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A novel mutation of *TMPRSS3* related to milder auditory phenotype in Korean postlingual deafness: a possible future implication for a personalized auditory rehabilitation

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Abstract

Appropriate customized auditory rehabilitation for hearing impaired subjects requires prediction of residual hearing and progression of hearing loss. Mutations in *TMPRSS3* encoding a transmembrane serine protease were reported to be associated with two different autosomal recessive nonsyndromic hearing loss (arNSHL) phenotypes, DFNB8 and DFNB10, in terms of residual hearing that may mandate different rehabilitation. We aimed to reveal the genetic contribution of *TMPRSS3* mutations among Korean populations and to correlate the clinical phenotype with *TMPRSS3* genotypes. Fifty families that segregated arNSHL and have visited our clinic recently for 2 years were recruited for *TMPRSS3 screening*. Novel *TMPRSS3* variants detected in our cohort were modeled using a predicted three-dimensional (3D) structure of the serine protease domain. The prevalence reached up to 11.2 % (3/27) among subjects with either prelingual hearing loss but retaining some degree of language development or with postlingual ski-slope hearing loss. We also found that a p.A306T allele is a founder allele in this population. Based upon the 3D modeling, we were able to correlate significant retention of residual low-frequency hearing and slower progression of its loss to this novel variant p.T248M that was predicted to have milder pathogenicity. A yeast-based protease assay confirmed a mild pathogenic potential of the p.T248M variant and a tight correlation between the protease activity and the residual hearing. Preservation of this low-frequency hearing should be of utmost importance when considering auditory rehabilitation. Our results significantly narrow down the candidate population for *TMPRSS3* sequencing for more efficient genetic diagnosis. More importantly, genotype–phenotype correlation of this gene observed in our cohort suggests

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that *TMPRSS3* can be an appropriate candidate for personalized and customized auditory rehabilitation.

Key message

- The prevalence of *TMPRSS3* mutations among Korean postlingual hearing loss is 8.3 %.
- The p.A306T variant of *TMPRSS3* is the common founder allele in Koreans.
- A novel variant, p.T248M of *TMPRSS3*, was predicted to have milder pathogenicity.
- There was a genotype–phenotype correlation of this gene in Koreans.
- Our data support implication of this gene for personalized rehabilitation.

Keywords DFNB8/10 · *TMPRSS3* mutations · Cochlear implantation · Ski-slope audiogram

Introduction

Autosomal recessive nonsyndromic hearing loss (arNSHL) is the most common form of hereditary hearing loss, accounting for about 70–80 % of congenital hereditary hearing loss. Extreme heterogeneity of arNSHL is reflected by the fact that more than 90 loci have been mapped and 40 causative genes have been identified to date (http://hereditaryhearingloss.org/). Despite this etiologic heterogeneity, the phenotype of autosomal recessive hearing impairment is typically prelingual, nonprogressive, and severe to profound. In contrast, autosomal recessive inheritance is rare in nonsyndromic hearing impairment with postlingual childhood onset [1, 2]. However, a deafness locus of DFNB8 family segregating childhood onset arNSHL (OMIM 601072) was mapped to chromosome 21q22 in large consanguineous Pakistani kindred [3], and, subsequently, a mutation in a gene encoding *TMPRSS3* was identified in this family [4]. Interestingly, *TMPRSS3* mutations were also revealed to account for the DFNB10 phenotype which was more severe and congenital (OMIM 605511) [4, 5]. Since then, this gene, *TMPRSS3*, has been spotlighted as a gene with two discrete phenotypes.

Weegerink et al. [6] recently proposed that *TMPRSS3* mutations can be classified into mild or severe mutations and that the phenotype would be dependent upon the combination of the two *TMPRSS3* mutant alleles. They predicted that a combination of two 'severe' mutations in a *trans* configuration leads to profound deafness with prelingual onset (DFNB10), while the severe mutation in combination with milder *TMPRSS3* mutations leads to a milder phenotype with postlingual onset (DFNB8) [6]. This differential phenotype may be accounted for by a differential proteolytic activity of mutant TMPRSS3 protein [7, 8].

Genetic load of this gene to arNSHL appeared to vary depending upon ethnicities. This gene contributed to less than 1 % of nonsyndromic childhood deafness in Caucasians [9, 10]. *TMPRSS3* variants accounted for 5–6 % of arNSHI among the Tunisian population and 12 % among the Turkish population, making this gene a significant contributor. Moreover, despite the rarity of *TMPRSS3* mutations among total arNSHL subjects, it was considered to be the candidate gene mutation accounting for postlingual form of progressive arNSHL in North European populations after exclusion of *GJB2* [6, 11].

Prevalence of *TMPRSS3* mutations has never been reported in East Asian populations. In this study, we estimated the prevalence of *TMPRSS3* mutations in a clinically homogenized Korean cohort in terms of onset of hearing loss and audiogram configuration. We also report a predominantly founder mutation of *TMPRSS3* in the Korean population and a novel *TMPRSS3* variant which showed a clear genotype–phenotype correlation. This information would be very helpful for genetic counseling of these patients and planning of personalized auditory rehabilitation.

Materials and methods

Ethical considerations

The Institutional Review Boards (IRBs) at the Seoul National University Bundang Hospital (SNUBH) (IRB-B-1007-105-402) and the Seoul National University Hospital (SNUH) (IRBY-H-0905-041-281) approved this study. We obtained a written informed consent from all the participants in this study. In the case of children participants, written informed consent was obtained from the parents or guardians on their behalf.

