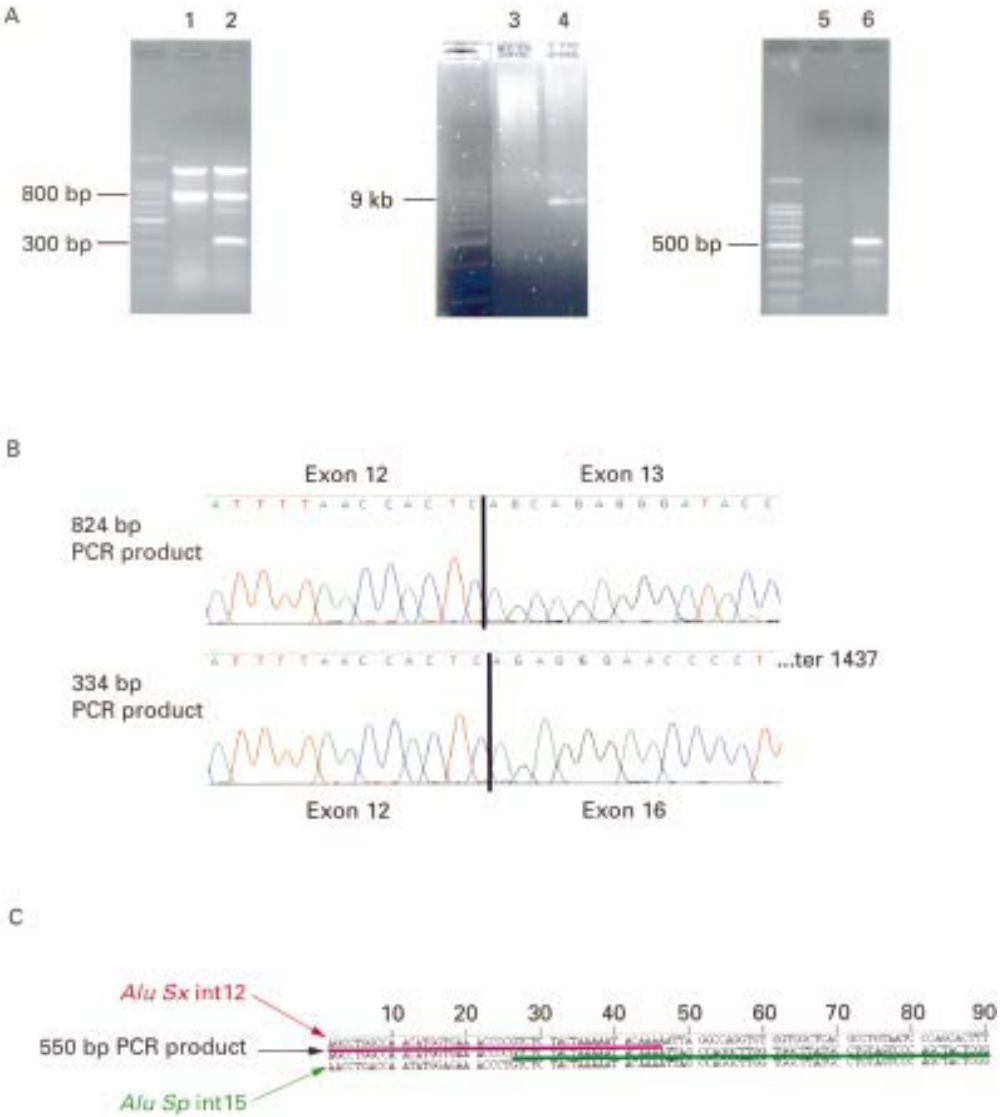

Figure 3 Characterisation of the IC2361BRCA1 rearrangement. (A) RT-PCR products with primers "exon 11 forward" and "exon 16 reverse" on (lane 1) control RNA (824 bp expected) and on (lane 2) IC2361 RNA: a product between 300 and 400 bp is observed and corresponds to the deletion of exons 13 to 15 (334 bp). RNA extraction was performed according to the TRIzol protocol (Gibco, Gaithersburg, MD, USA). Long range PCR products with primers "ex12 forward" and "ex16 reverse" on (lane 3) genomic control DNA showing, as expected, no product and on (lane 4) IC2361 genomic DNA showing the 8.5 kb abnormal product comprising the breakpoints. PCR products with primers "Alu intron 12 forward" and "Alu intron 15 reverse" on (lane 5) genomic control DNA showing as expected no product and on (lane 6) IC2361 genomic DNA showing the 550 bp narrowing the breakpoint region. (B) Sequences of IC2361 RT-PCR products. The 824 and 334 bp PCR products were gel extracted and sequenced with primers "exon 11 forward" and "exon 16 reverse". Sequence of the 824 bp product shows normal transition between exons 12 and 13, whereas sequence of the 334 bp product shows a junction of exons 12 to 16. (C) Box in which recombination has occurred, leading to a 11.6 kb deletion. The 550 bp was gel extracted and sequenced with primers "Alu intron 12 forward" and "Alu intron 15 reverse". Alignment of this sequence with Alu Sx in intron 12 and Alu Sp in intron 15 shows the recombination box located between nucleotides 44 377 and 44 397 in intron 12 and between nucleotides 55 980 and 56 000 in intron 15 (accession number Genbank L78833[15]), thus resulting in a 11 604 bp deletion.

primers "Alu intron 12 forward" and "Alu intron 15 reverse". No 550 bp fragment was observed, suggesting that this rearrangement is not frequent in the population studied (data not shown).

Discussion

Our report of a previously undescribed 11.6 kb deletion encompassing exons 13 to 15 of the *BRCA1* gene illustrates the diversity of large rearrangements and their contribution to the molecular pathology of the *BRCA1* gene. Few series of breast/ovarian cancer families have been systematically screened for large rearrangements of the *BRCA1* gene. The reported frequencies of *BRCA1* rearrangements range between 12% and 36%.[4 6 7 14] Even with a conservative estimate of 10%, it would be advisable to include a search for large rearrangements in *BRCA1* when analysing high risk breast/ovarian cancer families. The family reported here serves as a prime example of a case in which additional testing was warranted in the absence of a detectable point mutation with standard PCR methods. The prior probability of the index case being a *BRCA1/2* mutation carrier has been estimated at 95%. This value was obtained by using the MLINK program of the LINKAGE package, with the parameters of

the Claus segregation model modified by Easton and the estimated contributions of *BRCA1* and *BRCA2* mutations to breast/ovarian cancer predisposition.[1 19–21] In the absence of an identifiable mutation, closely related family members would have to be considered to be at high risk and would have to make decisions regarding cancer prevention on the basis of empirical data. With the identification of the familial *BRCA1* deletion, at risk family members can now consider testing for the identified familial mutation and can learn their mutation status with certainty.

The broad diversity of rearrangements, ranging from 0.5 to 23.8 kb and spread over the 81 kb of the *BRCA1* region, requires methods that allow for complete analysis of the gene. In this respect, colour bar coding on combed DNA appears useful. It allows for a panoramic view of the *BRCA1* region and for the detection of a rearrangement of about 6 kb (the size of a probe deleted or duplicated) at a glance. In addition, deletions and duplications as small as 2 kb can be detected with measurement of the probe signals.[16] Finally, more complex rearrangements involving inversions can also be detected. We think that software allowing for the automatic capture and analysis of signals would streamline the approach and, therefore, favour the use of colour bar coding on combed DNA. Searching for large gene rearrangements is a recurrent challenge for molecular geneticists. In addition to Southern blotting, other promising PCR based methods have recently been reported, including a long range PCR strategy and quantitative PCR.[11 22–24] Haploid conversion of human lymphocytes via a cell fusion strategy may be another alternative to these methods, as it allows for suppression of the normal allele, facilitating the detection of large rearrangements by standard PCR.[25] Comparative analysis of the different methods listed above, taking both sensitivity and cost into consideration, are now needed to improve genetic testing for breast and ovarian cancer predisposition.

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A unique form of autosomal dominant cataract explained by gene conversion between β-crystallin B2 and its pseudogene

Vanita, Virinder Sarhadi, André Reis, Martin Jung, Daljit Singh, Karl Sperling, Jai Rup Singh, Joachim Bürger

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Centre for Genetic Disorders, Guru Nanak Dev University, Amritsar, India
Vanita
V Sarhadi
J R Singh

Gene Mapping Centre, Max-Delbrück-Centre, Berlin, Germany
A Reis
M Jung

Institute of Human Genetics, Charité, Humboldt-Universität zu Berlin, Germany
A Reis
K Sperling
J Bürger

Dr Daljit Singh Eye Hospital, Amritsar, India
D Singh

Correspondence to:
Dr Bürger, Institut für Humangenetik, Charité, Augustenburger Platz 1, 13353 Berlin, Germany, joachim.buerger@charite.de or Prof Dr Singh, Centre for Genetic Disorders, Guru Nanak Dev University, Amritsar 143005, India, jairup@vsnl.com jairup@hotmail.com

EDITOR—Using linkage analysis, a large Indian family with autosomal dominant sutural cataract and cerulean opacities was mapped to chromosome 22 and two cosegregating sequence changes (475C→T and 483C→T) were identified in the *CRYBB2* gene. The first was previously described in two genetically unrelated families with other inherited forms of cataract. The two sequence alterations are identical to the sequence of the *CRYBP1* pseudogene that is 228 kb apart. Furthermore, the pseudogene-like fragment within the *CRYBB2* gene is flanked by chromosomal junction sequences. Therefore, we conclude that gene conversion is the most likely mechanism leading to this mutation. Alternatively, dual point mutation would explain our findings. In addition, since the three families with Q155X mutations all show different types of cataract, we conclude that mutant *CRYBB2* causes cataract formation but other modifying factors determine the type of cataract.

