Hum Genet (2005) 117: 528–535
DOI 10.1007/s00439-005-1332-x

ORIGINAL INVESTIGATION

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A novel TMPRSS3 missense mutation in a DFNB8/10 family prevents proteolytic activation of the protein

Received: 3 December 2004 / Accepted: 19 April 2005 / Published online: 14 July 2005

Abstract Pathogenic mutations in *TMPRSS3*, which encodes a transmembrane serine protease, cause non-syndromic deafness DFNB8/10. Missense mutations map in the low density-lipoprotein receptor A (LDLRA), scavenger-receptor cysteine-rich (SRCR), and protease domains of the protein, indicating that all domains are important for its function. TMPRSS3 undergoes proteolytic cleavage and activates the ENaC sodium channel in a *Xenopus* oocyte model system. To assess the importance of this gene in non-syndromic childhood or congenital deafness in Turkey, we screened for mutations affected members of 25 unrelated Turkish families. The three families with the highest LOD score for linkage to chromosome 21q22.3 were shown to harbor P404L, R216L, or Q398X mutations, suggesting that mutations in *TMPRSS3* are a considerable contributor to non-syndromic deafness in the Turkish population. The mutant TMPRSS3 harboring the novel R216L missense mutation within the predicted cleavage site of the protein fails to undergo proteolytic cleavage and is unable to activate ENaC, thus providing evidence that pre-cleavage of TMPRSS3 is mandatory for normal function.

Introduction

Congenital hearing loss is the most common sensory defect in humans, with an incidence of about one in 1000 births. One additional child in 1000 becomes deaf before adulthood. Hearing loss is classified as syndromic if

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deafness is associated with other manifestations, or as non-syndromic if deafness is the isolated phenotype. Approximately half of childhood deafness cases have a genetic origin (reviewed in Petit 1996). Pathogenic mutations in 38 different genes have already been described for non-syndromic deafness and 43 other loci have been described (http://www.uia.ac.be/dnalab/hhh/). Despite this extensive heterogeneity, mutations involving a single locus, DFNB1, account for most cases of genetic non-syndromic deafness in most populations (Nance and Kearsey 2004). DFNB1 corresponds to mutations in *GJB2* encoding connexin26. We have described the genetic cause of the recessive non-syndromic deafness DFNB8/10 that map on chromosome 21 (Bonne-Tamir et al. 1996; Veske et al. 1996), which is due to pathogenic mutations in the *TMPRSS3* gene (Scott et al. 2001). *TMPRSS3* encodes a type II transmembrane serine protease (TTSP) expressed in fetal cochlea. It can activate the sodium channel ENaC and has been shown to undergo proteolytic cleavage (Guipponi et al. 2002). We and others have identified ten different *TMPRSS3* mutations in deaf patients. The missense mutations affect all functional domains of the protein (Ben-Yosef et al. 2001; Masmoudi et al. 2001; Scott et al. 2001; Wattenhofer et al. 2002; Ahmed et al. 2004).

In the present study, we report the analysis of 25 Turkish families with non-syndromic autosomal recessive childhood deafness without mutations in *GJB2*. We found pathogenic mutations in *TMPRSS3* in three families, indicating that mutations in this gene are a significant cause of childhood non-syndromic deafness in the Turkish population (12% among the families negative for *GJB2* mutations). Two of the three families harbor novel mutations. Interestingly, one of the novel mutations identified in this study maps in the predicted TMPRSS3 cleavage site. We show that this mutant protein does not undergo proteolytic cleavage and fails to activate ENaC.

Materials and methods

Patients

Twenty-five Turkish families from Trabzon, Rize, and Ordu, which segregated either congenital or childhood deafness, with at least two affected members, were included in this study. A high frequency of consanguinity (77% of the families) suggests an autosomal recessive mode of inheritance in the majority of the families. There was no evidence for an autosomal dominant or X-linked mode of inheritance, or any obvious syndrome. Audiometric results of unaffected sibs were normal. Affected family members showed severe to profound sensorineural childhood non-syndromic deafness, with both males and females being affected. No information was available regarding walking age. Informed consent was obtained for all participating family members. Peripheral blood was obtained from members of all 25 families, and genomic DNA was isolated from blood lymphocytes (Grimberg et al. 1989). The Tunisian patients S17, S19, and S42 are members of family S, previously described (Masmoudi et al. 2001).

