication factor C subunit 2 | 1 | AF045555 | |
| ***Unknown*** | | | |
| CGI-18 protein | 1 | AF151814 | |
| cDNA DKFZp434H0115 | 1 | HSM801728 | |
| cDNA DKFZp434E2023 | 1 | AL512761 | |
| cDNA DKFZP566N1346 (= *DKFZp762K0911*) | 2 | AL110256 | |
| cDNA DKFZp586P1622 | 1 | *HSM800861* | |
| cDNA DKFZp686K0273 | 1 | AL833622 | |
| cDNA DKFZp686P072 | 1 | AL832213 | |
| cDNA DKFZp761F0621 | 1 | AL713742 | |
| cDNA FLJ 12116 (hypothetical protein) | 1 | AK022178 | |
| cDNA FLJ 13794 | 2 | AK023856 | |
| cDNA FLJ 21778 | 1 | AK025431 | + |
| cDNA FLJ 37657 | 1 | AK094976 | |
| Chromosome 6 open reading frame *1* | 1 | AY062936 | |
| Chromosome 11 open reading frame 15 | 2 | AJ400877 | |
| Clone IMAGE:4042587, mRNA | 1 | BC021694 | |
| Clone IMAGE:4052238, mRNA | 1 | BC014384 | + |
| Clone IMAGE:4097766, mRNA | 1 | BC032596 | |
| Clone IMAGE:5528612, mRNA | 1 | BC016735 | |
| Clone IMAGE:5752707, mRNA | 1 | BC036789 | |
| HepG 3′ region cDNA | 1 | D16932 | |
| Hypothetical protein CAB 56184 | 1 | BC014592 | |
| Hypothetical protein DKFZp564K0822 | 1 | NM_030796 | |
| Hypothetical protein FLJ21016 | 3 | XM_015303 | |
| Hypothetical protein FLJ21047 | 1 | BC019890 | |
| Hypothetical protein MGC 5350 | 1 | XM_086221 | |
| KIAA0100 gene product | 1 | XM_031460 | |
| KIAA0537 gene product | 2 | AB011109 | |
| KIAA0551 protein | 3 | AB011123 | |
| KIAA0828 protein | 1 | XM_045904 | |
| Nop 10p | 1 | AB043103 | |
| Novel protein similar to predicted chicken protein | 1 | AL078581 | |
| Similar to RIKEN cDNA 1110017I16 gene | 2 | BC018068 | + |

aspects in the fetal cochlea library database (http://hearing.bwh.harvard.edu/cochlearcdnalibrary.htm#EST production).

### Semiquantitative RT-PCR and inner-ear expression

We performed RT-PCR experiments for the 22 selected EST clones of unknown function to confirm expression in the vestibule and eight other tissues. Nine of 22 ESTs were confirmed to be expressed in the vestibular tissue, as shown in Table 3. The expression of the two representative ESTs (clones 297 and 402) was confirmed in the inner ear (vestibule) and other organs (Fig. 1).

## Discussion

We have reported here the successful construction of a human vestibular cDNA library by means of T7-based amplification of RNA. Although several methods were used previously for constructing mammalian inner-ear cDNA libraries, each library required a significant quantity of mRNA (Heller et al. 1998; Harter et al. 1999); for example, the well-established human fetal cochlear library required tissue from more than 100 fetal ears (Robertson et al. 1994; Skvorak et al. 1997). Because the collection of adequate amounts of inner ear tissue is so difficult, the number of clones obtained from a library will necessarily be limited.

**Table 3.** Expression profile analysis of nine novel ESTs by RT-PCR

| Clone no. | Accession no. | Vestibule | Brain | Heart | Lung | Liver | Placenta | Kidney | Skeltetal muscle | Pancreas |
|---|---|---|---|---|---|---|---|---|---|---|
| 18 | AB072818 | + | − | + | + | + | − | + | + | − |
| 35[a] | AB072817 | + | + | − | − | + | + | − | − | − |
| 36[a] | AB077323 | + | + | − | − | + | + | − | − | − |
| 124 | AB072821 | + | + | + | + | + | + | + | + | − |
| 129 | AB072822 | + | + | − | + | + | + | − | − | + |
| 229 | AB072823 | + | + | + | − | + | + | + | + | + |
| 297 | AB072825 | + | − | − | − | − | − | − | − | − |
| 360 | AB074133 | + | + | + | + | − | + | + | − | − |
| 402 | AB076958 | + | + | − | − | − | + | − | − | + |

ESTs, Expressed sequence tags; RT-PCR, reverse transcriptase-polymerase chain reaction; + detected; − not detected
[a]No. 35 and 36 ESTs share the same nucleotid sequences, and are considered to be derived from the same gene

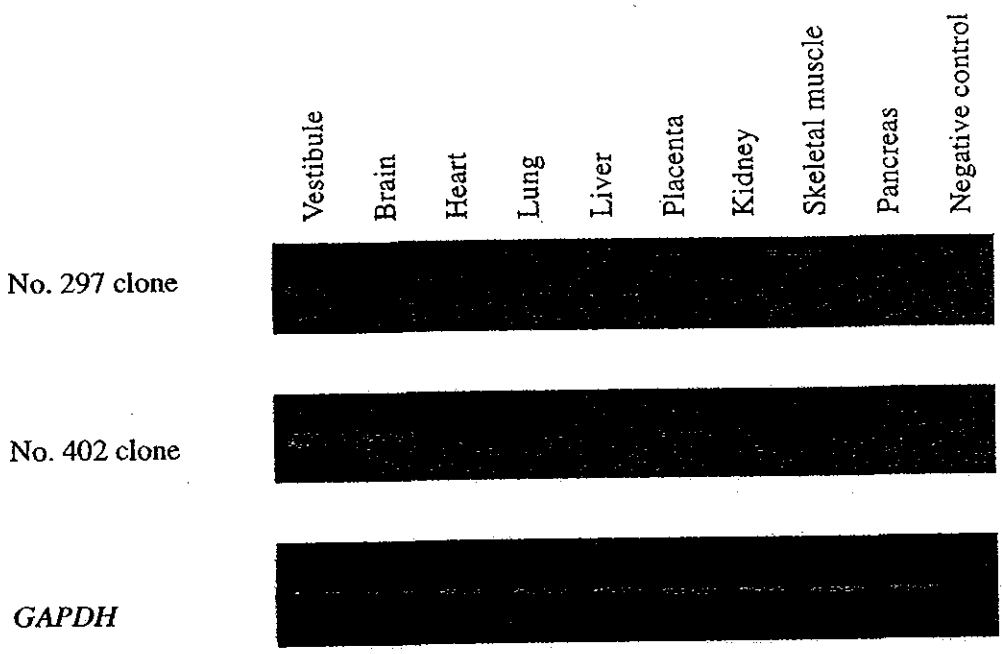


**Fig. 1.** Expression of vestibular cDNA clones (No. 297 and 402), estimated by reverse transcriptase-polymerase chain reaction (RT-PCR). A 2.5% agarose gel stained with ethidium bromide shows RT-PCR products obtained from aRNAs extracted from various tissues. One cycle of T7-based RNA amplification was performed in total RNA from the vestibule and eight other organs. Human glceraldehyde-3-phosphate dehydrogenase (*GAPDH*) serves as a positive control for the RNA