Autosomal dominant congenital cataract (ADCC) is a clinically and genetically heterogeneous group of disorders that cause blindness. More than 13 independent loci have been mapped, and 10 different genes identified so far. Five of them are crystallin genes that are categorised into the α, β, γ, μ, and ζ subgroups. The crystallins constitute the main lens proteins, whereby β-crystallin B2 is the only abundant protein in the adult lens fibre in man.[1 2] Causative mutations have been recognised in the α-crystallin A gene (zonular central nuclear cataract),[3] the β-crystallin A3/A1 gene (zonular cataracts with sutural opacities),[4] the γ-crystallin C gene (Coppock-like cataract),[5] and the γ-crystallin D gene (progressive juvenile onset punctate cataract).[6] These and all other ADCC mutations identified so far are private mutations, with one exception. Litt *et al*[7] described a nonsense mutation, Q155X, in the β-crystallin B2 gene leading to cerulean cataract. Exactly the same mutation was identified by Gill *et al*[8] in familial Coppock-like cataract. Here, we report the identical mutation in a large Indian family exhibiting sutural cataract with punctate and cerulean opacities. In addition, we present evidence that this mutation in the β-crystallin B2 gene is an independent event and most likely the result of gene conversion.

Identification and characterisation of the mutation

After obtaining informed consent, we performed linkage analysis in an Indian five generation family with 33 affected members, based on semi-automated genotyping with microsatellite markers from the Généthon linkage map; 48 members of this family, 25 of them affected, were selected for mapping (fig 1A). Assuming autosomal dominant inheritance with full penetrance and equal allele frequencies for each marker and using the LINKAGE program package, we calculated two point lod scores. After having excluded the autosomal dominant cataract loci on chromosomes 1, 2, 12, 13, 14, 16, 17, and 19, we detected linkage in our family to marker D22S315, with a lod score of Zmax = +8.500 at θmax = 0.05.

This region on chromosome 22 harbours four β-crystallin genes, *CRYBA4*, *CRYBB1*, *CRYBB2*, *CRYBB3*, and the pseudogene *CRYBP1*.[9] We amplified the translated exons 2-6 of the *CRYBB2* gene by PCR as described previously.[7] In addition, we designed primer sets for maximum discrimination between gene and pseudogene sequences: CRYBLg (5'-TGACCTTGTAGCTGGGCTTG-3'), CRYBLpsg (5'-TGACTTTGCAGCCAGGCTTG-3'), 596rg (5'-CACTGCATGTCGCGGATACG-3'), 596rpsg (5'-CCCTGCATGTCGTGGATGCA-3'). PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced directly using the Big-Dye-Terminator Cycle Sequencing Kit (PE Biosystems, Weiterstadt, Germany). Sequencing reactions were purified with a Dye-Ex Kit (Qiagen, Hilden, Germany) and run and analysed on ABI 310 and 377 sequencers (PE Biosystems, Weiterstadt, Germany).

Sequencing of exon 6 showed a C→T mutation at nucleotide position 475 (Q155X). This stop mutation truncates the protein by 51 residues and has previously been described.[7 8] However, our sequencing of exon 6 showed an additional variant, a C→T substitution at nucleotide position 483. This silent polymorphism was found exclusively in patients. Since

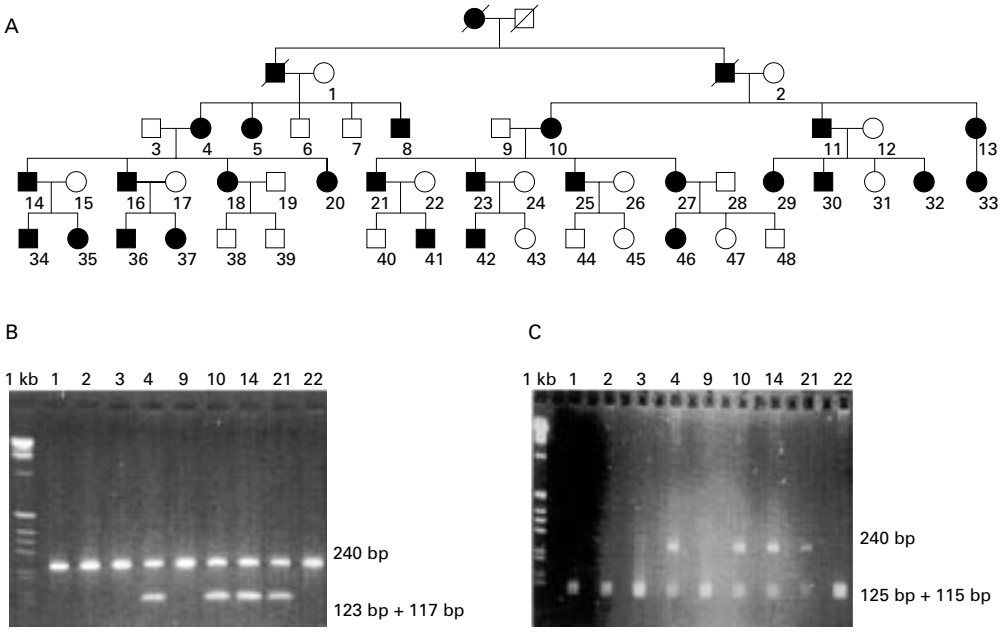

Figure 1 Pedigree of the family (A) and results of the restriction digestions of representative members. (B) CRYBB2 mutation Q155X creates a BfaI site. Restriction digestion with BfaI shows two unresolved digestion products of 117 bp and 123 bp in addition to the undigested 240 bp fragment in affected subjects. Unaffected persons show only an undigested 240 bp PCR product. Numbers above lanes correspond to individual numbers in the pedigree. 1 kb ladder is used as size standard. (C) CRYBB2 variant 483C→T destroys a MspI site. Restriction digestion with MspI shows the undigested 240 bp fragment in addition to two unresolved digestion products of 125 bp and 115 bp in affected subjects. Unaffected people show only two unresolved digestion products of 125 bp and 115 bp. Numbers above lanes correspond to subject numbers in the pedigree. 1 kb ladder is used as size standard.

the mutation Q155X creates a *Bfa*I restriction enzyme site and the variant 483C→T destroys an *Msp*I site, PCR products were *Bfa*I and *Msp*I (New England Biolabs, Frankfurt, Germany) digested and separated on agarose gels. The results showed complete cosegregation of mutation, variant, and disease in our family (fig 1B, C). Q155X and 483C→T were not found on 180 chromosomes of normal Indian subjects, excluding either from being a frequent polymorphism.

To address the question whether the mutations in our Indian family and the American family of Litt *et al*[7] are derived from a single mutational event or represent recurrent mutations, we established the haplotypes at the *CRYBB2* locus, based on closely linked microsatellite markers. Their order and genetic distance is *TOP1P2* - 3 cM - *CRYBB2* - 1 cM - D22S258.[10] The haplotypes were 133-173-180 in the Indian and 165-171-180 in the American family, indicating that the mutation Q155X arose independently on chromosomes with different haplotypes. This is confirmed by the "normal" cytosine at position 483 in the patients of Litt *et al*[7] and Gill *et al*,[8] as can be seen from the published sequences.

Gene conversion

Altogether these data point to a hot spot of mutation in exon 6 of the *CRYBB2* gene. Since the two sequence alterations in the Indian family, Q155X and 483C→T, are both cytosine to thymidine mutations, cytosine deamination as the mutational mechanism cannot be ruled out a priori. However, if one takes into account that the region harbours four closely related β-crystallin genes and a pseudogene (fig 2A), two other mutational mechanisms need to be considered, unequal crossing over and gene conversion.

By alignment of the DNA sequences of the β-crystallin genes, it became clear that *CRYBB2* is much more similar to the closely linked pseudogene *CRYBP1* than to the other β-crystallin genes (fig 2B). This finding suggests a recent duplication of a common precursor gene where one of the duplicated genes was maintained (*CRYBB2*) and the other one accumulated point mutations, turning it into a pseudogene (*CRYBP1*). The physical distance of 228 kb between *CRYBB2* exon 6 and the homologous sequence in the pseudogene *CRYBP1* (fig 2A) is compatible with both unequal crossing over and gene conversion. To our surprise, the alignment of the crystallin gene sequences showed that the alterations Q155X and 483C→T in the *CRYBB2* mutant are identical to the "normal" sequence in the pseudogene (fig 2B). Thus, the two alterations, Q155X and 483C→T, define a fragment of at least 9 bp of pseudogene-like sequence in the *CRYBB2* gene. These 9 bp are flanked upstream by 28 bp and downstream by 67 bp where the gene and pseudogene are identical (fig 2B). At the nucleotides 29 bp upstream and 68 bp downstream that differ between gene and pseudogene, the patients showed homozygosity for the *CRYBB2* sequence. Therefore, the mutant allele carries more than 9 bp but less than 104 bp of pseudogene-like sequence (fig 2B).