Audiometric ISO values for patients with a *TMPRSS3* mutation can be found in Table 2.

Genotyping, linkage analysis, and mutation search

PCR amplification of polymorphic microsatellite markers *D21S212* and *D21S1225* was carried out in 12.5 µl with 100 ng genomic DNA and 2 pmol of each primer. Amplification products were run on a 6% (19:1, acrylamide:bisacrylamide) denaturing polyacrylamide gel. Haplotypes were reconstructed for all persons in the 25 pedigrees by using chromosome 21 markers *D21S212* and *D21S1225* adjacent to *TMPRSS3* on 21q22.3. Linkage analyses were performed using the MLINK software. The disease was modeled as an autosomal recessive trait with complete penetrance at birth. Two-point LOD-scores (Z) in these families were calculated. Inbreeding loops were preserved in the pedigrees. Haplotype analysis of polymorphic markers adjacent to the *TMPRSS3* gene on chromosome 21q22.3 on DNA of Turkish patients 53-1, 53-2, and 53-3 and on the Tunisian patients S17, S19, and S42 was performed as previously described (Berry et al. 2000). All 12 coding exons of *TMPRSS3* and their splice junctions were PCR amplified as described elsewhere (Wattenhofer et al. 2002) and sequenced.

Functional analysis of TMPRSS3 missense mutations in *Xenopus* oocytes

The construction of plasmids pSD5TMPRSS3 Wt and pSD5TMPRSS3 P404L is described elsewhere (Guipponi et al. 2002). Mutant R216L was generated using the QuickChange mutagenesis system following the manufacturer's instructions (Stratagene). This construct was verified by sequencing. Transcription was performed on 1 µg vector linearized with *ScaI*, using 0.5 IU/µl SP6 RNA polymerase (Promega) in a reaction mix of: 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 1.5 mM each ATP, CTP, UTP, 0.3 mM GTP, 0.5 mM GpppG, 10 mM DTT, 1 IU/µl RNAsin, and 8 ng/µl BSA, 1 h at 40°C. Template was removed with RNAse-free DNAse I, and the cRNA purified on RNeasy mini spin-columns (Quiagen). Oocytes in stage V/VI from *Xenopus laevis* (Noerdhoek, South Africa) were injected with 0.25 ng of each cRNA coding for the rat α-, β-, and γ-ENaC subunits in the presence or absence of 2.5 ng of wild type or mutant TMPRSS3 cRNA in a total volume of 100 nl. Oocytes were incubated in modified Barth saline (MBS) solution, and 24 h after injection, electrophysiological measurements were performed. The amiloride-sensitive current (I_{Na}) was measured by two-electrode voltage clamp in the presence of 120 mM Na^+ in Frog Ringer with 5 µM amiloride at a holding potential of −100 mV. Three series of experi-

ments were performed, each with four to eight oocytes per condition. In each series, the individual current values measured were normalized to the average of the ENaC + H_2O values of that series. The results are reported as the means of all normalized values for each condition, ± SEM. Kruskal–Wallis test followed by a Dunn's multiple comparison post-test were performed to determine significance. The means of absolute I_{Na} values for ENaC + H_2O in the three series were 337 ± 50.6, 446 ± 46.4, and 4533 ± 980.8 nA. These values for ENaC + TMPRSS3 wild type were 4386 ± 1642, 2117 ± 717.4, and 13,517 ± 2414 nA.

Western blot analysis

Injected *Xenopus* oocytes were incubated in MBS for 24 h. Microsomal membrane protein extracts were obtained from oocytes lysed as previously described (Geering et al. 1989). Protein extract corresponding to 25% of the total protein content of a single oocyte was loaded in each lane. Proteins were separated on a 10% SDS–polyacrylamide gel under reducing and denaturing conditions and transferred to a nitrocellulose membrane (Protran from Schleicher & Schuell). The membrane was processed using rabbit anti-TMPRSS3 serum (Covalab, dilution 1/1000) and anti-rabbit IgG horseradish peroxidase linked antibody (Amersham Biosciences, dilution 1/10,000) according to standard procedures.