To overcome this primary disadvantage, we applied the T7-based RNA-amplification method (Van Gelder et al. 1993; Luo et al. 1999). Our results demonstrated that information about gene expression in the vestibule could be obtained from an extremely small amount of RNA. Although insert sizes of selected clones from our vestibular cDNA library were not as large as those of cochlear cDNA libraries reported previously, the almost complete nature of our genomic sequence information made it easy to define the transcription unit. With regard to repetitive elements, the insufficient sequence complexity, similar to that described in Morton's cochlear cDNA library, might have been enhanced to produce excess artifacts, and all repetitive elements were excluded in this study. The T7-amplification method was able to amplify the transcripts by maintaining the proportion of the original mRNA species, and the T7-amplified cDNA would be an almost complete representation of mRNA *in vivo*. Thus, identification of genes specifically expressed in the inner ear may be an effective approach to clarify which gene products play important roles in the auditory and vestibular systems and also to discover and characterize genes that carry mutations responsible for deafness. Characterization of our vestibular cDNA library showed a wide variety of molecules including many unknown function proteins, suggesting that yet additional gene products may be involved in the vestibular function. RT-PCR analyses of the selected 22 EST sequences showed that 9 ESTs were expressed in the inner ear. However, no expression of the other 13 ESTs was detected in any tissues, and the possibility cannot be excluded that this problem may be due to an artifact such as unexpected genomic sequence. To evaluate whether these 9 EST transcripts would be truly novel gene fragments, we need further investigation.

In the human, a few cochlear cDNA libraries have been reported (Robertson et al. 1999; Jacob et al. 1997), but until now no vestibular cDNA library was available. When we compared results from our vestibular cDNA library with those of the fetal cochlear library, we noted that many genes of known functions were common in both libraries. How-

ever, dozens of genes detected in our vestibular library were not found in the fetal cochlear library, indicating that a specific set of genes is expressed in the vestibule.

Developmentally, the cochlea and vestibule originate from one embryonic organ, the otic capsule. The vestibular organ is remarkably similar to the cochlea, although their functions are very different. Some structures, including sensory hair cells, neuroepithelia, tectorial or otolithic membranes, and supporting cells, are common in both organs, suggesting that genes expressed in these two parts of the inner ear would play similar roles. Therefore, it is not surprising that the cDNA profile of our vestibular library significantly overlapped with that of the cochlear library. In fact, we encountered the *COCH* gene four times, the mutations of which are causative to nonsyndromic deafness with vestibular symptoms (DFNA9); this gene is reported to be highly expressed in the human cochlea and vestibule (Robertson et al. 1998). It is also of interest to know how vestibular function remains intact for many patients whose deafness is caused by mutations of nonsyndromic deafness genes expressed in both the vestibular and cochlear organs. Compared with the cochlear ESTs, particularly with respect to the phenotypic differences, the lack of vestibular dysfunction among affected patients who are carrying mutations in the deafness genes may reflect neurobiological differences based on gene expression profiling. The present study revealed only a small part of the human vestibular cDNA sequences. Nevertheless, the vestibular cDNA library that we have reported here represents a valuable experimental tool for investigating genes related to deafness and should contribute important insights into the molecular basis for the vestibular function.

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ORIGINAL INVESTIGATION

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# *GJB2* deafness gene shows a specific spectrum of mutations in Japan, including a frequent founder mutation

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**Abstract** Mutations in the *GJB2* gene (connexin 26) are the major cause of autosomal recessive non-syndromic hearing impairment in many populations. In contrast to the volume of information regarding the involvement of *GJB2* mutations in hearing impairment in populations of European ancestry, less is known regarding other ethnic groups. In this study, we analyzed the *GJB2* gene for mutations in 1227 hearing-impaired Japanese individuals. This revealed a unique spectrum of *GJB2* mutations, different from that found in the Caucasian population. The most frequent mutation in Japanese, 235delC, has never been reported in Caucasians. To investigate a possible founder effect for the 235delC mutation, we analyzed single nucleotide polymorphisms in the vicinity of the *GJB2* gene. Results were consistent with inheritance of the 235delC mutation from a common ancestor. The results of this study have important implications for genetic diagnostic testing for deafness in the Japanese population.

Electronic database information: accession numbers and URLs for the data in this article are as follows:
GJB2-OMIM:121011, 220290 (DFNB1)
GenBank:M86849
HGMD:GJB2; http://www.iro.es/cx26deaf.html

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## Introduction

*GJB2* gene mutations have recently drawn much attention because they are involved in up to 50% of autosomal, recessively inherited, hearing-loss cases in populations of European ancestry (for a review, see Kalatzis and Petit 1998). More than 70 different *GJB2* mutations have been reported to be associated with recessive forms of non-syndromic hearing loss, whereas a few *GJB2* mutations leading to autosomal, dominantly inherited, hearing impairment have also been described (see the Connexins and Deafness Homepage of R. Rabionet, P. Gasparini, X. Estivill: http//www.iro.es/cx26deaf.html). Among the recessive mutations, a mutation deleting a G in a stretch of six at position 30–35 (35delG) is the most frequent in many Western countries. Although it has been proposed that a succession of six Gs may present a mutational hot spot (Denoylle et al. 1997; Kelley et al. 1998; Morell et al. 1998; Carrasquilo et al. 1997; Lench et al. 1998), a recent study has provided evidence that the high frequency of this mutation is the result of a founder effect (Van Laer et al. 2001). In a previous study analyzing Japanese deaf individuals for *GJB2* mutations, we have not found the 35delG mutation (Abe et al. 2000). Moreover, a different frequent mutation, 235delC, has been detected (Fuse et al. 1999; Abe et al. 2000; Kudo et al. 2000). The frequent presence of 235delC in Japanese patients suggests that, for this mutation, there may also be a founder effect. In this study, we have performed *GJB2* mutation analysis in 1227 Japanese individuals with hearing impairment. To distinguish between a possible founder effect and a mutational hot spot for the frequent 235delC mutation, we have analyzed single nucleotide polymorphism (SNP) markers close to the *GJB2* gene in mutation carriers and controls.

## Subjects and methods

### Subjects

In this study, 1227 Japanese subjects, who were unrelated to each other and visited outpatient clinics because of a variety of conditions involving sensorineural hearing loss, were analyzed. The composition of the subjects was as follows: 458 subjects from autosomal dominant or mitochondrial families (two or more generations affected), 226 subjects from recessive families (normal hearing parents and two or more affected siblings), and 543 subjects with sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss).

None of the patients had any other associated neurological signs. The control group consisted of 147 unrelated Japanese individuals without any noticeable hearing loss evaluated by auditory testing. All subjects gave prior informed consent for participation in the project. This study was approved by the Ethical Committees of Shinshu University and Hirosaki University.

### Mutation analysis

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was amplified by using the primer pair Cx48U/Cx1040L (Abe et al. 2000). Polymerase chain reaction products were sequenced and analyzed with an ABI sequencer 377XL (Perkin Elmer). Details of the methods for sequencing analysis were as described elsewhere (Abe et al. 2000).

### SNP analysis

SNP analysis was performed on 16 homozygous and 92 heterozygous patients for the 235delC mutation (some samples with the 235delC mutation were not included in Table 1, because they were from other institutes). Ninety normal hearing individuals without the 235delC mutation were chosen as controls. Six SNPs (SNP1–6; Van Laer et al. 2001) were used in this study. SNP analysis was carried out by using the ABI Prism SNaPshot ddNTP Primer Extension Kit as described previously (Van Laer et al. 2001).

