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A novel synonymous mutation causing complete skipping of exon 16 in the *SLC26A4* gene in a Korean family with hearing loss

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ABSTRACT

Introduction: Mutations in *PDS* (or *SLC26A4*) cause both Pendred syndrome (PS) and DFNB4, two autosomal recessive disorders that share hearing loss as a common feature. PS and DFNB4 are genetically homogeneous disorders caused by bi-allelic *SLC26A4* mutations. Here, we report a novel synonymous mutation (c.1803G>A, p.Lys601Lys), that caused aberrant splicing in two Korean family members who were clinically considered to have DFNB4, along with congenital hearing loss and dilated vestibular aqueducts (DVA).

Methods: After extracting DNA from whole blood using standard procedures, the 21 exons and flanking introns of *SLC26A4* were amplified with PCR. To evaluate the implication of a novel synonymous mutation (c.1803G>A), we used The Berkeley Drosophila Genome Project (BDGP) (http://www.fruitfly.org/) as a splice site prediction program and performed exon trapping analysis.

Results: In molecular analysis of the 21 exons of *SCL26A4*, we detected a known splicing mutation (c.919-2A>G, heterozygote) and a novel variant (c.1803G>A, heterozygote) in the patients (II-1 and II-2). According to *in silico* analysis, the novel variant (c.1803G>A) affects canonical splice donor nucleotide positioning. To define the transcript level effects of this novel 1803G>A variant, we performed exon trapping and confirmed that exon 16 is completely skipped in this variant type.

Conclusion: We report a novel synonymous mutation (c.1803G>A) causing complete exon 16 skipping in the *SLC26A4* gene in two Korean family members with hearing loss. This is the first case of a synonymous SNP (c.1803G>A) affecting vestibulocochlear organs through altering splicing accuracy by causing a complete skipping of exon 16. An important issue raised by this study is that synonymous mutations that have been previously ignored in clinical diagnoses must now be considered as potential pathogenic mutations.

1. Introduction

SLC26A4 (originally called *PDS*), a causative gene for Pendred syndrome, was identified in 1997 and consists of 21 exons and is located on chromosome 7 [1]. The pendrin is a member of the anion transporter family SLC26, and permits the exchange of anions between the cytosol and extracellular space. *SLC26A4* mutations, in an autosomal recessive manner, cause Pendred syndrome (PS, [MIM 274600]) or non-syndromic hearing loss (DFNB4, [MIM 600791]), which originally was thought to be a distinct entity from PS because of the absence of an enlarged thyroid [2–4]. Furthermore, mutations of *SLC26A4* are associated with malformations of the inner ear such as an enlarged vestibular aqueduct (EVA) and mondini malformations [5]. These abnormalities are detected using computed tomography or nuclear magnetic resonance of the temporal bones [4,6]. Published reports have made an effort to support a correlation between the type of *SLC26A4* mutation and the specific thyroid phenotype (PS versus DFNB4), but details are still uncertain [6]. Seventy to eighty-five percent of the mode of inheritance of neurosensory hearing impairment is in an autosomal recessive pattern [7,8]. Bi-allelic mutations of *SLC26A4* are detected at various proportions in Korea (5.4–81%), depending on the reports [8,9]. To date, more than 170 mutations within the pendrin

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gene have been identified (http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm, accessed on Sep 24, 2012) and about 13% of the total mutations of the *SLC26A4* gene affect splicing sites [10]. The pathological effects of synonymous nucleotide changes, affecting various organ systems, have been discussed in almost 50 diseases [11]. However, synonymous mutations affecting vestibulocochlear organs have not been reported. Here, we report a novel synonymous mutation in the *SLC26A4* gene that leads to alternative splicing on the *SLC26A4* gene.

2. Materials and methods

2.1. Subjects and audiometric evaluation

A boy (5 y old, II-1) and a girl (4 y old, II-2) (Fig. 1A) in a Korean family visited the Department of Otorhinolaryngology to evaluate hearing impairment in 2004 and 2005, respectively. Pure tone audiometry (PTA) tests were performed in both patients. The PTA test was performed in a sound-controlled room at frequencies ranging from 250 to 8000 Hz and the level of hearing loss was classified according to previously described methods [12,13]. These two probands with prelingual hearing loss and bilateral dilated vestibular aqueducts (DVAs) on magnetic resonance imaging (MRI) were diagnosed with NSHL. Patients II-1 and II-2 had bilateral severe to profound hearing impairment, and received cochlear implantations at ages 5 (II-1) and 4 (II-2). Mutations of the pendrin gene can lead to deafness that is usually severe to profound with an early onset, however, hearing impairments occasionally occur after head trauma in patients with *SCL26A4* mutations [14]. The hearing impairment level of patient II-1 was aggravated after head trauma when he was 11 y old. The parents of the two siblings have not shown any hearing impairment and/or symptoms of Pendred syndrome or DFNB4. We could not perform any genetic analysis for the parents due to their refusal. Written informed consent was obtained from the participants before blood sampling for molecular analysis.