Results

To evaluate the importance of mutations in *TMPRSS3* in childhood non-syndromic deafness in Turkey, we collected pedigrees with at least two affected individuals. We have ascertained 25 Turkish families comprising members likely to be affected by non-syndromic childhood hearing impairment. Affected and unaffected members of each family were examined by clinical evaluation and pure tone audiometry. The pedigrees were compatible with congenital/childhood onset, autosomal recessive, severe to profound sensorineural deafness. No additional phenotypes were observed.

The segregation of *D21S212* and *D21S1225*, two highly polymorphic microsatellite markers flanking *TMPRSS3*, was studied for linkage with the phenotype. We reconstructed the haplotypes of all available members of the 25 families. No recombinants were found between the two tested markers and the deafness phenotype in six of the families (Table 1). At $\theta = 0$, the LOD score at *D21S212*, which is 0.5 Mb centromeric to *TMPRSS3*, was between 0.125 and 1.646 in the six families with potential linkage to *TMPRSS3*. The DNA from one affected member of these six families was used for mutation analysis. We identified *TMPRSS3* pathogenic mutations in three patients and confirmed cosegregation of the mutation and the phenotype in these families by sequencing DNA of other family members. Consistently, linkage analysis in these families (families 40, 53, and 88) showed the highest LOD score (Table 1).

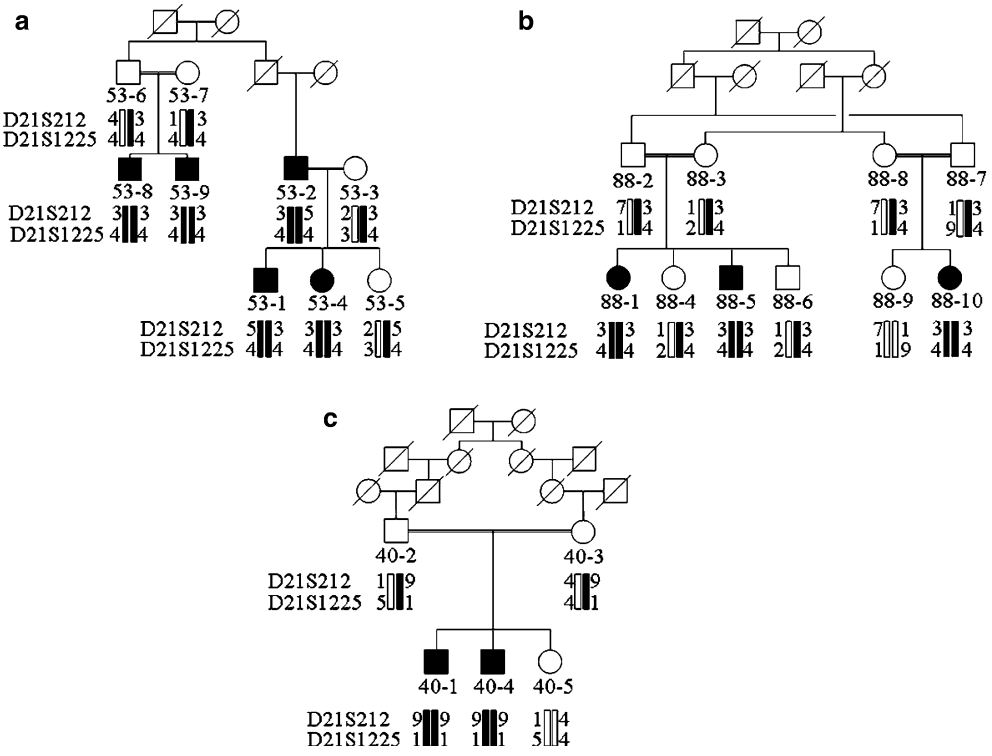
In family 53 (Fig. 1a), the proband 53-1 is homozygous for the transition c.1211C → T, a mutation previously identified in a Tunisian family that substitutes P404 by Leu (Masmoudi et al. 2001). The deaf father (53-2) and deaf father's cousin (53-9) are also homozygous for this transition, whereas the hearing mother (53-3) is heterozygous. All affected members of family 53 were hearing until the age of 6 or 7 years, whereas in the Tunisian family, deafness was congenital (Table 2). To assess if the P404L mutation in both families has a single origin, we analyzed microsatellite markers around the *TMPRSS3* locus in both families. The haplotypes in the two families are different at both the 5′ and 3′ of the mutation favoring a different origin hypothesis (Fig. 2).

