RPGR mutation associated with retinitis pigmentosa, impaired hearing, and sinorespiratory infections

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J. Med. Genet. 2003;40;609-615
doi:10.1136/jmg.40.8.609

LETTERS TO JMG

Characterisation of deletions of the *ZFHX1B* region and genotype-phenotype analysis in Mowat-Wilson syndrome

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J Med Genet 2003;**40**:601–605

In 1998, Mowat *et al*[1] delineated a syndrome with Hirschsprung disease (HSCR) or severe constipation, microcephaly, mental retardation, and a distinctive facial appearance.[1] Because two of the patients had a cytogenetically visible deletion of 2q22-q23,[1 2] and all patients were sporadic cases, a contiguous gene syndrome or a dominant single gene disorder involving this locus were suggested.[1] Two similar patients with cytogenetically balanced translocation t(2;13)(q22;q22) and t(2;11)(q22.2;q21), respectively, allowed Wakamatsu *et al*[3] and Cacheux *et al*[4] to narrow down the critical interval to 5 Mb and to one single gene respectively, which led both groups independently to the detection of intragenic mutations in the gene coding for Smad interacting protein-1 (formerly *SIP1*, now called zinc finger homeobox 1B (*ZFHX1B*)) in patients with so called "syndromic HSCR". However, because HSCR is not an obligatory symptom and patients with and without HSCR can be recognised by other features, especially their distinct facial gestalt,[5 6] we suggested that "Mowat-Wilson syndrome" (MWS) is a more appropriate name.[6]

Although the developmental *ZFHX1B* expression pattern fully explains the clinical spectrum observed in patients with Mowat-Wilson syndrome by haploinsufficiency of this gene alone,[5 7] Wakamatsu *et al*[3] initially stated that their deletion patient would have a more severe phenotype and therefore would have a contiguous gene syndrome. Amiel *et al*[8] reported that the phenotype was similar in patients with "syndromic HSCR" caused by mutations and cytogenetically non-visible large scale deletions of the *ZFHX1B* locus, respectively, but the deletion sizes were not delineated. We therefore analysed deletion size and genotype-phenotype correlation in four new patients with cryptic deletions of the *ZFHX1B* locus.

Key points

- Mowat-Wilson syndrome (MWS) is a distinct multiple congenital anomalies-mental retardation syndrome characterised by severe mental retardation, recognisable facial gestalt, pre- or postnatal microcephaly, and postnatal growth retardation, as well as seizures (82%) and malformations such as Hirschsprung disease (67.6%), congenital heart defects (47%), and agenesis of the corpus callosum (35%), caused by mutations or large scale deletions of the *ZFHX1B* gene in 2q22.
- Deletion sizes and breakpoints in Mowat-Wilson syndrome patients vary widely from 300 kb to at least 11 Mb, thus ruling out a true microdeletion syndrome.
- So far parental origin has only been determined in four patients and has always been paternal.
- In general, patients with deletions are very similar to those with truncating mutations. There was no correlation between the phenotype and size of deletion up to 5 Mb. However, one patient with a larger deletion (~11 Mb) had early seizures with a lethal course and hypoplasia of the big toes as additional features.

MATERIALS AND METHODS

Patients

The diagnosis of Mowat-Wilson syndrome was made in patients 3 and 4 (fig 1C) because of HSCR and associated features and in patients 1 and 2 because of mental retardation associated with the distinct facial gestalt (fig 1A, B) in the absence of HSCR. Clinical details are provided in table 1; patient 2 will be described in more detail elsewhere.[9]

METHODS

Conventional chromosome analysis was performed from cultivated peripheral blood cells after GTG and CBG banding at a 550-850 band level[10] according to standard protocols. FISH analysis was performed with directly labelled BAC probes on metaphase spreads as described previously.[11] In patient 3 and his parents, additional polymorphic markers D2S2184 (location 147.5 Mb), D2S2335 (147.2 Mb), D2S2277 (148.3 Mb), D2S2324 (148.6 Mb), D2S2275 (150.3 Mb), D2S2299 (152.2 Mb), D2S2241 (153.0 Mb), and a newly created marker from the *ZFHX1B* locus were analysed with an ABI 310 capillary sequencer as described previously.[11] The *ZFHX1B* marker is localised between bp 149003 and 149221 of BAC RP11-107E5 and was amplified with the following primers: ZFHX1Bms1f-gctgcagtagttgcctttga and ZFHX1Bms1r-gtcctttcgaggtccagttg.

RESULTS

Results of FISH and marker analysis are shown in fig 2. In patient 3 the distal border of the deletion was determined with polymorphic markers, which showed a distal breakpoint between markers D2S2275 (150.3 Mb) and D2S2299 (152.2 Mb), and a paternal origin of the deletion. Deletions were of differing sizes, approximately 300 kb in patient 4, 700 kb in patient 2, 5 Mb in patient 1, and 11 Mb in patient 3. The mothers of patients 2 and 4 and both parents of patient 3 were available for FISH analysis, which showed normal results. The phenotype observed in patient 3, with the largest deletion, showed early seizures, hypoplastic big toes, and premature death at the age of 4 months as additional features (table 1).

DISCUSSION

Our results indicate that deletion sizes and breakpoints in Mowat-Wilson syndrome patients vary widely, ruling out a true microdeletion syndrome with recurring breakpoints mediated by low copy repeat regions. There was generally no obvious correlation between the phenotype and the size of the deletion and the phenotypic spectrum was similar to that observed in patients with truncating mutations within *ZFHX1B* (table 1). The only remarkable difference was noticed

Figure 1 (Top row) Facial appearance of patient 1 in early childhood (A) and aged 8 years (B), and of patient 4 aged 10 years (C). (Bottom row) Note similarity to patients with *ZFHX1B* point mutation reported elsewhere.[6] (D, E) Patient with nt553-554insTG mutation aged 6 months and 6 years 10 months, respectively. (F) Patient with nt1892delA mutation aged 3 years 10 months.

in patient 3 with the 11 Mb deletion, who presented with seizures much earlier, had marked hypoplasia of the big toes, and who died in early infancy. Thus, genes within the close vicinity of the *ZFXH1B* gene seem not to be subject to gross haploinsufficiency. Parental origin was only determined in one of the present and three published patients,[3 8] and was of paternal origin in all cases investigated. As all investigated patients had HSCR and congenital heart defects, it is not possible to draw any conclusion about these symptoms, but agenesis of the corpus callosum was present and absent in two patients each, and thus shows no correlation with parental origin of the deletion. Similarly, the early onset of seizures in patient 3 is also not attributable to the parental origin of the deletion.

The most frequently observed major malformation in Mowat-Wilson syndrome is HSCR, which occurred in 21 of 30 (70 %) patients reported so far (table 1). As has been described for patients with *ZFHX1B* truncating mutations, two of our patients with deletions of approximately 700 kb and 5 Mb, respectively, did not have HSCR, while the two with the smallest and largest deletion (300 kb and 11 Mb deletions, respectively) did have it. Thus, our results suggest that the manifestation of HSCR is not influenced by deletion size. As *ZFHX1B* knockout mice also do not exhibit HSCR,[12] a non-allelic modifier might contribute to the manifestation of HSCR. The high rate of HSCR in humans is probably the result of recognition bias, as in our cohort (four patients reported earlier[6] and the present four patients) HSCR occurs in only 50%. Less frequent malformations include various congenital heart defects (for example, septal defects, pulmonary stenosis, or atresia), agenesis of the corpus callosum, urogenital anomalies, talipes, and strabismus.

Similarly, there is no difference in degree of mental retardation, facial appearance, and growth parameters. Regardless of the underlying defect, which may be a truncating mutation in *ZFHX1B* or a large scale deletion, psychomotor retardation is severe with a mean walking age of 4-5 years and speech starting at the age of 5-6 years, being restricted to single words. Personality is generally happy and affectionate. Although shortness of stature and low weight are characteristic in school age children, birth measurements are usually normal or even in the upper normal range. Only microcephaly was already evident at birth in eight out of 19 patients with reported measurements (table 1), and it has a tendency to occur before the decline of body length in our patients. Therefore, our findings do not support the initial statement by Wakamatsu *et al*[3] about the more severe phenotype in their deletion patient. Nevertheless, severe cerebral atrophy remains remarkable in this patient, but might be related to the other translocation breakpoint on chromosome 13.

A seizure disorder with varying age of onset is a very common feature which is found in 82% of all 34 patients (table 1). Severe neonatal seizures, however, have been reported only in our patient 3 and a patient with a cytogenetically visible deletion.[2] Thus a gene(s) responsible for early seizures with a lethal course and hypoplastic big toes might be located between BAC RP11-207O14 at 145.3 Mb and marker D2S2299 at 152.2 Mb where at least one gene related to epilepsy, *CACNB4* (OMIM 601949), is known to be located. However, detailed analysis in further patients is required for confirmation of this putative association.

The characteristic facial appearance was evident in all patients with deletion or truncating mutations and allows the distinction between Mowat-Wilson syndrome and other types of "syndromic HSCR" such as Goldberg-Shprintzen syndrome. The facial features are probably diagnosable in the neonatal period in the presence of HSCR, but the sunken eyes, broad, flared eyebrows, pointed nasal tip, short philtrum, and upturned ear lobes become more obvious in early childhood. Of 12 patients with the distinct facial gestalt of Mowat-Wilson

Table 1 Phenotype of previously published patients with Mowat-Wilson syndrome and mutation, deletion, or translocation breakpoint in the *ZFHX1B* gene and of the present patients

	MWS patients with mutations (n=22)] [1 3-6 8 14 15]	BP in *ZFHX1B* IVS2 [4 15]	Del[2]	Del[1]	Del[3 5 16]	Del [8]	Pat 1	Pat 2	Pat 3	Pat 4
Gender	16 M, 6F	1 M	1 M	1 M	1 F	1 M, 3 F	F	M	M	M
Sporadic occurrence	22	+	+	+	+	+	+	+	+	+
Birth length (centile)	5 × >90th 4 × >50th 4 × >10th 1 × rd3	50th	?	50th	?	?	?	10th	5th	90th
Birth weight (centile)	4 × >90th 9 × 50th 4 × >10th	90th	90th	50th	50th	?	50th	90th	60th	90th
OFC at birth (centile)	4 × >25th 4 × 10th-25th 8 × <3rd	50th	<3rd	25th	<3rd	?	<3rd	3rd	20th	?
Age at last investigation	13 mth-23 y	6 y	2y 6 mth	3 y	6 y	?	10 y	8 y 7mth	5 wks	13 y
Length (centile)	1 × 75th 2 × 10th-25th 9 × <3rd	<<3rd	3rd-10th	50th	<3rd	?	<10th	3rd	50th	?
Weight (centile)	4 × <3rd 5 × 3rd-10th 3 × <3rd	<3rd	10th	<3rd	<3rd	?	3rd	10th	50th	?
OFC (centile)	3 × 10th-50th 9 × <3rd	<3rd	<<3rd	3rd	<<3rd	?	<3rd	<3rd	3rd	40th
Microcephaly	18	+	+	+	+	4	+	+	+	-
Severe mental retardation	22	+	+	+	+	4	+	+	+	+
Walking at age	2-8 y Average 4-5 y	6 y	-	-	-	?	6 y	9 y	-	2½ y
Personality	Predominantly happy	?	?	Happy	?	Happy	Affectionate, happy	Happy	N/A	Affectionate, happy
Seizures	16	Febrile	Neonatal	2 y	11 mth	In 4	7 y	Childhood	Neonatal	4 y
Hypotonia	7	+	?	?	?	?	- Spasticity in lower limbs	+	+	?
Pes planus with calcaneovalgus	6	+	?	+	?	?	-	+	Hypoplasia of big toes	Walks on tiptoes
Agenesis of corpus callosum	5	+	?	+	- Cerebral atrophy	3	- MRI normal	+ Colpocephaly	-	+
Hirschsprung disease	13	+	+	+	+ SS	4 2SS, 1LS	-	-	+	+
Constipation	5						+	-		
Congenital heart disease	7	-	Ast, PDA, VSD, ASD	-	PDA	4	ASD	-	PFO, PS	VSD, PS
Renal anomalies	4	-	-	Duplex kidney	-	0	-	-	-	-
Hypospadias	3	+	-	+	?	1		+	Micro-phallus	+
Cryptorchidism	6		-		?			+	+	-
Cleft palate/lip	1 submucous	-	-	-	-	0	-	-	-	-
Ptosis	0	-	-	-	-	0	-	-	-	-
Iris coloboma	0	-	-	-	-	0	-	-	-	-
Strabismus	9	+	-	+	+	3?	-	+	?	+
Hypertelorism	20	+	-	+	+	2 of 2	+	+	+	+
Facial gestalt	All MWS	MWS	MWS	MWS	MWS	MWS	MWS	MWS	MWS	MWS
Deletion or translocation of 2q22		t (2;11) (q22.2;2q21)	del (2)(q22q23)	del (2) (q21q23)	5 Mb	Size?	5 Mb	700 kb	11 Mb	300 kb
ZFHX1B mutation (truncating)	1 × exon 3 1 × exon 5 1 × exon 7 1 × exon 10 2 × exon 6 16 × exon 8									

BP: breakpoint; IVS2: intron 2 ; colpocephaly: enlargement of the occipital horns of the ventricular system owing to an underdevelopment of the white matter in the posterior cerebrum[17]; Ast: aortic stenosis; ASD: atrial septal defect; PDA: persistent ductus arteriosus; VSD: ventricular septal defect; PFO: persistent foramen ovale; PS: pulmonary stenosis; SS: short segment HSCR; LS: long segment HSCR; VUR: vesicoureteric reflux. In deletion patients age of onset of seizures is given.

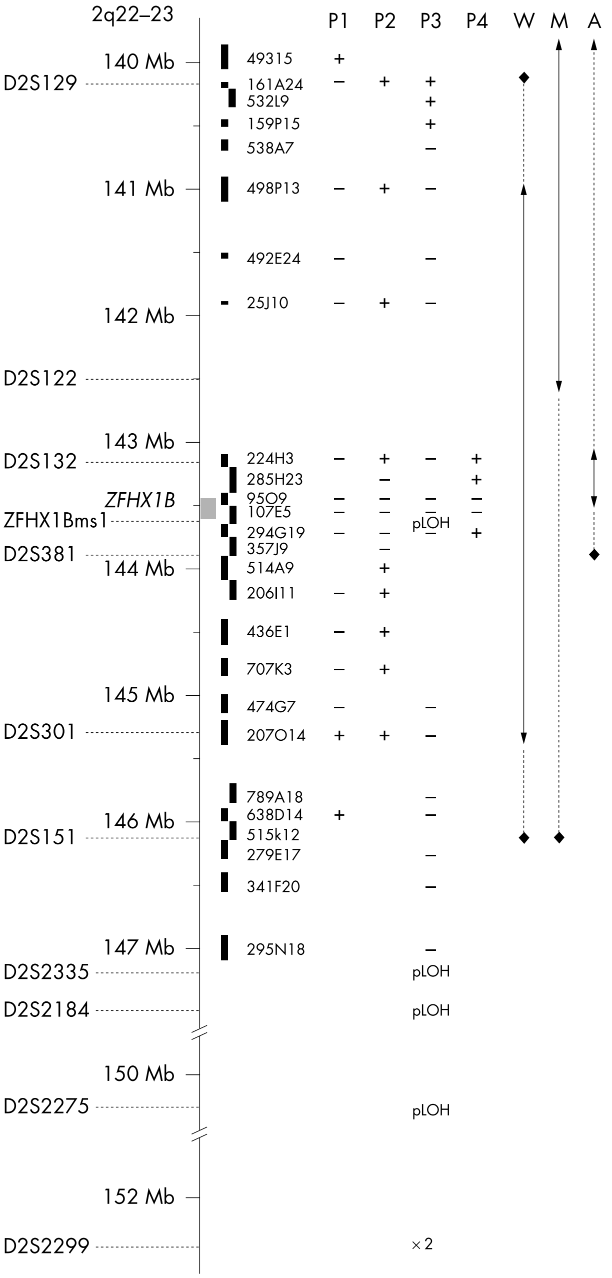

Figure 2 Results of FISH analysis with several BAC clones (RP11 library) in present patients P1-P4, informative results of analysis of polymorphic markers in patient 3, and deletion size in published cases (W = Wakamatsu *et al*,[3] M = Mowat *et al*,[1] A = S.203 from Amiel *et al*[8]). – lack of signal on one chromosome 2; + regular signals on both chromosomes 2; × 2: two alleles; pLOH: non-transmission of a paternal allele.

syndrome analysed in our laboratory so far (data not shown), eight had truncating mutations and four had large scale deletions, thus giving a *ZFHX1B* defect in 100% of patients and a deletion rate of 33%. However, it is possible that less severe cases are being missed following the work of Yoneda *et al*,[13] who described an atypically mild phenotype with late adult onset severe constipation and mild mental retardation in the absence of specific facial anomalies, seizures, and other malformations owing to a non-truncating 3 bp in frame deletion.