In principle, recombinational events resulting from unequal crossing over or gene conver-

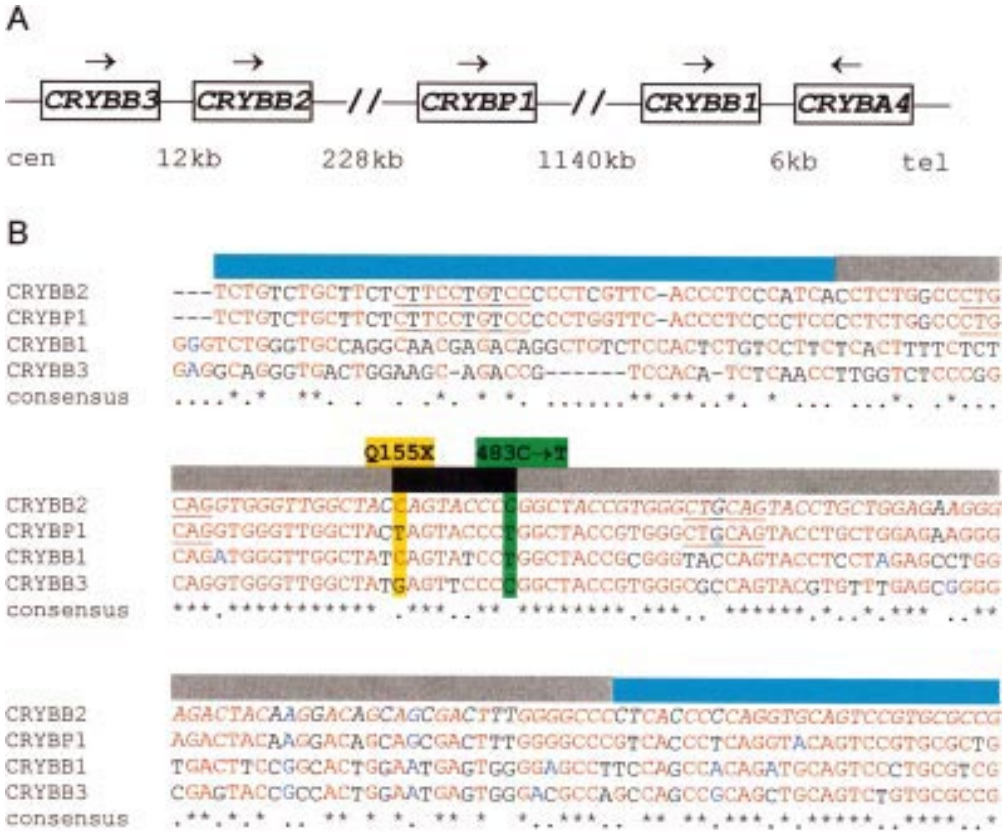

Figure 2 The crystallin genes on chromosome 22. (A) Position and orientation of crystallin genes and physical distances between them.[9]. (B) Alignment of CRYBB2 exon 6 (italicised) with the homologous sequences of CRYBP1, CRYBB1, and CRYBB3. The closest similarity is observed between CRYBB2 and CRYBP1. The sequence of the CRYBB2 gene with the Q155X mutation (highlighted yellow) and the 483C→T variant (highlighted green) is identical to the CRYBP1 pseudogene sequence. Sequences that promote recombination events are underlined. We identified one hypervariable minisatellite GGGCAGGA(A/G)G with 1 bp mismatch and two chromosomal junction sequences ATGCAG with one mismatch each. Black and dark grey bars indicate sequence of probable and possible gene conversion, turquoise shows where gene conversion is excluded.

sion could explain this situation because the distance of 228 kb between gene and pseudogene (fig 2A) is compatible with both mechanisms. Unequal crossing over should lead to relatively large duplications or deletions. However, Southern blotting of *Eco*RI digested genomic DNA with an exon 6 probe did not show any differences between affected and unaffected subjects (data not shown).

Thus, the most likely explanation is that gene conversion between *CRYBB2* exon 6 and its homologous *CRYBP1* sequence led to the pseudogene-like alteration in the *CRYBB2* gene. This assumption is in agreement with the shortness (≤104 bp) of the replaced fragment. It was estimated that gene conversions between human γ-globin genes are less than 300 bp in length[11] and between human β-globin genes less than 451 bp.[12] Our assumption of gene conversion is also supported by the presence of sequences that were described to promote gene conversion. Gene conversion is considered to be initiated at or near special sites.[13] The critical region in our patients is flanked by the chromosomal junction sequence ATGCAG[14] with one mismatch and also bears a hypervariable minisatellite GGGCAGGA(A/G)G[15] with one mismatch (fig 2B). Putative gene conversion between highly homologous genes, as in this family, was previously described at several other loci, for example, the β-globin genes,[16] the oxytocin vasopressin genes,[17] or the steroid 21-hydroxylase gene and its pseudogene.[18] Summarising all our data, we conclude that gene conversion between *CRYBB2* and its pseudogene *CRYBP1* is the most likely explanation for this mutation in exon 6 of the *CRYBB2* gene.

Phenotypic variation and modifiers

There are now three families described that carry the *CRYBB2* Q155X mutation. However, each of these shows a different phenotype. The clinical diagnosis in our Indian family is sutural cataract with punctate and cerulean opacities. The slit lamp examination (fig 3) showed prominent, dense, white opacification around the anterior and posterior Y sutures. The posterior Y sutures and the posterior pole of the lens were more severely affected than the anterior pole. It also showed greyish and bluish, sharply defined, elongated, spindle shaped, and oval punctate and cerulean opacities of various sizes arranged in lamellar form. The spots were bigger and more concentrated towards the peripheral layers. These did not delineate the embryonal or fetal nucleus. No pulverulent disc-like opacity was observed in the nuclear region. The sutural opacities appeared denser and whiter compared to the punctate and cerulean spots and were also more elongated and

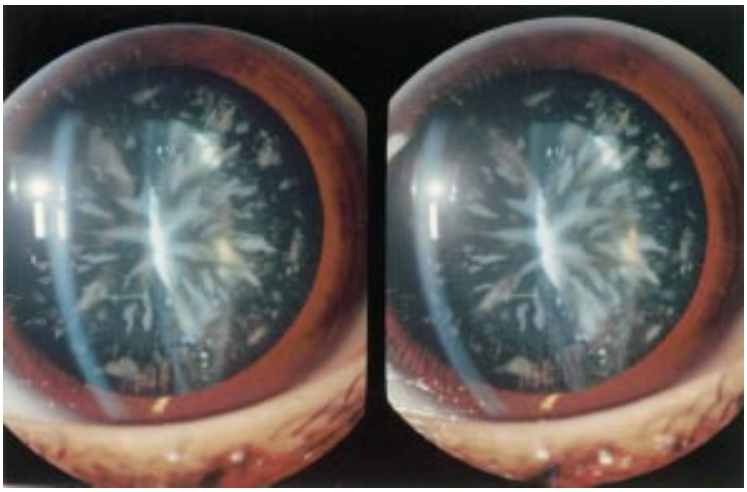

Figure 3 3-D photograph of the eyes of a 9 year old patient through slit lamp microscope. In the centre of the lens there is a dense, white, sharply defined sutural opacity with two of its branches showing fish tail-like division towards the periphery. A large number of sharply defined, elongated, spindle shaped, and oval punctate opacities are directed radially. Similar presence and distribution of opacities are seen posteriorly which are much larger and denser than the anterior ones.

larger in size. Phenotypic variation with respect to the size and density of the sutural opacities as well as the number and position of punctate and cerulean spots was observed among the affected members. Some subjects showed severely affected sutures with dense white opacifications spreading along the secondary divisions of the Y sutures. In some affected subjects the spots were present only as a single layer in the cortex while in the others the spots occurred in concentric layers involving the whole cortex.

The phenotype of this Indian family, sutural cataract with punctate and cerulean opacities, differs from all other reported forms of cataract. The American family of Litt *et al*[7] has pure cerulean cataract, and the Swiss family of Gill *et al*[8] shows Coppock-like cataract. The phenotype of our family overlaps with the American family, both showing cerulean opacities. However, the sutural cataract and the punctate opacities in our phenotype have not been reported in the American or Swiss families. Moreover, the prominent pulverulent central disc-like opacity involving the embryonal and fetal nucleus seen in the Swiss family is not present in the American or this Indian family. There is not even an overlap between the phenotype of the Swiss family and the other two families.

Hence, we conclude that the Q155X mutation causes cataract formation but the distinct type of cataract depends on modifying genetic and epistatic factors. The influence of modifiers would make it impossible to infer the mutant gene from the cataract phenotype. Consequently, ADCC patients would need to be analysed for much more than only one ADCC gene.

Cis acting major modifiers could explain the considerable phenotypic variability between families. Minor modifying factors, acting in *trans*, could cause the phenotypic differences within families. The minor modifier of our family is obviously not linked to the Q155X mutation, since there is considerable clinical variability within our patients who share an identical haplotype. Interestingly, this excludes three further crystallin genes, *CRYBA4*, *CRYBB1*, and *CRYBB3* (fig 2A), from being the minor modifier. On the other hand, exactly these crystallin genes are candidates for being the major modifier.

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Novel mutations of *TMPRSS3* in four DFNB8/B10 families segregating congenital autosomal recessive deafness

Tamar Ben-Yosef, Marie Wattenhofer, Saima Riazuddin, Zubair M Ahmed, Hamish S Scott, Jun Kudoh, Kazunori Shibuya, Stylianos E Antonarakis, Batsheva Bonne-Tamir, Uppala Radhakrishna, Sadaf Naz, Zahoor Ahmed, Sheikh Riazuddin, Arti Pandya, Walter E Nance, Edward R Wilcox, Thomas B Friedman, Robert J Morell

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Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, 5 Research Court, Rockville, MD 20850, USA
T Ben-Yosef
S Riazuddin
Z M Ahmed
E R Wilcox
T B Friedman
R J Morell

Graduate Program of Molecular and Cellular Biology, University of Geneva Medical School, 1 rue Michel Servet, 1211 Geneva, Switzerland
M Wattenhofer

Centre of Excellence in Molecular Biology, Punjab University, Lahore, Pakistan
S Riazuddin
Z M Ahmed
S Naz
Z Ahmed
S Riazuddin

Division of Medical Genetics, University of Geneva Medical School, 1 rue Michel Servet, 1211 Geneva, Switzerland
H S Scott*
S E Antonarakis
U Radhakrishna
M Wattenhofer

Department of Molecular Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
J Kudoh
K Shibuya

Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel-Aviv University, 69978 Ramat-Aviv, Israel
B Bonne-Tamir

Department of Human Genetics, Virginia Commonwealth University, Richmond 23298-0033, USA
A Pandya
W E Nance

Correspondence to:
Dr Friedman, 5 Research Court, Room 2A-19, NIDCD/NIH, Rockville, MD 20850, USA,
friedman@nidcd.nih.gov

*Present address: Genetics and Bioinformatics Division, Walter and Eliza Hall Institute, Royal Parade, Parkville, PO Royal Melbourne Hospital, Victoria 3050, Australia

EDITOR—Congenital deafness occurs in approximately 1 in 1000 live births and 50% of these cases are hereditary. Non-syndromic deafness is classified according to its mode of inheritance as DFN, DFNA, and DFNB (X linked, autosomal dominant, and autosomal recessive, respectively). Non-syndromic recessive deafness accounts for ~80% of congenital hereditary deafness cases.[1] At least 30 DFNB loci have been mapped in the past few years by genetic linkage studies, but the causative gene has been identified for only eight of these loci[2–4] (Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/ hhh).