Study participants

Evaluations of the clinical phenotype included developmental and medical history interviews, physical examinations, pure tone audiometry, and imaging studies (temporal bone CT and/ or MR images). To estimate the prevalence of the *TMPRSS3* mutation in Korean hearing impaired subjects, we focused on families that segregated nonsyndromic sensorineural hearing loss (SNHL) either in an autosomal recessive manner or sporadically (Fig. 1). Subjects with any syndromic feature or any radiologic marker such as inner ear anomaly were excluded. Then, sequencing of *GJB2* was performed as previously described (Fig. 1). Finally, 51 probands from 51 unrelated families with negative *GJB2* mutations were identified at the otolaryngology clinics of Seoul National University hospital (SNUH) and Seoul National University Bundang hospital (SNUBH) from May 2010 through April 2012. We also classified our cohort into two groups, prelingual and
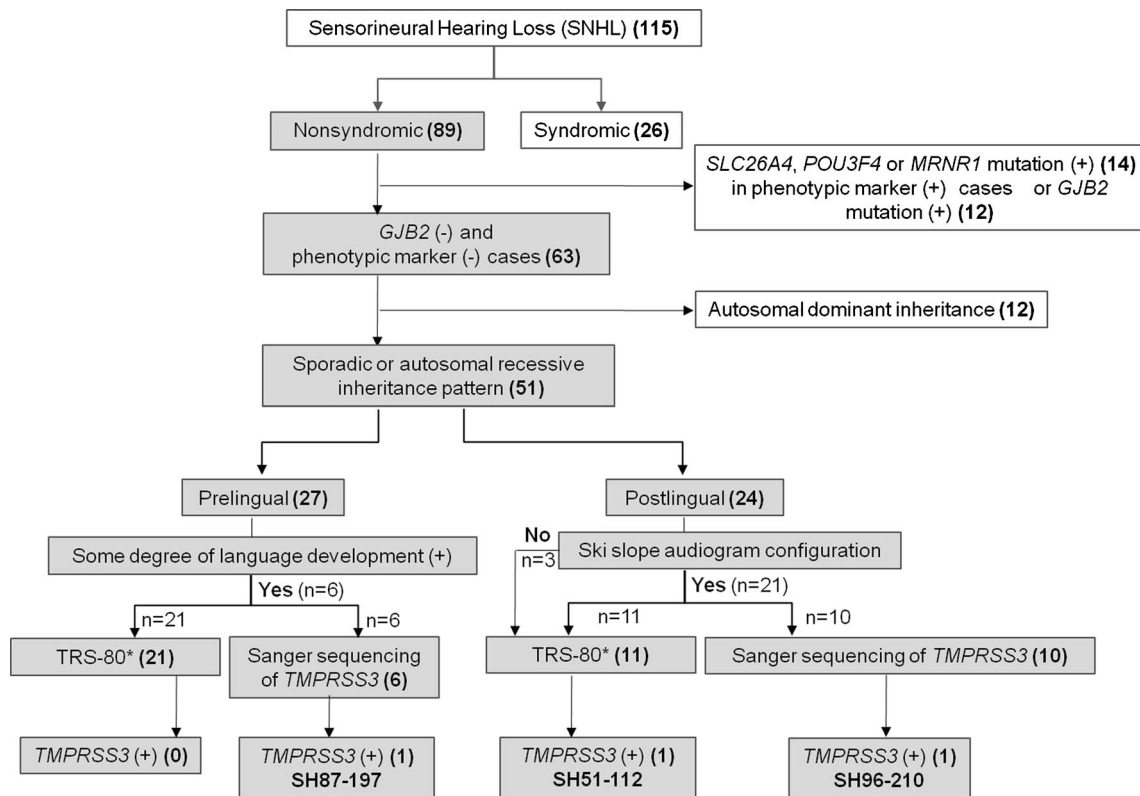
Fig. 1 Schematic diagnostic flow of this study *TRS-80*; targeted resequencing of 80 reported deafness genes

postlingual deafness, depending upon the onset of prominent hearing loss. Prelingual deafness in this study refers to hearing loss that begins before 3 years of age, the onset of developing spoken language [12]. In contrast, postlingual deafness refers to hearing loss that follows the onset of speech development. Our cohort was comprised of 27 probands segregating prelingual deafness and 24 probands manifesting postlingual progressive hearing loss. Patients/guardians and their relatives were interviewed to infer comprehensive clinical history in order to rule out perinatal, ototoxic, infectious and traumatic factors that could lead to nongenetic hearing loss.

Audiometric evaluation

Audiograms were also obtained. Pure tone audiometry (PTA) with air and bone conduction at frequencies ranging from 250 to 8,000 Hz was obtained from the recruited subjects according to standard protocols. The hearing loss range was described as follows depending on the PTA: low frequency, 250–500 Hz; mid frequency, 1–2 kHz; and high frequency, 4–8 kHz [13]. We calculated the mean level of hearing loss at all frequencies and at each frequency. We also calculated the mean hearing level at low frequency, mid frequency, and high frequency. We intended to find, if any, characteristic audiogram configurations that are more suggestive of *TMPRSS3* mutations among subjects manifesting high-frequency hearing loss. For this purpose, we compared audiologic findings

between subjects with *TMPRSS3* mutation and those without *TMPRSS3* mutation based on the mean±SD hearing level of low, mid, and high frequencies and speech discrimination score (percent). The serial audiograms were obtained at onset of progressive hearing loss, and before and after cochlear implantation (CI).

DNA samples and mutation analysis

Blood samples from 51 probands and, if necessary, their family members were obtained after informed consent had been obtained. Genomic DNA was extracted from peripheral blood cells and the samples using standard protocols. For ten postlingually hearing impaired probands with a typical ski-slope audiogram configuration and six prelingually deafened probands with some degree of language development, direct PCR Sanger sequencing of all 13 coding exons and exon–intron boundaries of *TMPRSS3* (GenBank No. NM_024022) was performed as suggested [14] (Fig. 1). This candidate gene approach was based upon the previous description that *TMPRSS3*-associated hearing loss was more prominent at the high frequencies, and the hearing at the low frequencies deteriorated with rapidity of variable degree depending upon the mutation [6]. For the remaining 35 probands including 11 probands with a typical ski-slope audiogram configuration, targeted resequencing of 80 reported deafness genes (TRS-80) including *TMPRSS3* was performed as previously described

[15] (Fig. 1). When a *TMPRSS3* mutation was identified, segregation analyses of the mutation were performed (Fig. 2).

Evaluation of CI outcomes by open set sentence test

Among three subjects with *TMPRSS3* mutations, two subjects underwent cochlear implantation. One subject underwent cochlear implantation at the age of 11, and the other underwent cochlear implantation at the age of five. Since we were afraid that cochlear implantation in subjects with *TMPRSS3*

mutations might not yield good postoperative performance considering its expression in the spiral ganglion cells as wells as in the hair cells, postCI outcomes were evaluated by open set sentence test. The postCI outcomes of subject with the *TMPRSS3* mutation and the age-matched control group were compared. The control group included 13 postlingually deafened subjects who underwent cochlear implantation at a similar age range with subjects with *TMPRSS3* mutations and had no inner ear anomalies and no evidence of calcified cochlea due to meningitis.
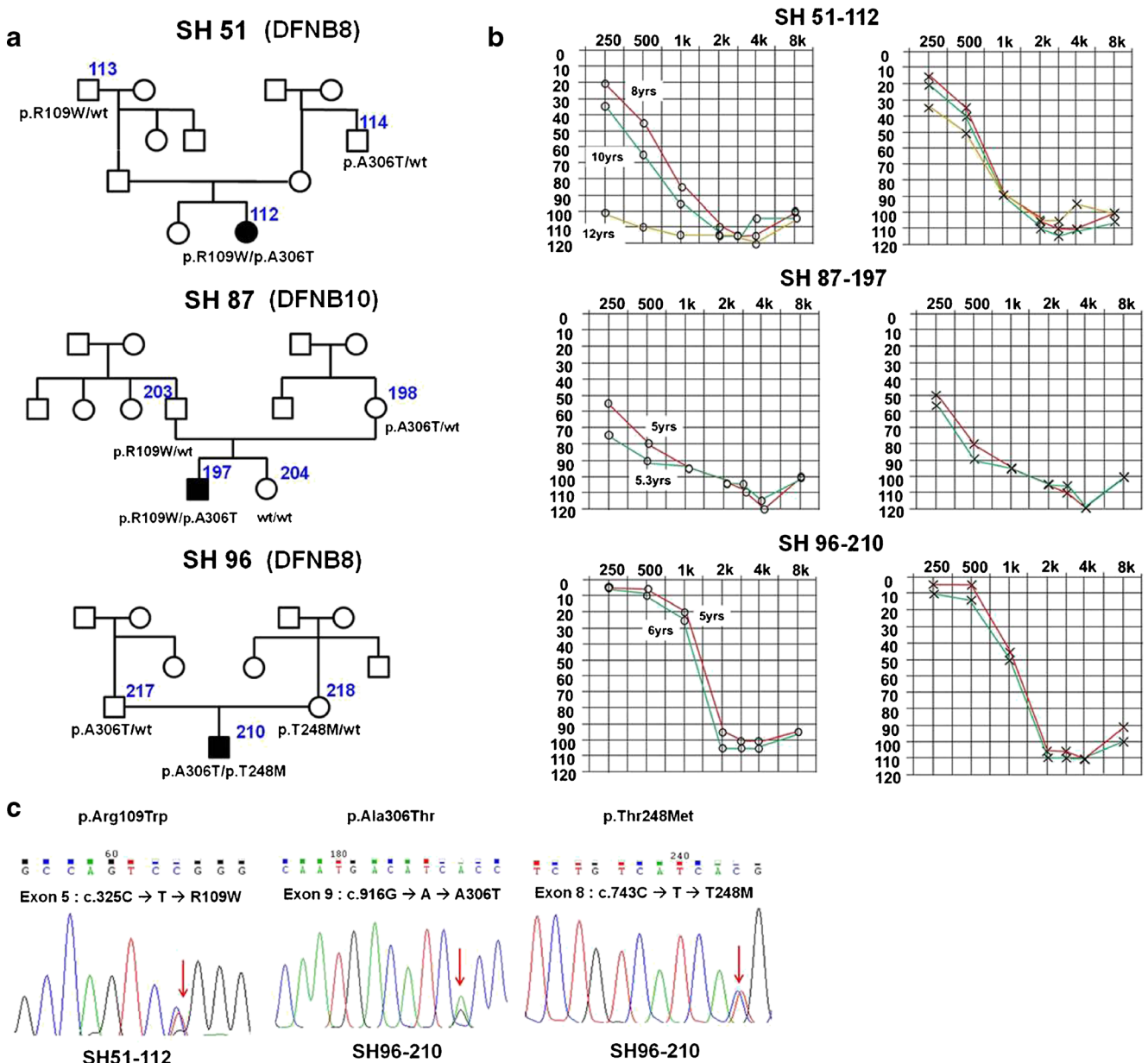