It seems likely that more patients will soon be described with Mowat-Wilson syndrome now that the clinical features are becoming increasingly recognised by clinical geneticists and in time it will be possible to elucidate the true clinical spectrum.

ACKNOWLEDGEMENTS

We thank Michaela Kirsch and Leonora Bille for their excellent technical assistance.

Data access. BAC position was obtained from map viewer at http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?org=hum&chr=2. Position of polymorphic markers within the sequence map were obtained from uniSTS at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists.

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Molecular study of three cases of odontohypophosphatasia resulting from heterozygosity for mutations in the tissue non-specific alkaline phosphatase gene

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J Med Genet 2003;**40**:605–609

Hypophosphatasia is an inherited disorder characterised by defective bone and tooth mineralisation and deficiency of serum and bone alkaline phosphatase activity. The bone symptoms are highly variable in their clinical expression and range from stillbirths without mineralised bone to pathological fractures developing only late in adulthood.[1] Odontohypophosphatasia is characterised by premature exfoliation of fully rooted primary teeth and/or severe dental caries, often not associated with abnormalities of the skeletal system.[2 3] The anterior deciduous teeth are more likely to be affected and the most frequently lost are the incisors.[4] Dental *x* rays show reduced alveolar bone and enlarged pulp chambers and root canals.[2 4] Although the only clinical feature is dental disease, biochemical findings are generally indistinguishable from those in patients with mild forms of hypophosphatasia (adult and childhood). While perinatal hypophosphatasia and infantile hypophosphatasia are transmitted as an autosomal recessive trait, both autosomal recessive and autosomal dominant transmission may be found in childhood, adult, and odontohypophosphatasia.[3 5–9] The distinction between recessive and dominant transmission may be difficult to determine conclusively by using familial analysis because expression of the disease is very variable, with parents of even severely affected children showing no or extremely mild symptoms of the disease.[9 10]

The tissue non-specific alkaline phosphatase (TNSALP) is a phosphomonoesterase anchored at its carboxyl terminus to the plasma membrane by a phosphatidylinositol-glycan moiety.[11] The enzyme cleaves extracellular substrates pyridoxal-5′-phosphate (PLP), phosphoethanolamine (PEA), and inorganic pyrophosphates (PPi). Its exact function in bone and dental mineralisation is still unclear but probably involves hydrolysis of Ppi[12] and perhaps mammalian specific activities such as collagen and calcium binding.[13]

The *TNSALP* gene is localised on chromosome 1p36.1[14] and consists of 12 exons distributed over 50 kb.[15] More than 127 distinct mutations have been described in the *TNSALP* gene,[16–33] in a relatively small number of North American, Japanese, and European patients,[34] indicating a very strong allelic heterogeneity in the disease. Most of them (82%) were missense mutations. This variety of mutations results in variable clinical expression even among the severe or moderate types. We report here the study of *TNSALP* gene mutations in three patients affected by odontohypophosphatasia and provide evidence that heterozygosity may produce clinical signs and symptoms that appear to be very variable in expression.

Key points

- Hypophosphatasia is an inherited disorder characterised by defective bone mineralisation and deficiency of tissue non-specific alkaline phosphatase (TNSALP) activity. We report here the molecular study of three cases of odontohypophosphatasia where the disease was the result of heterozygosity for *TNSALP* gene mutations.
- Three mutations were found, 323C>T (P91L), 346G>A (A99T), and 1240C>A (L397M). The mutation P91L has not been previously described and site directed mutagenesis experiments showed that it corresponded to a severe allele.
- In one family, the proband's mother carried the mutation P91L and was affected. In the two other families, dominant transmission was more difficult to determine, owing to variable expression of the disease in carriers, ranging from hypophosphatasaemia only to periodontal disease associated with multiple fractures.
- We show here that the convergence of clinical, biochemical, and molecular results may help to affirm the dominant effect of *TNSALP* mutations. Analysis of a 3D model of *TNSALP* indicated that residues affected by these mutations were located near the active site or in the mammalian specific crown domain, corroborating the functional effect of these mutations. This is consistent with the dominant effect of these mutations and the allosteric properties of the enzyme.

MATERIAL AND METHODS

Patients

Patient 1

The proband was a 9 year old boy affected by Down syndrome and odontohypophosphatasia. Loss of seven deciduous teeth,

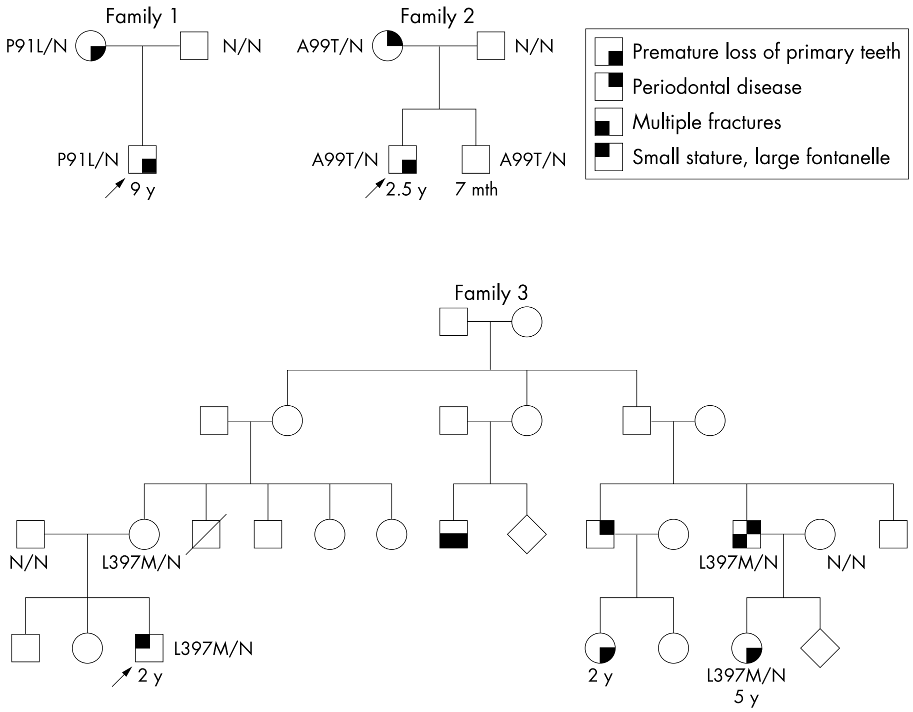

Figure 1 Pedigree data and mutation analysis results in the three families with odontohypophosphatasia. The arrow indicates the proband. N = normal allele as determined by the absence of any detectable mutation.

mostly incisors, began at the age of 2 years. Serum alkaline phosphatase was low (80 U/l, normal range >100). *X* rays showed normal growth plate development and normal long bones without evidence of fractures or rickets. The 39 year old mother of the proband had lost her permanent teeth. Her serum alkaline phosphatase level was low (18 U/l, normal range 30-120). The father did not show any symptoms and had a normal level of serum alkaline phosphatase (93 U/l, normal range 30-120).

Patient 2

The proband was a 2 year old male who has lost three teeth and was referred to the genetics department by his dentist. Serum alkaline phosphatase was low (64 U/l, normal range 100-320) and urinary phosphoethanolamine was high (583 µmol/g creatinine, normal range <350). The parents deny any problem with multiple fractures, bowing of legs, or loss of teeth but the 38 year old mother reported being affected by an unusual number of dental cavities and having had numerous treatments of dental root canals. Her serum ALP was low (29 U/l, normal range 47-137) while serum ALP of the proband's father was normal (66 U/l, 47-137). A second child was born in July 2002. At nearly 7 months of age, this baby boy has not shown any symptoms of hypophosphatasia to bring him to clinical attention.

Patient 3

The proband was a 2 year old boy diagnosed with hypophosphatasaemia. Serum alkaline phosphatase was repeatedly low (84 and 86 U/l, normal range 104-345) and urinary phosphoethanolamine was high (935 µmol/g creatinine, normal range 108-533). He had very slight leg bowing and was just below the 5th centile for height. The mother had a low ALP level (32 U/l, normal range 30-107) and high PEA (138 µmol/g creatinine, normal range 20-100). The father showed normal ALP and PEA levels. The proband's 5 year old maternal second cousin was diagnosed with hypophosphatasia because of early loss of primary teeth and low serum ALP (92 U/l, normal range 108-317). Her skeletal survey and growth/stature were normal for age. Her father had early loss of teeth, multiple caries, and had had four fractures. Another second cousin could not be tested but was reported to have lost her primary teeth at the age of 2.

METHODS

Primer sequences of the 12 *ALPL* gene exons have been previously reported[24] and allowed analysis of the whole coding sequence, including intron-exon borders and untranslated exons. PCR reactions were performed and analysed as previously described.[24] Site directed mutagenesis of the mutation P91L was performed with the Quikchange Site Directed Mutagenesis kit (Stratagene). Mutated and wild type plasmids were transiently transfected in COS-1 cells using the Lipofectamine PLUS reagent (Life Technologies) according to a methodology described previously.[9 26] The mutations were put into a 3D model of the TNSALP molecule[13] by using the molecular visualisation program RasMol (R. Sayle, Glaxo Research and Development, Greenford).

RESULTS

Pedigree data and mutation analysis results are shown in fig 1. In family 1, the proband's mother was affected by early loss of teeth, suggesting that the disease could not be put down to the proband's Down syndrome condition only[35] and that the disease was dominantly inherited.

Sequencing of the *ALPL* gene showed that the patient and her affected mother carried a 323C>T nucleotide substitution resulting in the missense mutation P91L (fig 2). The presence of this mutation in the affected parent and the absence of any other detectable mutation in the patient is consistent with dominant inheritance. The P91L mutation has not previously been reported in hypophosphatasia patients. We therefore introduced it into the expression plasmid pcDNA-3 by site directed mutagenesis and transfected COS-1 cells with the mutant plasmid. We found that the mutation exhibited 0.4% of wild type activity, that is, no or very low residual enzymatic activity, suggesting that this mutation is not a polymorphism and corresponds to a severe allele.[26] In family 2, we found in

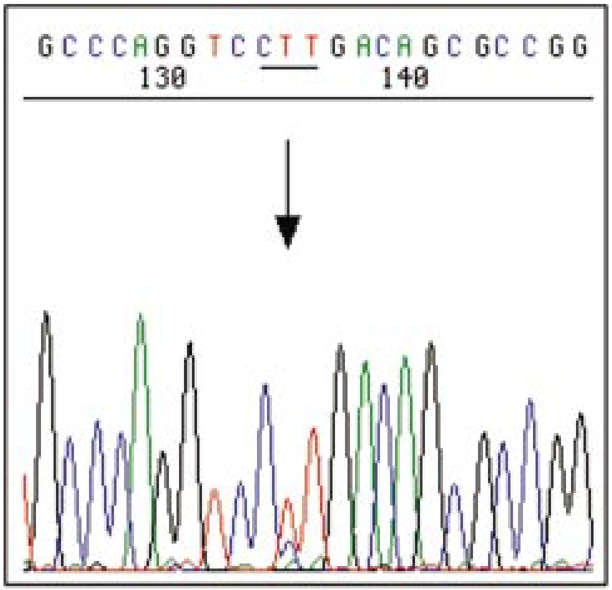

Figure 2 DNA sequencing electropherogram of the proband in family 1 showing the newly identified mutation P91L. The arrow indicates the position of the nucleotide substitution CCT (proline) > CTT (leucine).

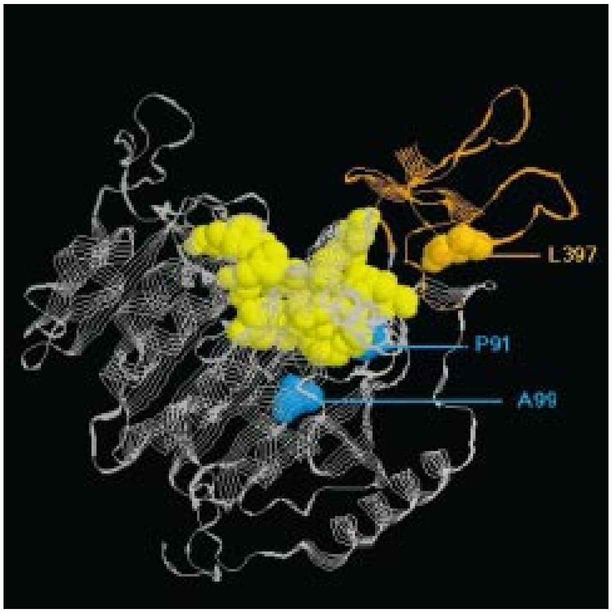

Figure 3 Location of the residues affected by the mutations P91L, A99T, and L397M in the 3D model. The active site is shown in yellow. The crown domain is shown in orange.

the proband the heterozygous nucleotide substitution 346G>A resulting in the missense mutation A99T. The mother reported being affected by dental problems. She showed a low level of serum ALP and carried the mutation A99T, while the father exhibited normal serum ALP and no ALPL gene mutation. The second child, born in July 2002, was prenatally found to be heterozygous for the A99T mutation. Although the mother did not exhibit the typical signs of odontohypophosphatasia (loss of teeth), these results suggest that in this family, heterozygosity for A99T resulted in clinical symptoms but that the disease was minimally expressed in the mother. The mutation A99T was previously described in a large family with dominant odontohypophosphatasia[8] and site directed mutagenesis and transfections of COS cells previously showed that A99T does not allow any significant in vitro residual activity and shows a negative dominant effect.[9] In family 3, the proband did not show any symptoms of hypophosphatasia and was referred to the genetics department because of hypophosphatasaemia. We found the heterozygous substitution 1240C>A resulting in the missense mutation L397M of maternal origin. Interestingly, the proband's second cousin and this cousin's father were affected by odontohypophosphatasia and carried the L397M mutation found in the proband. Exhaustive sequencing of the *ALPL* gene of the affected cousin did not show evidence of any other mutation. This suggests that in this family, heterozygotes for L397M may be affected by the disease and that its expression was subject to intrafamilial heterogeneity. The L397M mutation was previously reported by Mumm *et al*,[32] associated with the D277A mutation, in a patient affected by perinatal hypophosphatasia. This suggests that, like P91L and A99T, L397M is a severe allele. We finally concluded that the disease in these three families resulted from heterozygosity for a severe hypophosphatasia allele.

Localisation of the mutated residues in the 3D model of *TNSALP* based on the placental ALP structure[13] showed that L397M is located in the crown domain, a mammalian specific region observed for the first time in the placental alkaline phosphatase structure and containing a collagen binding loop[13] (fig 3). Alanine 99 is located in an alpha helix running from the active site to the surface of the molecule near the homodimer interface and supporting D92, S93, and A94, three residues of the active site involved in phosphate binding. By disturbing this helix, mutation A99T could therefore affect the active site. Proline 91 is in contact with the active site and there is no doubt that the change of this residue for leucine has an important effect on the catabolic activity. Thus, the study of the 3D model suggests that these mutations alter the function of the enzyme rather than have a structural effect resulting in the degradation of the molecule. This is consistent with the dominant effect of these mutations and the allosteric properties of the enzyme.[36]

DISCUSSION

Considerable variation occurs in the clinical expression of severe forms of hypophosphatasia, owing to the considerable allelic heterogeneity of the *ALP* gene.[33] Moderate forms of hypophosphatasia, especially odontohypophosphatasia, are not as well documented. Compared to bone forms of hypophosphatasia, only a few mutations responsible for odontohypophosphatasia have been published[8 22 28 31] (this study), but they suggest that similar variation exists in these forms, at both the clinical and genetic levels. In family 2, the patient was found to be heterozygous for the A99T mutation, a mutation also found in a large family with dominant hypophosphatasia.[8] The probands from the previously reported family were a 6 year old girl and her fraternal twin brother, both affected with premature loss of anterior teeth at the age of 3.5, and abnormal urinary PEA and serum PLP values. In addition to premature loss of teeth, the probands were affected by very slight bone symptoms, such as thin cortical bone of the cranium and multiple radiolucent spots in the cranial bones, but no additional skeletal abnormalities. In this article, the authors point out the intrafamilial clinical heterogeneity of the disease in carriers of A99T since the clinical signs were evident in eight carriers of the mutation and absent in subjects without the mutation and in five carriers. This intrafamilial heterogeneity was also observed in family 3 where the carriers of L397M showed variable expression of the disease, ranging from only hypophosphatasaemia to periodontal disease associated with multiple fractures. However, the absence of clinical symptoms in the proband could be because of his still young age (2 years), although another second cousin was reported to have lost her primary teeth at the age of 2 (fig 1). Finally, this report confirms that moderate forms of hypophosphatasia are also highly variable in their clinical expression, owing to allelic heterogeneity but also to other factors that remain to be determined, such as other sequence variations in the ALPL gene, a *trans* effect of other genes, or environmental factors.