In family 88 (Fig. 1b), patient 88-1 is homozygous for the transition c.1192C → T. This newly identified nucleotide change leads to a Q398X nonsense codon. In addition, patient 88-1 is heterozygous for the c.1367G → A SNP, excluding a large deletion as the second mutant allele in 88-1. In this family, the deafness phenotype is congenital for all affected members.

We identified another novel mutation in family 40 (Fig. 1c), a transversion c.647G → T. This nucleotide

Table 1 Linkage analysis in families with potential linkage of hearing impairment to the *TMPRSS3* locus

Family	Marker	*Z* at θ					
		0.00	0.05	0.1	0.2	0.3	0.4
40	D21S212	0.727	0.639	0.549	0.367	0.193	0.055
	D21S1225	0.727	0.639	0.549	0.367	0.193	0.055
50	D21S212	0.125	0.086	0.056	0.018	0.004	0.000
	D21S1225	0.125	0.086	0.056	0.018	0.004	0.000
53	D21S212	1.646	1.439	1.228	0.803	0.409	0.112
	D21S1225	0.857	0.744	0.630	0.408	0.208	0.057
69	D21S212	0.301	0.258	0.215	0.134	0.064	0.017
	D21S1225	0.301	0.258	0.215	0.134	0.064	0.017
70	D21S212	0.301	0.258	0.215	0.134	0.064	0.017
	D21S1225	0.602	0.515	0.430	0.267	0.129	0.034
88	D21S212	1.492	1.298	1.099	0.696	0.329	0.080
	D21S1225	1.492	1.298	1.101	0.705	0.344	0.088



substitution modifies an arginine-to-leucine codon at position 216 (R216L). The proband (40-1) and his deaf brother (40-4) are homozygous for this mutation, while both parents (40-2 and 40-3) are heterozygous. The hearing sister (40-5) is homozygous for the normal allele. Amino acid residue R216 is well conserved among serine proteases and is usually R or K (Fig. 3). This substitution was not observed in 10 and 383 Turkish and European Mediterranean unrelated individuals, respectively, either by SSCP or sequencing (Wattenhofer et al. 2002). These results are concordant with c.647G → T being a recessive pathogenic mutation. Deafness in patient 40-1 was detected when he was 1.5 years old because of speech delay. In contrast, deafness in his younger brother, 40-4, was diagnosed at birth. Because patient 40-1 was the first case of a deafness-affected child in this family and his parents were thus not aware of a deafness possibility, we cannot exclude the hypothesis that 40-1 was indeed deaf at birth.

In order to characterize more precisely the R216L mutant, we functionally characterized the predicted encoded protein. It has been suggested that TMPRSS3 is cleaved at the RIVGG zymogen activation site, between amino acids R216 and I217 (Guipponi et al. 2002). This sequence is well conserved among serine proteases (Fig. 3). Hence, we postulated that the transversion