Two of the previously reported loci for non-syndromic recessive deafness are DFNB8 and DFNB10, both located on chromosome 21q22.3 (MIM 601072 and 605316). The DFNB8 locus was originally identified in a large consanguineous Pakistani family, segregating childhood onset deafness,[5] while DFNB10 was identified in a large consanguineous Palestinian family, in which deafness was congenital.[6] Recently, the *TMPRSS3* gene was shown to be mutated in affected subjects of both families.[7]

TMPRSS3 belongs to a family of transmembrane serine proteases, also including TMPRSS1,[8] TMPRSS2,[9] and TMPRSS4.[10] The *TMPRSS3* gene extends over 24 kb and comprises 13 exons. It has four alternative transcripts (*TMPRSS3 a*, *b*, *c*, and *d*), encoding putative peptides of 454, 327, 327, and 344 amino acids, respectively.[7] *TMPRSS3a*, which contains all 13 exons, is the most abundant transcript and its expression could be detected in various tissues, including fetal cochlea.[7] In addition to the serine protease and the transmembrane domains, *TMPRSS3* also encodes low density lipoprotein receptor class A (LDLRA) and scavenger receptor cysteine rich (SRCR) domains, which are potentially involved in binding with extracellular molecules and/or the cell surface.[7]

Material and methods

To identify DFNB8/B10 linked families, we analysed a total of 159 consanguineous Pakistani families that segregate profound congenital deafness and are either large enough to support statistically significant linkage or have at least three affected subjects. Families were ascertained in schools for the deaf in Punjab and Karachi. IRB approval (OH93-DC-016) and informed consent were obtained for all participating family members. Genomic DNA was extracted from venous blood samples according to a standard protocol.[11] Linkage to known recessive deafness loci (DFNBs) and refinement of the DFNB8/B10 region in linked families was performed on an ABI-377 sequencer (PE Applied Biosystems) using marker information provided by the Hereditary Hearing Loss Homepage and by Berry *et al*.[12] Linkage analysis was conducted with the FASTLINK version of the LINKAGE program package.[13 14] Five of the families showed potential linkage to the DFNB8/B10 locus on chromosome 21q22.3 (fig 1). Medical history and pure tone audiometry testing indicated that all five families segregate congenital, profound, non-syndromic sensorineural deafness.

Results and discussion

To detect mutations in the *TMPRSS3* gene in DFNB8/B10 families, we determined the gene sequence in two affected subjects from each family by PCR amplification from genomic DNA of the 13 exons, including intron-exon boundaries, and cycle sequencing of the PCR products using *TMPRSS3* specific primers. Primer sequences and PCR conditions are summarised in table 1. PCR products were

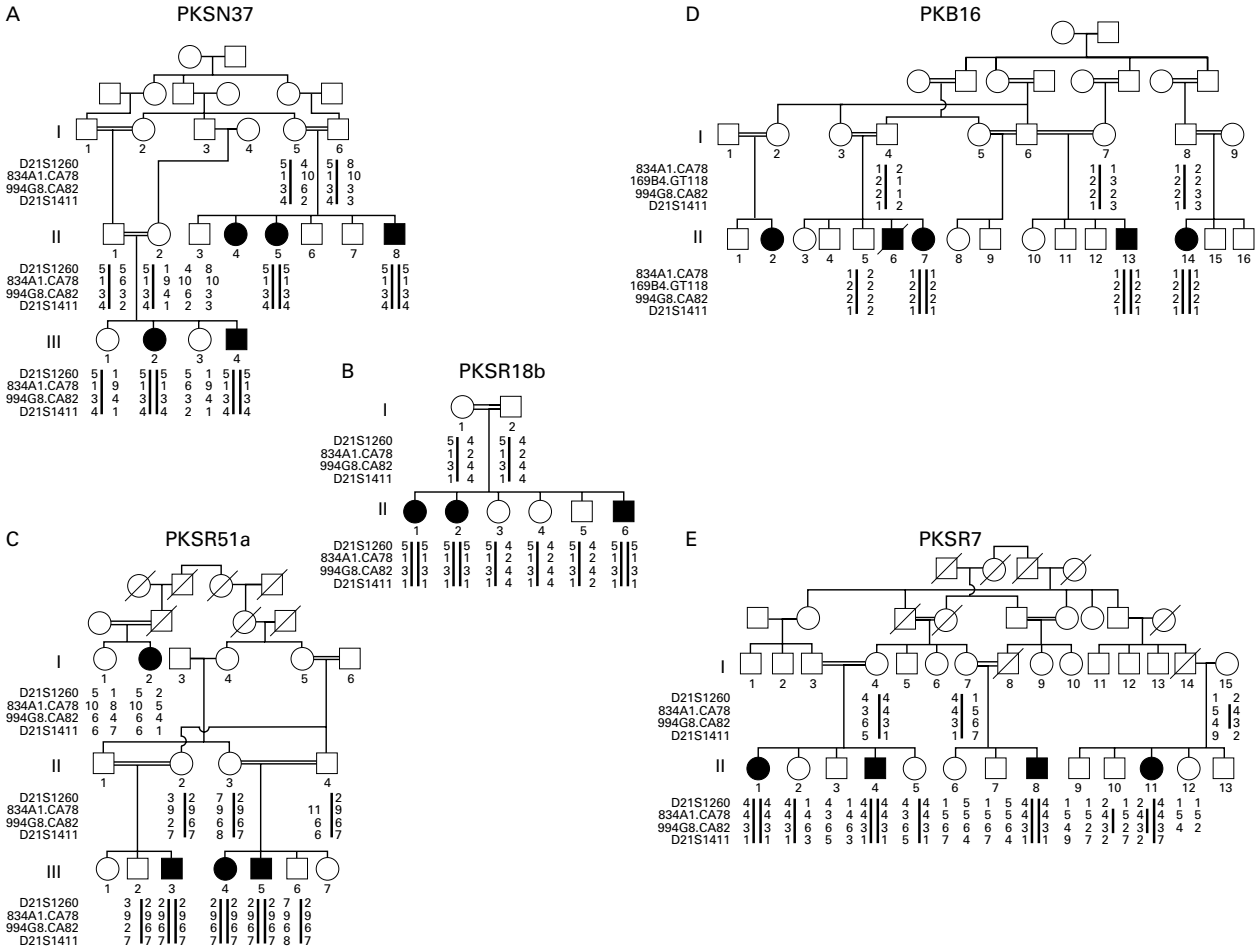

Figure 1 Pedigrees and genotype data for markers spanning the DFNB8/B10 interval. Markers are in order, from telomere to centromere. The region of homozygosity is marked by a black vertical bar.

purified with the QIAquick PCR purification kit (Qiagen). Sequence analysis using one of the amplification primers or an internal primer was performed with the Big Dye terminator cycle sequencing kit on an ABI-377 sequencer (PE Applied Biosystems). Novel *TMPRSS3* mutations were identified in four of the families. The relevant *TMPRSS3* exons in all available family members were then amplified by PCR and sequenced. This confirmed that in each family deaf subjects were homozygous for the *TMPRSS3* mutation and obligate carrier parents were heterozygous. Family and mutation data, including population study results for each mutation, are summarised in table 2. Several single nucleotide polymorphisms (SNPs) were also identified in both coding and non-coding sequences of the *TMPRSS3* gene (table 3).

Families PKSN37 and PKSR18b (fig 1A, B) were found to segregate the same *TMPRSS3* missense mutation, C407R (table 2). This mutation is a T to C transition at position 1420 (1219 from the first ATG) (GenBank accession number AB038157), located in exon 12, and leads to a cysteine to arginine substitution. C407 is located in the serine protease domain of TMPRSS3, only a few amino acids from the active site residue S401 within the substrate

Table 1 Synthetic oligonucleotide primers used for amplification and sequencing of TMPRSS3 exons

Exon	*Primer sequence (F) (5'→3')*	*Primer sequence (R) (5'→3')*	*Product size (bp)*	*Annealing temperature (°C)*	*Buffer**
1	CTCTGGTCTCCTTGGCATTGTG	CTCTCAAAGCCCTTTCCATTGC	387	60/50†	
2	GATGCACCTGATGCTACAAG	GGACAGTCAGTCACATTGGTC	286	55	12
3	GGACTGAACTAGAGAATGTGCC	GACAAAGCCATGAGCATGGC	510	55	
4	AGGGGGACAGTTGTTAGTGTTGC	TACAGATGGGAAGGGTCAGGGTTG	261	60	12
5	TGTGGAGAAACCCCTGCCTATG	GATGTGAGGATGTAATCTGAGAGCG	323	55	9
6	GACTCGCACATCGGTTGAATG	ATACTCCCTCAGGTTCTCACACCC	387	55	12
7	GTGTGACCTCATCCTCATGG	CTCTGAGGGCAAGGAGATAG	293	60	9
8	TAGAGCTGCTGTGAGCTCTG	AGACTCCTCTCCAACTGTAC	438	55	10
9	GGACCACATCTTGCCTGATAACC	AAAGCACACAGCCCACGAAG	694	60	12
10	CTCCTGCTGTGAGCTGATCG	CGAGCAGCTGACATGCACTC	393	55	10
11	GTCTCAGCATGCGCCTTCTG	CCCACGCAGAGCCAGATCAC	407	60	
12	TGGGTCATCCATTGGGACATC	TCTCTGTTTTCAGCACAAGCGTC	460	55	12
13	TACGGAAGTGACGGAACTGTCG	CTTGAAGGTTGTGCTGGAATCAG	442	60	

*The number of Opti-Prime buffer (Stratagene) used for PCR amplification. Other reactions were performed with a standard PCR buffer (PE Applied Biosystems), with 1.5 mmol/l $MgCl_2$.
†Touchdown.