Dominant transmission of moderate forms of hypophosphatasia has been documented in a few families.[3 5–9] We report here the case of one additional family with dominant odontohypophosphatasia (family 1) and two others in which heterozygotes for a *TNSALP* gene mutation show clinical symptoms, however variable in expression. In our experience, we failed to detect a second mutated allele in 18% of our hypophosphatasia patients, 70% of them being affected by moderate (childhood, adult, or odonto-) hypophosphatasia (E Mornet, unpublished data). In some of these patients, mutations of the *ALPL* gene may have not been detected because of their location in intronic or regulatory sequences, or because they correspond to large deletions undetectable by the methodology routinely used here. In others, however, the disease may be the result of heterozygosity and no other mutation needed to be sought. Analysis of the transmission of the odontohypophosphatasia phenotype, together with serum ALP level and presence or absence of the mutation, may help to distinguish between the two situations.

The mechanism of dominance remains unclear but probably involves interactions between monomers of the dimeric structure that disturb the allosteric properties of TNSALP. We and others have previously reported that some *ALPL* gene mutations result in a dominant negative effect owing to complete or partial inhibition of the normal monomer by the mutated monomer in the dimeric molecule.[7 9] Here, we show that residues mutated in these families are localised in the vicinity of functional regions such as the active site and the crown domain, suggesting that they may have a functional role. This is consistent with the expected localisation of mutations resulting in an inhibitory effect.

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(-195T, L-12X, 298-2G, T117 N, A159T, R229S, 997+2A, E274X, A331T, H364R, D389G, 1256delC, R433H, N461I, C472S) in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in patients with hypophosphatasia. *Hum Mutat* 2000;**15**:293.

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RPGR mutation associated with retinitis pigmentosa, impaired hearing, and sinorespiratory infections

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J Med Genet 2003;**40**:609–615

Retinitis pigmentosa (RP) is a progressive retinal degeneration that affects about 1 in 4000 of the population.[1] Approximately 15-30% of patients with RP have X linked retinitis pigmentosa (XLRP), which is the most severe form of RP consistently manifesting early in life.[2 3] Night blindness is usually present in early childhood with loss of peripheral visual fields and ultimately central vision, resulting in registered blindness by the end of the third decade. Female carriers display a broad spectrum of fundus appearances ranging from normal to extensive retinal degeneration.[4–6]

XLRP is genetically heterogeneous with two major loci, RP2 (Xp11.23) and RP3 (Xp21.1). Both disease genes have now been identified (respectively *RP2*[7] and *RPGR*[8–10]) with *RP2* mutations causing disease in approximately 15% of XLRP families,[11 12] while *RPGR* mutations are reportedly more common, accounting for up to 75% of XLRP.[10] Two other rare loci for XLRP have also been described on Xp22 and Xq26-27.[13 141]

Hong *et al*[15] described the phenotype and pathology of an *RPGR* knockout mouse model. They showed the subcellular localisation of RPGR to the photoreceptor connecting cilia, and in the absence of RPGR partial mislocalisation of essential outer segment proteins. These data suggest a putative role for RPGR in the retina, controlling movement of essential proteins from the inner to the outer segment of photoreceptors via the connecting cilia. Several groups have recently identified a retina specific RPGR interacting protein (RPGRIP1).[16–18] This protein also localises to the photoreceptor connecting cilium and is thought to be a structural component of the ciliary axoneme.[18] Subsequent mutation screening in patients suffering from retinal diseases has identified mutations in *RPGRIP1* as a cause of Leber congenital amaurosis.[19 20]

In this report, we present the phenotype of a family suffering from XLRP associated with hearing loss, sinusitis, and chronic recurrent respiratory tract infections. To identify the causative gene on the X chromosome, we performed haplotype analysis with subsequent mutation screening of candidate genes. The new phenotype described is associated with a mutation in the *RPGR* gene, and highlights the significance of RPGR protein kinociliary function in non-ocular tissue.

Key points

- We report a novel systemic phenotype associated with XLRP, with patients suffering from hearing loss, sinusitis, and chronic chest infections, suggesting a mutation in a gene involved in ciliary function.
- The phenotype overlaps those described for primary ciliary dyskinesia and Usher syndrome.
- Genetic analysis of this family has identified a frameshift mutation in exon 8 of the *RPGR* gene.
- A gene in close proximity to *RPGR*, *TCTEL1*, was also examined for cSNPs as a potential phenotypic modifier locus; none was detected.
- Our findings show that mutations in the *RPGR* gene are associated with a complex phenotype broadening the clinical spectrum of disease, and provide supporting evidence for an essential ciliary function for RPGR in the retina and other tissues.
- RPGR and interacting partners involved in kinociliary function in a variety of tissues may therefore represent attractive candidate genes for other diseases, such as primary ciliary dyskinesia or hearing loss.

PATIENTS AND METHODS

Patients and controls

Appropriate informed consent was obtained from the family and control volunteers under investigation. An X linked form of retinitis pigmentosa was established by pedigree analysis, clinical examination, and ophthalmological tests. Blood samples were collected from each available member of the family and from controls and DNA extracted using the Nucleon II Kit (Scotlab Limited) according to the manufacturer's instructions. Clinical characterisation included ophthalmic and systemic history, visual field testing, and fundus examination. In addition fundus photography was performed. Three

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The first two authors contributed equally to this work

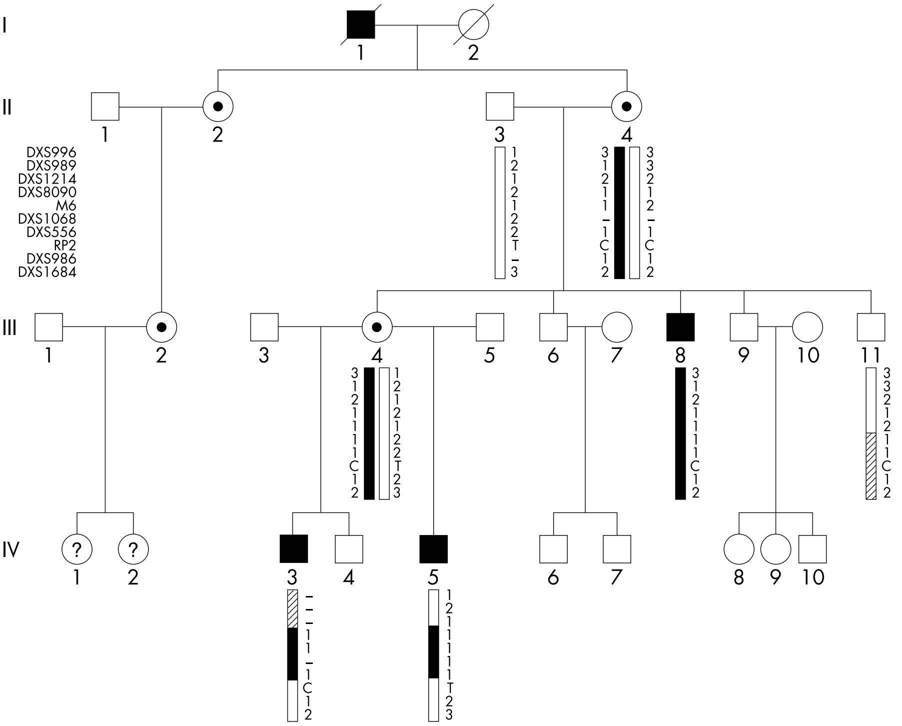

Figure 1 Pedigree of family 4462 showing haplotypes constructed around the RP2 and RP3 loci showing disease segregation with the RP3 locus. The polymorphic variant detected within the *RP2* gene is also shown and does not segregate with disease. The affected haplotype is shown as a shaded chromosome. Hatched chromosomes represent uninformative regions.

subjects (fig 1, II.4, III.4, and IV.5) underwent electrophysiological investigation; subject IV.3 was unable to attend because of renal dialysis. Electro-oculographic responses (EOG), full field electroretinography (ERG), and pattern electroretinograms (PERG) were recorded to incorporate the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.[21–23] Hearing loss was assessed in one affected male (III.8) by pure tone audiometry and systemic disease history was provided by the patients' physicians.

Genotyping

Microsatellite markers on the X chromosome were used to generate haplotypes of affected, carrier, and unaffected members of the family (primers and conditions available at http://www.gdb.org/). Haplotypes were constructed assuming the minimal number of recombination events. PCRs were carried out in 10 μl reactions in the presence of 1 μCi α^{32}P-dCTP, 0.5 U *Taq* polymerase, 200 μmol/l each of dATP, dGTP, dTTP, and 20 μmol/l of dCTP, 50 pmol of each primer, 30 mmol/l Tris-HCl, pH 8.5, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 0.01% gelatine. Amplification conditions were 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at the primer specific temperature for 15 seconds, and extension at 72°C for 30 seconds. A final extension followed for five minutes at 72°C. Amplified products were mixed with 6 μl of formamide sample buffer and 3 μl aliquots were electrophoresed in 6% denaturing polyacrylamide gels. Electrophoresis was carried out at a constant power of 90 W for between two and five hours depending on fragment size. The gels were then fixed in 10% methanol/10% acetic acid solution for five minutes, dried onto Whatman paper, and analysed by autoradiography.

Mutation screening

The coding sequence and intron/exon boundaries for the *RP2*, *RPGR*, and *TCTE1L* genes were amplified as described previously.[11, 24, 25] PCR products were examined by agarose gel electrophoresis before sequencing. The *TCTE1L* gene was amplified as described by Roux *et al*,[25] except exon 1 primers were redesigned (TCTE1L1F - TGAAGTGACGCCTGGCGTTG and TCTE1L1R - AGAGGAAGGCGGGAGTGGG) and annealed at 60°C. An aliquot of each amplification product (8 μl) was then purified by the addition of 1 U shrimp alkaline phosphatase (SAP, Amersham Life Science) and 1 U Exonuclease I (United States Biochemical) in SAP buffer, and incubated at 37°C for 30 minutes followed by 80°C for 15 minutes. Five μl of the purified DNA sample was then used for cycle sequencing using Big Dye Terminator cycle sequencing kit following the manufacturer's instructions. Reactions were then electrophoresed on an ABI 373A automated sequencer.

RESULTS

During the genetic and clinical analyses of families diagnosed with XLRP, a family with additional systemic features was identified. The four generation pedigree structure of this family is shown in fig 1.

Clinical assessment

Ophthalmic phenotype

Affected males manifested night blindness in early childhood, had constricted visual fields by early teens, and were registered as legally blind by 20 years of age. Gross fields to confrontation in affected males IV.3 (aged 25) and IV.5 (aged 16) were less than 10°. Intraretinal bone spicule pigmentation was observed in IV.3 (fig 2). Female subjects II.4 and III.4 were asymptomatic, had normal fields to confrontation, and showed sparse peripheral intraretinal pigmentation (fig 2). Carrier females and affected males were myopic. Visual acuities were as follows for the right and left eyes respectively: II.4, 20/30 and 20/20; III.4, 20/40 and 20/30; IV.3, 20/30 and 20/30; and IV.5, 20/30 and 20/30. Levels of myopia were recorded for carrier females II.4 (5.00/–1.25 diopters and –7.50 diopters) and III.4 (–10 diopters). Electrophysiological phenotype was ascertained in one affected male patient (IV.5) and two carrier females (II.4 and III.4, fig 3). No recordable ERG could be

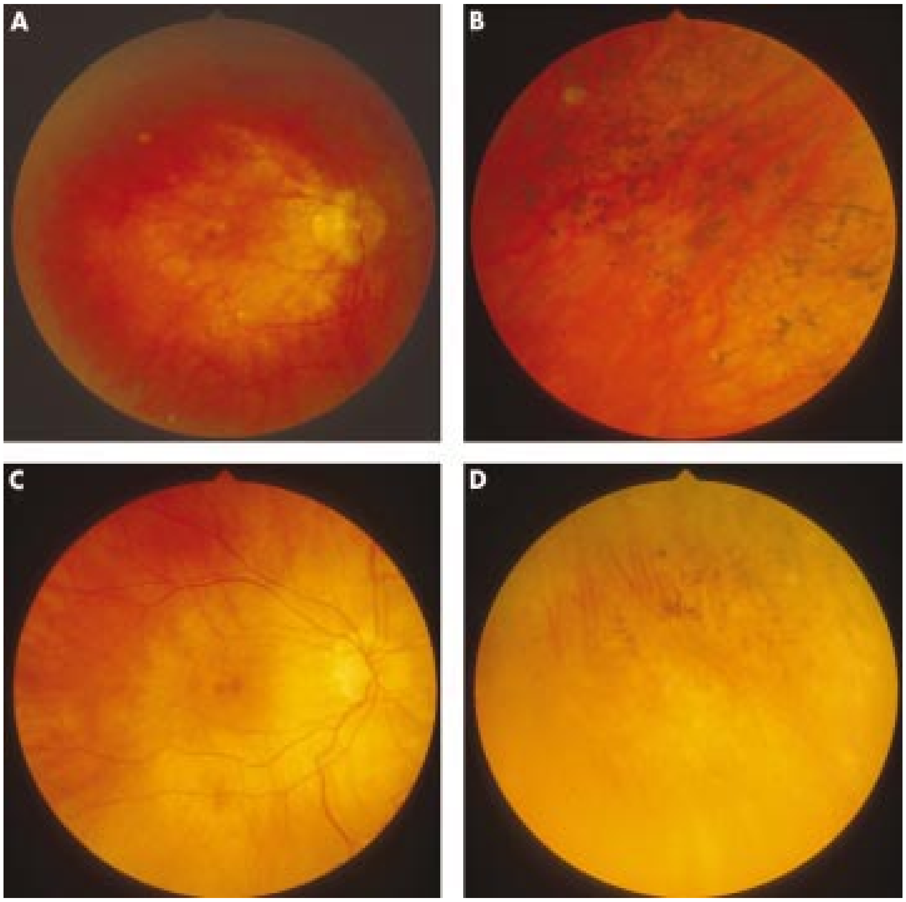

Figure 2 Fundus photos of (A) affected male IV.3, right macula showing retinal thinning and (B) typical intraretinal bone spicule pigmentation in peripheral retina, (C) right macula of carrier female III.4 showing mild retinal thinning, and (D) sparse intraretinal bone spicule pigmentation in the periphery.

detected in the affected male (IV.5) under any stimulus conditions; PERG was also undetectable confirming severe macular involvement. Both carrier females (II.4 and III.4) show delayed 30 Hz flicker ERG with mild delay also present in photopic single flash b wave. Rod specific ERGs do not show definite abnormality, but maximal response a wave is mildly subnormal in subject III.4. PERG is subnormal in both carrier females tested. EOG light rise was abolished in IV.5 and reduced in the carrier females. The ocular phenotype therefore fits the classical description of X linked retinitis pigmentosa, with very severe ERG abnormality in affected males and abnormal ERGs in female heterozygotes in keeping with carrier status.

Systemic phenotype

In this family, however, the classical ocular features of XLRP were associated with additional systemic symptoms in both hemizygous males and heterozygous females.

One of the most striking and obvious additional features was that affected subjects and carriers required hearing aids. Both males and females suffered severe recurrent ear infections from very early childhood continuing into adulthood. All affected males, and carrier female III.4 (aged 44), had progressive hearing loss and required hearing aids. Affected male I.1 (who had died) also had hearing loss and was described as deaf. The exception is carrier female II.4 (aged 68), who had recurrent ear infections, but did not require a hearing aid. The family had not been exposed to harmful environmental influences, such as excessive noise. Predominantly high frequency hearing impairment was recorded for affected male III.8 by pure tone audiometry (PTA, fig 4). Both left and right ear are similarly affected showing hearing loss between 4000 and 8000 Hz. Averaging the thresholds at these two frequencies is the convention to describe the degree of hearing loss predominant at high frequency. Thus, (20 + 25 + 40 + 45) ÷ 4 = 32.5, which indicates that hearing loss in this subject is mild, since the averaged threshold is less than 40 dB hearing loss. The audiogram could be consistent with a diagnosis of sensorineural hearing loss, in view of the hearing loss in the high frequencies. However, a conductive hearing component may also have contributed to this phenotype. Unfortunately, PTA data for other members of the family are not available.