Fig. 2 **a** Pedigree drawings of three families with autosomal recessive nonsyndromic hearing impairment segregating *TMPRSS3* mutations. *Filled symbols* are hearing-impaired individuals, whereas *clear symbols* denote unaffected family members. This family segregate the known two variants [c.325C>T(p.A109W), c.916G>A(p.A306T)]. **b** Selection of

binaural mean air conduction threshold values of three affected family members at different ages, ordered by age. **c** Sequence chromatograms showing the variants identified in the *TMPRSS3* gene segregating the p.R109W, p.A306T, and p.T248M variants

Comparative protein modeling of the TMPRSS3 Serine protease domain

To analyze how damaging the mutations are on the function of *TMPRSS3*, we built the four protein structural models, one model for the wild type and three models for the mutants (p.A306T, p.A426T, and p.T248M) that all reside in the serine protease domain [6]. Because the protein structure of *TMPRSS3* is not available, we first constructed the homology model of the wild type using the PDB structure 1Z8G as a template, and then built the three mutant structures by replacing the side chains. All protein models were designed with MODELLER software (http://salilab.org/modeller/) and visually inspected using PYMOL viewer (http://www.pymol.org). Next, we optimized the structures by performing the molecular dynamics (MD) simulations. We used GROMACS 4.5.4 with AMBER99SB-ILDN force field (http://www.gromacs.org). For each structure, the initial simulated annealing was performed for 3 ns and then subsequent MD simulations were performed at 300 K for 7 ns to obtain the optimized structure, which was used for our functional analysis.

Yeast-based protease assay

Proteolytic activity of TMPRSS3 and its variants (p.T248M and p.A306T) was evaluated as described [16]. The p.D103G and p.R109W previously documented to be defective in proteolytic activity [16] were used as null function controls. Primer sets designed to generate the two variants, p.T248M and p.A306T were as follows: p.T248M (5′-gtgcgggggctctgtcatcatgcccctgtggatcatcactg-3′ and 5′-cagtgatgatccacaggggcatgatgacagagcccc cgcac-3′) and p.A306T (5′-gaggctgggcaatgacatcactcttatgaagctggccgggc-3′ and 5′-gcccgg ccagcttcataagagtgatgtcattgcccagcctc-3′). Briefly, a yeast strain KSY01 (MATα, leu2 ura3 his3 trp1 lys2 suc2-Δ9 kex2::HIS3) was co-transformed with the wild type and mutant *TMPRSS3* expression vectors and the substrate vector by lithium acetate transformation. The Leu$^+$/Trp$^+$ transformants were selected on minimal media containing 2 % glucose but lacking Leu and Trp, and then serially diluted and spotted onto YPD (nonselective) or synthetic medium containing 2 % sucrose and 10 μg/ml anti-mycin A (selective). Ratios and relative ratios were basically obtained by dividing the number of colonies on selective plates by the number of colonies on nonselective plates. The quantification of number of colonies in the plates was performed by using the ImageJ program.

STR marker genotyping

Primers of six STR markers flanking the *TMPRSS3* gene were obtained from UCSG genome browser (www.genome.ucsc.edu)

(Fig. 3a). The sequences of the microsatellite markers were D21S266 (5′-GGGGACATTGAGTCATCACA-3′)(5′-AGGC AAATGAAGACCTGAAC-3′), D21S1260 (5′-TCCAAGGG GTTCATCC-3′)(5′-CCCAAGGCACTGTTCC-3′), D21S2092(5′-AATCCCTCCGGCCATCATGCAGG-3′)(5′-TTAACAGTCTGTTTCTAGCTTGG-3′), D21S1225(5′-AGAAGTGCCTGGATTCAGC)(5′-AGGTTTTACAAATA AGCAGAAAGG-3′), D21S1411 (5′-ATGATGAATGCATA GATGGATG-3′)(5′-AATGTGTGTCCTTCCAGGC-3′), and D21S1890(5′-GGTCTGACCACAGATTTCC-3′)(AAAAAC ACTCTGAACGATTAAGG-3′). Loci were amplified with AmpliTaq DNA polymerase by PerkinElmer 9700 thermal cyclers. Also, genotype at these loci on the 21 chromosome was defined with a Taq gold (PE Biosystems) touchdown protocol later under the following conditions: 10 min at 95 °C; 10 cycles of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C; and 20 cycles of the same conditions but dropping the annealing temperature from 0.5 °C to 55 °C, 15 cycles of annealing at 55 °C, and a 72 °C final extension for 10 min. STR marker genotyping was performed as previously described [17].

Statistical analysis

Wilcoxon rank-sum test was used for comparison of pure tone audiometry and speech discrimination score between subjects with *TMPRSS3* mutation and those without *TMPRSS3* mutation.

Differences in STR genotypes and distribution of haplotypes between mutant chromosomes harboring p.A306T and wild type Korean chromosomes from normal hearing control DNA samples were analyzed by Fisher's exact test. Some genotype contributions were compared among subjects, not chromosomes as described [18]. Statistical analyses were computed using SPSS 12.0K (SPSS Inc., Chicago, USA), and *p* values of less than 0.05 were chosen as the level of statistical significance.

