15 **Isasi CR**, Shea S, Deckelbaum RJ, Couch SC, Starc TJ, Otvos JD, Berglund L. Apolipoprotein ε2 is associated with an anti-atherogenic lipoprotein profile in children: the Columbia University Biomarkers Study. *Pediatrics* 2000;**106**:568-75.
16 **Garcés C**, Benavente M, Ortega H, Rubio R, Lasuncion MA, Rodriguez Artalejo F, Fernandez Pardo J, De Oya M. Influence of birth weight on the apo E genetic determinants of plasma lipid levels in children. *Pediatr Res* 2002;**52**:873-8.
17 **Infante-Rivard C**, Rivard GE, Yotov WV, Génin E, Guiguet M, Weinberg C, Gauthier R, Feoli-Fonseca JC. Absence of association of thrombophilia polymorphisms with intrauterine growth restriction. *N Engl J Med* 2002;**347**:19-25.
18 **Arbuckle TE**, Wilkins R, Sherman GJ. Birth weight percentiles by gestational age in Canada. *Obstet Gynecol* 1993;**81**:39-48.
19 **Spielman RS**, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993;**52**:506-16.
20 **Hixson JE**, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha I*. *J Lipid Res* 1990;**31**:545-8.
21 **Zietkiewicz E**, Yotova V, Jarnik M, Korab-Laskowska M, Kidd KK, Modiano D, Scozzari R, Stoneking M, Tishkoff S, Batzer M, Labuda D. Nuclear DNA diversity in worldwide distributed human populations. *Gene* 1997;**205**:161-71.
22 **Laird N**, Horvath S, Xu X. Implementing a unified approach to family based tests of association. *Genetic Epidemiol* 2000;**19**(suppl 1):S36-42.
23 **Rabinowitz D**, Laird N. A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered* 2000;**50**:211-3.
24 **Cleves M**. sg74: symmetry and marginal homogeneity test/transmission disequilibrium test. *Stata Technical Bulletin* 1997;**40**:23-7.
25 **Waldman ID**, Robinson BF, Rowe DC. A logistic regression based extension of the TDT for continuous and categorical traits. *Ann Hum Genet* 1999;**63**:329-40.
26 **Schaid DJ**, Sommer SS. Genotype relative risks: methods design and analysis of candidate-gene association studies. *Am J Hum Genet* 1993;**53**:1114-26.
27 **Schaid DJ**, Sommer SS. Comparison of statistics for candidate-gene association studies using cases and parents. *Am J Hum Genet* 1994;**55**:402-9.
28 **Schaid DJ**, Rowland CM. Genetic ASSOCiation analysis software for cases and parent. Version 1.06, 2001. http://www.mayo.edu/statgen.
29 **Wilson PW**, Schaefer EJ, Larson MG, Ordovás JM. Apolipoprotein E alleles and risk of coronary disease. A meta-analysis. *Arterioscler Thromb Vasc Biol* 1996;**16**:1250-5.
30 **Eichner JE**, Dunn ST, Perveen G, Thompson DM, Stewart KE, Stroehla BC. Apolipoprotein E polymorphism and cardiovascular disease: a HuGe review. *Am J Epidemiol* 2002;**155**:487-95.
31 **Hixson JE**. Pathobiological determinants of atherosclerosis in youth (PDAY) research group. Apolipoprotein E polymorphisms affect atherosclerosis in young males. *Arterioscler Thromb* 1991;**11**:1237-44.
32 **Davignon J**, Gregg RE, Sing CF. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis* 1988;**8**:1-21.
33 **Louhija J**, Miettinen HE, Kontula K, Tikkanen MJ, Miettinen TA, Tilvis RS. Aging and genetic variation of plasma apolipoproteins: relative loss of the apolipoprotein e4 phenotype in centenarians. *Arterioscler Thromb* 1994;**14**:1084-9.
34 **Schächter F**, Faure-Delanef L, Guénot F, Rouger H, Froguel P, Lesueur-Ginot L, Cohen D. Genetic associations with human longevity at the APOE and ACE loci. *Nat Genet* 1994;**6**:29-32.
35 **Araki S**, Moczulski DK, Hanna L, Scott LJ, Warram JH, Krolewski AS. APOE polymorphisms and the development of diabetic nephropathy in type 1 diabetes: results of case-control and family-based studies. *Diabetes* 2000;**49**:2190-5.
36 **Wierzbicki AS**, Hardman TC, Cheung J, Lambert-Hammill M, Patel S, Morrish Z, Lumb PJ, Lant AF. The apolipoprotein E2 allele modulates activity and maximal velocity of the sodium-lithium countertransporter. *Am J Hypertens* 2002;**15**(Pt 1):633-7.
37 **Geisel J**, Bunte T, Bodis M, Oette K, Herrmann W. Apolipoprotein E2/E2 genotype in combination with mutations in the LDL receptor gene causes type III hyperlipoproteinemia. *Clin Chem Lab Med* 2002;**40**:475-9.
38 **Athanasopoulos T**, Owen JS, Hassall D, Dunckley MG, Drew J, Goodman J, Tagalakis AD, Riddell DR, Dickson G. Intramuscular injection of a plasmid vector expressing human apolipoprotein E limits progression of xanthoma and aortic atheroma in apoE-deficient mice. *Hum Mol Genet* 2000;**9**:2545-51.
39 **Roberts D**, Schwartz RS. Clotting and hemorrhage in the placenta - a delicate balance. *N Engl J Med* 2002;**347**:57-9.