In addition to retinitis pigmentosa and hearing loss, affected males and carrier females also suffered from severe recurrent sinus infections resulting in sinusitis. The three affected males experienced chronic recurrent chest infections starting in early childhood, with episodes of bronchitis, which continued into adulthood. Affected male IV.3 had nasal polyps and renal failure. The patient's affected brother (IV.5) and affected uncle (III.8) did not have renal failure, and carrier females do not seem to suffer from milder renal insufficiency. Although renal failure does not, therefore, segregate with the visual and hearing loss in this family, it remains possible that renal failure in patient IV.3 is part of the spectrum of symptoms for this syndrome.

In summary, the three affected males in this family had classical XLRP associated with hearing loss, sinusitis, and chronic recurrent chest infections, with renal failure in one person. Carrier females (II.4, III.2, and III.4) had a milder systemic phenotype, suffering recurrent ear infections and sinusitis without the chronic chest infections. Obligate carrier

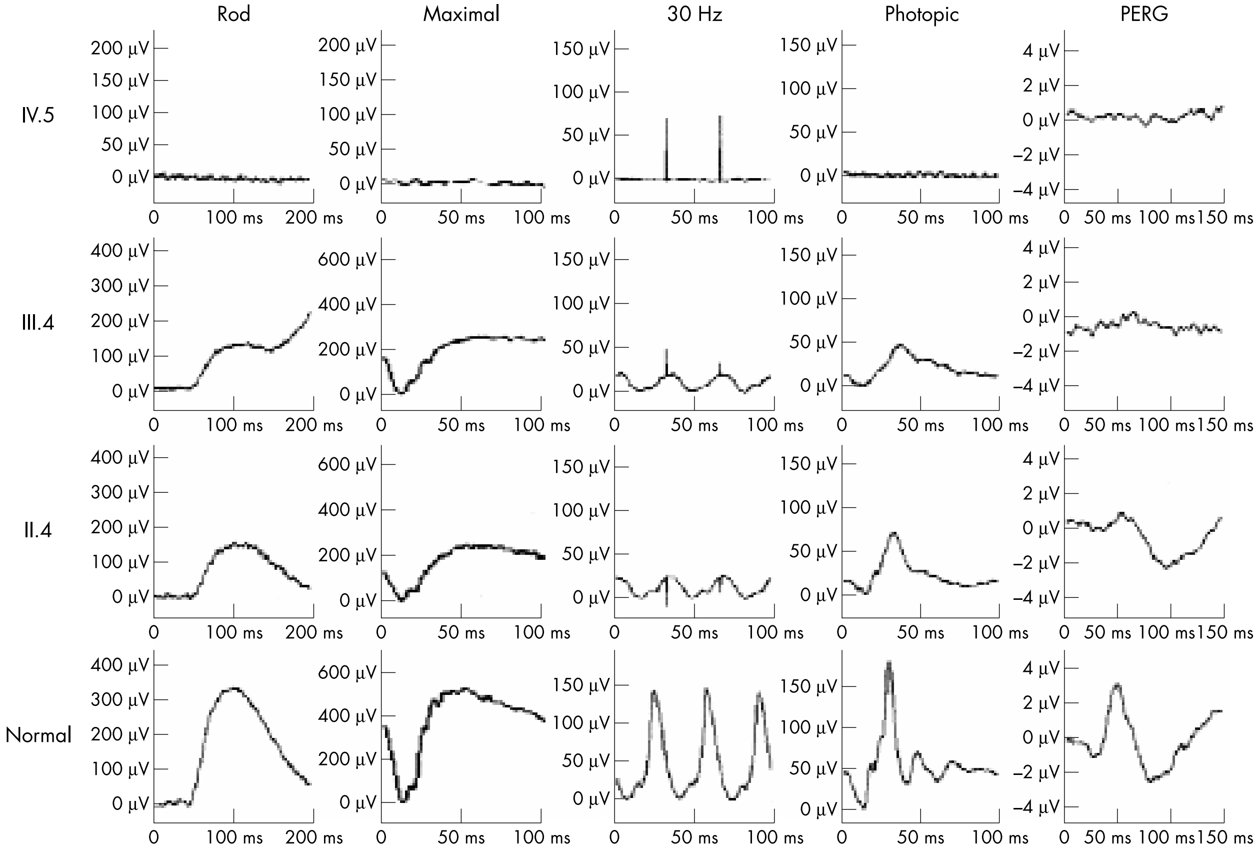

Figure 3 ERGs from an affected male (IV.5) and two carrier females (II.4 and III.4). The affected male shows no definite ERG under any stimulus conditions (note the changes in scale); PERG is also undetectable. The two carrier females show increased implicit time 30 Hz flicker ERGs, with mild change in photopic single flash b wave implicit time. Maximal response a wave is mildly subnormal in III.4. Both carriers show marked PERG reduction.

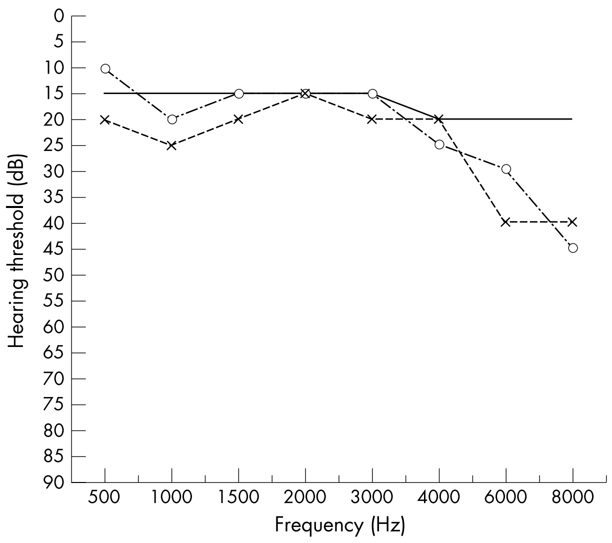

Figure 4 Pure tone audiometry for affected male III.8 showing bilateral hearing loss prominent at high frequencies. The continuous line represents the expected normal threshold for his age, patient right threshold = O, patient left threshold = X.

III.4 had progressive hearing loss and required a hearing aid. The ocular phenotype of II.4 and III.4 was consistent with carrier status for XLRP. Unaffected members of this family had no symptoms of either the ocular or systemic phenotype described.

The phenotype for this family is therefore XLRP associated with progressive hearing loss, sinusitis, and chronic recurrent chest infections.

Haplotype analysis and mutation screening

Since disease in this family appeared to be X linked through pedigree structure and phenotypic evaluation, we performed haplotype analysis using X chromosome markers to locate the disease gene interval. Haplotype analysis showed disease segregation between the markers DXS1214 and DXS986 spanning the *RP2* and *RPGR* genes. The *RP2* gene was screened for mutations by PCR exon amplification followed by direct sequencing.[11] *RP2* was considered a possible candidate since mutations were known to cause XLRP, and the encoded protein is ubiquitously expressed, with a potential role in tubulin biogenesis and hence ciliary function.[7 26] No mutations were identified in this gene. However, a polymorphism was identified in exon 3 (844C>T[18]) which did not segregate with the disease, thus refining the disease interval (fig 1).

Subsequently, *RPGR* was then analysed in our family.[8 9 10] We detected EST matches from olfactory and lung epithelial cells by BLAST and NIX analysis and UniGene database searches (http://www.ncbi.nlm.nih.gov/ and http://www.hgmp.mrc.ac.uk/) suggesting that this ubiquitously expressed gene is present in the tissues of interest. Sequence analysis of *RPGR* showed a 2 bp deletion in exon 8 (845-846delTG, fig 5). This frameshift mutation at residue 262, which segregates with disease, is predicted to introduce 19 new amino acids and a premature stop codon, resulting in a truncated protein of 280 residues. All exons upstream of exon 8 were also entirely sequenced to exclude the possibility of a second alteration, which may have a combinatory effect with the exon 8 mutation; no other alterations were detected.

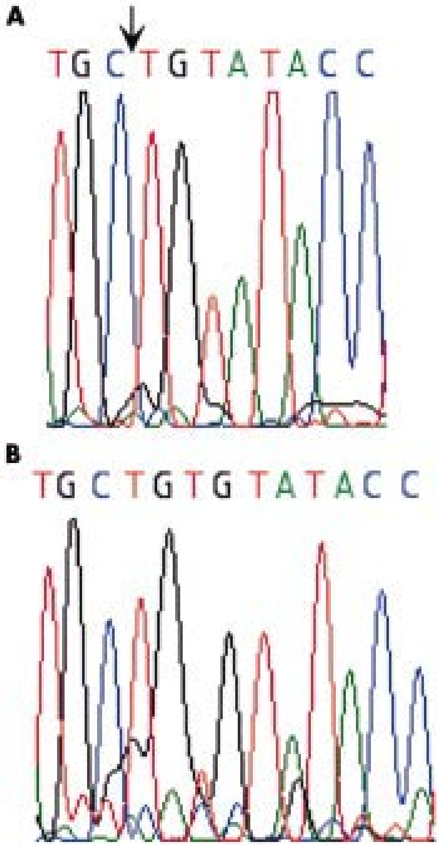

Figure 5 Electropherograms depicting patient mutation and wild type sequence in exon 8 of the *RPGR* gene. (A) TG deletion at nucleotide position 845-846 in an affected male from family 4462. (B) Wild type sequence from a population sample of the corresponding region of *RPGR*.

It is likely that the genetic background of subjects within this pedigree contributes to the additional systemic phenotypes observed. Since overlapping symptoms have been observed in numerous XLRP patients and at least one other XLRP pedigree (see Discussion), we hypothesised that a predisposing locus could be closely linked to *RPGR* on the X chromosome. The *TCTE1L* gene is approximately 500 kb distal to *RPGR* and has been shown to be expressed in lung, trachea, kidney, and brain, among other tissues and detects ESTs from olfactory and lung epithelial cells and the organ of Corti[25 28] (BLAST and UniGene searches at http://www.ncbi.nlm.nih.gov/). The TCTE1L protein forms part of the cytoplasmic dynein light chain of the microtubule motor complex, and may be involved in tissue specific cargo binding activities since other members of this protein family mediate specific interactions, for example, with rhodopsin.[29 30] The *TCTE1L* gene therefore presented an attractive positional and functional modifier locus for the phenotype described. To determine if a linked locus could predispose subjects to susceptibility to the systemic pathology observed, we screened the five exons of the *TCTE1L* gene for cSNPs and no polymorphic variants were detected in the affected males.

DISCUSSION

Phenotypic overlap with other syndromes

The systemic phenotype in this family has similarities with those observed in immotile cilia syndrome (ICS1) or primary ciliary dyskinesia (PCD, MIM 242650). PCD is a congenital respiratory disease characterised by impaired mucociliary clearance caused by cilia ultrastructural abnormalities.[31 32] PCD patients suffer from chronic bronchiectasis and sinusitis, usually associated with male infertility, but do not reportedly suffer from recurrent ear infections or deafness.[33] Approximately half of the patients with PCD also display situs inversus (Kartagener syndrome, MIM 244400). One causative gene for PCD has recently been identified, *DNAI1*, a dynein intermediate chain gene on chromosome 9p13-21, with mutations in this gene shown to cause axoneme ultrastructural abnormalities in two families.[34] Genetic and phenotypic heterogeneity are features of PCD, with a locus identified on chromosome 5p14-15,[34 35] and potential linkage to 11 other chromosomal intervals.[36] However, no linkage to the X chromosome or association with RP and deafness has previously been reported.

Usher syndrome is defined by an association of sensorineural deafness with RP, with three distinct clinical subtypes (I, II, and III) of variable severity and extensive genetic heterogeneity.[37] Usher syndrome is the most frequent cause of sensorineural deafness accompanied by blindness, although two of the causative genes have also been implicated in isolated deafness[38] or isolated retinitis pigmentosa.[39]

Although hearing loss in our family is associated with retinitis pigmentosa, the mode of inheritance, nature, and onset of hearing loss, and chronic infections leading to sinusitis and bronchitis distinguish the phenotype in this family from Usher syndrome types I, II, and III. Hearing loss in our family appears relatively mild and progressive with a sensorineural component, but the chronic infections suffered by the patients may also contribute to an acquired conductive hearing impairment. Unfortunately, we were not able to record bone conduction thresholds in this family, so we conclude that hearing loss is likely to be mixed, but not proven. It is difficult to say whether the patients have any loss of vestibular function without thorough neuro-otological evaluation, but family members did not report any dizziness/balance problems. The lack of reported symptoms may have been because of the progressive nature of the condition which allowed for vestibular compensation. The presence of an X linked form of Usher-like phenotype has been suggested, but no locus on the X chromosome has been described. In one report, however, retinitis pigmentosa with deafness (described as Usher syndrome) was associated with bronchiectasis and immotile cilia syndrome, and the possibility of an X linked mode of inheritance could not be excluded.[40]

The major sites of pathology in this new phenotype, causing hearing loss and other disabling systemic abnormalities in association with XLRP, suggest structural, degenerative, or developmental kinociliary defects.

RPGR mutations and ciliary abnormalities

Several lines of evidence support our findings that mutant *RPGR* causes XLRP with associated generalised cilia abnormalities. In 1992, Van Dorp *et al*[41] reported a family who suffered from XLRP with associated susceptibility to respiratory infections in the majority of affected males. The patients suffered from recurrent bronchitis and sinusitis, described as indistinguishable from immotile cilia syndrome, but did not suffer sterility or deafness. In a subsequent publication, a mutation was identified in this family in the *RPGR* gene, namely a G to T transversion at position +1 of the 5′ donor splice site of intron 5, predicted to result in aberrant splicing.[42] This additional phenotype, reported by Van Dorp *et al*,[41] overlaps the one described here with the exception of the associated hearing loss, present in our family. Independent studies describing the prevalence of deafness in association with RP[43] identified a group of patients that did not fit into previously described clinical categories (that is, not Usher syndrome). Hearing impairment in three families with XLRP was reported by Rosenberg *et al*,[43] and a mutation in *RPGR* had previously been identified in one of these families.[9 44] This mutation is described as a 6.4 kb deletion which disrupts the

3′ end of *RPGR* removing the last six exons. Both affected males and carrier females from this family had hearing difficulties.[43]

Other studies have centred around examination of cilia in patients with RP. Several reports examining nasal mucosa and sperm in heterogeneous groups of patients suggest increased incidence of abnormal cilia in XLRP patients.[45–47] However, the molecular basis for these observations remains undetermined.

Although the physiological role of RPGR in retina and other tissues is not fully understood, compelling evidence for the importance of RPGR in ciliary function comes from recent studies of animal models and the identification of RPGR interacting proteins.[15–20] It is possible, therefore, that other cell specific ciliary proteins exist in the lung, trachea, inner ear, and nasal passages which bind RPGR, and that this interaction may be compromised in the family described in this report.

Mutation type and modifiers of phenotype

The novel mutation we have identified results in partial loss of the RCC1 domain (exon 8, 845-846delTG) and downstream sequence. This protein truncation mutation occurs upstream of many other protein truncation and missense mutations previously reported to cause XLRP. It is unclear, however, why the phenotype of this protein truncation mutation is different from others reported to result in only an ocular phenotype.[48] Perhaps persistent but milder systemic infections in other families remain undetected or may not have been reported, and exposure to infections and subsequent disease manifestation varies widely.

Mutations in the *RPGR* gene have also been detected in families with X linked cone-rod dystrophy and X linked macular degeneration (as opposed to the rod-cone degeneration observed in classical XLRP), widening the clinical spectrum associated with mutant *RPGR* and highlighting the fact that other factors modulate the phenotype.[49–51] The factor(s) underlying the significant variability of the pathogenic expression of *RPGR* remain to be identified.

The genotype at a particular locus may account for an inter-individual susceptibility that can both increase or decrease the risk to develop the pathology by modulating mechanisms involved in the pathogenesis. We hypothesised that a closely linked gene which segregated with the primary *RPGR* mutation could be acting as a modifier gene in this family, since association with the symptoms described in this report are more common than previously suspected. *TCTE1L* lies approximately 500 kb distal to RPGR and presents an interesting functional candidate which is expressed in the tissues involved in the systemic disease associated with XLRP. No cSNPs were identified. Predisposing SNPs may lie outside those gene regions tested, and other loci on the autosomes can not be excluded as predisposing factors; however, if autosomal SNPs are involved in disease expression, they are predicted to be common owing to the occurrence of disease in more than one pedigree. It is now essential to collect a cohort of families with these overlapping phenotypes to determine the factors involved in disease expression.