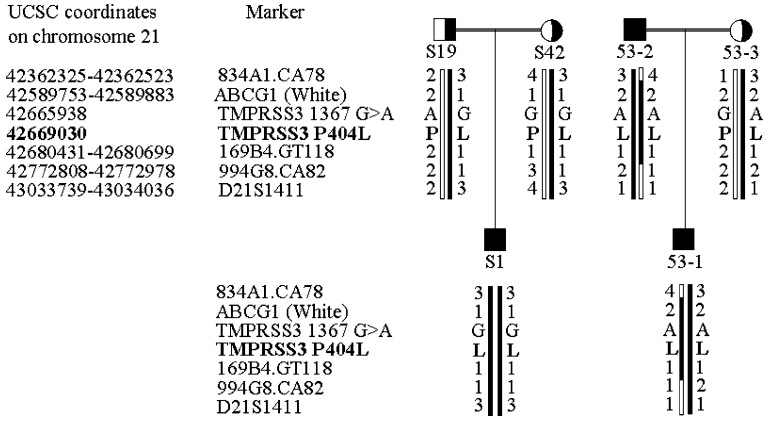


Fig. 1 Pedigrees of the three families harboring mutations in TMPRSS3. *Black symbols* represent deaf patients whereas *white symbols* represent hearing individuals. The haplotypes of each tested individual is indicated. *Black bars* indicate the haplotypes linked to the deafness phenotype. **a** Family 53. **b** Family 88. **c** Family 40

Fig. 2 Haplotype analysis of polymorphic markers on 21q22.3 in Tunisian family S (*left*) and Turkish family 53 (*right*). *Complete black symbols* represent deaf patients, whereas *complete white* or *black and white* symbols represent hearing individuals, homozygous or heterozygous at the *TMPRSS3* locus, respectively. *Black bars* indicate the haplotypes linked to the deafness phenotype. At the *left*, position of the polymorphic marker in the UCSC Genome Browser (http://genome.ucsc.edu/) is indicated (assembly of May 2004)

Table 2 Clinical phenotype of patients with a *TMPRSS3* mutation. Audiometric ISO values are average of hearing threshold values obtained for four different pure tone frequencies (500, 1000, 2000, and 4000 Hz) on the better hearing ear

Family	Patient	Audiometric ISO value (dB)	Age at deafness detection (years)
40	40-1	91	1.5
	40-4	87	Birth
53	53-1	94	6
	53-2	99	6–7
	53-4	85	6–7
	53-8	92	6–7
	53-9	86	6–7
88	88-1	98	Birth
	88-5	87	Birth
	88-10	96	Birth

present in family 40 would result in a TMPRSS3 mutant protein that cannot be cleaved. To test this assumption, we expressed both wild type and mutant TMPRSS3 in *Xenopus* oocytes, isolated the membrane fraction, and performed a Western blot using anti-TMPRSS3 serum (Guipponi et al. 2002). The P404L mutant was previously shown not to undergo proteolytic cleavage (Guipponi et al. 2002). In contrast to the wild type TMPRSS3, and similarly to the P404L mutant, the R216L variant fails to undergo proteolytic cleavage (Fig. 4a). To investigate whether this non-cleaved zymogen was functionally active, we tested its capacity to activate ENaC. As expected (Guipponi et al. 2002), co-expression of the human wild type TMPRSS3 and rat ENaC subunits led to an increase in ENaC mediated current (I_{Na}) compared to oocytes expressing only the ENaC subunits ($P < 0.01$) (Fig. 4b, lane 2 versus lane 1). In contrast, and similarly to the P404L mutation, the R216L mutant was totally inactive (Fig. 4b). Thus, we can conclude that the R216L missense mutation impairs TMPRSS3 proteolytic activation, thereby resulting in an inactive protein.