2.2. Mutation analysis

Peripheral blood samples from II-1 and II-2 were obtained and genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. All the 21 exons and adjacent intron sequences of *SLC26A4* were amplified by polymerase chain reaction (PCR) and sequenced as described [8]. Sequences were compared with the database sequence in GenBank (http://www.ncbi.nlm.nih.gov) assessed June, 2012. The GenBank accession number for *SLC26A4* is NM_000441.

2.3. Exon trapping analysis

The Berkeley Drosophila Genome Project (BDGP) (http://www.fruitfly.org/) [15] was used as a splice site prediction program to analyze the novel variant. For the *in vitro* splicing assay, approximately 300 bp of the 5′- and 3′-intronic flanking regions, including *SLC26A4* exon 16, with normal or splicing mutant (c.1803G>A) alleles from the genomic DNA of patientII-2 were amplified. PCR products were inserted between exons A and B in the pSPL3 and the pSPL3b splicing vectors [16,17]. These hybrid minigenes were transfected into HeLa cells using FuGENE HD Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Ger-

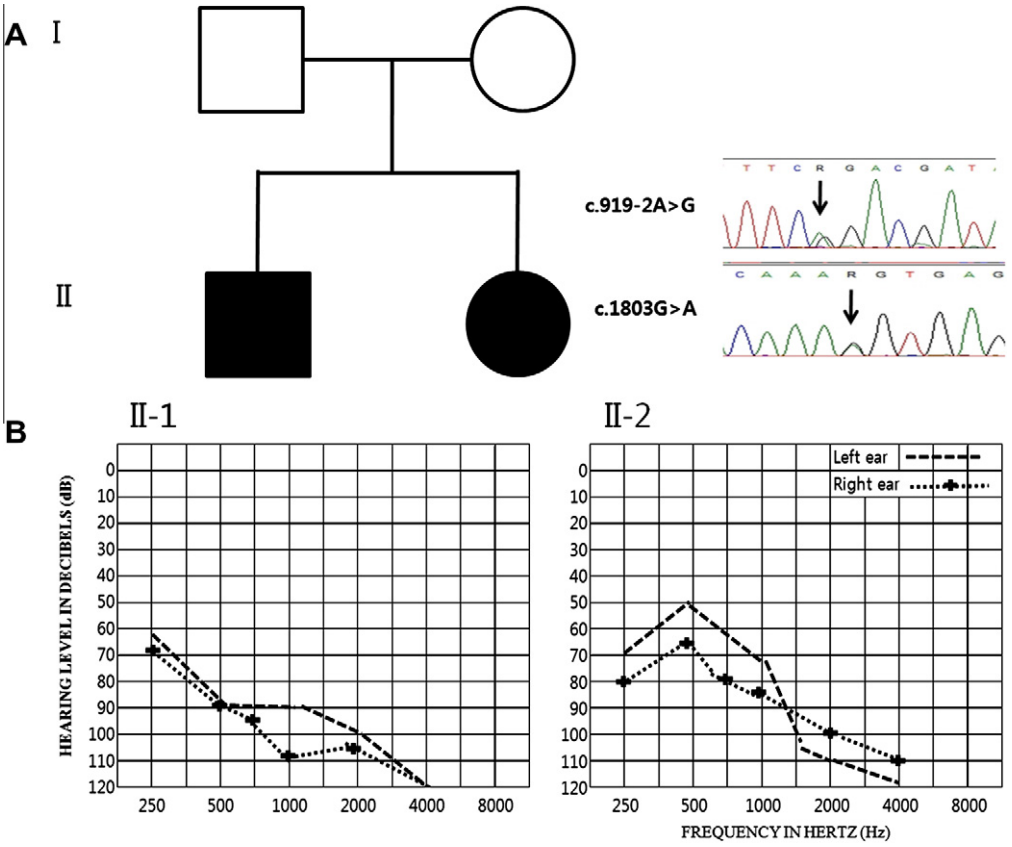

Fig. 1. Clinical findings of the patients with non-syndromic hearing loss. (A) Pedigree and genotypes of the family is shown. Filled symbols represent affected individuals. IVS7-2A>G (c.919-2A>G) and c.1803G>A, present in two family members II-1 and II-2 simultaneously, are indicated with black arrows. (B) Pure tone audiograms of the affected members (II-1 and II-2) are shown.