Results

Prevalence of TMPRSS3 mutations in our cohort

The overall genetic load of *TMPRSS3* to arNSHL is 5.9 % (3/51) in Koreans. In more detail, the prevalence of *TMPRSS3* mutations among prelingual and postlingual progressive arNSHL subjects was calculated to be 3.7 and 8.3 %, respectively (Fig. 1). The prevalence reached up to 11.2 % (3/27) among subjects either with prelingual onset hearing loss but retaining some degree of language development or with postlingual hearing loss manifesting ski-slope audiogram configuration. In contrast, none of the 21 prelingual, profoundly deafened subjects without any language development carried *TMPRSS3* mutations.

Fig. 3 Conservation of the p.T248 residue (*red*) among various TMPRSS3 orthologs and one paralog (TMPRSS2) as compared with the p.I253 residue in which a known polymorphism, p.I253V, is located. The p.T248 residue is well conserved among orthologs from various vertebrate species in contrast with the minimally conserved p.I253 residue (*black*)
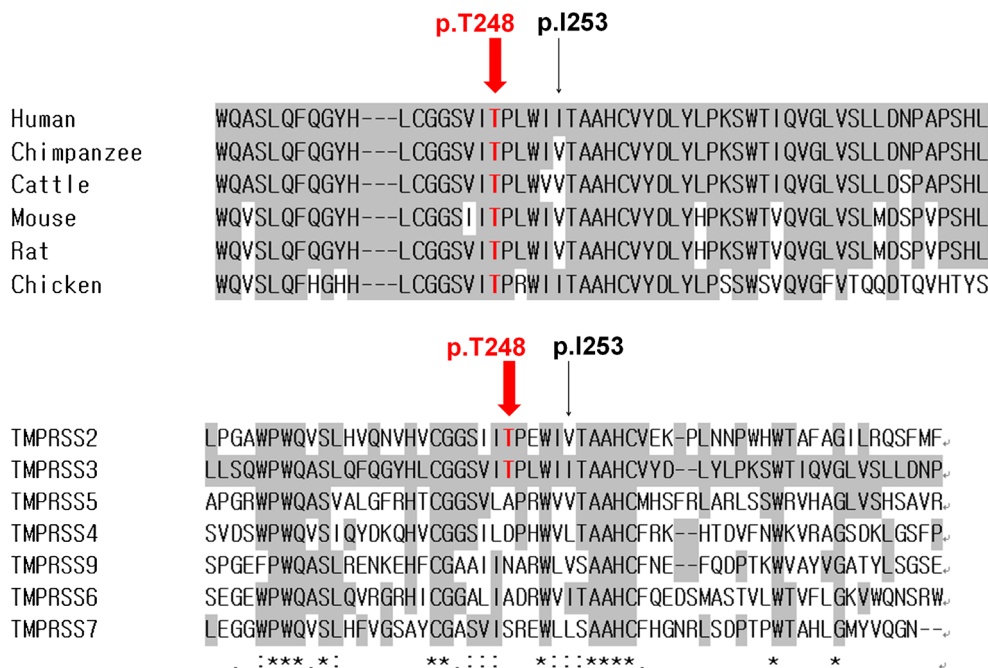
As for the three subjects with *TMPRSS3* mutations, two subjects (SH51-112 and SH87-197) segregated known *TMPRSS3* mutant alleles [c.325C>T(p.A109T),c.916G>A(p.A306T)] in a compound heterozygous state (Fig. 2a), and the other subject (SH96-210) had a novel variant c.743C>T(p.T248M) in a *trans* configuration with the c.916G>A(p.A306T) (Fig. 2a). To our interest, the c.916G>A(p.A306T) mutation was detected in all three subjects in our cohort, implying that this mutation is the main contributor of the DFNB8/DFNB10 phenotype in Koreans. p.T248M was not detected in 160 normal hearing Korean chromosomes, and the p.T248 residue was well conserved in human, rat, mouse, and chicken *TMPRSS3* orthologs in contrast with the nonconserved p.I253 residue where a known polymorphism p.I253V resided. This p.T248 residue was conserved also in one paralogs, *TMPRSS2* (Fig. 3). All of the *TMPRSS3* variants detected in our cohort were also listed in Table 1. We have submitted novel *TMPRSS3* variants to the publicly available LOVD database (https://research.cchmc.org/LOVD2/variants.php?select_db=TMPRSS3&action=view&view=0000810).

Haplotype analyses of p.A306T allele of TMPRSS3 in Koreans

All three DFNB/DFN10 Korean subjects carried p.A306T. Therefore, we investigated if the p.A306T allele in Koreans is a common disease associated haplotype (founder mutation). We analyzed and constructed p.A306T linked haplotype of four STR markers (D21S2092, D21S1225, D21S1411, and D21S1890) (Fig. 4a) in three unrelated probands and 80 normal hearing control subjects. Two of the three p.A306T carriers showed an identical haplotype (Fig. 4b). Meiotic

phase and chromosome in the father of SH51-112 and in some normal hearing controls could not be assigned due to lack of parental DNA samples. Nevertheless, we observed a statistically significant association of p.A306T with a single haplotype by Fisher's exact test in Koreans (p=0.00209 by Fisher's exact test) (Table 2).

Predicted effect of the *TMPRSS3* mutation in the serine protease domain

Previously studied *TMPRSS3* mutation site, p.A306T, is located at the core region very close to the active site (Asp304), which reflects the evidence of severe to profound hearing loss. The other mutation that was previously studied is p.A426T, which was reported to have milder effect than p.A306T [7]. The residue 426 is relatively far from the active site and is located at the peripheral region which is part of a flexible loop. Therefore, it is expected that a mutation of hydrophobic alanine into hydrophilic threonine at this site (p.A426T) has small effect on the entire protein stability.

Interestingly, our novel mutation site at 248 is not part of the protein core, but seems to be important in stabilizing the beta-sheet that directly interacts with the active site (Fig. 5A). The structural analysis revealed that the mutation of threonine to methionine at this site altered the hydrogen bond characteristics between the residue at 248 and the beta-sheet, which could affect the protein functionality by disturbing the protein stability. However, the effect is predicted to be mild, since the mutation site is at the surface region. In addition, based on the fact that the residue at 248 is located farther to the active sites than the residue at 306 but closer than the residue at 426

Table 1 All the *TMPRSS3* variants detected in our cohort

Nucleotide	Protein	Exon	Proband	Reference
Pathogenic				
c.C325T	p.R109W	exon5	SH51-112, SH87-197	[13]
c.C743T	p.T248M	exon8	SH96-210	This study
c.G916A	p.A306T	exon9	SH51-112, SH87-197, SH96-210	[11]
Nonpathogenic				
c.G157A	p.V53I	exon3	SH27-61, SH60-136, SH63-148, SH93-206, SH96-210, SB77-133, SB84-149, SH87-197	rs928302
c.447-13A>G		intron6	SH62-147, SH64-149, SB77-133, SB105-197	rs8130564
c.G453A	p.V151V	exon6	SH63-148, SB77-133, SB84-149, SB105-197	rs2839501
c.617-3TAins		intron8	SH60-136, SH62-147, SH63-148, SH64-149, SH93-206	chr21:43,803,310-43,803,311
				Korean specific SNP
c.A757G	p.I253V	exon8	SH60-136, SH62-147, SH63-148, SH92-205, SH93-206	rs2839500
UTR+2G>A		3′ UTR	SH27-61, SH60-136, SH62-147, SH63-148, SH92-205, SH93-206, SH96-210, SH106-224, SB69-119, SB77-133, SB115-207	rs13047838
Indeterminate				
c.133C>A	p.P45T	exon3	SH62-147	chr21:43,810,108
c.1195-6A>T		intron12	SH62-147	chr21:43,795,983

(Fig. 5B), it is predicted that the overall effect of our novel variant (p.T248M) would be somewhere in between the severe mutation p.A306T and milder variantp.A426T, that is, milder than p.A306T but more severe than A426T.