Discussion

TMPRSS3 encodes a serine protease with LDLRA and SRCR domains and is the cause of deafness in DFNB8/10 families (Ben-Yosef et al. 2001; Masmoudi et al. 2001; Scott et al. 2001; Wattenhofer et al. 2002). Mutations identified so far map in all TMPRSS3 domains (reviewed in Wattenhofer et al. 2002), and all three domains have been shown to be important for the ability of TMPRSS3 to activate ENaC and for its catalytic activity (Guipponi et al. 2002; Lee et al. 2003). ENaC is a sodium channel known to be regulated by serine protease activity (Vallet et al. 1997). It is expressed in various tissues, including the inner ear, where it has been proposed to be involved in maintaining a low Na^+ concentration in the endolymph (Couloigner et al. 2001). By RT-PCR, we observed co-expression of *TMPRSS3* and the three *ENaC* genes both in the stria vascularis and in the modiolus of P5 rats. In addition, in a *Xenopus* oocyte model, TMPRSS3 wild type, but not all mutants identified in DFNB8/10, is able to activate ENaC (Guipponi et al. 2002). Based on these results, we have proposed ENaC as a potential substrate for TMPRSS3 in the inner ear (Guipponi et al. 2002). Here, we report the characterization of a new deafness-causing mutation, R216L, which fails to activate the ENaC channel although harboring non-mutated LDLRA, SRCR, and serine protease domains. In addition, R216L does not undergo proteolytic cleavage. We postulated that TMPRSS3 requires a proteolytic cleavage in order to become active (Guipponi et al. 2002). Similar to other serine proteases, the predicted cleavage site is between

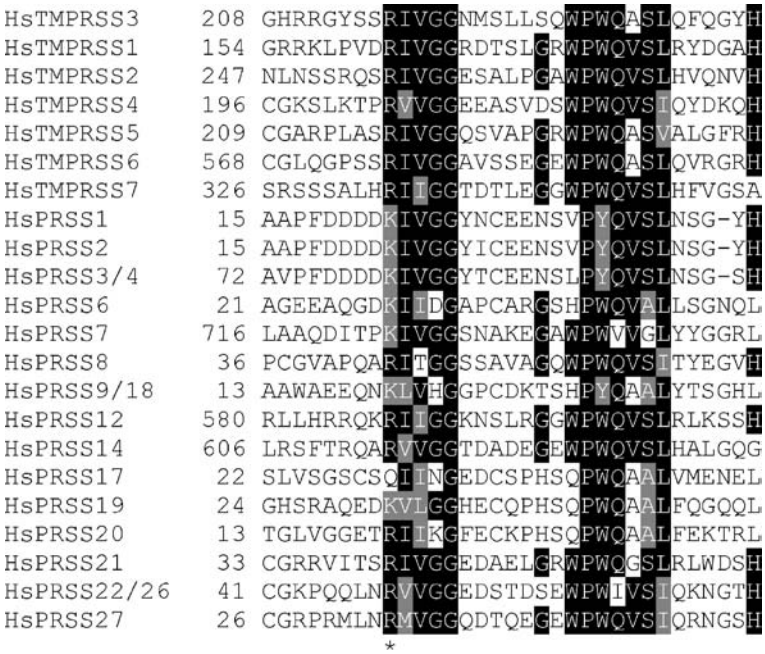


Fig. 3 Partial alignment of selected human serine proteases around the consensus cleavage site. An *asterisk* denotes the position corresponding to the TMPRSS3 R216 residue