### Statistical analysis

Statistical analysis was performed by chi-square testing on the basis of 3×2 contingency tables (three genotypes for cases and controls) with Sigmastat v2.0 software (Jandel Scientific Corporation). If expected values were too low for reliable chi-square testing, the two least frequent genotypes were added to give a 2×2 table, and Yates's correction for continuity for 2×2 tables was used. If the expected values remained too low in the 2×2 table, the Fisher's exact test was used. *P*-values were taken to be significant at <0.05.

## Results

### *GJB2* mutations in the Japanese population

Thirteen mutations, including five novel ones, were identified in 1227 affected individuals (Table 1). Eight of them were missense mutations, one was a nonsense mutation, and four were frameshift mutations. Of these mutations, 235delC was the most prevalent mutation detected. Figure 1 shows a schematic representation of Cx26 and the positions of mutations found in the Japanese and in other populations. These mutations were either homozygous, compound heterozygous, or heterozygous with no other mutations being detectable.

We detected five inactivating mutations: one stop mutation (Y136X), three deletion frameshift mutations (235delC, 176–191del16, 299–300delAT), and one insertion frameshift mutation (605 ins46). The eight missense mutations were: V37I (109G→A), G45E (134G→A), A49V (146C→T), I71T (212T→C), T86R (257C→G), T123N (368C→A), R143W (427C→T), and F191L (570T→C). To evaluate the evolutionary conservation of the amino acids affected by these missense mutations, we made an alignment of the *GJB2* amino acid sequence of five species (not shown): human (AAL87696), mouse (M63803), rat (P21994), cow (CAC19883), and sheep (P46691). On the basis of this alignment, all missense mutations had changed evolutionary conserved amino acids, except for T123N. Moreover, at position 123, the sheep sequence had an N.

V37I, G45E, T86R, and R143W were found in the homozygous or compound heterozygous state, with one mutation being inherited from the father and the other from the mother. In combination with these mutations not being found in controls, this represented a strong argument for

**Table 1** *GJB2* variants detected in Japanese subjects with sensorineural hearing loss. Names of mutations are according to the HUGO nomenclature recommendations (Antonarakis 1998). Previously reported polymorphic variants V27I, E114G and I203T were not included (*del* deletion, *ins* insertion, *NA* not applicable)

| Amino acid change | Nucleotide change | Allele *n*=2454 | Evolutionary conservation | Reference |
|---|---|---|---|---|
| – | 235delC | 96 (34%) | NA | Fuse et al. 1999 |
| V37I | 109G→A | 61 (21%) | Yes | Abe et al. 2000 |
| G45E | 134G→A | 45 (16%) | Yes | Fuse et al. 1999 |
| Y136X | 408C→A | 30 (10%) | NA | Fuse et al. 1999 |
| – | 176–191del16 | 12 | NA | Abe et al. 2000 |
| T123N[a] | 368C→A | 11 | No | Park et al. 2000 |
| – | 299–300delAT | 8 | NA | Abe et al. 2000 |
| R143W | 427C→T | 8 | Yes | Brobby et al. 1998 |
| I71T[b] | 212T→C | 4 | Yes | This study |
| F191L[a] | 570T→C | 4 | Yes | This study |
| T86R | 257C→G | 3 | Yes | This study |
| A49V[b] | 146C→T | 1 | Yes | This study |
| – | 605ins46 | 1 | NA | This study |

[a]Variants probably representing polymorphisms
[b]Variants possibly representing polymorphisms

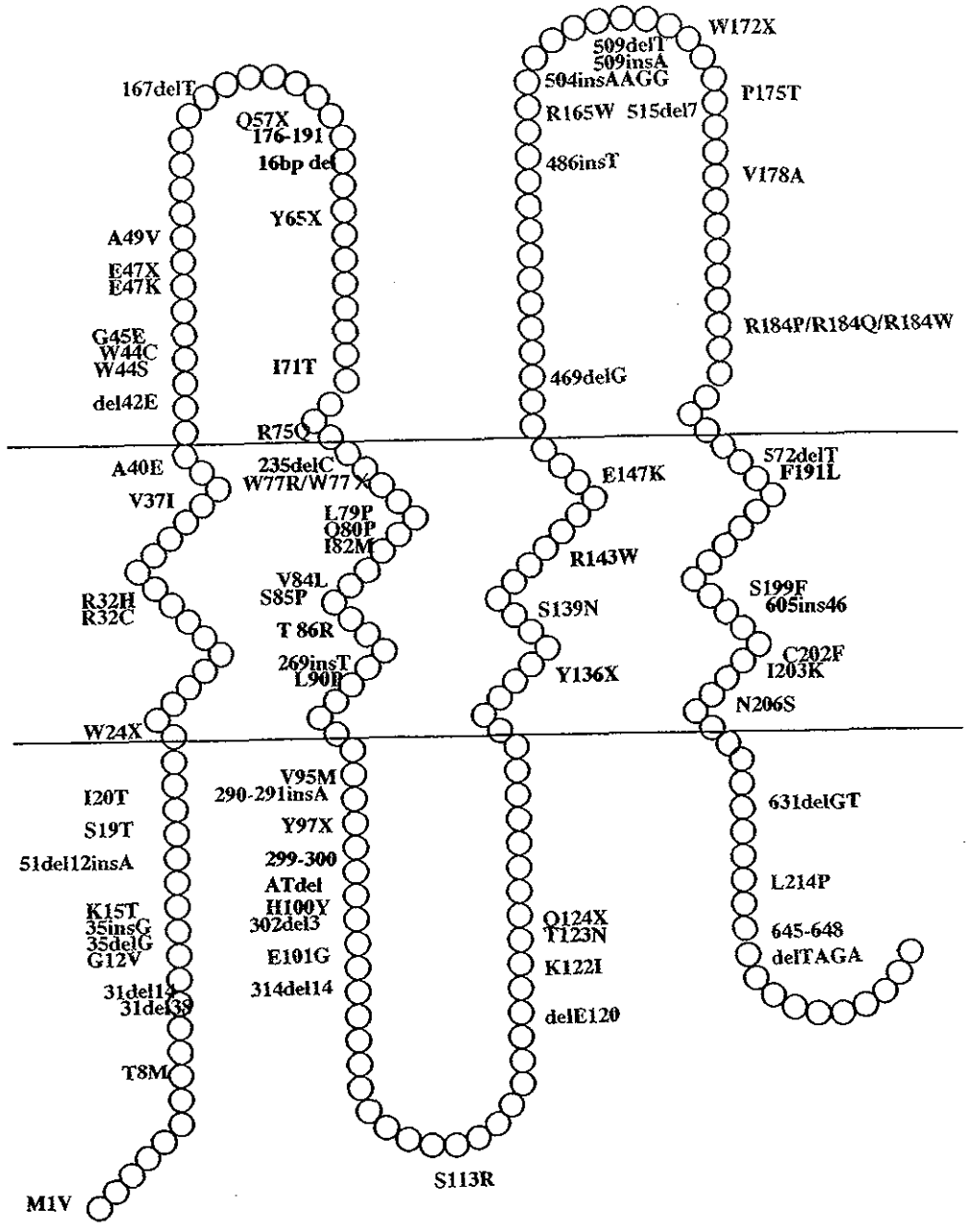


**Fig. 1** Differences in *GJB2* mutation spectrum between Japanese and other populations. Schematic representation of connexin 26 protein and the approximate positions of mutations. The *GJB2* mutation spectrum in Japanese (*red*) is markedly different from that reported in other populations (*green*). Only two mutations (*blue*) are found in both populations

their disease-causing nature. In contrast, T123N, I71T, F191L, and A49V were found to be heterozygous with no other mutations being detectable; it therefore remains a possibility that they are rare polymorphisms.