Table 2 TMPRSS3 mutations in DFNB8/B10 families

Family	Maximal simulated lod score*	Mutation in exon	Mutation†	Amino acid substitution	Presence in normal control chromosomes‡	
					Pakistani	Muslim Indian
PKSN37	3.4	12	1219T>C	C407R	0/160	1/200
PKSR18b	2.2	12	1219T>C	C407R	0/160	1/200
PKSR51a	2.4	5	325C>T	R109W	0/122	0/196
PKB16	2.6	7	581G>T	C194F	0/184	NT§

*Maximal simulated lod scores were calculated using the FAST SLINK program.[15] The disease allele frequency was set at 0.00001 and four equally frequent alleles were assumed for any marker. The disease was coded as fully penetrant, genetically homogeneous, and recessive.
†The nucleotide changes follow the nomenclature rules of Dunnen and Antonarakis.[18] Nucleotide position relates to *TMPRSS3* cDNA (GenBank accession number AB038157).
‡Pakistani controls were collected in Lahore, Pakistan. Muslim Indian controls were collected in northern India, near the Pakistani border.
§Not tested.

Table 3 Single nucleotide polymorphisms (SNPs) detected in the TMPRSS3 gene

SNP*	Location	Amino acid
157G>A†‡§	Exon 3	V53I
331G>A‡	Exon 5	G111S
IVS5-13 A>G†‡	Intron 5	
453G>A†‡§	Exon 6	V151V
IVS7-3 ins TA†	Intron 7	
757 A>G†‡	Exon 8	I253V
1128C>T‡	Exon 11	Y376Y
1367 G>A†‡§	Exon 13	
1451A>T‡	Exon 13	

*The nucleotide changes follow the nomenclature rules of Dunnen and Antonarakis.[18] Position of coding SNPs (cSNPs) relates to *TMPRSS3* cDNA (GenBank accession number AB038157). Position of SNPs located in introns of *TMPRSS3* as previously described.[7]
†Observed in Pakistani DFNB8/B10 families.
‡Observed in North American deaf subjects.
§Previously reported.[7]

pocket, and thus may be important in substrate specificity. Although C407 is not highly conserved, three out of the other four known TMPRSS proteins (mouse and human TMPRSS2 and human TMPRSS1) have either a cysteine or a threonine at this position, both of which are polar, uncharged amino acids, while the mutation changes the amino acid at this position to the positively charged arginine. All affected subjects were homozygous for the C407R mutation and obligate carriers were heterozygous (fig 1A, B and 2A). C407R was also found in one of 200 normal control Muslim Indian chromosomes. The haplotype for markers flanking the *TMPRSS3* gene (834A1.CA78 and 994G8.CA82, located approximately 300 kb and 100 kb from the mutation, respectively) in both families and in the Muslim Indian subject harbouring the C407R mutation was identical (fig 1A, B and data not shown).

Another missense mutation, R109W (table 2), was found in family PKSR51a (fig 1C). This mutation is a C to T transition at position 526 (325 from the first ATG) (GenBank accession number AB038157), located in exon 5, and leads to an arginine to tryptophan substitution. Three out of the other four known TMPRSS proteins have either an arginine or the similar positively charged histidine at this position (mouse and human TMPRSS2 and human TMPRSS4). R109 is located within the SRCR domain of TMPRSS3. The SRCR is an adhesive extracellular domain (PROSITE Database of Protein Families and Domains, http:// www.expasy.ch/prosite, accession number PDOC00348), which is potentially involved in

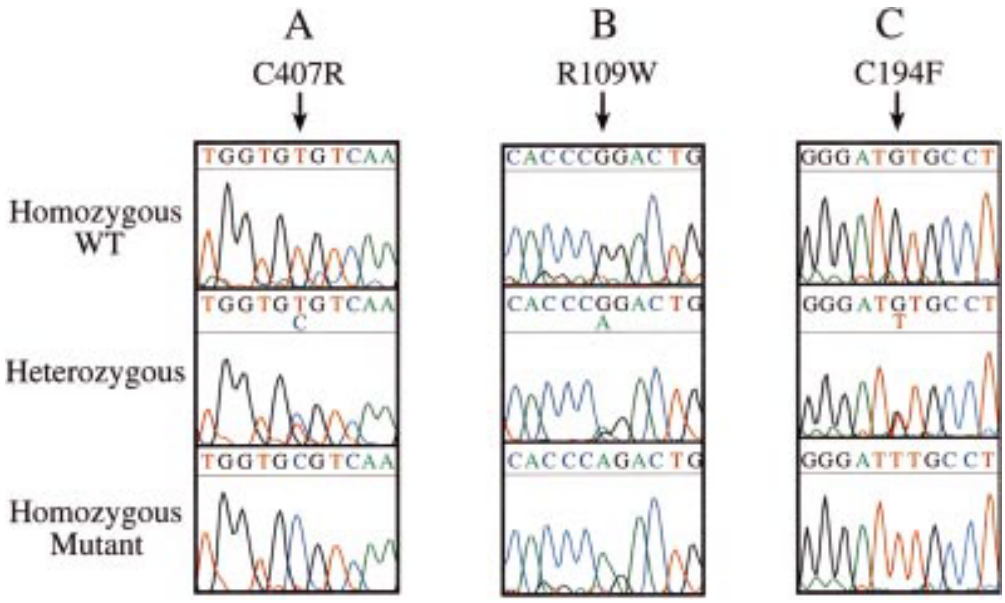

Figure 2 Sequence traces of exons 12, 5, and 7 of the TMPRSS3 gene in DFNB8/B10 families segregating the C407R, R109W, and C194F mutations (A, B, and C, respectively). Non-carriers are homozygous for the wild type (WT) alleles, while obligate carriers are heterozygous for the 1219T>C, 325C>T, and 581G>T substitutions, respectively, and affected subjects are homozygous for the mutant alleles. Sequence traces for the R109W mutation are shown on the reverse complement strand.

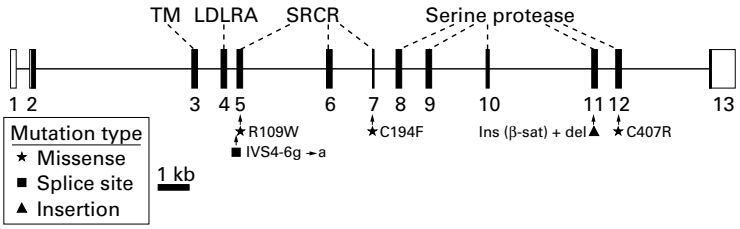

Figure 3 TMPRSS3 mutations in DFNB8/B10 families. The location of two mutations described by Scott et al[7] and the three novel mutations described in this report are shown on a schematic representation of the TMPRSS3 gene (drawn to scale). Four domains of TMPRSS3 encoded by different gene exons, including the transmembrane (TM), low density lipoprotein receptor class A (LDLRA), scavenger receptor cysteine rich (SRCR), and serine protease domains, are indicated. Non-coding exons (exon 1 and most of exon 13) are represented by white boxes.

binding of TMPRSS3 with extracellular molecules and/or the cell surface.[7] All affected subjects belonging to the main branch of the family were homozygous for the R109W mutation and obligate carriers were heterozygous (fig 1C and 2B). Yet, subject I.2, who is deaf, was found to be homozygous for the normal allele. This finding is not surprising, since our initial genotyping data indicated that I.2, who belongs to a remote branch of the family, has a different haplotype at the DFNB8/B10 region than the haplotype shared by the other deaf subjects in the family (fig 1C). Thus, her deafness might be the result of a mutation in a different gene or the result of non-genetic factors.

Family PKB16 (fig 1D) was found to segregate a third missense mutation, C194F (table 2). This mutation is a G to T transversion at position 782 (581 from the first ATG) (GenBank accession number AB038157), located in exon 7, which encodes part of the SRCR domain, and leads to the substitution of the cysteine at position 194, which is highly conserved among all TMPRSS proteins, to phenylalanine. All affected subjects were homozygous for the C194F mutation and obligate carriers were heterozygous (fig 1D and 2C).

Family PKSR7 (fig 1E) supports a simulated maximal lod score of 3.8 (FAST SLINK[15]), and deaf subjects are homozygous for markers spanning the DFNB8/B10 region (fig 1E). The region of homozygosity is shared by all affected subjects, but is more restricted in one of the sibships (fig 1E, II.9-13). However, none of the markers is fully informative, resulting in a maximal calculated lod score of 2.9. The disease allele frequency was set at 0.0011 (upper limit for recessive deafness based on estimates from the Indian population[16]) and the disease was coded as fully penetrant and recessive with a 1/1000 phenocopy rate. Allele frequencies of 0.1 and 0.2 for each allele of markers 834A1.CA78 and 994G8.CA82, respectively, were assumed based on observations in the other analysed Pakistani DFNB8/B10 families. The lod score was not significantly changed by omitting subjects II.9-13 (2.7), nor by increasing 834A1.CA78 and 994G8.CA82 allele frequencies to 0.4 each (2.3). Since linkage analysis for this family did not obtain a significant lod score, it is possible that deafness in family PKSR7 is not actually linked to the DFNB8/B10 locus, and the homozygosity observed in this region is incidental. However, it was previously estimated that 60% of all Pakistani marriages are consanguineous.[17] It is unknown whether I.15 is related to family PKSR7. Recalculation of the lod score under the assumption that I.14 and I.15 are first cousins resulted in a maximum two point lod score of 4.2. Thus, this family may have a mutation in a regulatory element of the *TMPRSS3* gene, or alternatively it might carry a mutation in a different gene located in the same region.

R109W and C194F were not found in any of the normal control Pakistani or Muslim Indian chromosomes tested, while the C407R mutation was found in 1 of 200 Muslim Indian control chromosomes. In addition, C407R was found in two of our DFNB8/B10 families. Taken together, these findings imply that the carrier frequency for C407R in the Muslim Indian-Pakistani population is higher than for the other *TMPRSS3* mutations described in this manuscript. Moreover, finding of pathogenic *TMPRSS3* mutations in four out of a total of 159 Pakistani families segregating profound congenital recessive deafness indicates that *TMPRSS3* mutations contribute to approximately 2.5% of the recessively inherited deafness cases in the Pakistani population (95% confidence interval 0.7-6.3). This is a significant contribution, considering the high level of genetic heterogeneity of recessively inherited deafness in general, and in this population in particular.