many), and then the transfected cells were harvested after 24 h. RNA isolation of the cultured cells was done with an RNeasy Mini Kit (Qiagen, Hilden, Germany). Synthesis of the complimentary DNA (cDNA) was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR amplification and sequencing on cDNA was performed using SD6 (5′-TCTGAGTCACCTGGACAAC-3′) and SA2 (5′-ATCTCAGTGGTA TTTGTGAG-3′) primers [17,18]. The fragment sizes of the normal and mutant PCR products were detected on 2% agarose gels. Purified PCR products obtained using a QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany) were used for sequencing with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using an ABI 3500dx system (Applied Biosystems).

3. Results

In the molecular analysis of the 21 exons on *SCL26A4*, we detected a known splicing mutation (c.919-2A>G, heterozygote) and a novel variant (c.1803G>A, heterozygote) in the patients (II-1 and II-2) (Fig. 1A and B). We considered this a novel synonymous single nucleotide change (sSNP) from G to A at position 1803 in mono-allele, which had the potential to alter the splicing site and pendrin production. According to *in silico* analysis with prediction programs, the novel variant (c.1803G>A) affected the canonical splice donor nucleotide position. To investigate the RNA splicing pattern of a novel 1803G>A variant, wild type and variant type RNA were extracted from HeLa cells that had been transfected with the pSPL3 splicing vectors (Fig. 2A). The PCR product of wild type showed a band size of 359 bps size to be expected of a normal transcript, but the variant type revealed a complete skipping of exon16 by alternative transcripts and showed 96 bps shorter product (263 bps) than the normal transcript product (359 bps) on agarose gel electrophoresis (Fig. 2B). We confirmed that the entirety of exon 16 was skipped in the variant type using sequence analysis, and exon B sequence was observed by a series of exon A sequence in the variant type sequence analysis (Fig. 2C).

4. Discussion

IVS7-2A>G (c.919-2A>G) is well known to be a mutation that causes PS/DFNB4 with temporal bone abnormalities [19]. IVS7-2A>G, along with H723R, is one of the most commonly found mutations among Korean sensorineural hearing loss patients [20]. However, IVS7-2A>G on one allele is not enough to cause the PS/DFNB4 phenotypes seen in our subjects (II-1and II-2), who had severe to profound hearing impairment and bilateral EVA. A novel synonymous SNP (c.1803G>A) of unknown significance may be affecting our subjects. Owing to the dogma that the structure of proteins is determined by amino acid sequence changes, synonymous SNPs were usually referred to as ‘silent’ [21]. Over the past decade, however, considerable evidence has accumulated to show the mechanistic and conceptual framework of synonymous SNPs in human disease. Around up to 10% of human genes contain at least one region in which synonymous mutations could be deleterious or disease-associated [22,23]. Synonymous SNPs can lead to changes in the phenotype by affecting the rate of translation, mRNA stability, protein folding, and splicing accuracy [11,24,25]. Some reasonable cases of functional synonymous SNPs have been reported; a synonymous SNP (C3435T) in exon 26 of the