Three previously described common sequence changes, viz., V27I (79G→A), E114G (341A→G), and I203T (608T→C), were frequently found in patients and in controls but have been omitted because these base changes are thought to be non-pathological polymorphic changes (Abe et al. 2000). Four of the mutations found in the patient group were also found in the control group: 235delC, V37I, T123N, and F191L (Table 2). The 235delC and V37I mutations are both well-established autosomal recessive deafness alleles, and their finding in the control group most likely represents the detection of carriers. Both mutations also have a much higher frequency in the patient group compared with the control group. However, this is not the case for T123N and F191L. These two variants are equally frequent in patients and controls. There-

**Table 2** *GJB2* variants detected in the Japanese control group without hearing loss. Previously reported polymorphic variants V27I, E114G, and I203T are not included

| Codon | Nucleotide change | Control (294 alleles) |
|---|---|---|
| L79FS | 235delC | 2 (0.7%) |
| V37I | 109G ÆA | 2 (0.7%) |
| T123N | 368C ÆA | 2 (0.7%) |
| F191L | 570T ÆC | 1 (0.3%) |

fore, T123N and F191L probably represent polymorphisms. For the T123N variant, this is in contrast with the finding of Park et al. (2000) who have reported it as a disease-causing mutation. The lack of evolutionary conservation at position 123, however, does not support a disease-causing nature. The other mutations identified in the subjects with hearing loss were not detected in the control samples.

### SNP analysis of 235delC homozygotes and heterozygotes

To investigate a possible founder effect for the 235delC mutation, we analyzed six SNPs in 16 homozygous and 92 heterozygous 235delC patients and in 90 controls without the 235delC mutation. Only two of the six SNPs, viz., SNP1 and SNP6, were informative in the Japanese population. SNP1 is located in the 3'UTR (untranslated region, Genbank accession no. M86849) at a distance of 931 bp from nucleotide position 235 of *GJB2*. SNP6 is positioned upstream of *GJB2*, at a distance of 62,959 bp from position 235 of *GJB2*. For SNP1, we see a complete association of the C allele in the homozygous patients, whereas the allele frequency of the C allele in controls is 55%. Comparison of genotype frequencies between patients and controls is significant for SNP1 with *P*-values below 0.001. For SNP6, the 235delC mutation is associated with allele A, but not completely. Comparison of genotypes between the 16 homozygotes and controls is not significant, but the comparison of homozygotes and heterozygotes with controls is significant ($P$=0.038). Tables 3 and 4 summarize the SNP analysis.

**Table 3** SNP1 analysis (*hom* patients homozygous for the 235delC mutation, *het* patients heterozygous for the 235delC mutation)

| Subjects | CC | CT | TT | *P*-value |
|---|---|---|---|---|
| Patients (hom) | 16 | 0 | 0 | $P<0.001$[a] |
| Controls | 3 | 12 | 1 | – |
| Patients (hom+het) | 49 | 18 | 0 | $P<0.001$ |
| Controls | 22 | 49 | 14 | – |

[a]Rows were combined for chi-square analysis or Fisher's exact test

**Table 4** SNP6 analysis (*hom* patients homozygous for the 235delC mutation, *het* patients heterozygous for the 235delC mutation)

| Subjects | GG | GA | AA | *P*-value |
|---|---|---|---|---|
| Patients (hom) | 1 | 1 | 14 | $P$=0.394[a] |
| Controls | 0 | 4 | 11 | – |
| Patients (hom+het) | 2 | 7 | 92 | $P$=0.038[a] |
| Controls | 1 | 15 | 60 | – |

[a]Rows were combined for chi-square analysis or Fisher's exact test

## Discussion

The present study of Japanese hearing-impaired patients has revealed a unique spectrum of *GJB2* mutations quite different from that found in populations of European ancestry. Of a total of 13 mutations found in Japanese, only two have previously been found in other populations.

Previous reports (Denoyelle et al. 1997; Kelley et al. 1998; Morell et al. 1998; Carrasquilo et al. 1997; Lench et al. 1998) have suggested that the high prevalence of 35delG, the most frequent *GJB2* mutation in Caucasians, is attributable to a mutational hot spot. However, several facts throw doubt on this hypothesis. First, prevalent *GJB2* mutations depend on ethnic origin: 167delT is reported as being prevalent in Ashkenazi Jews, (Morell et al. 1998; Sobe et al. 1999), R143W in a restricted village in Africa (Brobby et al. 1998), and 235delC in Asian populations (Fuse et al. 1999; Abe et al. 2000; Kudo et al. 2000; Park et al. 2000). Second, in spite of the high prevalence of the 35delG mutation in some populations, it has never been reported in Japanese (Fuse et al. 1999; Abe et al. 2000; Kudo et al. 2000) and is very rare in Koreans (Park et al. 2000). Third, Japanese and Koreans share a similar spectrum of *GJB2* mutations/polymorphisms, suggesting a common origin. Fourth, there are regional differences in carrier rates of 35delG even in Europe, i.e., they are high in southern Europe and low in some northern European countries (Gasparini et al. 2000). Finally and most importantly, a recent study has concluded, by means of SNP analysis, that the 35delG mutation was derived from a common ancestor (Van Laer et al. 2001).

Although it is theoretically possible that 235delC could have arisen through multiple independent mutational events on the same haplotype, it is more likely that 235delC was derived from a common ancestor. Kudo et al. (2000) have previously investigated a possible founder effect for 235delC, which seems to be a unique mutation in Asian populations, including Japanese, Korean, and Chinese (Fuse et al. 1999; Abe et al. 2000; Kudo et al. 2000; Park et al. 2000; Liu et al. 2002), and which is the most frequent *GJB2* mutation in Japanese. They have classified the mutation-bearing chromosomes into seven haplotypes by using four polymorphisms and have shown that all 235delC alleles from four patients belonged to the type 1 allele. Because this haplotype is the most common type in Japanese and because of the limited number of patients, they could not however draw a definite conclusion (Kudo et al. 2000). In a recent study, Liu et al. (2002) have also postulated a possible founder effect of this mutation in Chinese; they report that homozygous 235delC mutations share the same haplotype for three microsatellite markers, although no details are given, and they do not report whether these findings are statistically significant. The SNP analysis in our study is the first to establish a founder event for the origin of the 235delC mutation in Japanese.

For children with congenital hearing loss associated with *GJB2* mutations, cochlear implantation has brought remarkable improvement in auditory skills and develop-

ment of speech production compared with children with other types of hearing impairment (Fukushima et al. 2001; Matsushiro et al. 2001). It is therefore evident that genetic analysis to identify the origin of deafness will help to predict the prognosis of language development.

The results of this study should facilitate the development of DNA diagnostics. Clearly, different mutation spectrums in specific populations will have to be considered when designing genetic testing procedures.