To estimate the contribution of *TMPRSS3* mutations to genetic deafness in North America, we sequenced all *TMPRSS3* coding exons (exons 2-13) in a panel of 64 deaf North American subjects. DNA samples of North American deaf subjects were obtained from the National DNA Repository for Research on Deafness (NDRRD) based at the Virginia Commonwealth University. None of the subjects included in the screened panel had any obvious syndromic or environmental cause for their deafness based on their medical history. Subjects with identifiable mutations in *GJB2* (*Cx26*) (based on complete sequencing of exon 2 of the *GJB2* gene) or with known mitochondrial deafness related mutations were also excluded from this panel. The panel includes subjects from both multiplex (25) and simplex (38) families, with the following ethnic origins: 54 whites, two African-Americans, two Hispanic, three Asians, one Indian, and two of unknown origin. We identified several common SNPs in both coding and non-coding sequences of the *TMPRSS3* gene (table 3). Interestingly, no *TMPRSS3* mutations were detected. Comparison of this finding to another deafness related gene, *GJB2* (*Cx26*), which accounts for approximately 20% of non-syndromic, recessive hearing loss,[4] indicates that *TMPRSS3* is not a major contributor to genetic deafness in North America. Direct comparison between our findings in the North American panel and in the Pakistani population is difficult, since some of the subjects included in the panel are sporadic cases. This is unlike the Pakistani families we analysed, which are all consanguineous families with

multiple affected subjects. Thus it is possible that some of the North American deaf analysed do not actually have a genetic cause for their deafness, while deafness in the Pakistani families is most probably genetic.

The five *TMPRSS3* deafness related mutations identified to date include missense (current report), splice site, and insertion mutations,[7] which affect various domains of the TMPRSS3 protein (fig 3). Finding of *TMPRSS3* mutations in several families segregating autosomal recessive deafness DFNB8/B10 indicates that TMPRSS3 is essential for hearing. It remains to be determined whether TMPRSS3 has serine protease activity, as suggested by its conserved serine protease domain. Further work is needed to identify its substrates and the role it plays in the hearing process.

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Department of Neurology, University of Oulu, PO Box 5000, FIN-90014 University of Oulu, Oulu, Finland
S Finnilä
K Majamaa

Department of Medical Biochemistry, University of Oulu, Oulu, Finland
S Finnilä
K Majamaa

Biocenter, University of Oulu, Oulu, Finland
S Finnilä
K Majamaa

Department of Neurology, University of Kuopio, Kuopio, Finland
J Autere
M Lehtovirta
P Hartikainen
H Soininen

DNA and Chromosome Laboratory, Kuopio University Hospital, Kuopio, Finland
A Mannermaa

Correspondence to:
Dr Majamaa,
kari.majamaa@oulu.fi

Increased risk of sensorineural hearing loss and migraine in patients with a rare mitochondrial DNA variant 4336A>G in tRNA^{Gln}

Saara Finnilä, Jaana Autere, Maarit Lehtovirta, Päivi Hartikainen, Arto Mannermaa, Hilkka Soininen, Kari Majamaa

EDITOR—Selectively neutral polymorphisms in mitochondrial DNA (mtDNA) have accumulated during human evolution along mtDNA lineages that correlate with ethnic and geographical origin. By contrast, deleterious mutations have arisen repeatedly and occur against various genomic backgrounds. Since they reduce the fitness of carriers, however, the affected maternal lineages eventually become extinct. Mutations of intermediate severity also occur along mtDNA lineages and have become fixed in the population.[1] Although they do not substantially reduce fitness, they may interact with nuclear or environmental factors, predisposing people to an increased risk of developing neurodegenerative diseases later in life.[1]

1555A>G in the 12S rRNA gene may be considered a mutation of intermediate severity. It has been shown to predispose carriers to maternally transmitted sensorineural hearing impairment, the expression of which requires additional environmental or genetic factors.[2] Similarly, 14484T>C and 11778G>A have been shown to cause Leber's hereditary optic

Table 1 Frequency of 4336A>G in the patient cohorts

	Age (y)	Total No of samples	No (%) of samples with 4336A>G	Odds ratio	95% CI
Group 1					
Sensorineural hearing loss	44 ± 11	110	4 (3.6)	6.0	1.3–27
Migraine	44 ± 7	42	2 (4.8)	8.0	1.3–49
Diabetes mellitus	48 ± 10	122	1 (0.82)	1.3	0.14–13
Hypertrophic cardiomyopathy	50 ± 14	20	1 (5.0)	8.4	0.83–84
Other phenotypes*	50 ± 15	281	0	NA	NA
Controls	41 ± 12	480	3 (0.63)	NA	NA
Group 2					
Alzheimer's disease	76 ± 9	175	3 (1.7)	2.8	0.55–14
Parkinson's disease	69 ± 9	262	1 (0.38)	0.61	0.06–5.9
Non-AD dementia	62 ± 12	60	1 (1.7)	2.7	0.28–26
Controls	75 ± 6	107	0	NA	NA

Age denotes the age at the time of obtaining the blood sample. CI, confidence interval; Non-AD dementia, non-Alzheimer type dementia; NA, not applicable.
*Other phenotypes include patients with epilepsy (n=165), ataxia (n=38), ophthalmoplegia (n=23), intracranial calcification (n=11), white matter disease (n=15), and occipital stroke (n=29).

neuropathy, but expression of the disease phenotype is significantly higher when 14484T>C occurs in mtDNA belonging to haplogroup J[3] and a preferential association with this haplogroup has also been observed for the 11778G>A mutation.[4] The homoplasmic transition A to G at nt 4336 (4336A>G) in the mtDNA $tRNA^{Gln}$ gene has been found at low frequency in populations of European origin. This nucleotide connects the amino acid acceptor stem with the TψC stem of $tRNA^{Gln}$ and is moderately conserved between species.[5] It is thought to entail an increased risk of Alzheimer's disease (AD) and Parkinson's disease (PD),[1 5–7] but its possible role in diseases manifesting in middle life has not been evaluated. We therefore set out to study the frequency of this mutation among middle aged patients with various clinical phenotypes and in healthy, age matched controls (group 1), and also in patients with late onset neurodegenerative diseases and elderly, cognitively normal controls (group 2). We determined the entire mtDNA sequence in 10 patients and three controls with 4336A>G by conformation sensitive gel electrophoresis (CSGE) and subsequent sequencing.

Material and methods

Group 1 contained 575 patients and 480 controls (table 1). Patients with diabetes mellitus, epilepsy, sensorineural hearing loss, occipital stroke, ophthalmoplegia, intracranial calcification, white matter disease, ataxia, and migraine were ascertained as described previously.[8–11] The patients with diabetes mellitus, epilepsy, or sensorineural hearing loss had a family history of similar diseases in maternal relatives. Furthermore, we obtained blood samples from 20 consecutive patients with hypertrophic cardiomyopathy. To be included as a control in group 1 it was required that the subjects and their mothers should be free of diabetes mellitus, sensorineural hearing impairment, and neurological ailments and that their mothers should have been born in central or northern Finland. After obtaining this information, the samples were anonymised.

Group 2 included 497 patients with AD, non-Alzheimer dementia, or PD. The inclusion criteria for patients with AD were those of NINCDS-ADRDA.[12] All the patients with PD satisfied the PD Brain Bank (London) criteria for idiopathic PD, that is, akinetic rigid syndrome with asymmetrical onset, resting tremor, and a good response to L-dopa.[13] The non-Alzheimer dementia group included patients with Parkinson plus syndrome or frontotemporal dementia.[14] The control samples in group 2 were from 107 elderly subjects recruited from the same population as the patients with AD and assessed for cognitive performance to exclude dementia. The research protocol was approved by the local Ethics Committees and the Finnish Red Cross. Permission for the chart review was obtained from the Finnish Ministry of Social Affairs and Health.

Blood samples were obtained from the patients after written informed consent. Total DNA was isolated from the blood cells using a QIAamp Blood Kit (Qiagen, Hilden, Germany), and restriction fragment length polymorphisms (RFLPs) were used to identify the most informative polymorphic sites. The subsequent definitions of the various mtDNA haplogroups conformed to the published criteria.[15]

The $tRNA^{Gln}$ 4336A>G mutation was detected by restriction fragment analysis using *Nla*III. The mtDNA region was amplified in PCR using a forward primer spanning nucleotides 3951 to 3970[16] and a reverse primer spanning nucleotides 4508 to 4489. The template DNA was amplified in a total volume of 50 μl by PCR in 30 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, with a final extension at 72°C for 10 minutes. The amplified DNA fragment (557 bp) was then digested overnight at 37°C with 10 U of *Nla*III (New England Biolabs, Beverly, MA, USA) and electrophoresed through a 1.5% agarose gel. The PCR fragment remained undigested when the wild type nucleotide was present, but was cleaved into fragments of 388 and 169 bp when the 4336A>G mutation was present. The PCR fragment also encompassed a nucleotide variant 4216T>C, which is a common variant in haplogroups T and J[15] that creates a novel *Nla*III site gain. The PCR fragment was cleaved into fragments of 289 and 268 bp when 4216T>C was present.