Pathogenic mutations but not polymorphisms in congenital and childhood onset autosomal recessive deafness disrupt the proteolytic activity of TMPRSS3

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Congenital hearing loss occurs in approximately 1 in 1000 live births and 60% of these cases are hereditary.[1 2] Non-syndromic autosomal recessive deafness accounts for about 70% of congenital hereditary hearing loss cases. To date, at least 33 genetic loci have been mapped for non-syndromic deafness, and the causative genes for 17 of these loci have been identified.[3] Mutations in the *TMPRSS3* gene, which encodes a transmembrane serine protease, were originally identified in Pakistani DFNB8 and Palestinian DFNB10 families, and later in two Tunisian families and several white patients.[4–9]

TMPRSS3 belongs to a family of transmembrane serine proteases, which includes TMPRSS1-5.[5 10] Like the other members of this family, TMPRSS3 contains a short amino terminus, a transmembrane domain and a large extracellular or lumenal carboxyl terminal segment characterised by a stem region containing *LDLRA* (low density lipoprotein receptor class A), *SRCR* (scavenger receptor cysteine rich) domains, and a catalytic domain. In addition to a deleterious β-satellite repeat insertion and splice acceptor site substitution,[6] six pathogenic missense mutations have been isolated.[7] Since

Key points

- Mutations in the *TMPRSS3* gene, which encodes a transmembrane serine protease, are responsible for non-syndromic autosomal recessive deafness, DFNB8 and 10. Pathogenic mutations were found in the *LDLRA* (low density lipoprotein receptor class A) and *SRCR* (scavenger receptor cysteine rich) domains as well as the serine protease domain.
- We have assayed the proteolytic activity of the TMPRSS3 proteins containing pathogenic mutations or polymorphisms using a yeast based protease assay.
- All the six tested pathogenic missense mutations disrupted the proteolytic activity of TMPRSS3, while two non-pathogenic polymorphisms did not affect the activity.
- The disruption of proteolytic activity of TMPRSS is tightly correlated with the pathogenesis of deafness.

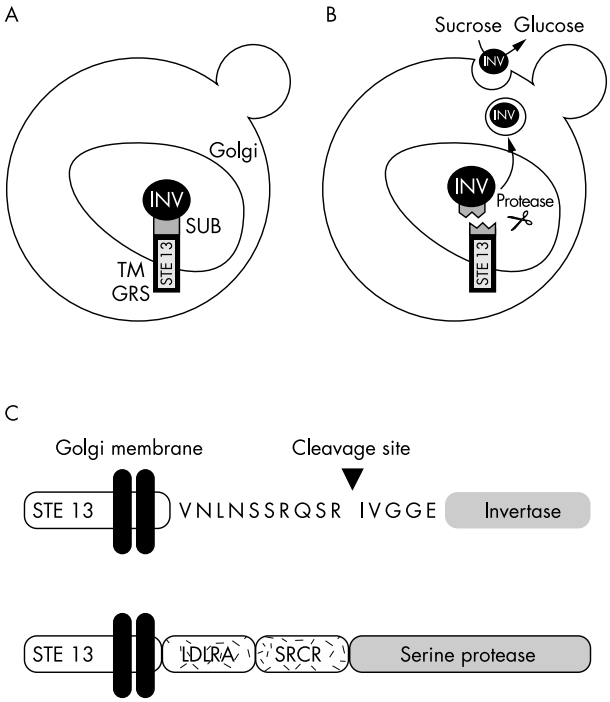

Figure 1 Principle of sGASP. In a yeast strain lacking invertase activity (suc2), a fusion protein is expressed in which invertase is linked to the truncated lumenal domain of an integral Golgi membrane protein, STE13, by a short substrate sequence containing linker. In the absence of cleavage of the substrate sequence, the invertase moiety remains anchored to the Golgi membrane (A). However, upon cleavage of the substrate sequence by a specific protease, invertase is released into the periplasmic space where it degrades sucrose to glucose and fructose. As a result, transformants are able to grow on selective plates containing sucrose as the sole carbon source (B). The structures of the STE13-substrate-invertase fusion protein and STE13-TMPRSS3 are shown in (C). Targeting proteases to Golgi apparently augments the protease-substrate interaction and hence the proteolysis.