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ORIGINAL ARTICLE

# Origins and frequencies of *SLC26A4* (*PDS*) mutations in east and south Asians: global implications for the epidemiology of deafness

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Recessive mutations of *SLC26A4* (*PDS*) are a common cause of Pendred syndrome and non-syndromic deafness in western populations. Although south and east Asia contain nearly one half of the global population, the origins and frequencies of *SLC26A4* mutations in these regions are unknown. We PCR amplified and sequenced seven exons of *SLC26A4* to detect selected mutations in 274 deaf probands from Korea, China, and Mongolia. A total of nine different mutations of *SLC26A4* were detected among 15 (5.5%) of the 274 probands. Five mutations were novel and the other four had seldom, if ever, been identified outside east Asia. To identify mutations in south Asians, 212 Pakistani and 106 Indian families with three or more affected offspring of consanguineous matings were analysed for cosegregation of recessive deafness with short tandem repeat markers linked to *SLC26A4*. All 21 *SLC26A4* exons were PCR amplified and sequenced in families segregating *SLC26A4* linked deafness. Eleven mutant alleles of *SLC26A4* were identified among 17 (5.4%) of the 318 families, and all 11 alleles were novel. *SLC26A4* linked haplotypes on chromosomes with recurrent mutations were consistent with founder effects. Our observation of a diverse allelic series unique to each ethnic group indicates that mutational events at *SLC26A4* are common and account for approximately 5% of recessive deafness in south Asians and other populations.

Approximately one half of childhood hearing loss is thought to have a genetic aetiology, the majority of which is non-syndromic and not associated with abnormalities of other organ systems. Estimates from clinical and epidemiological studies suggest that 80-85% of hereditary, non-syndromic, prelingual deafness is autosomal recessive, 15% is autosomal dominant, and a few percent is inherited as an X linked trait or via matrilineal transmission.[1] There are at least 30 distinct genetic loci (known as DFNB loci) at which mutations can cause non-syndromic recessive deafness (NSRD).[2] In the absence of syndromic features to guide genetic diagnosis, efficient molecular diagnosis requires a detailed knowledge of the distribution of mutant alleles at specific loci for individual populations.

Recessive mutations of the *SLC26A4* (*PDS*) gene on chromosome 7q can cause sensorineural deafness with goitre[3] (Pendred syndrome, OMIM 274600) or NSRD without goitre[4] (at the DFNB4 locus, OMIM 600791). These mutations disrupt in vitro transmembrane anion/base exchange activity of the SLC26A4 polypeptide, pendrin.[5] Pendrin is expressed in non-sensory epithelia of the inner ear,[6] thyroid folliculocytes,[7] renal cortical collecting ducts,[8 9] placental trophoblasts,[10] and uterine endometrium.[11] In vivo, pendrin is likely to mediate iodide transport across the apical membrane of thyroid folliculocytes[7] and bicarbonate secretion by intercalated cells of renal cortical collecting ducts,[8 9] but its critical transport substrate(s) in the inner ear has not been defined.[12]

Both DFNB4 and Pendred syndrome phenotypes are associated with enlargement of the vestibular aqueduct (EVA) as detected by radiological imaging of the temporal bones.[13] It is unknown whether the occurrence of goitre is attributable to the underlying *SLC26A4* genotype,[5] to modifying genetic or environmental factors, or to a combination of these mechanisms. Moreover, deaf subjects may have goitre that is unrelated to Pendred syndrome,[14] although these phenocopies can often be distinguished with a perchlorate discharge test. Fraser[15] used this test to estimate that Pendred syndrome accounts for 5.6-7.8% of hereditary deafness, but it is now clear that molecular genetic diagnostic techniques can provide a more accurate estimate of the prevalence of *SLC26A4* deafness.

Most published studies of *SLC26A4* mutations have dealt with western populations.[3 16–18] There are only a few reported cases from Asia[19–21] with no estimates of mutation or phenotype frequencies in deaf Asian populations. The epidemiology of *SLC26A4* deafness may vary among Asian and western populations, as has already been reported for recessive *GJB2* (*Cx26*) mutations at the DFNB1 locus.[22 23] Up to 50% of NSRD is associated with *GJB2* mutations in some western populations,[24] whereas *GJB2* mutations only account for 5% of deafness in Korea[22] and 20-30% in Japan.[25 26] Since Asia contains approximately one half of the global population,[27] the origins and frequencies of *SLC26A4* mutations among its populations have important implications for a global understanding of the genetic epidemiology of deafness.

## MATERIALS AND METHODS

### Subjects

Approval for this study was obtained from institutional review boards (IRBs) at the National Institutes of Health (NINDS/NIDCD joint IRB), Medical College of Virginia (Western IRB), Ajou University (Suwon, Korea), Shinshu University School of Medicine (Matsumoto, Japan), Hirosaki University School of Medicine (Japan), All-India Institute of Medical Sciences (Delhi, India), and the Centre of Excellence in Molecular Biology (Lahore, Pakistan). Informed consent was obtained for all participants.

The east Asian study subjects included one large Korean family (K-87) segregating severe to profound prelingual deafness, and 92 Korean, 86 Chinese, and 195 Mongolian

probands with sporadic or familial severe to profound prelingual deafness. The south Asian subjects comprised 106 Indian and 212 Pakistani families with three or more affected subjects segregating severe to profound, prelingual, recessive deafness. Subjects were ascertained through schools and outpatient referral clinics for the deaf in Korea,[27] Ulaanbaatar, Mongolia,[28] Sichuan province, China,[29] the Maharashtra, Karnataka, and Tamil Nadu provinces of India, and Punjab province, Pakistan. With the exception of goitre in Korean family K-87, a subset of Pakistani families, and eight Mongolian probands, subjects with syndromic features or known exposure to ototoxic agents (for example, aminoglycosides) were excluded. Korean, Mongolian, and Chinese probands with known mitochondrial or biallelic *GJB2* mutations, comprising 2-15% of the original cohorts, were also excluded from this analysis.

Japanese probands known to have either the splice site mutation IVS7-2A>G or the missense mutation H723R of *SLC26A4*[19] were included in STR genotype and haplotype analyses to detect evidence of founder effects for these alleles. Samples from sibs and parents were analysed, when possible, in order to assign meiotic phase for *SLC26A4* mutations and linked marker genotypes. Control DNA samples were obtained from normal hearing adults from Korea, Mongolia, China, India, and Pakistan. Indian and Pakistani control samples were grouped and analysed according to province of origin to match the origin of deaf subjects.

## METHODS

Peripheral venous blood samples were obtained for preparation of genomic DNA. DNA preparations and genotype analyses were performed as described previously.[30] Genotypes of STR markers linked to known NSRD (DFNB) loci were determined for affected members of Indian and Pakistani families, and all members of Korean family K-87.

*SLC26A4* exons were PCR amplified and sequenced as described previously.[3] Novel intronic primers were designed to flank: exon 2, 5′-CTCCGATCGTCCTCGCTTA-3′ and 5′-CTCCGCTTCTCTCTACGCA-3′; exon 4, 5′-CGCTTAGGCTATC CTACCTGAAATGTGC-3′ and 5′- CACTGAAATCCCATTTCC CTGACAACA-3′; exon 6 , 5′-GGCAGGCTACTAGTGTTTTCA-3′ and 5′-GGCCCAGACTCAGAGAATGA-3′; exon 16, 5′-AGCTTT AGGTGCCAGGCATT-3′ and 5′-GACCCTCTAACTGCTCTCA TCA-3′; and exon 20, 5′-TTCACCTTTCAATGTGCAAAA-3′ and 5′-TGCATTTGGGGGAATTATGT-3′. The genomic deletion mutation IVS2_IVS3del4kb was detected by PCR amplification and sequencing with primers 5′-CTCTTGACCAGGAG GAGTCC-3′ and 5′-CCTCTTCCTCCCTGGAAAATGC-3′.

### Statistical analysis

Differences in STR genotype and haplotype distributions between mutant and wild type chromosomes from ethnically matched, normal hearing control DNA samples were analysed by Fisher's exact test. Some genotype distributions were compared among subjects, not chromosomes.