Yeast-based protease assay

The p.A306T showed a significant defect in protease activity comparable to that of p.D103G and p.R109W (Fig. 6). In contrast, the protease activity of p.T248M at days 3 and 5 corresponded to 92 and 81 %, respectively, of wild type activity, exhibiting only mildly diminished activity (Fig. 6).

Audiologic phenotype

The serial pure tone audiograms of three affected subjects carrying *TMPRSS3* mutations are shown in Fig. 2b. Two probands (SH51-112 and SH96-210) were considered to have a DFNB8 phenotype, whereas SH87-197 manifested a DFNB10 phenotype (Fig. 2). The low-frequency hearing ability of up to or at 1 kHz was preserved in SH96-210 (M/6 year) segregating p.T248M with a predicted milder pathogenicity than the p.A306T. SH51-112 (F/12 years) who manifested DFNB8 had a bilateral progressive hearing loss that aggravated for recent 5–6 years. Finally, she required cochlear implantation (Fig. 2b). Hearing loss of SH87-197 (M/5.3 years) was initially detected before the age of three and became prominent at the age of four. Finally, his hearing ability deteriorated abruptly for the recent years, necessitating cochlear implantation (Fig. 2b). He passed a newborn hearing screening at birth and his language

development, despite hearing loss, was not significantly delayed until the age of four according to their parents' recollection.

Then, we focused upon the auditory phenotype of 22 subjects that showed prominent high-frequency hearing loss so called ski-slope hearing loss. These 22 subjects comprised of 21 postlingual cases and one prelingual case with *TMPRSS3* mutations and downsloping audiogram configuration (Fig. 1). The mean threshold level of 4 kHz was the worst among the six frequencies followed by that of 8 kHz, most significantly involving high frequency. The binaural mean± SD hearing level of low, mid, and high frequencies was 49.7± 4.7, 81.3±4.4, and 89.0±3.4, respectively (Table 3 and Fig. 7). There are no statistically significant differences of hearing level in the low, mid, and high frequencies between subjects with *TMPRSS3* mutation ($n=3$) and those without *TMPRSS3* mutation ($n=19$) ($p>0.05$). However, the high-frequency hearing threshold in subjects with *TMPRSS3* mutations tended to be slightly worse than those without *TMPRSS3* mutations (Fig. 7A). To our interest, the mean speech discrimination score (11.3±8.5 %) of three subjects with *TMPRSS3* mutations was remarkably lower than those of subjects without *TMPRSS3* mutations even though pure tone thresholds of two groups did not differ significantly. The difference in discrimination scores did not reach a statistical significance mainly due to a small sample number of subjects with *TMPRSS3* mutation (Table 3).

Breaking down the three subjects with *TMPRSS3* mutations into two groups (severe versus mild) based upon the predicted pathogenic potential described above, the one (SH96-210) with the predicted mild pathogenic potential
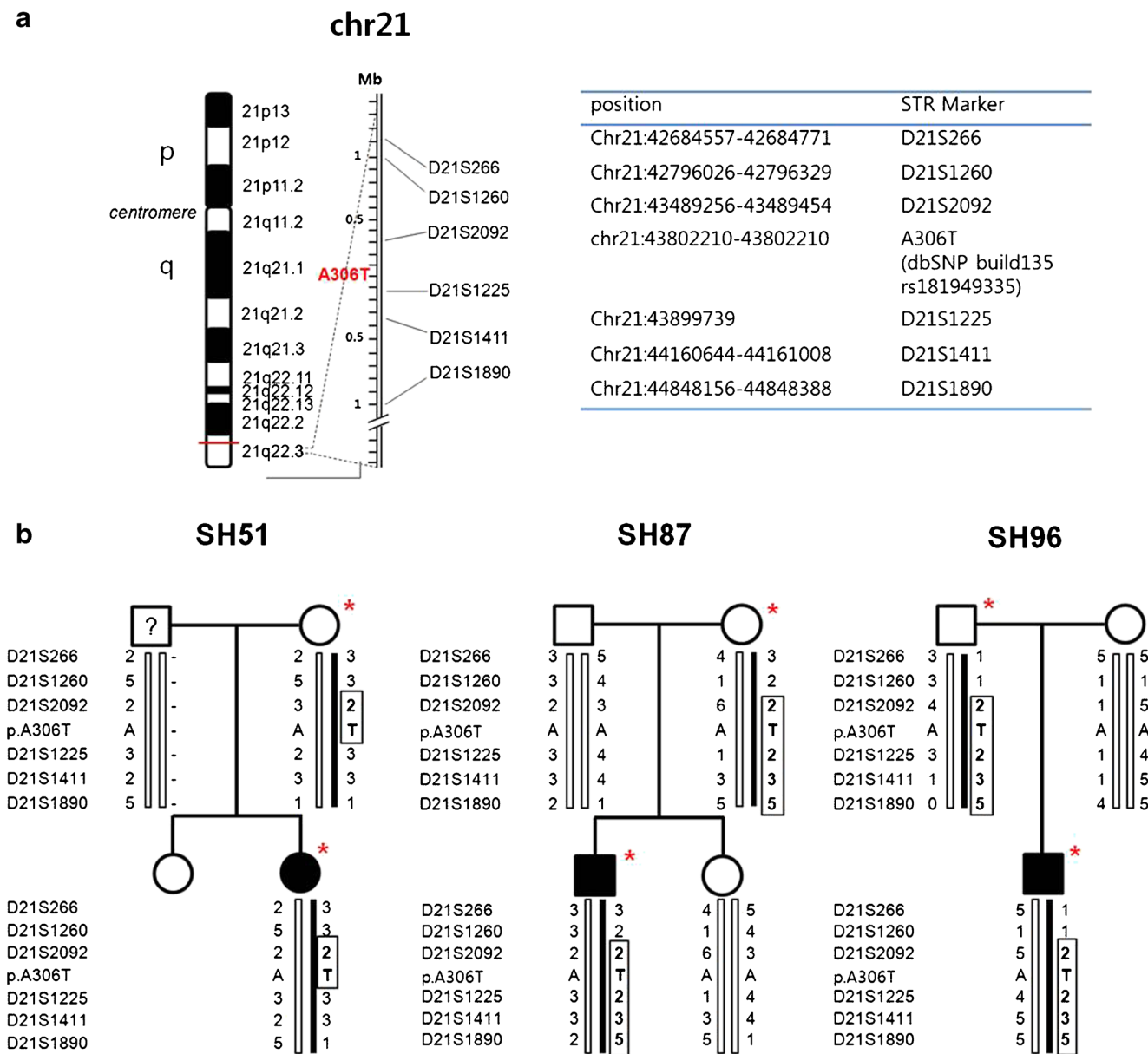
Fig. 4 **a** Physical map and location of p.A306T mutation of *TMPRSS3* and linked STR markers on chromosome 7q. **b** Pedigrees showing segregation of hearing loss and p.A306T mutation. Haplotypes for STR markers linked to the p.A306T (T) are boxed. A haplotype for four STR markers (D21S2092, D21S1225, D21S1411, and D21S1890) is shared by deaf subjects from SH87 and SH96, indicating that this is a common founder allele in Koreans

retained a significant degree of hearing in lower frequencies up to 1 kHz, manifesting abrupt drop of hearing threshold from 1 to 2 kHz (Fig. 7B-a). In contrast, the hearing thresholds of two subjects (SH87-197 and SH51-112) carrying two severe *TMPRSS3* mutations progressed rapidly to the point where they had to undergo conventional cochlear implantation (Fig. 7B-c).