Further evidence for this new syndrome being primarily an *RPGR* gene disorder comes from colleagues who have identified a family with an almost identical phenotype, XLRP, hearing loss, and recurrent respiratory tract infections. On the basis of our findings, they examined the *RPGR* gene and found a missense mutation in exon 6 (Iannaccone *et al*, in preparation). The data show that the families are unrelated and that different mutations in *RPGR* can result in overlapping phenotypes implicating ciliary dysfunction in a variety of tissues. In addition, Iannaccone *et al* describe expression of RPGR in human cochlea and bronchial and sinus epithelial lining.

Future studies towards unravelling the function of RPGR in the retina will need to be expanded to include analyses of multiple ciliated epithelial tissues. The identification of RPGR binding partners within these tissues may identify other specific proteins capable of interacting with RPGR. It would be of interest to evaluate the mouse and dog models of *RPGR* disease[15 52] with a view to examining structure/function and development of the ciliated epithelium of the respiratory tract, sinuses, and inner ear, for example, in addition to re-evaluating the patients already described as harbouring *RPGR* mutations as a cause of XLRP.

SUMMARY

In conclusion, we describe a new phenotype of typical X linked retinitis pigmentosa associated with hearing loss, chronic respiratory tract infections, and sinusitis caused by a mutation in *RPGR*. The systemic phenotypes are predicted to be variable, accentuated by repeated infections of the respiratory tract and consequent upon impaired mucociliary clearance (as described for PCD). Phenotypic variation between families may be caused by *RPGR* mutation type, genetic background, environmental effects, or a combination of these factors. Additional families will need to be investigated for SNPs on the X chromosome in proximity to *RPGR* to explore fully any phenotypic modification caused by adjacent loci. *RPGR* and interacting partners involved in kinociliary function in a variety of tissues may also represent attractive candidate genes for other phenotypes such as primary ciliary dyskinesia or isolated hearing loss.

ACKNOWLEDGEMENTS

The authors would like to thank the family for participating, and The Wellcome Trust (AJH) and the British Retinitis Pigmentosa Society for their support.

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Evidence of *RPGRIP1* gene mutations associated with recessive cone-rod dystrophy

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J Med Genet 2003;**0**:616–619

Cone-rod dystrophies (CRD) are forms of inherited retinal dystrophy which characteristically lead to early impairment of vision. An initial loss of colour vision (cone mediated functions) and of visual acuity, usually from the first or second decade of life, is followed by night blindness (largely rod mediated) and loss of peripheral visual fields.[1] CRD patients suffer from severe photophobia and show reduced ERG responses. In later life, vision may be reduced to a bare perception of light. CRD is a milder condition compared to Leber congenital amaurosis (LCA) which is the most severe form of all the inherited retinal dystrophies and is diagnosed as bilateral congenital blindness, with a diminished or absent electroretinogram (ERG). Cone-rod dystrophy loci have been mapped to chromosomes 17q,[2] 19q,[3] 18q,[4] 17p13,[5 6] 6q,[7] 1q12,[8] and 8p11.[9] Mutations in the *peripherin/RDS*,[10] *CRX*,[11 12] and *RetGC-1*[13 14] genes have been shown to cause autosomal dominant CRD. Mutations in the ATP binding cassette transporter rim protein (*ABCR*) gene have been shown to be associated with autosomal recessive CRD.[15] Mutations in the *CNGA3* gene encoding the α-subunit of the cone photoreceptor cGMP gated channel have also been reported to cause cone photoreceptor disorders.[16]

The RPGRIP1 protein (retinitis pigmentosa GTPase regulator interacting protein 1, MIM 605446) is encoded by the gene located on chromosome 14q11. It consists of 24 exons and the predicted size of its protein product is 1259 amino acids. It is expressed specifically in the rod and cone photoreceptors and is a structural component of the ciliary axoneme. One of its functions is to anchor the RPGR protein within the photoreceptor connecting cilium.[17] Recently, in an in vivo investigation of RPGRIP1 function and its physical interaction, it has been shown that RPGRIP1 is essential for RPGR function and is also required for normal disk morphogenesis.[18] Mutations in *RPGRIP1* have been reported to be a cause of LCA.[19 20] Here we report the first observation of the involvement of *RPGRIP1* gene mutations as a cause of CRD in four Pakistani families.

SUBJECTS AND METHODS

We studied 20 members of a two generation and 19 members of a three generation consanguineous Pakistani families, 1CRD and 4CRD, respectively. The 1CRD family consists of eight affected and 12 unaffected subjects and the 4CRD family consists of eight affected and 11 unaffected subjects (fig 1A, B). One of the authors (A Aziz) clinically examined all the patients and their unaffected family members. The deterioration in central vision and colour blindness was from an early age in all the patients and there was a rapid loss of vision between the ages of 14 and 16 years (visual acuity 1/60, 0.01). Patients had also had severe photophobia since their childhood. Fundoscopy showed a variable degree of fundus granularity and macular degeneration. The affected subject IV.7 (aged 14 years) has a characteristic macular bull's eye lesion in both her eyes. Full field flash ERG was used to measure functions of both cones and rods. Both photopic and scotopic full field ERG amplitudes were reduced, showing involvement of both photoreceptor systems. However, among the young patients, cone response was reduced more than that of their rod response. Based on family history and clinical diagnosis, the disease was classified as autosomal recessive cone-rod dystrophy (arCRD).

For genetic analysis peripheral blood samples were collected with informed consent from all members of the two families and from 100 ethnically matched control subjects.

Linkage analysis

Genomic DNA was extracted from whole blood using the Nucleon II extraction kit (Scotlab Bioscience). DNA was amplified using primers (Research Genetics) for polymorphic microsatellite markers specific for known loci/genes associated with various retinal degenerations according to the conditions described previously.[8]

Mutation detection

Exon specific intronic primers were designed from the genomic sequence of the *RPGRIP1* gene (NM 020366). PCR was performed in a 50 µl reaction volume using 1 µg of genomic DNA. The resulting product was allowed to cool slowly to room temperature to maximise the formation of heteroduplexes. Heteroduplex analysis was performed using an automated DHPLC instrumentation (WAVE DNA fragment analysis system, Transgenomic, Crewe, UK). Sample preparation for heteroduplex analysis was carried out by denaturing and reannealing of unpurified PCR products of the carriers (heterozygotes). The temperature conditions required for the successful resolution of heteroduplexes were obtained from the website (http://insertion.stanford.edu/melt.html).

On identification of heteroduplex peaks in the carriers, all family members were sequenced in the forward and reverse directions using a commercially available kit (Big Dye, ABI) and the products were analysed on an ABI Prism 377 automated DNA sequencer. Subsequently, six small families with CRD (5, 6, 7, 8, 9, and 10CRDs) and two with LCA were also included for mutation screening of the *RPGRIP1* gene. To exclude the possibility that the mutations are polymorphisms, 100 ethnically matched control samples were also screened for heteroduplexes. The unpurified PCR products of the control

Key points

- *RPGRIP1* gene mutations have previously been reported to cause Leber congenital amaurosis.
- Here we report two novel mutations in the *RPGRIP1* gene that cause cone-rod dystrophy in four Pakistani families.
- A homozygous G to T point mutation was identified in exon 16 at nucleotide 2480 in all the affected members of family 1CRD.
- In the affected members of another CRD family (4CRD), a G to T substitution was found in exon 13 at nucleotide 1639.
- The same G 1639 T mutation was found in two more small families, 5CRD and 10CRD.

The first two authors contributed equally to this work.

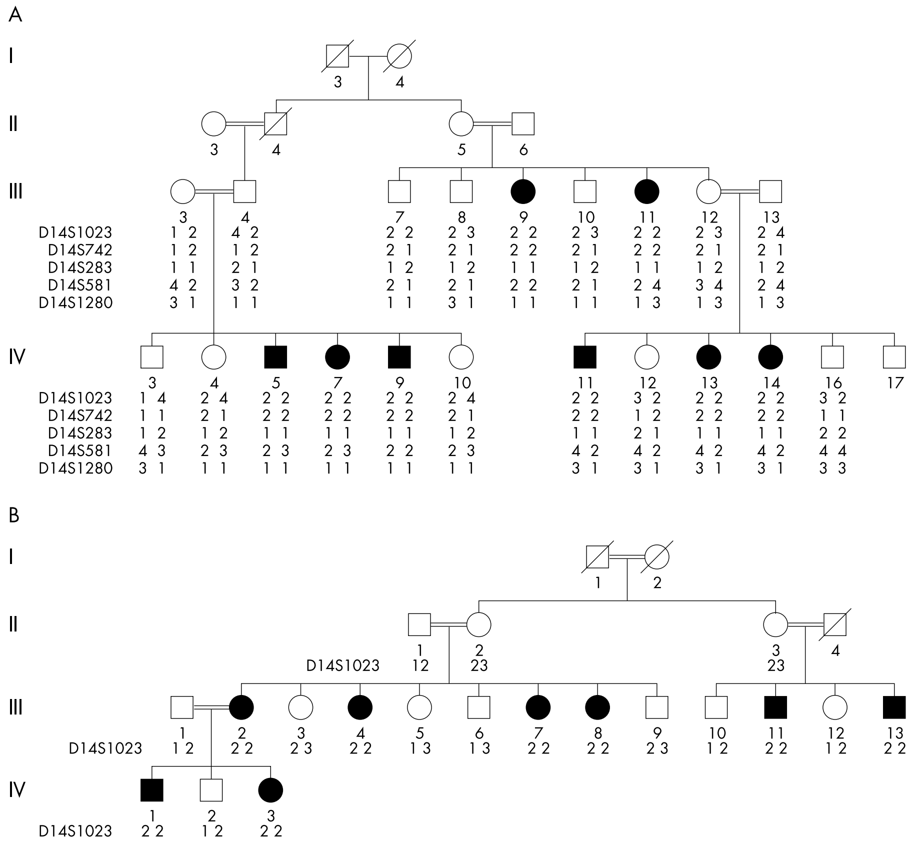

Figure 1 Pedigrees of Pakistani families with cone-rod dystrophy along with their genotypes. (A) Family 1CRD. (B) Family 4CRD.

samples and the homozygous wild type reference DNA sample were mixed in an equimolar ratio. The mixture was then subjected to a three minute, 95°C denaturing step followed by gradual reannealing from 95-65°C over 30 minutes. The heteroduplex mismatches were detected using the WAVE DNA fragment analysis system.[21]

RESULTS

Exclusion studies on both the large families (1CRD and 4CRD) showed linkage with the microsatellite marker D14S1023 (Zmax = 5.17 and 4.21 for the two families respectively at $\theta = 0.00$) at chromosome 14q11, a locus for the *RPGRIP1* gene (fig 1A, B). Mutation screening of the candidate gene, *RPGRIP1*, was carried out to identify the disease associated mutations.

Initially, DHPLC analysis was performed with samples from unaffected carriers of the families. Heteroduplex mismatches were recognised by the appearance of more than one peak in the elution profile. The presence of heteroduplex peaks in the unaffected carriers was convincing enough to do sequencing to identify the exact mutational change.

Sequence analysis of the *RPGRIP1* gene for family 1CRD showed a homozygous G to T point mutation in exon 16 in all the affected subjects (fig 2A). This substitution at nucleotide 2480 changes codon 827 from CGC (Arg) to CTC (Leu). This change in exon 16 was not found in any other Pakistani family studied here, the 100 control samples, nor any of the unaffected subjects of family 1CRD.

In the affected members of family 4CRD, a G to T substitution was found in exon 13 at nucleotide 1639 (fig 2B). This point mutation changes codon 547 from GCT (Ala) to TCT (Ser). The same mutation was found in two other small families, 5CRD and 10CRD (fig 2C). No disease associated mutation was observed in the *RPGRIP1* gene sequence for the remaining CRD and LCA families examined.

In addition, three polymorphisms were also identified in the *RPGRIP1* gene, which include a CTC to CTT (Leu427Leu) polymorphism in exon 16, a G to A sequence change in intron 9 and a deletion (9 base pair) in the intronic region of exon 13, 32 bp downstream from exon 13.

DISCUSSION

Homozygous mutations in the *RPGRIP1* gene have been reported in a panel of unrelated patients with Leber congenital amaurosis (LCA).[20] In most of these cases, the mutations result in a premature termination codon. To date *RPGRIP1* is the only gene that has not been associated with any other retinal disease phenotypes except LCA. LCA represents the severe end of a spectrum of inherited retinal dystrophies while cone-rod dystrophy is a milder condition. It has been suggested that mutations that cause residual RPGRIP1 activity may lead to phenotypes such as RP or CRD which are less severe compared to LCA.[22] Mapping of two Pakistani families with cone-rod dystrophy to the *RPGRIP1* locus supports this hypothesis. The identification of two novel disease associated mutations also indicates allelic heterogeneity of the *RPGRIP1* gene. Both novel mutations are in exons encoding domains of RPGRIP1 that are reported to be involved in interaction with the *RPGR* gene product.[20] Prosite (http://us.expasy.org/tools/scanprosite/) scan predicted the occurrence of an additional, more efficient, casein kinase II phosphorylation site in the Ala547Ser mutated protein, the significance of which is unknown. The second mutation (Arg827Leu) occurs in the major calcium dependent membrane binding module, the CK2 domain of the *RPGRIP1* protein. However, the prediction does not indicate any change in the 3D structure of the domain. Functional analysis of this protein would be required to demonstrate the role of these mutations in retinal dystrophies.

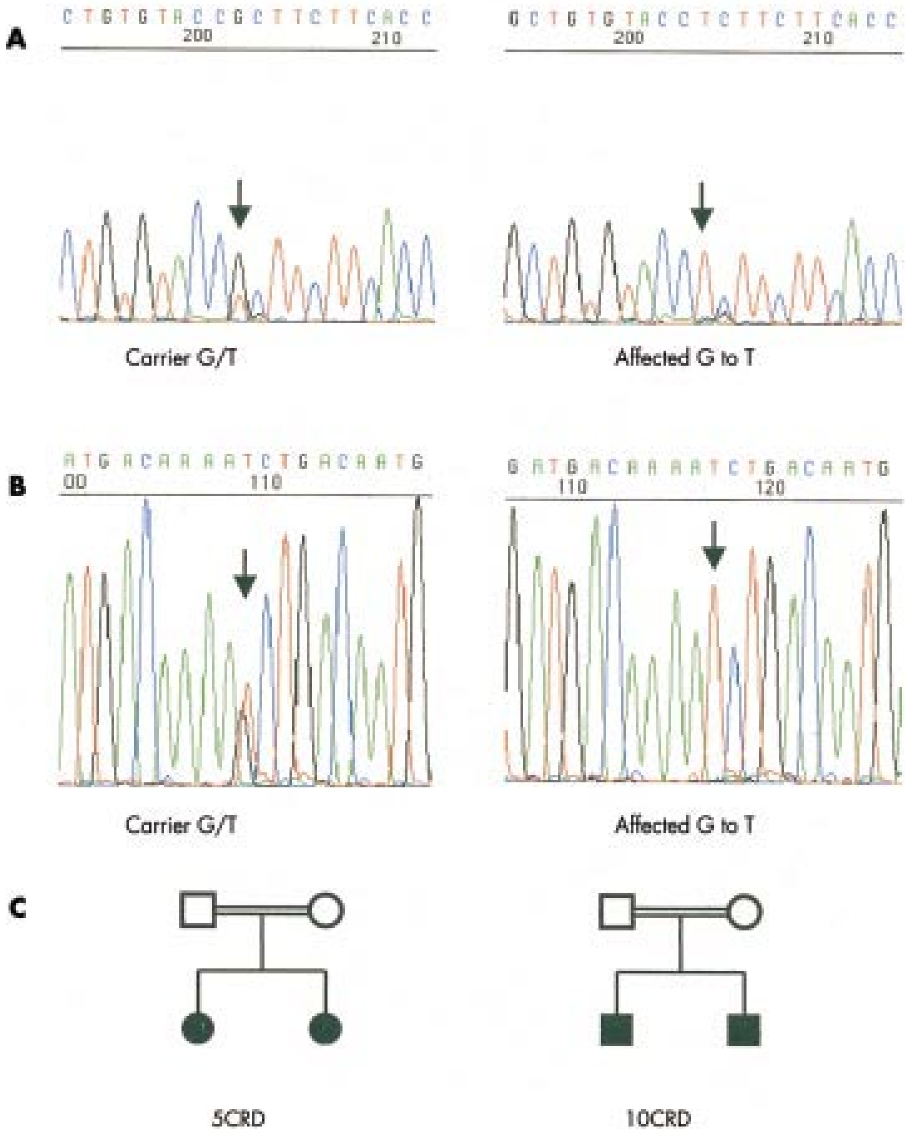

Figure 2 Selected electropherograms of members of the 1CRD and 4CRD families. In each panel, the left electropherogram is for the heterozygous carriers and the right electropherogram is for the patients who are homozygous for the respective mutation. (A) III.4 (carrier, left) and IV.5 (patient, right) from family 1CRD showing a G-T transversion in exon 16 of the *RPGRIP1* gene, (B) III.1 (carrier, left) and IV.1 (patient, right) from family 4CRD showing a G-T transversion in exon 13 of *RPGRIP1* gene. (C) Two small families, 5CRD and 10CRD, that carried the same G-T mutation in exon 13 of *RPGRIP1* gene.