## RESULTS

### A common locus for recessive deafness in Koreans

In Korean family K-87 (fig 1A), there are deaf offspring from two different matings between unrelated deaf subjects, suggesting that their hearing loss is caused by mutations of a gene in which mutant alleles are a common cause of recessive deafness in Korea. Computed tomography showed EVA in II.6 and II.7 (fig 1B), and ultrasonography showed a goitre in II.7. STR haplotype (D7S496, D7S2459, and D7S2456; fig 2) and *SLC26A4* mutation analyses showed the deafness cosegregates with homozygosity or compound heterozygosity for two *SLC26A4* missense substitutions, L676Q (exon 17) and H723R (exon 19), in a subset of family members that includes the subjects with EVA (fig 1A). Deafness was not linked to *SLC26A4* in II.3 and III.1, who do not have EVA and are thus deaf from another aetiology. Nucleotide sequence analysis of *GJB2* did not identify pathogenic mutations in II.3 or III.1 (not shown).

### *SLC26A4* mutations in East Asians

To determine the contribution of L676Q, H723R, and other *SLC26A4* mutations to recessive deafness in east Asia, exons 17 and 19 were selected for sequence analysis of DNA samples from deaf probands from Korea, China, and Mongolia (table 1). We did not analyse all *SLC26A4* exons in all of the probands owing to limitations of DNA sample volumes and the expense of nucleotide sequencing. One H723R homozygote and three H723R heterozygotes were detected among the 92 deaf Korean probands, and two heterozygous H723R carriers were identified among 120 normal hearing Korean controls, confirming that H723R is a common allele in the Korean population. Four L676Q heterozygotes were identified among 195 deaf Mongolian probands and one L676Q carrier was observed in the Mongolian normal hearing controls, indicating that L676Q is a common allele in this population.

The 19 remaining *SLC26A4* exons were analysed in the eight deaf mutation carriers since their deafness was possibly associated with a second *SLC26A4* mutation in *trans* configuration. Mutations were detected in exons 6, 8, 10, 14, and 15 or their adjacent splice sites (table 1). These exons were sequenced in additional probands from each cohort, including a final total of 96 Mongolian probands. Four novel mutations (1548insC, IVS14-7A>G, S252P, and N392Y) and two previously reported mutations[16 20 21 32] (IVS7-2A>G, T410M) were identified. None of these mutations was detected in 44 to 120 ethnically matched, normal hearing control samples. The pathogenic potential of IVS14-7A>G is unknown since its effect on splicing has not been experimentally determined. All of the missense mutations detected in the probands and south Asian families are non-conservative substitutions of amino acid residues that are conserved in the rat and mouse orthologues of *SLC26A4*. We did not detect any of the four mutations (L236P, IVS8+1 G>A, E384G, and T416P, table 1) most commonly reported among western patients.[16 17] Six (6.5%) of 92 Korean, five (5.8%) of 86 Chinese, and four (2.1%) of 195 Mongolian probands had at least one detected *SLC26A4* mutation (table 1).

### *SLC26A4* mutations in south Asians

In south Asia, we ascertained 212 families from Pakistan and 106 families from India with three or more deaf offspring of consanguineous matings or, in one case, six affected offspring of a non-consanguineous Indian mating (family DKH-5). All 21 *SLC26A4* exons were sequenced in affected probands from 15 families cosegregating deafness with homozygosity for STR markers linked to *SLC26A4* (table 2). Homozygous *SLC26A4* mutations were identified in all of these probands, and nucleotide sequence analysis of the mutated exons in remaining family members confirmed cosegregation of the mutations with deafness. The affected subjects of a sixteenth family, DKH-5, cosegregated deafness with compound heterozygosity for the nonsense mutation S57X and IVS2_IVS3del4kb, a 4 kb genomic deletion encompassing exon 3. The existence of this novel deletion was initially manifested by hemizygosity for the *trans* mutant allele S57X in the exon 3 PCR product from affected members of family DKH-5 (not shown).

A total of 10 different mutations, all of which are novel, were detected among the south Asian families (table 2). I455F was detected in 2/90 Pakistani normal hearing control samples, but otherwise none of the other nine mutations were identified in any of 53 or more ethnically matched control samples. The previously reported Indian ISL-1 family segregating the [G497S; I490L] mutant allele was ascertained through this same study.[4] If ISL-1 is included, seven (6.6%) of 106 Indian families segregated *SLC26A4* mutations (95% CI