these mutations occur not only in the catalytic domain, but also in the *LDLRA* and *SRCR* domains involved in interactions with extracellular molecules, the molecular mechanism for pathogenesis is unclear. In this study, we determine whether these missense mutations affect the proteolytic activity of TMPRSS3.

MATERIALS AND METHODS

Generation of wild type and mutant *TMPRSS3* constructs

The lumenal region of *TMPRSS3* (accession number AB038157) was amplified by PCR and fused to the truncated STE13. The PCR primers used in this amplification (5′-ATG CCT CGA GTT CGA CTG CTC AGG GAA GTA C-3′ and 5′-ATG CGC GGC CGC TCA GGT TTT TAG GTC TCT CTC-3′) was also used for generation of mutated *TMPRSS3.* Missense mutations and polymorphisms were introduced by PCR based mutagenesis.[11] Primer sets containing the nucleotide alterations are as follows: D103G (5′-CAA AGA CGG GGA GGG CGA GTA CCG CTG TG-3′ and 5′-CAC AGC GGT ACT CGC CCT CCC CGT CTT TG-3′), R109W (5′-CCG CTG TGT CTG GGT GGG TGG-3′ and 5′-CCA CCC ACC CAG ACA CAG CGG-3′), C194F (5′-AGG GAG GGA TTT GCC TCT GGC-3′ and 5′-GCC AGA GGC AAA TCC CTC CCT C-3′), W251C (5′-CAT CAC GCC CCT GTG CAT CAT CAC TGC TG-3′ and 5′-CAG CAG TGA TGA TGC ACA GGG GCG TGA TG-3′), P404L (5′-GAC AGC GGG GGG CTC CTG GTG TGT CAA G-3′ and 5′-CTT GAC ACA CCA GGA GCC CCC CGC TGT C-3′), C407R (5′-GCC CCT GGT GCG TCA AGA GAG G-3′ and 5′-CCT CTC TTG ACG CAC CAG GGG C-3′), G111S (5′-CGC TGT GTC CGG GTG AGT GGT CAG AAT GCC GTG-3′ and 5′-CAC GGC ATT CTG ACC ACT CAC CCG GAC ACA GCG-3′), I253V (5′-ACG CCC CTG TGG ATC GTC ACT GCT GCA CAC TGT-3′ and 5′-ACA GTG TGC AGC AGT GAC GAT CCA CAG GGG CGT-3′), D173N (5′-GAG TTT GTG TCC ATC AAT CAC CTC TTG CCA GAT-3′ and 5′-ATC TGG CAA GAG GTG ATT GAT GGA CAC AAA CTC-3′), and A426T (5′-TTT GGC ATC GGC TGC ACA GAG GTG AAC AAG CCT-3′ and 5′-AGG CTT GTT CAC CTC TGT GCA GCC GAT GCC AAA-3′).

Yeast based protease assay

Proteolytic activity of TMPRSS3 and its variants were assayed as described previously.[12] Briefly, a yeast strain KSY01 (MATα, leu2 ura3 his3 trp1 lys2 suc2-Δ9 kex2::HIS3) was cotransformed with the wild type and mutant TMPRSS3 expression vectors and the substrate vector. The Leu^+/Trp^+ transformants were selected on minimal media containing 2% glucose but lacking Leu and Trp, and then replica plated onto YPD media containing 2% sucrose and 10 μg/ml antimycin A. Colonies usually appeared in seven days at 30°C.