Conformation sensitive gel electrophoresis (CSGE) was carried out as described previously.[17] In short, 63 pairs of primers were designed for amplification of the mtDNA coding region (nt 574-16023). The template DNA was amplified in a total volume of 50 μl by PCR in 30 cycles of denaturation at 94°C for one minute, annealing at a primer specific temperature for one minute, and extension at 72°C for one minute, with a final extension at 72°C for 10 minutes. The quality of the amplified fragment was estimated visually on a 1.5% agarose gel, and a suitable amount of the PCR product, usually 3-10 μl, was then taken for heteroduplex formation. Each amplified fragment of mtDNA from the patients with 4336A>G was mixed with the corresponding fragment amplified on two control templates,

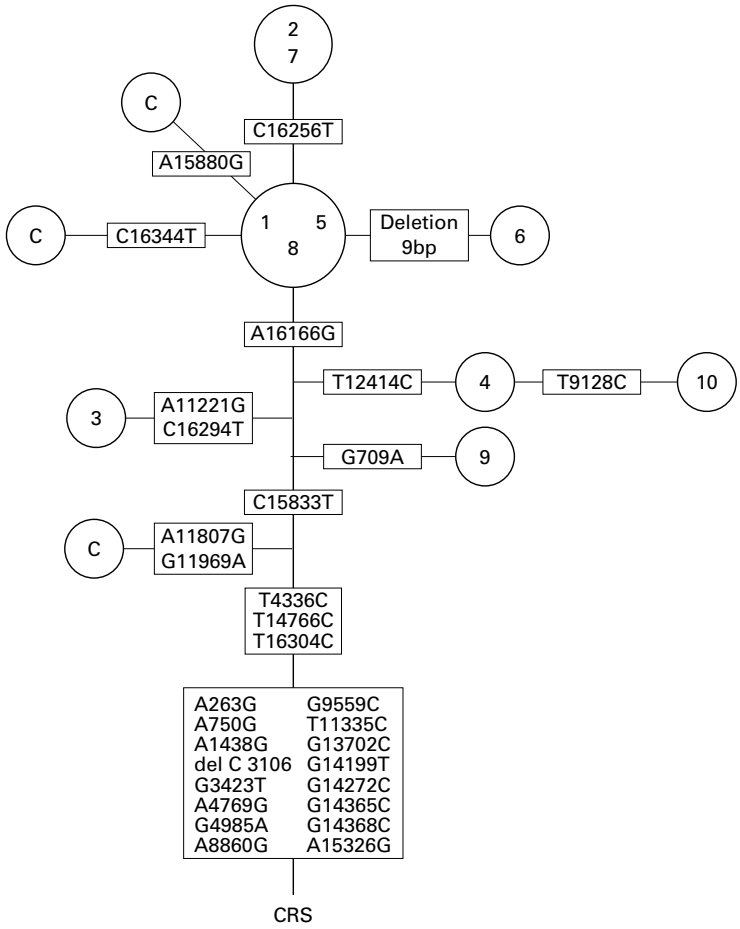

Figure 1 Phylogenetic network of patients and controls harbouring 4336A>G in mtDNA. The network was constructed on the basis of variations in the sequence of the coding region (nt 574-16023), and the polymorphisms in the HVS I (nt 16024-16400) were incorporated afterwards. The polymorphic variants are shown on the lines connecting the nodes. The symbol inside the node denotes patient (number) or control (C) identification. The mtDNA sequence was not determined in the three patients with AD. CRS=Cambridge reference sequence. The polymorphic variants shown in the box next to CRS include sequence errors in the CRS or rare polymorphisms.[20]

respectively, the complete sequences of which were known.[17] The amplified fragments were denatured at 95°C for five minutes and the heteroduplexes were subsequently allowed to anneal at 68°C for 30 minutes.

The polyacrylamide gel was prepared as described previously,[17] pre-electrophoresed for 30 minutes, and the heteroduplex samples were electrophoresed through it at a constant voltage of 400 V overnight at room temperature. After electrophoresis, the gel was stained on a glass plate in 150 μg/l of ethidium bromide for five minutes followed by destaining in water. It was then transferred to an UV transluminator and photographed (Grab-IT Annotating Grabber 2.04.7, UVP Inc, Upland, CA).

PCR fragments within the coding region and with differential mobility in CSGE, together with the hypervariable segment I (HVS I) in the D loop of the patients and the population samples, were analysed by automated sequencing (ABI PRISM™ 377 Sequencer using Dye Terminator Cycle Sequencing Ready Kit, Perkin Elmer, Foster City, CA) after treatment with exonuclease I and shrimp alkaline phosphatase.[18] The primers used for sequencing the coding region were the same as those used in the amplification reactions for CSGE. The HVS I was amplified in a fragment spanning nts 15714 and 16555 and the sequence was determined between nts 16024 and 16400.

Results

Eight middle aged patients in group 1 (1.4%) with various clinical phenotypes and three of the group 1 controls (0.63%) carried the 4336A>G mutation (table 1), this being most frequent among the patients with sensorineural hearing impairment and migraine. In addition, the mutation was found in occasional patients with diabetes mellitus or hypertrophic cardiomyopathy. The odds ratios (OR) were higher than unity for many patient groups (table 1), but calculation of the 95% confidence intervals suggested a significant increase only in the case of migraine and sensorineural hearing loss.

Five patients in group 2 (1.0%) but none of the controls harboured 4336A>G. The mutation was found in three patients with Alzheimer's disease, one with non-Alzheimer dementia, and one with Parkinson's disease, but it was not found among the 107 cognitively normal elderly subjects.

The mtDNAs harbouring the 4336A>G mutation belonged to haplogroup H. Furthermore, we determined the entire sequence in the mtDNA coding region and HVS I in 10 out of the 13 patients with 4336A>G, the three AD patients being excluded because of an insufficient amount of sample. The data were used to construct a phylogenetic network[19] (fig 1). There were eight mtDNA substitutions in the coding sequence that differed from the revised Cambridge reference sequence.[20] Three of the variants were silent mutations and three were common polymorphisms (709G>A in 12S rRNA, a 9 bp deletion in a non-coding region

Table 2 Evolutionary conservation of amino acids in ATPase subunit 6. The transition T to C at nt 9128 in patient 10 replaces the moderately conserved hydrophobic isoleucine (*) with hydrophilic threonine

										*									
Patient 10	I	F	T	I	L	I	L	L	T	**T**	L	E	I	A	V	A	L	I	Q
Human, *Homo sapiens*	I	F	T	I	L	I	L	L	T	**I**	L	E	I	A	V	A	L	I	Q
Chimpanzee, *Pan paniscus*	I	F	T	I	L	I	L	L	T	**V**	L	E	I	A	V	A	L	I	Q
Orangutan, *Pongo pygmaeus*	T	L	T	I	L	T	L	L	T	**I**	L	E	I	A	V	A	L	I	Q
Gorilla, *Gorilla gorilla*	I	F	T	V	L	I	L	L	T	**M**	L	E	I	A	V	A	L	I	Q
Dog, *Canis familiaris*	T	F	I	I	L	I	L	L	T	**I**	L	E	F	A	V	A	L	I	Q
Sheep, *Ovis aries*	T	F	I	I	L	I	L	L	T	**V**	L	E	F	A	V	A	M	I	Q
Mouse, *Mus musculus*	T	F	I	I	L	L	L	L	T	**I**	L	E	F	A	V	A	L	I	Q
White stork, *Ciconia ciconia*	T	A	S	I	L	L	L	L	T	**I**	L	E	V	A	V	A	M	I	Q
Frog, *Xenopus laevis*	T	S	I	V	L	F	L	L	T	**L**	L	E	I	A	V	A	M	I	Q
Latimeria, *Latimeria chalumnae*	T	T	L	V	L	F	L	L	T	**L**	L	E	I	A	V	A	M	I	Q
Fruit fly, *Drosophila melanogaster*	L	L	M	A	Q	I	A	L	L	**V**	L	E	S	A	V	A	M	I	Q

Table 3 Clinical features of the patients with 4336A>G

Patient	*Age*	*Gender*	*Clinical features**	*Family history*
1	47	M	HL, DM, HA	HL
2	45	F	HL, HA, PS, CD	HL
3	43	M	DM, MI	DM
4	31	M	CM, EP, HA, PS, CD	DM
5	42	F	MI	MI
6	45	M	MI	
7	42	F	HL	HL
8	48	M	HL	HL
9	43	F	PD	
10	66	F	Non-AD	
11	88	F	AD	
12	80	F	AD, DM, HL	
13	56	M	AD, DM	DM

AD, Alzheimer's disease; CD, cognitive decline; CM, cardiomyopathy; DM, diabetes mellitus; EP, epilepsy; HA, headache; HL, hearing loss; MI, migraine; non-AD, non-Alzheimer type dementia; PD, Parkinson's disease; PS, psychiatric symptoms.
*The phenotype that led to the identification of the proband is mentioned as the first clinical feature.

between nts 8272 and 8280, and 14766T>C in the cytochrome b gene). The remaining two substitutions, 4336A>G and 9128T>C, were considered rare variants. The 9128T>C mutation, found in a patient with frontal lobe dementia, leads to an isoleucine to threonine amino acid replacement in ATPase subunit 6, and this amino acid was found to be moderately conserved in evolution according to information from the Entrez-Protein data bank (http://www.ncbi.nlm.nih.gov). This site is almost invariably occupied by a hydrophobic aliphatic amino acid, the only exception being in *Gorilla gorilla*, where it contains methionine (table 2).

A 5 bp insertion between nts 956 and 965 in the 12S rRNA gene and 3397A>G in the *ND1* gene have been reported previously in patients with AD and 4336A>G.[5] Neither of these variants were found in our 10 patients harbouring 4336A>G.

The 13 patients harbouring 4336A>G were not clinically distinct, although six of them presented with more than one phenotype (table 3). Syndromic features were seen only in patient 4, who presented with hypertrophic cardiomyopathy, epilepsy, non-migrainous headaches, psychiatric symptoms, and cognitive decline. Age at the onset of the leading phenotype among the 13 patients did not differ from that among the patients without 4336A>G, but, interestingly, the age at onset for the patient with Parkinson's disease (patient 9) was 40 years.

Discussion

The 4336A>G mutation has previously been found at an increased frequency among patients with neuropathologically defined AD,[5–7] but other studies have failed to detect any difference in the frequency of this mutation between AD patients and controls[21] or have shown a decreased frequency.[22 23] We found 4336A>G in three out of 175 patients with AD (1.7%), whereas none of the 107 cognitively normal elderly subjects harboured the mutation. A total of 748 patients with AD have been screened for 4336A>G in the six previous studies and the present study combined, and 17 (2.3%, 95% confidence interval 1.20-3.34%) have been found to harbour the mutation, suggesting that the frequency of 4336A>G may be increased among patients with AD. Our results, moreover, show a similar finding among middle aged patients with phenotypes commonly associated with mitochondrial diseases, as patients with migraine harboured 4336A>G at a frequency of 4.8% and patients with matrilineal sensorineural hearing impairment at a frequency of 3.6%.