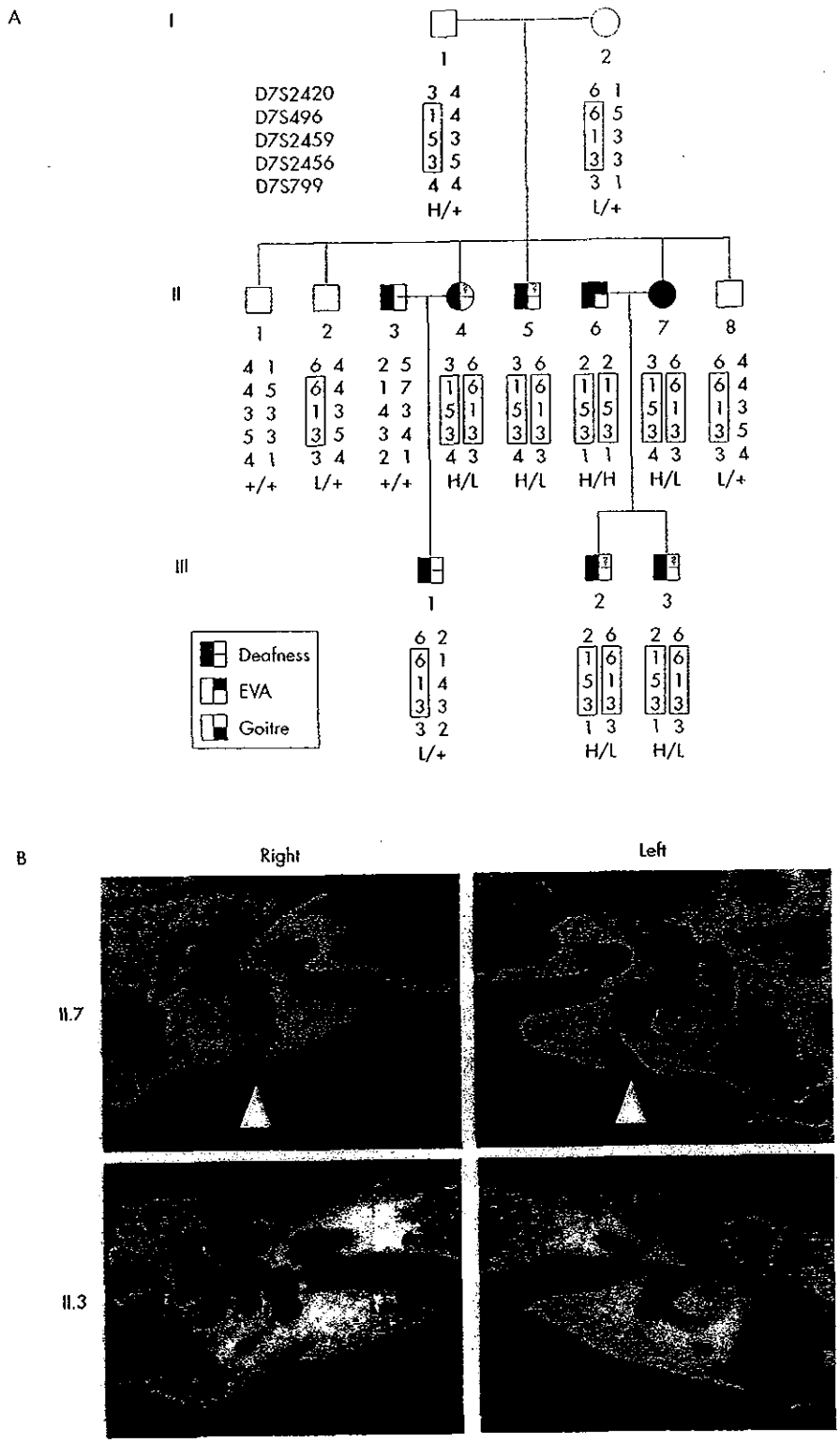


**Figure 1** Korean family K-87 segregating recessive deafness. (A) Pedigree showing segregation of severe prelingual deafness, enlarged vestibular aqueducts (EVA), chromosome 7q31 STR genotypes, and *SLC26A4* mutations. STR haplotypes linked to missense mutations of *SLC26A4*, H723R (H) and L676Q (L), are boxed. In II.3 and III.1, deafness is not linked to *SLC26A4* and must have another aetiology. (B) Axial computed tomographic images of the temporal bones showing bilateral EVA (indicated by white arrowheads) in II.7 and normal appearing vestibular aqueducts in II.3.

2.9 to 13.6%). In combination with 10 (4.7%) of 212 Pakistani families (95% CI 2.4 to 8.8%), *SLC26A4* mutations were detected in a total of 17 (5.4%) of 318 families from south Asia (95% CI 3.3 to 8.6%).

### Origins of recurrent *SLC26A4* mutations

The detection of IVS7-2A>G, L676Q, H723R, and S90L in multiple probands from different Asian populations suggested that they may have arisen on ancestral founder chromosomes.

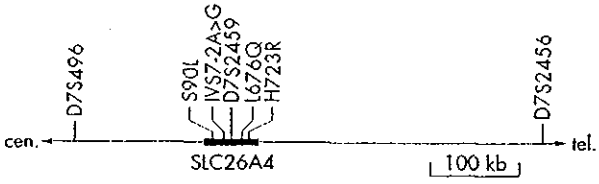


**Figure 2** Physical map of *SLC26A4* mutations and linked STR markers on chromosome 7q.

H723R is the most commonly reported *SLC26A4* mutation in Japanese subjects,[19 20] so we analysed H723R linked haplotypes of three STR markers in 26 unrelated probands from Korea and Japan. We observed an association of H723R with a single haplotype by Fisher's exact analysis in Korean ($p=0.00000002$) and Japanese chromosomes ($p=0.009$) (table 3), suggesting its derivation from a common founder.

Meiotic phase and chromosome 7q31 haplotypes could not be assigned for some chromosomes with L676Q or IVS7-2A>G owing to a lack of parental DNA samples. Nevertheless, L676Q was significantly associated with the 119 bp allele of the centromeric flanking marker D7S496 ($p=0.0006$, online supplementary table 1) and the 139 bp allele of the intragenic marker D7S2459 ($p=0.00005$, online supplementary table 1). The 240 bp allele of the telomeric flanking marker D7S2456 was present in all six subjects with L676Q (online supplementary table 1), but its detection in 54-65% of controls precluded statistical significance ($p=0.057$ for Mongolian subjects). IVS7-2A>G was weakly associated with the 145 bp allele of D7S2459 on Korean chromosomes ($p=0.046$, online supplementary table 2), but similar comparisons of Chinese or Japanese chromosomes or subjects with IVS7-2A>G did not reach statistical significance (not shown). S90L was associated with a single two marker haplotype comprising the 145 bp allele of D7S2459 and the 240 bp allele of D7S2456 in all five unrelated Pakistani families in which it was detected ($p=0.0001$, table 2).

## DISCUSSION

There are over 50 published mutant alleles of *SLC26A4*,[15] most of which originated in western countries. Our study shows that overall frequencies and diversities of *SLC26A4* mutations are similar in western and various Asian populations, although the mutations are different. This conclusion is consistent with recent reports of diverse, novel mutant *SLC26A4* alleles in small numbers of Japanese,[19 20] Mexican,[31] Spanish, and Italian patients.[32] We observed several examples of more closely related Asian ethnic groups sharing common *SLC26A4* mutations, which may have been derived from shared ancestral founders. Mutations arising before the divergence of related ethnic groups could have been vertically transmitted to each of the groups or, alternatively, mutant alleles may have been shared through more recent genetic admixture.

H723R and IVS7-2A>G are prevalent alleles accounting for a majority of observed *SLC26A4* mutations in our Korean study

**Table 1** Numbers of east Asian probands with detected *SLC26A4* mutations

| Mutation | Exon | Genotype | Korean | | Mongolian | | Chinese | | Reported ethnic origins |
|---|---|---|---|---|---|---|---|---|---|
| | | | Deaf (n=92)* | Control (n=120) | Deaf (n=195) | Control (n=94) | Deaf (n=86) | Control (n=44) | |
| S252P (754T>C) | 6 | Homo | 0 | – | 0† | – | 0 | 0 | |
| | | Hetero | 0 | – | 0† | – | 1 | 0 | |
| IVS7-2 A>G | (8) | Homo | 1 | 0 | 0† | – | 1 | 0 | China[21] |
| | | Hetero | 2 | 0 | 0† | – | 2 | 0 | |
| N392Y (1174A>T) | 10 | Homo | 0 | 0 | 0† | – | 0 | 0 | |
| | | Hetero | 0 | 0 | 0† | – | 2 | 0 | |
| T410M (1229C>T) | 10 | Homo | 0 | 0 | 0† | – | 0 | 0 | Japan,[20] Pakistan,[16] Italy[32] |
| | | Hetero | 1 | 0 | 0† | – | 0 | 0 | |
| 1548insC (frameshift) | 14 | Homo | 0 | – | 0† | 0 | 0 | – | |
| | | Hetero | 0 | – | 11 | 0 | 0 | – | |
| IVS14-7A>G‡ | (15) | Homo | 0 | – | 0† | 0 | 0 | – | |
| | | Hetero | 0 | – | 11 | 0 | 0 | – | |
| L676Q (2027T>A) | 17 | Homo | 0 | 0 | 0 | 0 | 0 | – | |
| | | Hetero | 0§ | 0 | 4 | 1 | 0 | – | |
| T721M (2162C>T) | 19 | Homo | 0 | 0 | 0 | 0 | 0 | – | Japan,[19] Italy[32] |
| | | Hetero | 1 | 0 | 0 | 0 | 0 | – | |
| H723R (2169A>G) | 19 | Homo | 1 | 0 | 0 | 0 | 0 | – | Japan,[19 20] Netherlands[17] |
| | | Hetero | 3 | 2 | 0 | 0 | 0 | – | |
| Totals | | Homo | 2 | | 0 | | 1 | | |
| | | Comp hetero | 3 | | 2 | | 1 | | |
| | | Hetero | 1 | | 2 | | 3 | | |
| | | Total | 6 | | 4 | | 5 | | |
| | | Percentage (95% CI) | 6.5% (2.7 to 14.2) | | 2.1% (0.7 to 5.5) | | 5.8% (2.2 to 13.7) | | |

The numbers of deaf or normal hearing control subjects who are homozygous (Homo), heterozygous (Hetero), or compound heterozygous (Comp hetero) for *SLC26A4* mutations in exons 6, 8, 10, 14, 15, 17, or 19 and adjacent splice sites are indicated.
*Does not include family K-87 owing to lack of a single representative *SLC26A4* genotype among affected family members.
†n=96.
‡Unknown pathogenicity.
§Detected in family K-87.