ACKNOWLEDGEMENTS

The authors thank the family members for taking part in this study. We also thank Dr Q Ayub for his valuable help. This work was supported by Wellcome Trust grant number 063406/Z/2000/Z to SQM.

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Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (*MTHFR*): findings from over 7000 newborns from 16 areas world wide

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J Med Genet 2003;**40**:619–625

Since its biochemical characterisation in 1991[1] and its genetic identification in 1995,[2] 677C>T allele (T allele) of the 5,10 methylenetetrahydrofolate reductase (*MTHFR*) gene has been a focus of increasing interest from researchers world wide. The expanding spectrum of common conditions linked with the 677C>T allele now includes certain adverse birth outcomes (including birth defects), pregnancy complications, cancers, adult cardiovascular diseases, and psychiatric disorders.[3–8] Although several of these associations remain unconfirmed or controversial,[4] their scope is such that it becomes of interest to explore the geographical and ethnic distribution of the allele and associated genotypes.[9] Accurate information on such distribution can contribute to studies of gene-disease associations (by providing reference population data) and population genetics (by highlighting geographical and ethnic variations suggestive of evolutionary pressures),[10] as well as help to evaluate health impact (by allowing estimates of population attributable fraction).

Current population data, however, show gaps and even for some ethnic groups or large geographical areas (for example, China) few data are available.[3] Our aim was to supplement the available data by collecting a large and diverse sample of newborns from different geographical areas and ethnic groups, and to examine international variations in the distribution of the 677C>T allele. We present findings relating to more than 7000 newborns from 16 areas around the world.

MATERIALS AND METHODS

The study was conducted under the auspices of the International Clearinghouse for Birth Defect Monitoring Systems (ICBDMS) and was coordinated through its head office, the International Center on Birth Defects (ICBD).

Sample selection

Participating programmes, in consultation with the coordinating group, identified a population sampling approach that would be simple yet minimise sampling bias with respect to the *MTHFR* genotype. We made an explicit attempt to sample systematically the newborn population. Details of each programme's approach are listed below, and further information is available on request.

Generally, programmes chose one of two approaches. The first approach used regional newborn screening programmes as the source of samples. Typically, such an approach used a

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Authors are listed by alphabetical order of country with coordinating group and corresponding author last. *Coordinating group.

Key points

- Our objectives were to characterise the geographical and ethnic distribution of the 677C>T allele (T allele) of the *MTHFR* gene and its associated genotypes among newborns around the world, using newborn screening programs and birth hospitals. The participants were 7130 newborns of different ethnicities from 16 areas in Europe, Asia, the Americas, the Middle East, and Australia.
- The distribution of the allele showed marked ethnic and geographical variation The homozygous TT genotype was particularly common in northern China (20%), southern Italy (26%), and Mexico (32%). There was also some evidence for geographical gradients in Europe (north to south increase) and China (north to south decrease). The TT genotype frequency was low among newborns of African ancestry, intermediate among newborns of European origin, and high among newborns of American Hispanic ancestry. Areas at the extremes of the frequency distribution showed deviations from Hardy-Weinberg expectations (Helsinki, Finland, southern Italy, and southern China).
- This study, the largest to date, suggests the presence of selective pressures leading to marked geographical and ethnic variation in the frequency of the 677C>T allele. Geneticists can benefit from these reference data when examining links between the 677C>T allele and health outcomes in diverse populations.

geographically defined birth population. In Atlanta, for example, researchers visited the Georgia newborn screening programme on different days over several weeks and selected a 1 day collection of blood spots received by the laboratory from children whose mothers resided in one of five counties in Atlanta. Discussions with the director of the newborn screening programme indicated that day to day variability in the flow of specimens from birth hospitals to the state laboratory was negligible. The second approach relied on systematic sampling directly from birth hospitals that were part of an established network. In Spain, for example, staff collected specimens from 15 consecutive newborns at each hospital participating in the ECEMC monitoring programme, which includes birth hospitals from across Spain. Details for specific areas are summarised below.

Australia, New South Wales

Specimens were obtained from the New South Wales newborn screening programme, by selecting 100 consecutive newborn screening cards on each of five consecutive days, excluding repeat specimens, for a total of 500 specimens. All maternity units in the state of New South Wales send their specimens to the programme. Specimens consisted of blood remaining after routine newborn screening tests had been completed.

Canada, Alberta

Specimens were taken from consecutive newborns from the provincial newborn screening programme in Alberta. Specimens consisted of the remaining blood spots used by the newborn screening programme. The first 100 specimens of the month were collected each month for four months.

China, northern and southern

Umbilical cord blood samples were collected from newborns from major hospitals in 12 cities in China from March to November 1998. One hundred consecutive samples were requested from each hospital. The hospitals were in cities from southern China (Wuhan, Nanjing, Guangzhou, and Chengdu) and northern China (Yanbian, Urumchi, Changchun, Jinan, Xi'an, Shenyang, Beijing, and Jilin). For homogeneity, only newborns of Han ethnicity were included in the study.

Finland, Helsinki

Specimens originated from newborns in the major maternity hospital in Finland, at the Helsinki University Hospital. Sampling was restricted to babies whose parents were both Finns. Specimens consisted of the remainder of umbilical blood specimens for hypothyroidism screening. The latter are collected for every newborn in Finland.

France, Strasbourg

Specimens were taken from consecutive newborns from newborn screening centres in Département du Bas-Rhin, whose births are also covered by the Strasbourg Birth Defects Registry. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Hungary

Specimens were taken from consecutive newborns. Specimens were collected from the remainder of the blood spots from the two newborn screening centres that operate in Hungary. For twin pairs, only one of the pair, selected at random, was included.

Israel, Tel Aviv

Specimens were taken from consecutive newborns from one major university hospital in Tel Aviv and consisted of blood spots.

Italy, Campania

Specimens were taken from consecutive newborns at three hospitals in Campania (two in Avellino, one in Benevento). Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Italy, Sicily

Specimens were taken from consecutive newborns from the newborn screening programme in south east Sicily. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Italy, Veneto

Specimens were taken from consecutive newborns at one hospital outside the town of Vicenza. The hospital was chosen because it is an area hospital with 1200 births per year that has good obstetric care but does not select for high risk pregnancies. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Mexico

Specimens were randomly selected from blood spots from newborns born in hospitals that are part of the RYVEMCE birth defect monitoring network in Mexico. Samples were obtained from the remainder of the blood spot specimens collected for hypothyroidism screening. Selection was stratified to include equal numbers of males and females in the final sample.

The Netherlands, northern region

Specimens were randomly chosen from newborns whose mothers resided in the northern Netherlands. Specimens consisted of the remainder of blood spots used by the newborn screening programme.

Spain

Specimens were taken from consecutive newborns in 67 hospitals of the National Health Service throughout Spain. Essentially all babies in Spain are born in such hospitals. These hospitals are part of ECEMC (Spanish Collaborative Study of

Table 1 Allele and genotype frequency of the 677C>T allele of the *MTHFR* gene by area and ethnic origin

		Genotype (No)*			Genotype (%)				Allele frequency
					CC	CT	TT		T
Area	Sample No	CC	CT	TT	(%)	(%)	(%)	95% CI	(%)
Europe									
Italy, Sicily	468	138	236	94	29	50	**20.1**	16.7 to 24.0	**45.3**
Italy, Campania	500	172	196	132	34	39	**26.4**	22.7 to 30.4	**46.0**
Italy, Veneto									
Italian whites	385	128	198	59	33	51	**15.3**	12.1 to 19.3	**41.0**
Others	47	27	15	5	57	32	**10.6**	4.6 to 22.6	**26.6**
Spain, multicentre									
Spanish whites	601	265	265	71	44	44	**11.8**	9.5 to 14.6	**33.9**
Others	51	24	20	7	47	39	**13.7**	6.8 to 25.7	**33.3**
France, Strasbourg	178	72	85	21	40	48	**11.8**	7.8 to 17.4	**35.7**
The Netherlands	188	97	79	12	52	42	**6.4**	3.7 to 10.9	**27.4**
Finland, Helsinki	545	293	230	22	54	42	**4.0**	2.7 to 6.0	**25.1**
Hungary	378	165	171	42	44	45	**11.1**	8.3 to 14.7	**33.7**
Russia	587	312	234	41	53	40	**7.0**	5.2 to 9.3	**26.9**
Middle East									
Israel	210	120	72	18	57	34	**8.6**	5.5 to 13.1	**25.7**
China									
North, Han ethnicity	643	201	315	127	31	49	**19.8**	16.9 to 23.0	**44.2**
South, Han ethnicity	430	167	228	35	39	53	**8.1**	5.9 to 11.1	**34.7**
Oceania									
Australia									
Whites	288	146	119	23	51	41	**8.0**	5.4 to 11.7	**28.6**
Others	75	40	27	8	53	36	**10.7**	5.5 to 19.7	**28.7**
Americas									
Mexico	500	91	248	161	18	50	**32.2**	28.3 to 36.4	**57.0**
USA, Atlanta									
Whites	300	142	126	32	47	42	**10.7**	7.7 to 14.7	**31.7**
Blacks	298	231	59	8	78	20	**2.7**	1.4 to 5.2	**12.6**
Hispanics	62	22	29	11	35	47	**17.7**	10.2 to 29.0	**41.1**
Asian	26	16	9	1	62	35	**3.8**	0.7 to 18.9	**21.2**
Other, unknown	100	63	32	5	63	32	**5.0**	2.2 to 11.2	**21.0**
Canada, Alberta									
Whites	240	136	90	14	57	38	**5.8**	3.5 to 9.6	**24.6**
Others	30	17	13	0	57	43	**0.0**	0.0 to 11.4	**21.7**
Total	**7130**								

*TT, two 677C>T alleles; CT, one 677C>T allele; CC, no 677C>T allele.

Congenital Malformations), which monitors one quarter of all births in Spain. Each hospital contributed specimens for 15 consecutive newborn infants during three selected months. Specimens were collected at the same time as the blood spots for the newborn screening programme.

Russia

Specimens were selected from the neonatal screening programme that collects and banks specimens from 54 maternity hospitals in the Moscow area. All selected babies were apparently free from congenital anomalies.

USA, Atlanta (Georgia)

Specimens were chosen from newborns whose mothers resided in one of the five counties in metropolitan Atlanta, as ascertained from information on the newborn screening card. Staff visited the newborn screening programme four times over two months. At each visit researchers selected at random the specimens collected during one day and collected specimens from the blood spots left over from newborn screening.

Sample determination and data collection

We determined that a sample size of approximately 400 to 500 specimens per area would provide a reasonably precise estimate (plus or minus 3%) for a genotype with 10% frequency. Such a frequency is within the range reported for the homozygous 677C>T (TT) genotype in many European countries and among North Americans of European descent, and is intermediate between the lower frequencies reported in some populations of African descent, and the higher frequencies reported in specimens from Mexico, Italy, and Hispanics in the USA.[3] Although 400 to 500 was the targeted number of specimens per area, smaller sample sizes were accepted, recognising that such samples would provide less precise estimates. For each sample, researchers collected information on sex and race/ethnicity. Ethnicity was determined from the blood spot card (for example, in Atlanta), from maternal interview (for example, Italy, Veneto, Spain), or from the last name or birth place of the parent (for example, Australia). Not all programmes collected all variables.

Human subject protection

Local review boards approved the study. In most cases specimens were anonymised before testing. In all cases, personal identifiers were removed before data were provided to ICBD for epidemiological analysis.

Laboratory testing

Genomic DNA was isolated from blood spots collected on filter paper. The presence of the C>T change within the *MTHFR* gene creates a *Hin*fI restriction site that can be detected by restriction enzyme digestion followed by electrophoresis. Amplification of the *MTHFR* gene by the polymerase chain reaction and detection of the T allele was performed using protocols based on the method of Frosst *et al*.[2]

Five programmes (USA, China, Israel, Mexico, The Netherlands) tested their own specimens. All other specimens were tested at a single laboratory (Naples, Italy). The laboratories participated in proficiency testing to ensure inter-laboratory

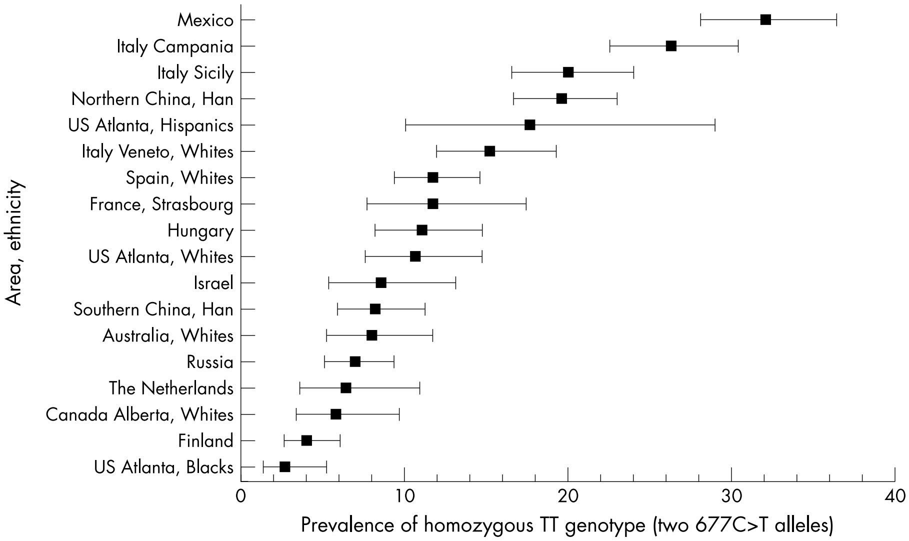

Figure 1 Prevalence of homozygous TT genotype (two 677C>T alleles) among newborns by area and ethnic background, ICBDMS 2003.

consistency. The proficiency testing consisted of the preparation of punches from 12 blood spots in one laboratory (CDC) with a mix of genotypes (CC, CT, and TT). These genotypes were confirmed by sequencing. Punches from these blood spots were then sent to the other laboratories, which performed DNA extraction followed by genotype assay. The laboratories were blinded to the genotype of these specimens as well as to the relative proportion of the genotypes. Results from each laboratory were sent back to CDC for evaluation. With the exception of one sample, which could not be amplified by four of the six laboratories, results showed complete agreement across laboratories.

Statistical analysis

We computed the confidence intervals for proportions using the Wilson score method without continuity correction.[11 12] Deviation from Hardy-Weinberg equilibrium was tested by chi-square analysis. In addition to allele frequencies, we present genotype frequencies for the homozygous TT genotype (two 677C>T alleles), the heterozygous CT genotype (one 677C>T allele), and the homozygous CC genotype (no 677C>T alleles).

RESULTS

We present data on 7130 newborns from 16 areas in the Americas, Europe, Russia, China, and Australia. Amplification rates for blood spots by geographical source were the following: Italy, Sicily 89%, Italy, Campania 76%, Italy, Veneto 73%, Spain 69%, France, Strasbourg 78%, Finland, Helsinki 95%, Hungary 95%; Russia, Moscow 95%, Australia, New South Wales 79%, Canada, Alberta 77%, USA, Atlanta 96%. By comparison, a large Irish population based study using newborn blood spots successfully genotyped 85% of collected samples.[13]

Prevalence by geographical area and ethnicity

The allele and genotype distribution, by area and ethnicity, is presented in table 1. The prevalence of the homozygous TT genotype (two 677C>T alleles) is visually summarised in fig 1 for those groups with at least 50 subjects tested.