Comparison of CI results between a patient with *TMPRSS3* mutation and normal control group

The mean open set sentence score of patients with *TMPRSS3* (SH51-112, SH87-197) that had been followed up for more than 6 months after CIs was 88.5 %. This result is comparable to the CI results of the age matched control group (n=13) which demonstrated the mean open set sentence score of 85.7 % after 6 months or more of follow-up.

Discussion

The prevalence of 5.9 and 8.3 % of *TMPRSS3* mutations in total arNSHL and postlingual arNSHL patients, respectively, in Koreans clearly indicates that this gene contributes significantly to arNSHL patients in this population. This figure is similar with that in Tunisian [19] and Turkish populations [20]

Table 2 Chromosome 21q22 STR haplotypes linked to p.A306T of *TMPRSS3*

Haplotype	STR marker genotype				Korean chromosome	
	D21S2092	D21S1225	D21S1411	D21S1890	A306T (*n*=3)	Control (*n*=80)
1	194	217	292	167	2[*]	1
2	194	219	292	151	1	0
3	194	217	292	155	0	2
4	194	215	292	163	0	2
5	194	217	292	153	0	2
6	194	217	296	167	0	2
7	194	215	296	167	0	2
8	206	221	292	167	0	2
9	210	217	292	159	0	2
10	192	217	300	167	0	1
11	194	217	296	169	0	1
12	194	221	288	167	0	1
13	194	217	292	167	0	1
14	194	215	292	165	0	1
15	194	217	292	163	0	1
16	194	217	296	165	0	1
17	196	217	292	165	0	1
18	196	217	292	163	0	1
19	198	217	292	163	0	1
20–38	–	–	–	–	0	55

[*] *p*=0.00209 by Fisher's exact test

but higher than the prevalence of 2.5 % from another Korean arNSHL cohort [21]. When we focused upon subjects manifesting postlingual hearing loss with ski-slope audiogram configuration or subjects with prelingual hearing loss but retaining some degree of low-frequency residual hearing and speech development, the prevalence of *TMPRSS3* mutations seemed to reach 11.2 %. In contrast, the contribution of this gene to congenital profound deafness without any language development seemed negligible. Our analyses upon the audiogram configurations of our cohort provide us with more probable situations where a candidate gene approach targeting *TMPRSS3* would be successful. These situations include abrupt decline of hearing threshold by more than 30 dB between 1 and 2 kHz (Fig. 7A, B). *TMPRSS3* sequencing should also be considered when 100 dB or more of high-frequency hearing threshold and relatively low speech discrimination score is observed despite 70 dB or better mean hearing threshold at 250 and 500 Hz (Fig. 7).

The p.A306T mutation that affects a residue in the serine protease domain (Fig. 6) was detected in all three DFNB8/DFNB10 families in this study. Our haplotype analyses of four *TMPRSS3* linked STR markers have revealed that two of the three subjects are of a single mutational origin, suggesting that this p.A306T variant is likely to be a founder mutation as well as being a hotspot mutation in the Korean population (Table 3).

Screening of this p.A306T mutant allele of *TMPRSS3* may be an efficient way to detect DFNB8/10 in Koreans.

It is interesting to notice that this p.A306T allele, a founder mutation in the Korean population, had also been identified in one German family [11] and four families in a Dutch series [6]. This suggests that p.A306T is likely to be a hotspot mutation in many ethnicities.

This p.A306T variant has been classified as a severe mutation in terms of pathogenicity, based upon its close proximity to one of the active residues, Asp304 and also upon the proposed genotype–phenotype correlation [6]. The p.R109W variant detected from SH51-112 and SH87-197 was originally reported in a Pakistani family segregating congenital profound deafness [14]. This variant resides in the LDLRA domain (Fig. 8). Based upon the complete absence of proteolytic activity (0 %) in a yeast-based protease assay as compared with significant retention of the activity (20 %) for the p.A426T variant with a questionable pathogenicity [7], this p.R109W variant can also be classified as 'severe'. To predict the pathogenicity of the novel variant p.T248M from SH96-210, we performed a comparative protein modeling of the *TMPRSS3* serine protease domain and focused specifically on three variants (p.A306T, p.T248M, and p.A426T). p.A306T and p.A426T were included as controls with severe and mild pathogenicity, respectively. Our protein modeling
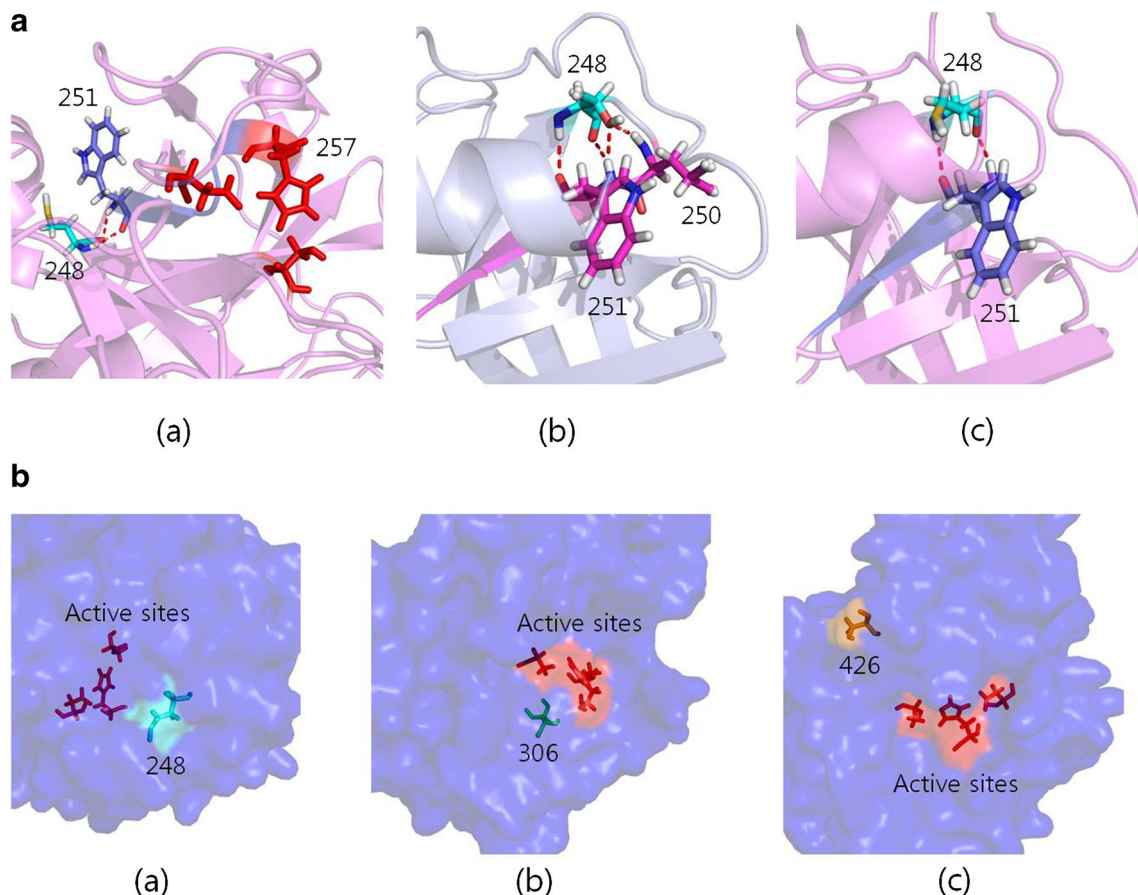
a