RESULTS AND DISCUSSION

In this study, we determined whether the missense mutations affect the proteolytic activity of TMPRSS3 by using a yeast based protease assay, designated sGASP (secretory Genetic Assay for Site specific Proteolysis), which is effective for the study of secretory proteases.[12] The principle of sGASP is illustrated in fig 1A and B. Using this method, proteolysis can be monitored simply by the growth of yeast cells on selective plates. Assays were performed with the substrate sequence VNLNSSRQSR_IVGGE and the extracellular domain of TMPRSS3 expressed as a fusion protein with STE13 (fig 1C). The structure of TMPRSS3, locations of pathogenic missense mutations, and polymorphisms are depicted in fig 2A and the results of assays are shown in fig 2B. The substrate sequence alone was not cleaved (data not shown), but cleavage was evident upon coexpression with wild type TMPRSS3, as shown by the growth of transformants on sucrose plates (panel I). The mutation in the potential activation cleavage site, R216A, greatly diminished the proteolytic activity, and the mutations in the serine residue of the catalytic triad, S401A, completely abolished the activity, as evaluated by the failure of yeast cells transformed with these mutants to grow on sucrose plates (panels II and III). Pathogenic missense mutations (D103G, R109W, C194F, W251C, P404L, and C407R), were all defective in protease activity (panels IV-IX). In contrast, the two polymorphisms G111S and I253V did not affect the proteolytic activity of TMPRSS3 (panels X and XI). Our data indicate that the pathogenesis is strongly correlated with the defective proteolytic activity of TMPRSS3.

We additionally examined two reported polymorphisms, D173N and A426T, which could not be clearly categorised as non-pathogenic.[9] Our results showed that the D173N mutant possesses full proteolytic activity, while that of A426T is significantly diminished (panels XII and XIII). We suggest that D173N is a non-pathogenic polymorphism, while A426T could be pathogenic in certain circumstances (for example, when the critical substrates of TMPRSS3 become slightly less cleavable owing to gene alterations, the A426T mutation in TMPRSS3 could worsen the situation and eventually lead to pathogenesis). It is interesting to note that D173 is not conserved, while A426 is highly conserved among TMPRSS proteases.

Recently, the epithelial amiloride sensitive sodium channel (ENaC) was suggested to be a potential substrate of TMPRSS3.[13] It was suggested that TMPRSS3 proteolytically activates ENaC, which might control important signalling pathways in the inner ear. Consistent with this report, our data indicate that disruption of the proteolytic activity of TMPRSS3 is tightly correlated with the pathogenesis of hearing loss. It remains to be seen how the mutations in the *LDLRA*

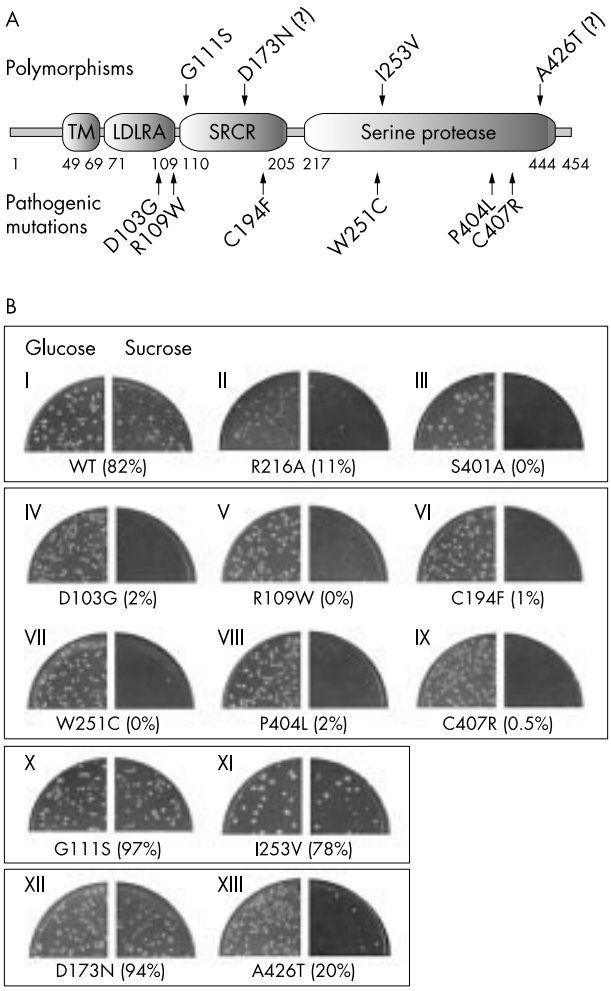

Figure 2 Protease assays for pathogenic mutations and polymorphic alterations. (A) Schematic representation of TMPRSS3 with pathogenic mutations and polymorphisms. (B) Transformants expressing both the STE13-substrate-invertase fusion protein and STE13-TMPRSS3 (wild type or variants) were plated on non-selective (glucose) and selective (sucrose) plates. I, wild type TMPRSS3; II, R216A; III, S401A; IV, D103G; V, R109W; VI, C194F; VII, W251C; VIII, P404L; IX, C407R; X, G111S; XI, I253V; XII, D173N; XIII, A426T. The numbers in parentheses are plating efficiencies, which represent relative proteolytic activity. They are calculated by dividing the number of colonies on sucrose plates by the number of colonies on glucose plates.

and *SRCR* domains affect the proteolytic activity of TMPRSS3. It may be possible that these domains are necessary for proper folding or assembly of the catalytic domain or protease substrate recognition and binding.