The distribution of the 677C>T allele showed regional and ethnic variations. For example, the prevalence of the homozygous TT genotype was 10-12% in several areas in Europe (for example, Spain, France, and Hungary). However, the prevalence appeared to be lower (4% and 6%, respectively) in Finland, Helsinki and the northern Netherlands, whereas in some areas in southern Europe it was much higher (26% and 20% in Campania and Sicily, respectively). In the Americas, the frequency of the homozygous TT genotype was higher in Mexico (32%), intermediate in Atlanta (11% among whites), and somewhat lower in Alberta (6%). In Australia, TT prevalence was 7.5% among whites.

Genotype varied by ethnicity as well as by geographical location. For example, TT homozygosity was more common among newborns from Mexico or those born in Atlanta of Hispanic origin, intermediate among newborns of European ancestry (for example, in Europe and North America), and lower among newborns of African ancestry (for example, in Atlanta and Veneto, Italy). However, a range of genotype frequencies was evident even within broad ethnic groups. For example, TT homozygosity among whites ranged from as low as 6% in Alberta (Canada), to 7.5% in New South Wales (Australia), to 11% in Atlanta (USA), to the high values already noted for Italy. For other ethnic and racial groups, such estimates are more unstable because of the smaller number of specimens, but it is worth noting an apparently low frequency of TT homozygosity among newborns of Asian origin from Australia and Atlanta.

Hardy-Weinberg equilibrium

The observed distribution of the three genotypes (CC, CT, TT) in most areas was similar to that expected under Hardy-Weinberg equilibrium (table 2). This was true also among males and females separately (data not shown). We found a relative excess of TT homozygotes in Campania, Italy, and an excess of heterozygotes in Helsinki, Finland and southern China ($p<0.05$).

DISCUSSION

We documented distinctive geographical and racial/ethnic variation in the prevalence of the 677C>T allele of the *MTHFR* gene among a large international sample of newborns. The several fold variation in the prevalence of the TT homozygous genotype across the study areas (fig 1) was also consistent, in some areas, with the presence of geographical gradients. In Europe, for example, the prevalence of the TT genotype increased in a roughly southerly direction, from low values in the north (4-7% in Finland, Helsinki, northern Netherlands, and Russia), to intermediate values (8-10%) in France and Hungary, to higher values in southern Europe (12-15% in Spain and northern Italy), peaking in southern Italy (20-26% in Campania and Sicily). In North America, the frequency of

Table 2 Distribution of *MTHFR* genotypes, Hardy-Weinberg expectations, and p value

Area	Sample No	Genotype (No)			Observed/expected			p value, χ^2
		CC	CT	TT	CC	CT	TT	
Europe								
Italy, Sicily	468	138	236	94	1.0	1.0	1.0	
Italy, Campania	500	172	196	132	1.2	0.8	1.2	p <0.05
Italy, Veneto								
Italian whites	385	128	198	59	1.0	1.1	0.9	
Spain, multicentre								
Spanish whites	601	265	265	71	1.0	1.0	1.0	
France, Strasbourg	178	72	85	21	1.0	1.0	0.9	
The Netherlands	188	97	79	12	1.0	1.1	0.9	
Finland, Helsinki	545	293	230	22	1.0	1.1	0.6	p <0.05
Hungary	378	165	171	42	1.0	1.0	1.0	
Russia	587	312	234	41	1.0	1.0	1.0	
Middle East								
Israel	210	120	72	18	1.0	0.9	1.3	
China								
North, Han	643	201	315	127	1.0	1.0	1.0	
South, Han	430	167	228	35	0.9	1.2	0.7	p <0.05
Oceania								
Australia								
Whites	288	146	119	23	1.0	1.0	1.0	
Americas								
Mexico	500	91	248	161	1.0	1.0	1.0	
USA, Atlanta								
Whites	300	142	126	32	1.0	1.0	1.1	
Blacks	298	231	59	8	1.0	0.9	1.7	
Hispanics	62	22	29	11	1.0	1.0	1.0	
Asian	26	16	9	1	1.0	1.0	0.9	
Other, unknown	100	63	32	5	1.0	1.0	1.1	
Canada, Alberta								
Whites	240	136	90	14	1.0	1.0	1.0	

TT homozygotes increased from western Canada (Alberta) to south eastern United States (Atlanta) and peaked in Mexico.

Ethnic variation was apparent among and within geographical areas. In metropolitan Atlanta, for example, TT homozygosity was common among newborns of Hispanic origin (15%), intermediate among those of European origin (11%), and low among African-American newborns (3%). These data are consistent with the high prevalence of TT homozygosity among newborns from Mexico in this study and with published data from the population based sample of babies of Mexican ancestry from California.[14] The low prevalence among US blacks is similar to that reported in pooled estimates of five studies on US blacks and three studies from sub-Saharan Africa[3] as well as in later studies from South Africa and Zimbabwe.[15 16] The intermediate prevalence among whites in Atlanta is consistent with similar rates observed in several European areas in this and several other studies.[3] However, more detailed comparisons are difficult because of the misclassification and imprecision of such ethnic labels.

Of note is the finding in Australia of a lower prevalence of the TT genotype among whites (7.5%) compared to previous reports.[17] Also, we noted a relatively low prevalence of the TT genotype (5.8%) among the random sample of white newborns in Alberta (Canada), compared to the frequency (11%) reported in a previous study from Quebec (Canada).[18] The latter study differed from ours in that newborns were enrolled from a single university hospital in Montreal and were selected, by design, so that their birth weights were at or above the 10th centile.[18]

The high frequency of TT homozygotes observed in this study among newborns from Mexico, northern China, and southern Italy was notable. These findings confirm and extend those previously reported from Mexico[19] and southern Italy.[20] Why such high rates of TT homozygosity occur in these regions is unclear, given the apparently limited ethnic, genetic, or environmental commonalities among such areas. Researchers have suggested the possibility of heterozygote advantage with respect to the risk for neural tube defects.[21] However, such a hypothesis remains unconfirmed. Nevertheless, further exploration of gene-gene and gene-environment interaction might help to identify the evolutionary pressures favouring a high prevalence of this gene variant in certain areas and ethnic groups.

The impact of such geographical and ethnic variation on the distribution of disease in the population is unclear. For example, one would predict high rates of neural tube defects, whose risk appears to be increased nearly two-fold in the presence of 677C>T homozygosity[3] in those geographical areas or ethnic groups with a high frequency of this genotype. The evidence supporting such relations is mixed. For example, the data are consistent for Mexico and northern China, which not only have a very high frequency of the TT genotype but also high rates of neural tube defect.[22 23] Furthermore, within China, rates of neural tube defect are higher in the north (where the TT homozygous genotype is more common) than in the south.[23] In the United States, the rates of neural tube defects historically have been higher among Hispanics, intermediate among non-Hispanic whites, and lower among African-Americans, a trend that follows the relative frequency of the TT homozygous genotype.

There are, however, notable exceptions. In southern Italy, for example, the TT genotype is common, but the rate of neural tube defects is not particularly high.[22] Nevertheless, such exceptions are not entirely unexpected, because environmental and nutritional factors are likely to modulate considerably the genetic risk for neural tube defects. In fact, these exceptions might prove particularly valuable when investigating the aetiological heterogeneity and the role of interactions in the occurrence of neural tube defects.

Similar analyses are possible with respect to other outcomes. For example, recent meta-analyses showed associations of the TT genotype with ischaemic heart disease, deep venous thrombosis, and perhaps stroke.[24] Like neural tube defects, these health outcomes are subject to interacting risk factors and therefore the relation between genotype and outcome at a group level is likely to be complex. Nevertheless, researchers seeking to understand such relations might find data such as these on the geographical and ethnic variation of the 677C>T allele helpful.

On a population level, the genotype distribution associated with the T allele was generally consistent with Hardy-Weinberg expectations. However, a few significant deviations did occur, mostly at the ends of the frequency spectrum. An excess of TT homozygotes was observed in southern Italy (Campania), where the allele was common, whereas the reverse was observed in Finland (Helsinki) where the allele was uncommon. Though these two deviations from Hardy-Weinberg expectations could be the result of chance and multiple statistical testing, they might also suggest the presence of local selective pressures.

In interpreting the findings of this study, one should consider its strengths and limitations. Although we attempted to draw unbiased, systematic samples of newborns from defined populations, sampling strategies varied across areas, and one cannot be certain that the efforts were always entirely successful. We provide details on sampling procedures as guidance to readers who wish to use part or all of these data. Dealing with race and ethnicity was also a difficult but inescapable challenge. Classifications based on self report and particularly on the birth place of the parent or last name are unsatisfactory to varying degrees. Thus, we present our data (table 1) either stratified in two groups (the main ethnic group and all other groups combined), or present data only for the major ethnic group (for example, Han Chinese). While this approach does not solve the difficulties entirely, it decreases the misclassification inherent in defining the many smaller ethnic groups that coexist in many areas. Other limitations of this study include the lack of coverage from many areas of the world, including most of Africa, the Middle East, Latin America, and the Indian subcontinent.[25]

Another challenge of this study was addressing measurement error in genotyping. One might speculate, for example, that deviations in Hardy-Weinberg equilibrium may be the result of genotyping errors. However, inter-laboratory consistency and quality control measures showed remarkable agreement among laboratories. In addition, the same laboratory that assayed samples from areas showing deviations from Hardy-Weinberg equilibrium also assayed the samples from many areas not showing such deviations, suggesting no systematic laboratory error.

A strength of the study was the ability to assemble systematically relatively large samples from newborns using explicit sampling protocols. Measures were also taken to ensure the reliability and comparability of genotypic data across laboratories, including quality control protocols that involved blind retesting of results and exchange of specimens.

Data from studies such as these can serve several purposes. Geneticists could find them useful when evaluating the distribution of genetic variation in human populations and its role in genetic susceptibility to disease. For example, population data might help geneticists reassess controversial associations such as that between *MTHFR* genotypes and risk for Down syndrome,[26–29] for which the evidence favouring the association was largely derived from comparisons with convenient samples of controls. As discussed previously, these genetic data can help to interpret prevalence gradients of disease, such as the well known geographical gradients of neural tube defect occurrence. Similarly, huge amounts of data on other outcomes, such as other birth defects, pregnancy complications, certain cancers, adult cardiovascular disease, and certain psychiatric disorders,[3–8] could be called upon to interpret the prevalence gradients noted in this and other studies. Our data are offered as a contribution to such investigation.

Population data on the 677C>T variant might also help population and public health geneticists assess the potential impact of preventive measures based on environmental modifications. For example, some adverse biochemical effects of the thermolabile enzyme coded by the T allele, such as the increase in total plasma homocysteine, appear to be reversible by increasing the consumption of the B vitamin folic acid.[30] If the effect of folic acid varies by genotype, then the overall impact in the population of fortification or supplementation programmes might vary predictably once the genotype distribution is known.

Finally, a practical outcome of this collaborative study was to show the feasibility of conducting such genetic surveys using existing networks of hospitals, birth defect registries, and research institutions. Other research groups have carefully selected and examined large and representative samples of newborns from single states or countries (for example, California[14] and Ireland[13]) and generated genotype prevalence data. We tried to expand such efforts to an international scale, and suggest that, with appropriate planning, such international networks can use their access and experience in community based studies to provide core data on the population distribution of common gene variants. These data in turn can serve as the foundation for studies of genetic variation and its role in increasing or decreasing disease risk.

ACKNOWLEDGEMENTS

The study was supported in part by grant U50/CCU207141 from the US Centers for Disease Control and Prevention. The work by Generoso Andria (Napoli) was partially supported by grants No 97.03983, 98.02936, and 99.2368 from CNR Rome. We also gratefully acknowledge the contribution of the following researchers: Australia: Paul Lancaster; Canada (Alberta): Brian Lowry; China: Wanyin Shen, Youjun Gao, Fei Deng, Chunna He, Shuqin Zhang, Yabin Liu, Guangfeng Ju, Hua Dong, Zhongyue Yuan, Dongping Ye, Ping Bai, Yuqin Zhang, Li Jin, Qian Gao; Hungary: László Tímár (National Centre of Health Promotion, Budapest); Israel: Paul Merlob (Beilinson Medical Center, Petah Tikva); Italy (Campania): Roberta Arsieri and Carmela Cafasso (Birth Defects Register of Campania); Italy (Napoli): Roberto Brancaccio and Anna Buoninconti (Department of Paediatrics, Federico II University, Napoli); Italy (Veneto): Luciano Marcazzo' (Paediatrics Unit, Arzignano Hospital, Vicenza); Mexico: Marcela Vela (Reproductive Health Agency of Ministry of Health); Russia (Moscow): Swetlana Kalinenkova (Neonatal screening programme); Spain: P Aparicio, F Ariza, I Arroyo, A Ayala, F Barranco, M Blanco, J M Bofarull, M J Calvo, R Calvo, A Cárdenes, S Castro, C Contessotto, M T Cortés, F Cucalón, J Egüés, M J Espinosa, V Felix, E Fernández, A Foguet, J M Gairi, E Galán, A García, M J García, M M García, J L Gomar, H Gómez, F Gómez, J Gómez-Ullate, J González de Dios, P Gutiérrez, F Hernández, H Huertas, N Jiménez-Muñoz, M M Lertxundi, A Lara, J A López, E Mancebo, J J Marco, A Martínez, N Martínez, G Martínez, S Martínez, V Marugán, C Meipp, A Moral, M C Morales, A Moussallem, I Mújica, M J Oliván, L Paisán, M Pardo, A Peñas, J L Pérez, I Puig, I Riaño, C Ribes, M J Ripalda, J Rosal, L Rota, J Rubio, A Sanchis, M Silveira, M E Suárez, J M de Tapia, M C Tauler, L Valdivia, M S Vázquez; USA (Atlanta, Georgia): Jennifer Rapier and Vicki Brown (CDC), Muthukrishnan Ramachandran (Georgia Public Health Laboratory, Atlanta).

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Small babies receive the cardiovascular protective apolipoprotein ϵ2 allele less frequently than expected

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J Med Genet 2003;40:626–629

A newborn whose weight for gestational age and sex is less than expected, based on population standards, is considered as having intrauterine growth restriction (IUGR); a cut off at less than the 10th centile is often used to define IUGR. Causes of IUGR remain unclear although a number of fetal and maternal risk factors have been identified.[1 2] Increased early morbidity and mortality, as well as, possibly, less than optimal neuropsychological development, have been reported as consequences of IUGR.[2 3] In addition, small size at birth has been associated with health problems in adulthood such as coronary heart disease and dyslipidaemia.[4 5] The association between restricted fetal growth and adult chronic diseases (often referred to as the Barker hypothesis) is now considered robust and possibly causal.[6]

Apolipoprotein E (apoE) is one of the key regulators of plasma lipid levels as it affects hepatic binding, uptake, and catabolism of several classes of lipoproteins.[7] The apolipoprotein E gene *(APOE)* codes for the apoE protein; in animal models, underexpression of the *APOE* gene and lack of the apoE protein result in increased susceptibility to atherosclerosis,[8 9] whereas gene overexpression displays anti-inflammatory, antiproliferative, and atheroprotective properties.[10] ApoE has also emerged as a central factor in various biological processes such as immunoregulation, control of cell growth and differentiation,[11] and brain development.[12] The three common allelic variants at the *APOE* locus (ϵ2, ϵ3, ϵ4) code for three major apoE protein isoforms (E2, E3, E4). These isoforms differ from one another only by single amino acid substitutions, yet these changes exhibit functional consequences at both the cellular and molecular levels.[13 14] In previous studies, children who carry the ϵ4 allele and those who carry the ϵ2 allele have been shown to have, respectively, higher and lower total cholesterol and low density lipoprotein (LDL) cholesterol than those with the ϵ3/ϵ3 homozygous genotype.[15 16]

Key points

- A newborn whose weight for gestational age and sex is less than expected, based on population standards, is considered as having intrauterine growth restriction (IUGR).
- The *APOE* gene has three common allelic variants (ϵ2, ϵ3, ϵ4), which result in functional consequences at both the cellular and molecular levels. The ϵ2 allele has been associated with lower total cholesterol and low density lipoprotein cholesterol.
- We studied the transmission of the three *APOE* alleles from heterozygous parents to newborns with IUGR and found a significantly reduced transmission of allele ϵ2.
- Because the ϵ2 allele has been associated with a lower risk of cardiovascular disease, while babies born with growth restriction are reported to be at higher risk for such disease later in life, our data seem to reconcile these two observations.

Despite mounting evidence indicating the participation of *APOE* polymorphisms in various developmental processes involving cell growth and differentiation, atherosclerosis, brain development, and other disorders, we found no report on the relation between *APOE* polymorphisms and IUGR. Given the data suggesting changes in cardiovascular disease (CVD) risk with the different apo ϵ alleles, and the data showing that growth restricted babies are at higher risk of CVD, we thought it justified to examine if there is or is not preferential transmission of the apo ϵ alleles from parents to IUGR cases.