(a)   (b)   (c)

b

(a)   (b)   (c)

Fig. 5 Pathogenicity of TMPRSS3 mutations in terms of protein structure. **a** Molecular model comparison between wild type and p.T248M. *(a)* p.T248M—active site 257 *(red)* is influenced by the β-sheet *(blue)* which interacts with mutated residue 248 (cyan). *(b)* Wild type—residue 248 (cyan) has four hydrogen bonds with nearby β-sheet *(magentas)* through residues 250 and 251. *(c)* p.T248M—the number of hydrogen bonds with β-sheet *(blue)* is reduced to two. Only residue 251 has hydrogen bonds with residue 248. **b** Location of active sites *(red)* and residue 248 *(cyan)*, 306 *(green)*, and 426 *(orange)* for each mutation in TMPRSS3 protein. *(a)* p.T248M—active site is present at the opposite surface of the protein which is not visible but physical distance between with active sites is somewhere between p.A306T and p.A426T. *(b)* p.A306T—mutated residue is placed inside the protein near active sites. *(c)* p.A426T—residue 426 is relatively far from the active site and is located at the peripheral region

results strongly suggest that the pathogenic potential of p.T248M would be definitely milder than that of p.A306T and imply that this variant is likely to have a residual proteolytic activity (Fig. 5). In accordance with the 3D modeling prediction, the auditory phenotype of SH96-210 carrying p.T248M in *trans* with p.A306T is significantly milder than the other two subjects (SH51-112 and SH87-197) where two 'severe' mutations of *TMPRSS3* segregate. Intriguingly, the yeast-based protease assay confirmed tight correlation between the proteolytic activities of the *TMPRSS3* variants that we detected from our cohort and the audiologic phenotype. The proteolytic activity of the p.A306T variant was not previously measured, but the change was predicted to directly disturb the function of the serine protease domain, thereby probably causing a 'severe' effect upon the protein function [6]. The proteolytic activity of the p.A306T mutant protein was indeed severely decreased, being nearly comparable to the known null function allele controls, p.D103G and

p.R109W. In contrast, the p.T248M mutant protein demonstrated mildly diminished proteolytic activity. SH96-210 showed significantly better and only subtle aggravation of hearing thresholds in low to mid frequencies. This mild phenotype would probably result from the compound heterozygosity for the mild p.T248M and the severe p.A306T as suggested [6]. This genotype–phenotype correlation is clinically important since the low-frequency hearing status would significantly affect decision upon the method of auditory rehabilitation in these patients. Combined electric and acoustic stimulation (EAS) should be very cautiously considered in cases where there are two "severe" mutations since it is likely that the residual low-frequency hearing would aggravate very rapidly as you can see in the SH51-112. Genetic counseling should be provided accordingly. This possible abrupt aggravation was perfectly recapitulated in the knock-in mouse model (*Tmprss3^{Y260X}*) carrying a severe a homozygous nonsense mutation in *Tmprss3* [22]. In the mouse model carrying

Fig. 6 Protease assays for known pathogenic mutations and the variants of *TMPRSS3* detected in this study. **a** Transformants expressing wild type and each mutant TMPRSS3 are serially diluted and spotted onto nonselective (glucose) and selective (sucrose) plates. The number of colonies in the selective plates is counted at days 3 and 5. **b** Ratios and relative ratios are obtained by dividing the number of colonies on selective plates by the number of colonies on nonselective plates at days 3 and 5. The number of colonies represents proteolytic activity. p.A306T shows significantly reduced protease activity comparable to known null function mutations, p.D103G and p.R109W, while p.T248M exhibits mildly diminished activity
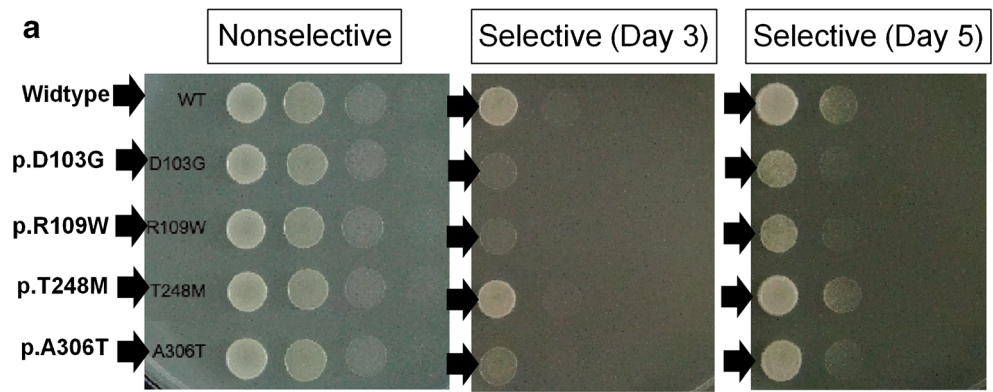
		Day 3		
	Non selective	Selective	Ratio*	Relative Ratio (vs WT**)
Wildtype	158216.877	156706.463	0.9905	1.0000
p.D103G	168198.170	24185.078	0.1438	0.1452
p.R109W	156278.756	20217.543	0.1294	0.1306
p.T248M	154054.756	141368.149	0.9176	0.9265
p.A306T	178263.605	38352.149	0.2151	0.2172
		Day 5		
	Non selective	Selective	Ratio*	Relative Ratio (vs WT**)
Wildtype	158216.877	182597.362	1.1541	1.0000
p.D103G	168198.170	93726.806	0.5572	0.4828
p.R109W	156278.756	84131.149	0.5383	0.4665
p.T248M	154054.756	144587.635	0.9385	0.8132
p.A306T	178263.605	129279.463	0.7252	0.6284

*Ratio: Selective/Nonselective, **WT: Wildtype

homozygous truncation mutations, hair cells in the organ of Corti rapidly degenerated within only 2 days between P12 and P14, a period that corresponds to the onset of hearing in mice [22]. In contrast, possibility of maintenance of a substantial portion of low-frequency hearing even for a couple of decades should be taken into account when deciding the auditory rehabilitation in subjects with at least one mild *TMPRSS3* mutation. Based upon the tight relationship between the

impaired extent of activity and the severity of hearing loss/progression shown in this study, in vitro functional study of unknown *TMPRSS3* variants using the yeast-based protease assay would serve as a good model system to precisely predict the prognosis of hearing status. Combination of two "mild" *TMPRSS3* mutations has never been reported in association with human hearing loss in literature. The phenotype resulting from two "mild" *TMPRSS3* mutations would be very