The frequency of 4336A>G among the controls, comprising middle aged Finnish blood donors, was 0.63%, which is similar to that reported for controls previously,[5 6] although this mutation has been found at frequencies as high as 2.0 to 3.8% in control samples from British,[23 24] German,[25] French-Canadian,[21] and US populations.[22] The variation in frequency found in the three studies with more than 200 subjects is 0.34-0.71%[5 6] (present study), whereas that in the six studies with fewer than 200 subjects[7 21–25] is much larger (0-3.8%), suggesting that a sampling error may be involved, although true differences between the populations cannot be ruled out. In the eight previous studies,[5–7 21–25] a total of 2751 subjects had been examined for 4336A>G, yielding 27 cases of the mutation (proportion of carriers 0.98%, 95% confidence interval 0.79-1.17%). The frequency of 4336A>G in the Finnish population may thus be lower than that in other white populations. Interestingly, we have previously found that a haplotype harbouring 5656A>G within haplogroup U is more than 30-fold more common among the Finns than elsewhere in Europe.[26]

The mtDNAs bearing 4336A>G belonged to haplogroup H, which is the most common European specific haplogroup, being found at an average frequency of 50%.[27] Since haplogroup H has been found less than three times among 1175 non-whites,[15] the lack of the 4336A>G mutation in Japanese patients[28] is not surprising. Sequence analysis of our patients and controls indicated that 4336A>G occurs together with 14766T>C and 16304T>C, suggesting that it occupies a specific branch in the phylogenetic network. Nine patients and 15 controls had also been characterised previously by the 16304T>C variant[6 22] or the corresponding *Rsa*I site loss or *Ava*II site gain at nt 16303,[5] suggesting that they also belong to the same branch. We determined the mtDNA sequence of the coding region and the HVS I segment in 13 samples with 4336A>G (10 patients and three controls), but although the samples belonged to 10 different haplotypes, the polymorphisms characterising the haplotypes were more peripheral in the network in every case, suggesting that 4336A>G had arisen earlier. Interestingly, one control[22] with 4336A>G had been found to harbour not 16304T>C but 16356T>C, a variant which characterises haplogroup U4[27] but which has also been found in a sample belonging to haplogroup H harbouring 16189T>C and 16223C>T.[29] Comparison of the latter haplotype with those in the 4336A>G network based on our samples nevertheless suggests that 4336A>G has indeed arisen at least twice in human evolution.

It has been suggested that 4336A>G may not contribute to the pathogenesis of AD in itself but may serve as a marker of a haplotype harbouring a pathogenic mutation. Our results showed, however, that the mtDNAs with 4336A>G harboured nine different substitutions, none of which was held in common. The additional substitutions included three polymorphisms in the HVS I, four in the protein coding sequence, one in the 12S rRNA gene, and one in a non-coding segment between the COX II gene and the $tRNA^{Lys}$ gene. Only 9128T>C in the ATPase6 gene was considered potentially pathogenic, as it was not found among 480 controls and sequence comparison showed that it changed a moderately conserved isoleucine to threonine. This substitution was found in a patient with frontotemporal dementia (patient 10), the clinical features of which did not conform to the clinical criteria for AD.

The patients with sensorineural hearing impairment or migraine and with 4336A>G were not clinically distinct. Interestingly, one of those with AD (patient 12) had insulin dependent diabetes mellitus and another (patient 13) had sensorineural hearing impairment. Both of these disorders are common phenotypes of mitochondrial disorders.[30] Patient 10 was diagnosed with clinically typical frontotemporal dementia at the age of 66 years. Apathy and reduced speech were the main symptoms at onset and single photon emission computed tomography with ^{99m}Tc-hexamethylpropyleneamine oxime as the tracer isotope showed left temporoparietal hypoperfusion. Patient 9, with PD and harbouring 4336A>G, was clinically unremarkable except for the early onset.

Previous studies have implied a role for 4336A>G in late onset neurodegenerative diseases. We found that the frequency of 4336A>G in the $tRNA^{Gln}$ gene was significantly higher in patients with matrilineal sensorineural hearing impairment or migraine than in the controls, suggesting that the mutation may be involved in diseases already manifest in middle life. 4336A>G was the only mtDNA variant that was common to the patients, and it could therefore have a causal role in sensorineural hearing impairment or migraine and is not simply a marker linked to another, more significant mtDNA mutation.

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Department of Paediatrics, University Hospital of Innsbruck, Austria
E Haberlandt
H Fischer
P Heinz-Erian
T Müller

Institute of Medical Biology and Human Genetics, University of Innsbruck, Schöpfstrasse 41, 6020 Innsbruck, Austria
J Löffler
G Utermann
A R Janecke

Departments of Hearing, Speech and Voice Disorders/ENT, University Hospital of Innsbruck, Austria
A Hirst-Stadlmann

Department of Orthopaedics, University Hospital of Innsbruck, Austria
B Stöckl

Institute of MR Imaging and Spectroscopy, University Hospital of Innsbruck, Austria
W Judmaier

Department of Otolaryngology, University of Iowa Hospitals, Iowa City, USA
R J H Smith

Correspondence to:
Dr Janecke,
Andreas.Janecke@uibk.ac.at

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Split hand/split foot malformation associated with sensorineural deafness, inner and middle ear malformation, hypodontia, congenital vertical talus, and deletion of eight microsatellite markers in 7q21.1-q21.3

Edda Haberlandt, Judith Löffler, Almut Hirst-Stadlmann, Bernd Stöckl, Werner Judmaier, Helmut Fischer, Peter Heinz-Erian, Thomas Müller, Gerd Utermann, Richard J H Smith, Andreas R Janecke

EDITOR—The split hand/split foot malformation (SHFM, MIM 183600) is a central reduction defect of the hands and feet and occurs both as an isolated malformation and as part of several syndromes including the EEC syndrome (MIM 129900). We report on a 2 year old boy with SHFM associated with features of ectodermal hypoplasia, a submucous cleft palate, congenital vertical talus, malformations of the middle ear, profound sensorineural hearing loss resulting from Mondini dysplasia, and a de novo deletion of the paternal chromosome 7q21.1-q21.3. This patient with syndromic SHFM represents a case of atypical EEC syndrome, but also displays abnormalities previously not associated with SHFM or EEC syndrome.

The classical features of the autosomal dominant inherited EEC syndrome are ectrodactyly, ectodermal dysplasia, and clefting of the lip/palate. In most patients, there are additional anomalies typically affecting the urogenital and lacrimal systems.[1 2] Some patients also have dysmorphic facies, a tendency to infectious disease, endocrine disorders, and mental retardation. This phenotypic variability has become increasingly apparent over the last 15 years[3 4] and numerous related and overlapping syndromes have been delineated by many investigators.[5] In an attempt to clarify classification, major and minor criteria for the diagnosis of EEC syndrome have been elaborated.[3 4]

Dominant inheritance of EEC has been documented in several large multigenerational

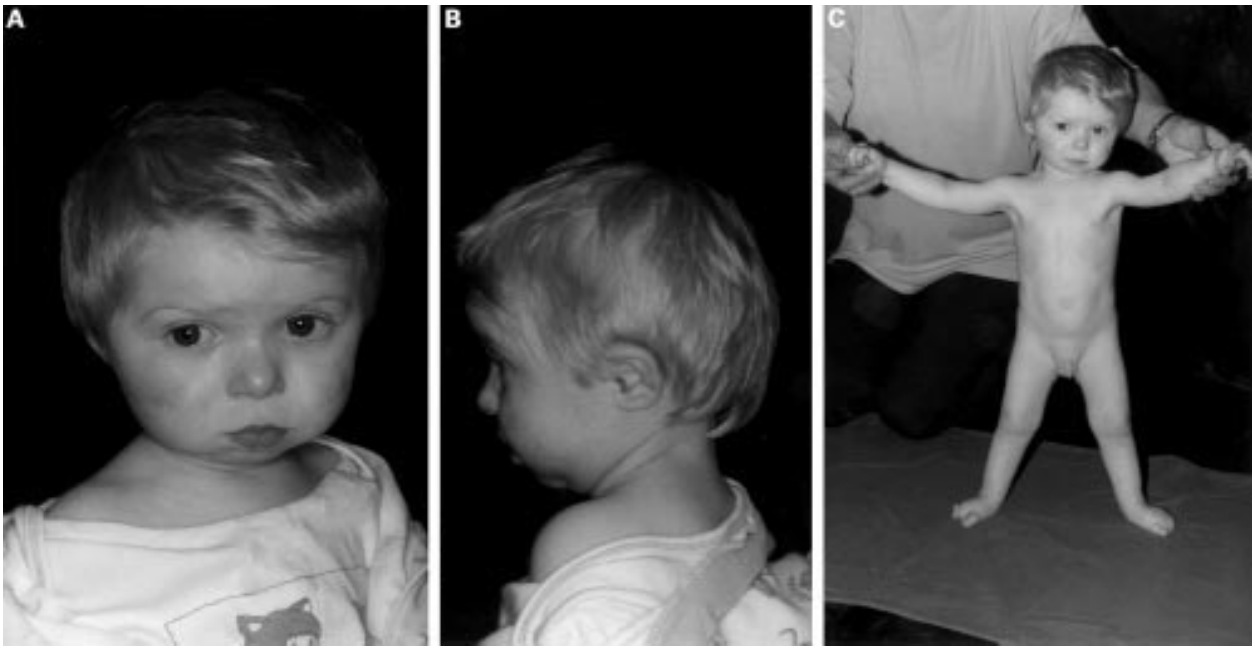

Figure 1 The proband aged 18 months. (A, B) Note facial dysmorphism (see text). (C) He cannot stand unsupported.