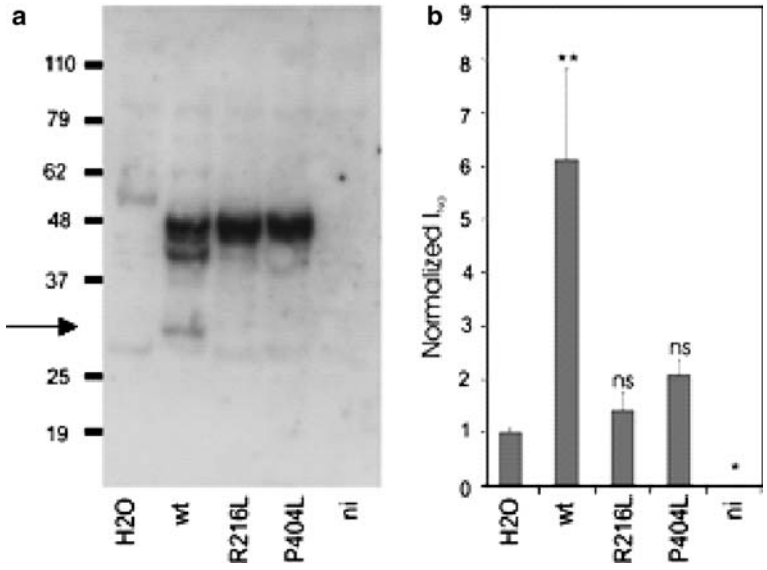


Fig. 4 Functional analysis of the R216L mutant in *Xenopus* oocytes. Oocytes were injected with rat ENaC subunits in the presence of water (H_2O, lane 1), TMPRSS3 wild type (*wt*, lane 2), or missense mutations (R216L, lane 3 and P404L, lane 4). *Ni* non-injected oocytes. **a** Biochemical analysis of wild type and mutant TMPRSS3 by Western blot. Proteolytic cleavage of TMPRSS3 is partial. Both mutants R216L and P404L proteins did not show the cleaved product indicated by an *arrow*. **b** Comparison of the effect of Wt-TMPRSS3 and mutants on I_{Na} (sodium current) in *Xenopus* oocytes. $*P < 0.05$ versus lane 1; $**P < 0.01$ versus lane 1; *ns* non-significant versus lane 1. $n = 15$–18 for each condition, performed in a total of three series of experiments

R216 and I217 in the context of the R/K-I-V-G-G consensus sequence. The engineered mutant S401A of TMPRSS3 catalytic site, which is unable to undergo proteolytic cleavage (Guipponi et al. 2002), showed that this cleavage is dependent on TMPRSS3 catalytic capacity. This suggests that TMPRSS3 cleavage occurs as the result of autocatalytic cleavage. Similar results have been obtained for TMPRSS2: Western blot on cells expressing a TMPRSS2 protein harboring a mutation of the catalytic serine S441 to alanine showed that the proteolytic cleavage is dependent on catalytic activity (Afar et al. 2001). The non-cleavage of the R216L mutant protein is consistent with our hypothesis of TMPRSS3 as the protease responsible for this cleavage.

Pathogenic mutations in the R/K-IVGG consensus have already been identified in several serine proteases, including factor VII (Chaing et al. 1994; Wulff and Herrmann 2000) and factor IX (Sommer et al. 1992), both implicated in coagulation, and protein C (Grundy et al. 1989). The mutation can affect any amino acid of the consensus site and in all cases impairs the activating cleavage. In contrast, the PRSS1 (*pro*tea *se* *s*erine 1) pathogenic mutation N21I, which maps six amino acids after the cleavage site, results in an increased propensity to autoactivation, thus causing pancreatitis (Sahin-Toth 2000; Sahin-Toth and Toth 2000). All these observations underscore the biological importance of activation cleavage. Zymogen protease activation by proteolytic cleavage is a regulatory mechanism to prevent undesired proteolysis. The current accepted view is that all proteases are synthesized as inactive zymogens. In the majority of the cases, catalytic cleavage of the zymogen induces a conformational change of the protease domain, allowing the formation of the active site and thus an active protease (Lazure 2002). By analogy with other proteases (Hedstrom 2002), we can surmise that in the case of TMPRSS3, I217 would form a salt bridge with R400, allowing the formation of the active site and the oxyanion hole. The identification and characterization of the R216L mutation demonstrates the importance of the activation cleavage for TMPRSS3 function and emphasizes the currently accepted view of activation sequences as major players in protease function. The level of TMPRSS3 R216L mutant in our *Xenopus* model system does not seem lower than TMPRSS3 wild type. Thus, our data do not argue in favor of an obvious altered turnover of the mutant protein compared with the wild type. The possibility that decreased ENaC activation activity is due to increased degradation by the ER protein quality control system in collaboration with the proteosome is thus unlikely. Nevertheless, we cannot exclude the possibility that the R216L mutation induces a drastic conformational change thus masking the cleavage activation site.