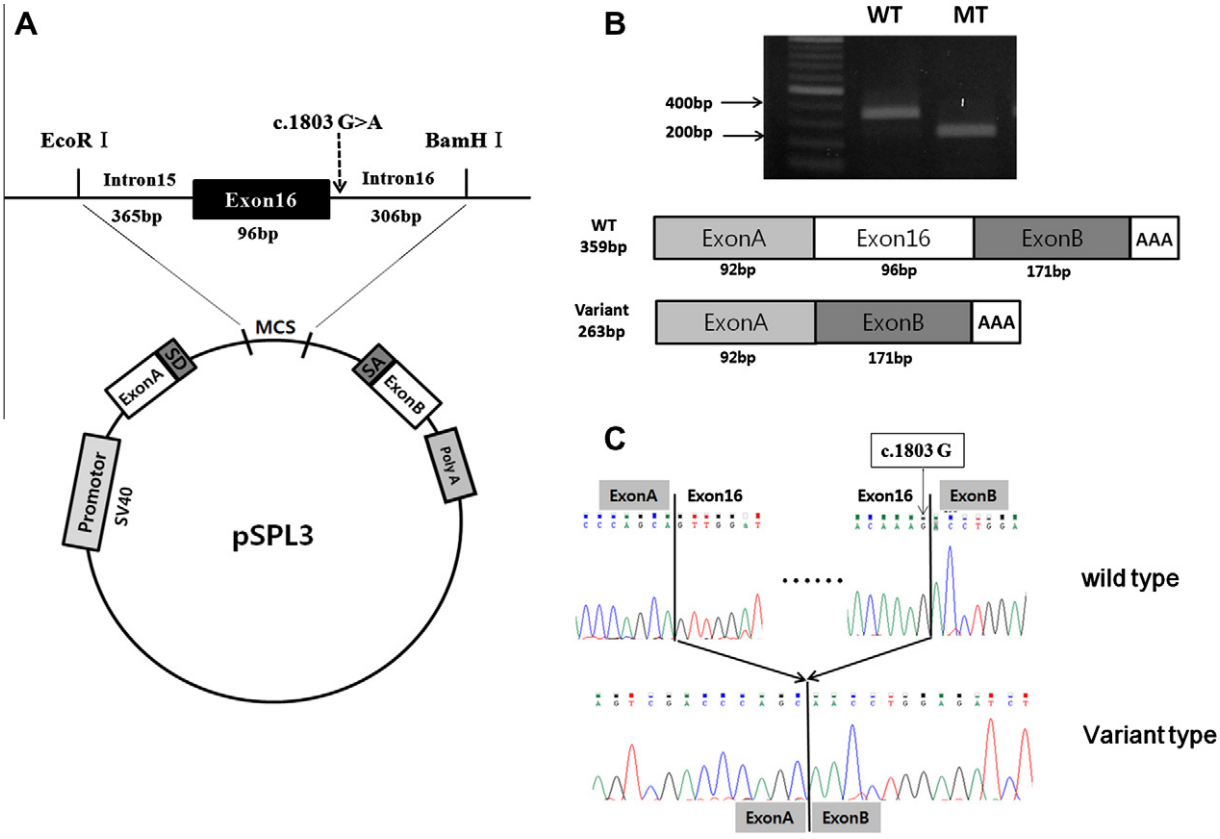

Fig. 2. Exon trapping analysis of *SCL26A4* exon 16 in transfected HeLa cells. (A) Construction of the pSPL3-hybrid minigenes that harbored exon 16 and flanking intronic sequences from wild type or variant types (c.1803G>A) of *SCL26A4* gene. (B) The isolated RNA of transfected cells was amplified by RT-PCR analysis. The different splicing product for wild type (WT lane, 359 bps) and variant type (MT lane, 263 bps) are shown on a 2% agarose gel electrophoresis and represented graphically. (C) Partial DNA sequences of the *SCL26A4* gene showing the complete skipping of exon 16 in the variant type.

Multidrug Resistance 1 (MDR1) gene altered the levels of cell surface P- glycoprotein activity by affecting co-translational folding [26] and synonymous SNPs (C/T; rs4633, C/G; rs4818) in the *catechol-O-methyltransferase* (*COMT*) gene affected pain sensitivity by changing mRNA stability [11]. Synonymous mutations that affected splicing accuracy have also been presented in reports from Italian patient with cystic fibrosis and patients with combined deficiency of factor V and factor VIII [25,27,28]. Now, synonymous changes have been discussed in almost 50 diseases and in most organ systems in humans [11]. However, a synonymous SNP related to hearing impairment and affecting vestibulocochlear organs has not yet been reported. This study presents the first case of a synonymous SNP (c.1803G>A) that has a phenotype of hearing impairment by affecting splicing accuracy. This novel SNP occurred at the terminal nucleotide of exon 16 and lead to a transcript with exon 16 skipped. The phenomenon of exon skipping corresponds to previous reports, for example, last nucleotide changes of exon 8 in the *ATM* gene and exon 11 in the *TAT* gene showing skipped exons 8 and 11, respectively [29,30]. We could not find any information about c.1803G>A from studies that reported novel mutations in exon 16 and performed sequencing analysis with 184 non-affected controls for exon 16 on *SCL26A4* gene [4,31].

In summary, we confirmed a novel synonymous mutation in the *SLC26A4* gene in a Korean family with severe to profound sensorineural hearing. This is the first case of a synonymous SNP (c.1803G>A) affecting vestibulocochlear organs by altering splicing accuracy and causing a complete skipping of exon 16. We must consider novel synonymous SNPs as potentially pathogenic mutations, where before they have often been ignored as silent mutations.

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