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REFERENCES

1 **Morton NE**. Genetic epidemiology of hearing impairment. *Ann NY Acad Sci* 1991;**630**:16-31.
2 **Cohen MJ**, Gorlin R. Epidermiology, aetiology and genetics patterns. In: Gorlin R, Toriello H, Cohen MJ, eds. *Hereditary hearing loss and its syndromes*. Oxford: Oxford University Press, 1995:9-12.
3 **Steel KP**, Kros CJ. A genetic approach to understanding auditory function. *Nat Genet* 2001;**27**:143-9.
4 **Bonne-Tamir B**, DeStefano AL, Briggs CE, Adair R, Franklyn B, Weiss S, Korostishevsky M, Frydman M, Baldwin CT, Farrer LA. Linkage of congenital recessive deafness (gene DFNB10) to chromosome 21q22.3. *Am J Hum Genet* 1996;**58**:1254-9.
5 **Veske A**, Oehlmann R, Younus F, Mohyuddin A, Muller-Myhsok B, Mehdi SQ, Gal A. Autosomal recessive non-syndromic deafness locus (DFNB8) maps on chromosome 21q22 in a large consanguineous kindred from Pakistan. *Hum Mol Genet* 1996;**5**:165-8.
6 **Scott HS**, Kudoh J, Wattenhofer M, Shibuya K, Berry A, Chrast R, Guipponi M, Wang J, Kawasaki K, Asakawa S, Minoshima S, Younus F, Mehdi SQ, Radhakrishna U, Papasavvas MP, Gehrig C, Rossier C, Korostishevsky M, Gal A, Shimizu N, Bonne-Tamir B, Antonarakis SE. Insertion of beta-satellite repeats identifies a transmembrane protease causing both congenital and childhood onset autosomal recessive deafness. *Nat Genet* 2001;**27**:59-63.
7 **Ben-Yosef T**, Wattenhofer M, Riazuddin S, Ahmed ZM, Scott HS, Kudoh J, Shibuya K, Antonarakis SE, Bonne-Tamir B, Radhakrishna U, Naz S, Ahmed Z, Riazuddin S, Pandya A, Nance WE, Wilcox ER, Friedman TB, Morell RJ. Novel mutations of *TMPRSS3* in four DFNB8/B10 families segregating congenital autosomal recessive deafness. *J Med Genet* 2001;**38**:396-400.
8 **Masmoudi S**, Antonarakis SE, Schwede T, Ghorbel AM, Gratri M, Pappasavas MP, Drira M, Elgaied-Boulila A, Wattenhofer M, Rossier C, Scott HS, Ayadi H, Guipponi M. Novel missense mutations of TMPRSS3 in two consanguineous Tunisian families with non-syndromic autosomal recessive deafness. *Hum Mutat* 2001;**18**:101-8.
9 **Wattenhofer M**, Di Iorio MV, Rabionet R, Dougherty L, Pampanos A, Schwede T, Montserrat-Sentis B, Arbones ML, Iliades T, Pasquadibisceglie A, D'Amelio M, Alwan S, Rossier C, Dahl HH, Petersen MB, Estivill X, Gasparini P, Scott HS, Antonarakis SE. Mutations in the TMPRSS3 gene are a rare cause of childhood nonsyndromic deafness in Caucasian patients. *J Mol Med* 2002;**80**:124-31.
10 **Hooper JD**, Clements JA, Quigley JP, Antalis TM. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J Biol Chem* 2001;**276**:857-60.
11 **Ho SN**, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989;**77**:51-9.
12 **Kim SY**, Park D, Oh M, Sellamuthu S, Park WJ. Detection of site-specific proteolysis in secretory pathways. *Biochem Biophys Res Commun* 2002;**296**:419-24.
13 **Guipponi M**, Vuagniaux G, Wattenhofer M, Shibuya K, Vazquez M, Dougherty L, Scamuffa N, Guida E, Okui M, Rossier C, Hancock M, Buchet K, Reymond A, Hummler E, Marzella PL, Kudoh J, Shimizu N, Scott HS, Antonarakis SE, Rossier BC. The transmembrane serine protease (TMPRSS3) mutated in deafness DFNB8/10 activates the epithelial sodium channel (ENaC) in vitro. *Hum Mol Genet* 2002;**11**:2829-36.