In the frame of this study we identified the c.1221C → T transition that leads to the P404L missense, previously identified in a Tunisian family (Masmoudi et al. 2001). Although the Turkish and the Tunisian families harbor the same mutation, the deafness phenotype is different: in the Tunisian family deafness was congenital (Masmoudi et al. 2001), whereas in the Turkish family the onset of deafness was at age 6–7 years. Consistently, the haplotypes of polymorphic sites located in a region of 500 kb encompassing the *TMRSS3* mutation are different in the two families, indicating an independent origin of the mutation

(Fig. 4). We postulate that besides the c.1221C → T mutation, other genetic factors influence the severity of the disease, leading to the phenotypic variability. A similar situation has already been observed in the case of sickle cell disease (Wood et al. 1980; Kulozik et al. 1987; Serjeant 1989). Phenotypic variability among patients harboring the same *CFTR* mutation has also been observed in families with cystic fibrosis (Mekus et al. 2000). It becomes evident that phenotypic variability of monogenic disorders is modified by complex genetic and environmental factors. The hearing phenotype associated with the TMPRSS3 P404L mutation is consistent with the proposed idea that the current classification of disorders into monogenic vs multifactorial diseases is an oversimplification (Scriver and Waters 1999).

A significant part of recessive non-syndromic deafness can be attributed to mutations in the Connexin26 (*GJB2*) gene. However, this contribution varies between populations. Forty-two percent of Americans (Green et al. 1999), 42–46% of European Mediterraneans (Estivill et al. 1998; Rabionet et al. 2000; Pampanos et al. 2002), 32% of Turks (Uyguner et al. 2003), 27% of Japanese (Fuse et al. 1999), and 8% of Koreans (Park et al. 2000) affected by non-syndromic recessive deafness show *GJB2* mutations, raising the possibility that other gene(s) may contribute significantly to childhood non-syndromic recessive deafness in the Turkish, Japanese, and Korean populations. We collected 25 unrelated Turkish families in schools for deaf. As we studied non-syndromic deafness, we can consider that having collected only families with children in deaf schools did not include a bias in the enrolling procedure. Thus, the 25 families we collected can be considered as a representative sample of childhood non-syndromic onset deafness in Turkey. In this manuscript, we present evidence suggesting that in the Turkish population studied, *TMPRSS3* mutations significantly contribute to non-syndromic recessive deafness. Hence, *TMPRSS3* appears to be an important locus, in addition to *GJB2*, involved in a substantial fraction of non-syndromic deafness cases in a defined population. In the Turkish population, we observe a prevalence of 11% of *TMPRSS3* mutations among patients with childhood hearing loss and negative for *GJB2* mutations, which enables us to estimate that this locus plays a role in about 8% of the total childhood deaf Turkish population. Since we did not identify a recurrent mutation, we can consider that there is no obvious founder effect. Similar to *GJB2*, the contribution of *TMPRSS3* differs in different populations. Previously reported *TMPRSS3* mutations have been identified in one Palestinian, five Pakistani, and two Tunisian families, as well as in two sporadic Caucasian patients from Spain and Greece (Ben-Yosef et al. 2001; Masmoudi et al. 2001; Scott et al. 2001; Wattenhofer et al. 2002). All these analyses reported very low percentages of *TMPRSS3* mutations in the studied deaf populations, i.e. 2.5% (4/159) in Pakistani, 0.4% (2/448) in European Mediterranean, and even 0% (0/64) in North American populations.

Acknowledgements We thank the families and the administrative bodies of the schools for the deaf for their kind participation in this study. We also thank N. Akarsu, J.-L. Blouin, I. Bouchardy, C. orel, Ö. Refik Çaylan, L. Excoffier, M. Friedli, M. Gagnebin, J.-D. Horisberger, E. Kalay, N. Lin-Marq, M. Morris, L. Pignat, C. Rossier and J.D. Vassali for critical comments, helpful discussions, or technical assistance. This work was supported by grants from the Swiss National Science Foundation (31.57149.99), the European Union (QLRT-2001-00816), the NCCR frontiers in Genetics, the Jérôme Lejeune Foundation, the Child Care Foundation to AR and SEA, from the Swiss National Science Foundation (31-061966.00) and the Danish Research Council (22-05-0535) to BR and DA. BB was supported by the Geneva Research Programme for Medical Students (PREM). This study was supported by the Karadeniz Technical University Research Fund (Project no: 2002.114.001.3), Stichting Irene Kinderziekenhuis and Stichting Vrienden van Effatha.

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