**Table 2** *SLC26A4* mutations and chromosome 7q31 haplotypes in south Asian families

| Mutation | Exon | Family | Country | Linked STR haplotype D7S496 | D7S2459 | D7S2456 |
|---|---|---|---|---|---|---|
| S28R (84C>A) | 2 | DKMy10 | India | | | |
| S90L (269C>T) | 3 | PKDF026 | Pakistan | 131 | 145 | 240 |
| | | PKDF074 | Pakistan | 133 | 145 | 240 |
| | | PFDF106 | Pakistan | 133 | 145 | 240 |
| | | PKSRE | Pakistan | 131 | 145 | 240 |
| | | PKSN23 | Pakistan | 131 | 145 | 240 |
| S57X (170C>A) | 3 | DKH-5 | India | | | |
| IVS2_IVS3del4kb* | 3 | DKH-5 | India | | | |
| V239D (716T>A) | 6 | I-56 | India | | | |
| | | PKDF032 | Pakistan | | | |
| | | DKH-10 | India | | | |
| IVS8+4A>G | 8 (splice donor) | PKSR21 | Pakistan | | | |
| R409P (1226G>C) | 10 | DKMy-8 | India | | | |
| I455F (1363A>T) | 12 | PKSR5 | Pakistan | | | |
| | | PKSR6 | Pakistan | | | |
| N457K (1371C>A) | 12 | DKH-9 | India | | | |
| 1863delT† | 17 | PKSRD | Pakistan | | | |

STR genotypes are given as allele sizes (bp). Underlines indicate the haplotype significantly associated with S90L (p=0.0001).
*4017 bp genomic deletion of IVS2-713 to IVS3+3164, with 3 bp insertion of CAT.
†Predicted to result in a frameshift and premature translation termination.

**Table 3** Chromosome 7q31 STR haplotypes linked to H723R

| Haplotype | STR marker genotype* D7S497 | D7S2459 | D7S2456 | Korean chromosomes H723R (n=6) | Control (n=52) | Japanese chromosomes H723R (n=20) | Control (n=9) |
|---|---|---|---|---|---|---|---|
| 1 | 119 | 147 | 240 | 6* | 0 | 10† | 0 |
| 2 | 119 | 145 | 240 | 0 | 8 | 0 | 0 |
| 3 | 131 | 143 | 240 | 0 | 7 | 0 | 0 |
| 4 | 135 | 145 | 240 | 0 | 6 | 0 | 0 |
| 5 | 135 | 143 | 143 | 0 | 6 | 0 | 0 |
| 6 | 119 | 143 | 143 | 0 | 4 | 0 | 0 |
| 7 | 121 | 145 | 240 | 0 | 3 | 0 | 0 |
| 8 | 121 | 147 | 240 | 0 | 2 | 0 | 0 |
| 9 | 119 | 147 | 242 | 0 | 0 | 3 | 0 |
| 10 | 119 | 147 | 238 | 0 | 0 | 3 | 0 |
| 11 | 131 | 147 | 238 | 0 | 0 | 2 | 0 |
| 12 | 137 | 145 | 240 | 0 | 0 | 0 | 3 |
| 13–35‡ | – | – | – | 0 | 16 | 2 | 6 |

STR genotypes are given as allele sizes (bp). Numbers of mutant (H723R) and wild type chromosomes with each STR haplotype are shown.
*Haplotype 1 is significantly associated with Korean H723R chromosomes (p=0.00000002).
†Haplotype 1 is significantly associated with Japanese H723R chromosomes (p=0.009).
‡Haplotypes each observed on one chromosome.

population. H723R is also prevalent among the Japanese, in whom it has been identified in 12 of 19 patients (17 of 38 chromosomes) with hearing loss and EVA.[19 20 33] In contrast, the carrier frequency was reported to be 1/96 in normal hearing Japanese control subjects,[19] which is consistent with our results in Koreans and confirms the specific association of H723R with hearing impairment. IVS7-2A>G is another recurrent *SLC26A4* mutation in multiple east Asian study populations. We detected IVS7-2A>G in multiple probands from the Korean and Chinese cohorts (table 1), as well as Japanese patients with hearing loss and EVA (SU, unpublished observations). The lack of a common STR haplotype linked to IVS7-2A>G on different chromosomes may reflect that this is a hot spot for recurrent mutational events, although this allele has not been observed in western populations. Alternatively, IVS7-2A>G may be an older founder mutation which has undergone ancestral recombination events with the flanking STR markers D7S496, D7S2459, and D7S2456 (fig 2). Analysis of single nucleotide polymorphisms more proximal to IVS7-2A>G might be required to identify a smaller region of linkage disequilibrium.

Our results indicate that *SLC26A4* mutations account for approximately 5% of all prelingual deafness in east Asia (table 1) and 5% of recessive deafness in south Asia. These approximations include heterozygotes that may not have a *trans* *SLC26A4* mutation, which would have led to significant

overestimates in the Chinese and Mongolian populations. The presence of an undetected *trans* mutation would be supported by radiological evidence of EVA, but CT and MRI imaging data were not available for those subjects. Conversely, the estimate of *SLC26A4* deafness in east Asia may be low, since we analysed only seven of 21 *SLC26A4* exons in those panels of samples, and the K-87 family was not included in the estimate for the Korean cohort owing to the lack of a single representative genotype for the affected members of the family.

The countries sampled in this study contain nearly one half (42%) of the global population.[27] It is possible that ethnic heterogeneity in the two most populous countries, India and China,[34] led to sampling bias. Our study subjects were from south west China and south and west India, where the specific mutations may differ from those in other provinces inhabited by distinct but related ethnic groups.[34] Moreover, we did not attempt to differentiate Pendred syndrome from NSRD since the goitrous phenotype is incompletely penetrant and not usually evident until adolescence,[35] phenocopies are common,[14] and intermediate perchlorate discharge results are non-diagnostic.[35]

The epidemiology of *SLC26A4* deafness is different from that of *GJB2* mutations at the DFNB1 locus, which is the most common cause of hereditary deafness in many western populations.[24] DFNB1 deafness in those populations is most frequently associated with a single ancient founder allele of *GJB2*,[24 36 37] whose high prevalence has been postulated to have arisen through assortative mating among the deaf.[38] *GJB2* mutations are much less common in Koreans,[22] Middle Eastern Arabs,[39] and African-Americans.[40] *SLC26A4* mutation frequencies may be more constant among different populations since they arise from multiple, newer mutational events.

The expense and inaccessibility of temporal bone radiology and perchlorate discharge testing are prohibitive for the clinical diagnosis of *SLC26A4* deafness in many populations. In those cases, *SLC26A4* mutation analysis may be the only available diagnostic test since blood or other tissues can be collected locally and sent elsewhere for testing. Our results indicate that rigorous molecular diagnosis will require an analysis of all *SLC26A4* exons unless the patient's ethnicity and the *SLC26A4* mutation spectrum for that population are well defined. Mutation analyses should include strategies to detect specific genomic deletion mutations, such as IVS2_IVS3del4kb, in populations where they are identified. Future studies are therefore needed to define further the identities and distributions of *SLC26A4* mutations in Asian and other global populations.

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Supplementary tables 1 and 2 can be found on our website at www.jmedgenet.com/supplemental

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