Table 3 Audiologic evaluation of subjects with high-tone hearing loss and comparisons between subjects with and those without *TMPRSS3* mutations

	High-frequency hearing loss (n=22)	Without *TMPRSS3* mutation (n=19)	With *TMPRSS3* mutation (n=3)	*p* value
Low frequency (250–500 Hz)	49.7±4.7 dB HL	49.9±5.0 dB HL	48.3±14.3 dB HL	0.855
Mid frequency (1–2 Hz)	81.3±4.4 dB HL	79.9±4.8 dB HL	90.8±11.8 dB HL	0.652
High frequency (−8 kHz)	89.0±3.4 dB HL	86.6±3.7 dB HL	104.2±3.8 dB HL	0.122
Speech discrimination score (%)	37.2±7.9 %	41.3±8.7 %	11.3±8.5 %	0.265

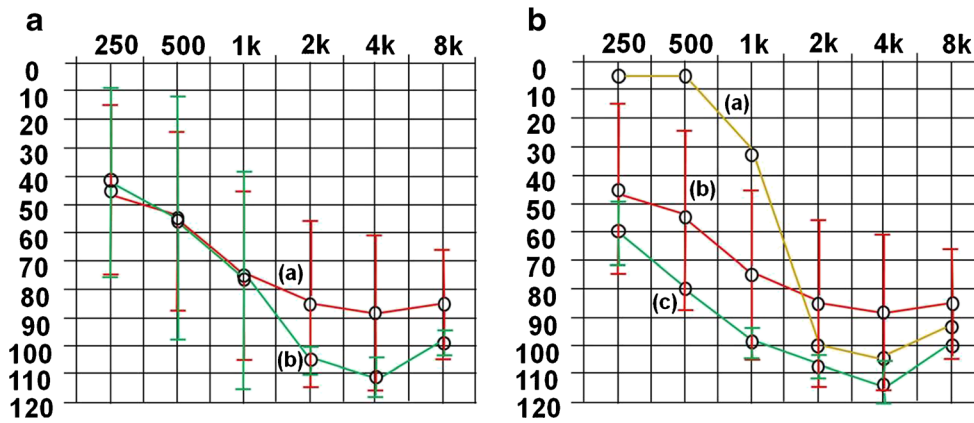Statistical analyses by Wilcoxon rank-sum test

Fig. 7 Audiogram of patients with high tone hearing loss. **a** *(a)* Audiogram of 19 patients with high-frequency hearing loss and no *TMPRSS3* mutation, *(b)* audiogram of three patients with *TMPRSS3* mutation. **b** *(a)* Audiogram of a patient with one mild [c.743C>T(p.Thr248Met)] variant of *TMPRSS3* mutation, *(b)* audiogram of 19 patients with high-frequency hearing loss and no *TMPRSS3* mutation, and *(c)* audiogram of two patients with two severe [c.325C>T(p.Arg109Trp), c.916G>A(p.Ala306Thr)] variants of *TMPRSS3* mutation. *Error bar* indicates ±one standard deviation

subtle or even close to normal or might take a different audiogram configuration. Generation of knock-in mouse model carrying homozygous "mild" mutations would answer this question. Of course, other genetic factors may cause the phenotypic variability from the same mutation, considering the significantly different phenotype of the same mutation p.P404L between Tunisian [19] and Turkish [20]. However, the phenotypic variability from other genetic factors is not likely to be significant due to relatively homogenous genetic background in Korean population.

Tmprss3 has been reported to be expressed in the inner hair cells and supporting cells of the organ of Corti, inner and outer sulcus cells and the cell bodies of the spiral ganglion neurons [8, 22]. Spiral ganglion neuronal loss starting at P90 in the *Tmprss3*[Y260X/Y260X] mouse reported by Fasquelle et al. [22]

implies that human patients carrying *TMPRSS3* mutations may also have a significantly reduced spiral ganglion cell population, thereby showing diminished speech discrimination scores. The spiral ganglion cell populations were reported to be correlated with auditory thresholds and with speech discrimination scores in previous cadaveric studies [23]. Their observation was revisited by our finding that the mean speech discrimination score of three subjects with *TMPRSS3* mutations was remarkably lower than those of subjects without *TMPRSS3* mutations even though pure tone thresholds of two groups did not differ significantly. This may account for the poor outcomes after two cochlear implantation cases with *TMPRSS3* mutations reported by Eppsteiner et al. [24]. However, there was no difference of speech perception after cochlear implantation between subjects with *TMPRSS3*
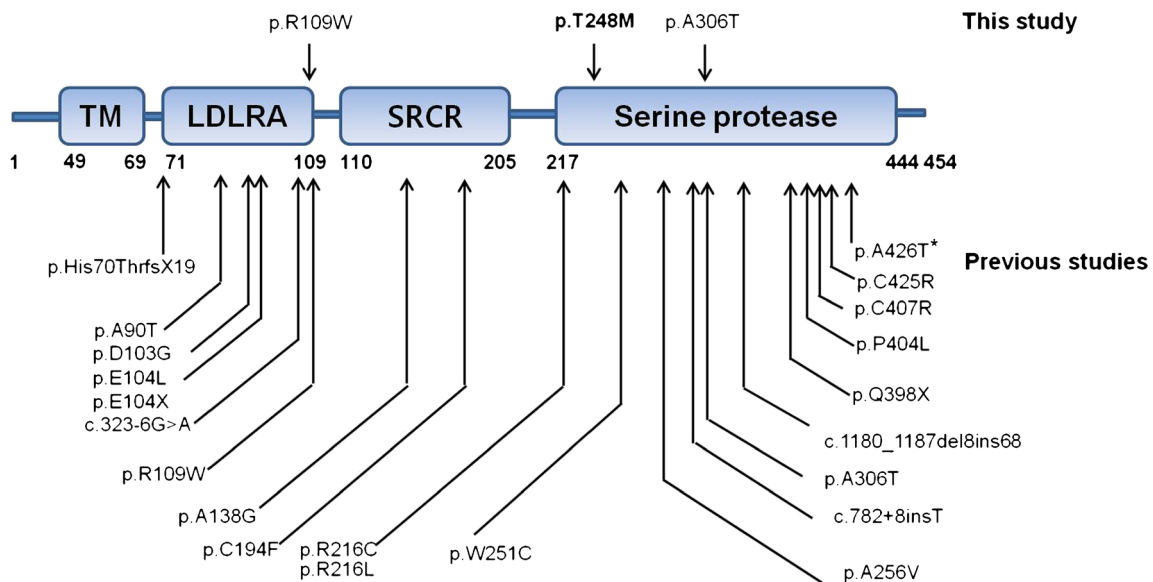

Fig. 8 Schematic representation of *TMPRSS3* mutations identified in this study and previous studies

mutations and the age-matched control subjects in our study. Our finding, together with previously reported satisfactory results after CI [6, 11], suggests that decrease in the spiral ganglion cell population in DFNA8/10 is significant enough to cause deterioration of speech discrimination score but usually not to the extent where postCI outcomes are adversely affected. Therefore, CI appears to be a nice option for auditory rehabilitation in many DFNA8/10 subjects despite expression of this gene in spiral ganglion neurons and significant reduction of the cell population in *Tmprss3* deficient mice.

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