METHODS

Study subjects

We carried out a study of IUGR in relation to thrombophilic polymorphisms.[17] Cases were newborns whose birth weight was below the 10th centile for gestational age and sex, based on Canadian standards.[18] All cases seen at our centre between May 1998 and June 2000 who were born alive after the 24th week of gestation and without severe congenital anomalies were eligible for the study if the mother agreed to participate. The project was approved by the Institutional Review Board of the hospital. Informed consent was signed by the mother to collect cord and maternal blood. During that period, 505 newborns met the criteria for cases and 493 participated in the study (97.6%). In the original study, we also included controls, which are not used in the present report. Midway through this study, we started to collect buccal swabs from the fathers of babies to be included in the final phase of the study. The goal was to analyse case-parental trios (mother, father, newborn) to test for association and linkage.[19] Among the fathers contacted, 86% provided genetic material. We genotyped 449 newborns and 440 mother-newborn pairs for *APOE* (89% of all case pairs); there was enough DNA from the buccal swabs remaining to genotype *APOE* in 194 fathers (78% of fathers providing DNA). Genotyping of all three family members was complete for 170 trios.

Laboratory investigation

Human genomic DNA was extracted from whole blood samples (mothers and newborns) or from buccal swabs (fathers), as previously described.[17] Briefly, PCR reactions were performed with a final reaction volume of 50 l, using 50-100 ng of DNA template per tube under the following conditions: an initial DNA denaturation step at 94°C for three minutes before adding the mixture containing the *Taq* DNA polymerase enzyme; this was followed by a 40 cycle sequence of primer annealing at 62°C for 30 seconds, extension at 72°C for one minute, and denaturation at 94°C for 30 seconds with a 10 minute final extension step at 72°C. The PCR *APOE* primer sequences were as follows: 5′ CGGGCACGGCTGTCCAAGGA 3′ (forward) and 5′ CGGGCCCCGGCCTGGTACAC 3′ (reverse).[20] Allele specific oligonucleotide hybridisation assays were performed as described by others.[21] PCR products were denatured, divided into aliquots, and blotted onto nitrocellulose membranes. Positive and negative controls were included on each membrane. Specimens of family members were assigned randomly to membranes. After hybridisation and washing, the membranes were read using PhosphoImager (Molecular

Dynamics, Sunnyvale, CA) and an automatic scanning program. Membranes were also read visually by two independent observers and again by three observers together. Disagreements after this step were resolved by reamplification, digestion with *Hha*I restriction enzyme, and gel electrophoresis.[20]

Statistical analysis

The general goal of the analysis was to determine if transmission of an allele from the parents to the newborn departs from the expected probability (50%). A statistically significant departure from the expected (or preferential transmission) is indicative of linkage and association. First, we used the transmission disequilibrium test (TDT) that analyses the transmission of alleles from heterozygous parents to their affected child. To analyse the trait defined as presence of IUGR, we used the Family Based Association Test (FBAT) program.[22] FBAT replaces missing values for the unobserved parental genotype using the distribution of offspring genotype.[23] We also used the TDT program from STATA,[24] which does not replace missing values; both programs use a McNemar test for matched pairs and gave exactly similar statistical test results and p values. We report the results of the former. The FBAT test provides correct results regardless of population admixture, the true genetic model, and the sampling strategy.[22 23] With FBAT, we also analysed the trait as quantitative, using birth weight, adjusted for sex, gestational age, and race; the residuals standardised to variance equal to 1 were used as the quantitative trait in this analysis. In each instance (using IUGR or birth weight as the trait), biallelic tests were carried out (each allele against the others) as well as a global chi-square test for all the alleles (multi-allelic test); an additive model, which counts the number of alleles the offspring have, was used. In the second approach, we used a logistic regression model as an extension of the TDT. Here, the outcome is defined as the transmission of the high risk allele (coded as 1) versus the transmission of the other allele (coded as 0).[25] With a multi-allelic locus, one can define one of the alleles as high risk and contrast its transmission with that of the other alleles. We chose the ϵ2 allele as the high risk allele. In this analysis, a model including only the intercept is equivalent to a TDT test. Other factors can be included in the model to determine if the transmission probabilities are modified by these factors. The former TDT analyses treat transmissions from parents independently. Schaid and Sommer[26 27] proposed a method to study the association of a candidate gene with disease using case and parental data; the program GASSOC,[28] which was developed to test for the transmission of alleles from both parents together, was used in our third analysis.

Finally, we carried out an analysis using FBAT without 32 trios where the placenta, on routine pathological examination showed, as previously reported,[17] signs of infarction and where the proband was genotyped for *APOE*. The second and third analyses (logistic regression and GASSOC) use only complete trios; there were 10 trios where a proband with placental infarction was involved and we removed these.

RESULTS

Table 1 shows the allele and genotype frequency distributions in 449 probands genotyped for *APOE* according to maternal racial groups. Allele ϵ2 was found more frequently in blacks than in whites as was allele ϵ4. The other racial groups included small numbers of subjects. The average birth weight in the genotyped group of newborn cases was 2425 g (±577); 54.8% were girls and 20.5% were born before the 37th week of gestation.

Results of the TDT analysis using the FBAT program[22] with the observed and expected numbers of transmitted alleles are shown in table 2. There was a significantly reduced transmission of allele ϵ2 and a marginally significant excess transmission of allele ϵ3. Transmission of allele ϵ4 did not depart from the expected. Overall, transmission of apo ϵ significantly departed from the expected probabilities. Removing the 32 trios where probands had signs of placental infarction did not change any of the conclusions: for allele ϵ2, 16 transmissions were observed while 27 were expected (p=0.002), whereas for allele ϵ3 these were 132 and 199, respectively (p=0.02), and 44 and 44.5, respectively, for allele ϵ4 (p=0.74). The global chi-square was 9.78 (p=0.007).

Table 1 Frequencies of *APOE* alleles and genotypes in 449 newborn probands with intrauterine growth restriction according to racial groups

	White (n=295)	Black (n=109)	Asian (n=24)	Amerindian/ Hispanic (n=21)
Alleles				
ϵ2	40 (6.8)	24 (11.1)	4 (8.8)	3 (7.1)
ϵ3	469 (79.5)	141 (65.6)	33 (79.3)	38 (90.4)
ϵ4	81 (13.7)	50 (23.2)	8 (17.7)	1 (2.3)
Genotypes				
ϵ2/ϵ2	0	0	1 (4.2)	0
ϵ2/ϵ4	7 (2.4)	3 (2.7)	0	0
ϵ2/ϵ3	33 (11.2)	21 (19.2)	5 (20.8)	3 (14.3)
ϵ3/ϵ3	188 (63.7)	41 (37.6)	11 (45.8)	17 (80.9)
ϵ3/ϵ4	60 (20.3)	38 (34.8)	6 (25.0)	1 (4.7)
ϵ4/ϵ4	7 (2.4)	6 (5.5)	1 (4.2)	0

Table 2 Transmission of the *APOE* alleles to the probands with intrauterine growth restriction using FBAT

	ϵ2	ϵ3	ϵ4
Observed	19	143	48
Expected	29	131.5	49.5
Z test*	–2.63	2.06	–0.31
p value	0.008	0.04	0.75

*Each allele is compared to the others.
Global χ^2 test (2 df) = 7.56; p=0.02.

Table 3 shows the results of the analysis with the FBAT program when using the birth weight of the probands as the trait. Although there was a tendency towards a deficit in transmission of allele ϵ2, the results were not statistically significant. Removing the 32 trios where probands had signs of placental infarction did not change any of the conclusions (data not shown).

A logistic regression analysis with transmission of allele ϵ2 as the outcome of interest confirmed the results of all previous analyses showing a significantly reduced transmission of allele ϵ2 (results not shown). Introducing race, sex, birth weight, gestational age, and maternal smoking, each in turn in the model, did not affect the transmission probabilities as measured by the likelihood ratio (LR) chi-square statistic comparing nested models. Removing from this analysis, which uses complete trios, the 10 trios where probands had signs of placental infarction did not materially change the results.

Finally, using the GASSOC program, we rejected the null hypothesis that the genotype relative risks were null based on the LR statistic (7.7, 2 df, p=0.02). Under the additive model, allele ϵ2 was associated with half the risk of allele ϵ3, used as the reference allele (relative risk (RR) = 0.44, p=0.008), while the risk was close to null for allele ϵ4 (RR=0.82, p=0.39). The dominant model was also compatible with the data (score statistic = 6.8, 2 df, p=0.03). The recessive model could not be estimated well with only one case carrying the ϵ2/ϵ2 genotype.

Table 3 Results of the FBAT on birth weight among probands with intrauterine growth restriction

Allele	S*	E(S)†	Z score‡	p value
ϵ2	−2.55	3.41	−1.57	0.11
ϵ3	17.46	12.24	1.08	0.27
ϵ4	9.96	12.91	0.20	0.83

Using a global χ^2 (2 df) = 2.35, p=0.31.
*Test statistic for the observed number of transmitted alleles.
†Expected value of S under the null hypothesis of no linkage and no association.
‡Each allele is compared to the others.

Removing from the analysis the 10 trios where the proband had signs of placental infarction did not materially change the results.

DISCUSSION

Using a family based study design and related statistical tests, we consistently found a significantly reduced transmission of allele ϵ2 to newborns affected with intrauterine growth restriction; in other words, allele ϵ2 seems protective against IUGR. Overall, results indicated significant linkage disequilibrium (linkage and allelic association) between the *APOE* polymorphisms and IUGR. On the other hand, using the probands' birth weight as the trait, we did not observe significant deviation in the number of transmissions from the expected. One possible explanation for the latter results is the relatively limited variation in birth weight among cases. Removing the trios where placental infarction was found on routine pathological examination did not alter any of the conclusions, although the results for the presence of IUGR were even more statistically significant. This could point to a different cause for the growth restricted newborns with placental infarction because their inclusion in the analysis seems to dilute the effect; however, there was only a small number of such newborns and placental infarction reported on gross examination was not confirmed histologically in this study.

If we assume that allele ϵ2 is protective against later cardiovascular disease, the fact that it is less often transmitted to babies who are born small gives support to the Barker hypothesis. Indeed, the hypothesis suggests that newborns with small body size are more prone to later cardiovascular diseases. To our knowledge, there are no previous reports showing linkage disequilibrium between the *APOE* locus and IUGR.

The relation between the apo ϵ2 allele and cardiovascular disease is complex. A protective role for the allele in the development of CVD has been reported[29 30] and this seems particularly marked in younger people.[31] The apo ϵ2 allele is also associated with lower LDL cholesterol levels,[15 16 32] as well as with a survival advantage[33 34] which could be the result of a reduced risk of cardiovascular disease. However, the apo ϵ2 allele is also associated with higher triglyceride levels and possibly with other adverse outcomes such as diabetic nephropathy in type I diabetes[35] and hypertension.[36] In addition, homozygosity for the apo ϵ2 allele predisposes to the development of type III hyperlipidaemia.[37] Our data suggested a small excess in the transmission of allele ϵ3, for which chance is still an explanation. Long term follow up studies conducted in mice overexpressing apoE3 showed clear retardation of atherosclerotic and xanthomatous lesions.[38] Finally, the role of the apo ϵ4 allele on CVD seems clearer: its presence has been associated with an increased risk in CVD as well as with higher total and LDL cholesterol[30] compared with the other apoE isoforms. We found no departure from expected in the transmission of allele ϵ4. It is possible that functions of the apoE isofoms other than those associated with lipid abnormalities are involved in IUGR. However, this study cannot address these issues. The group of IUGR newborns in this study and their mothers were very typical in their clinical and personal characteristics of similar published groups.[17] There were population substructures in the studied group, but the family based analysis we used is robust against such a bias.

In conclusion, our results are indicative that the apo ϵ2 allele is transmitted significantly less often than expected among babies whose birth weight for gestational age and sex was below the 10th centile. Because the apo ϵ2 allele has been associated with a lower risk of cardiovascular disease, and babies born with growth restriction have been found to be at higher risk of cardiovascular disease, our data reconcile these two observations. IUGR is a complex disease about which we know little in terms of mechanisms. In a previous study, we have excluded the role of thrombophilic polymorphisms as potential contributing causes for IUGR status.[17 39] The results of the present study may suggest an underlying atherosclerotic mechanism for IUGR. Despite the plausibility of our results, they need to be replicated in independent studies.

ACKNOWLEDGEMENTS

The study was supported by grants from the Canadian Institutes of Health Research (MA-14705 and MOP-53069) and the Research Foundation of CHUME Sainte-Justine. Claire Infante-Rivard holds a Canada Research Chair (James McGill Professorship).

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

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J Med Genet 2003;**40**:629–631

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.

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

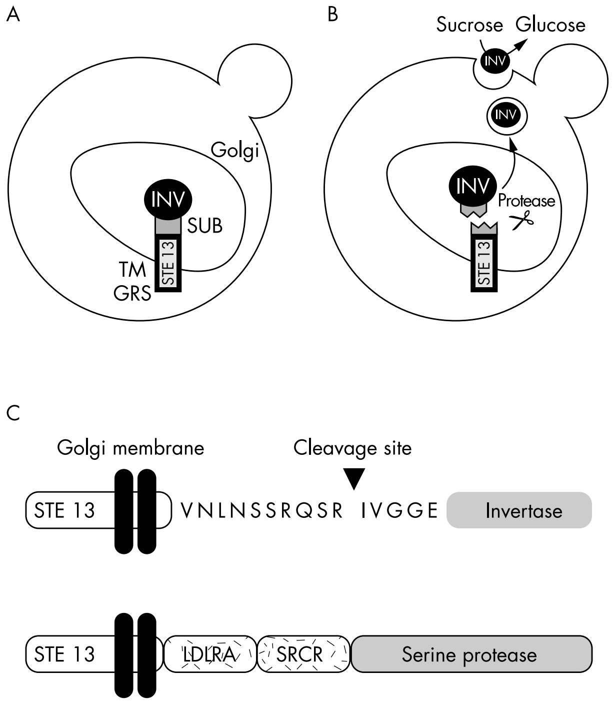

Figure 1 Principle of sGASP. In a yeast strain lacking invertase activity ($suc2^-$), a fusion protein is expressed in which invertase is linked to the truncated lumenal domain of an integral Golgi membrane protein, STE13, by a short substrate sequence containing linker. In the absence of cleavage of 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*

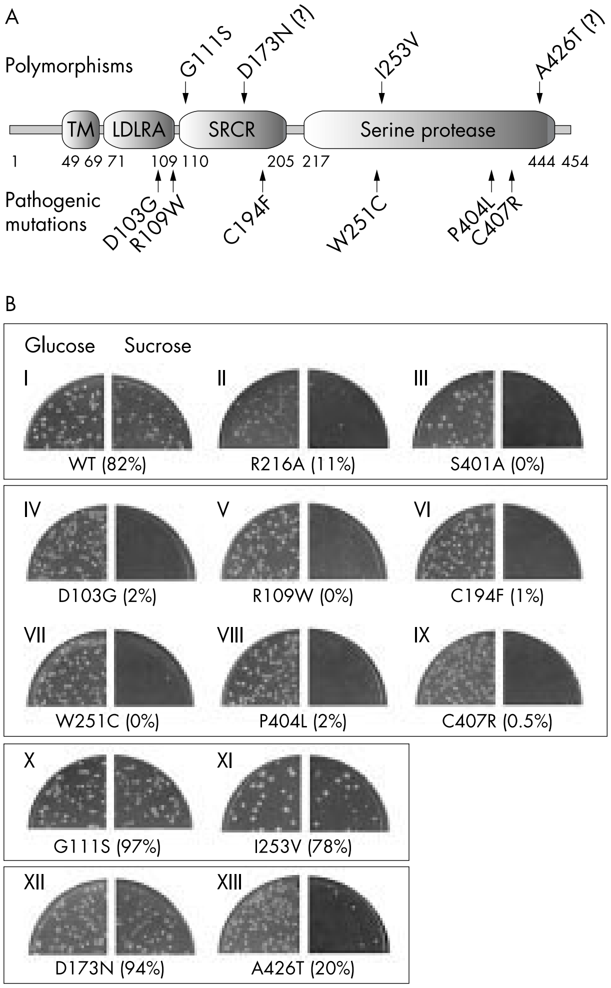

Figure 2 Protease assays for pathogenic mutations and polymorphic alterations. (A) Schematic representation of TMPRSS3 with pathogenic mutations and polymorphisms. (B) Transformants expressing both the STE13-substrate-invertase fusion protein and STE13-TMPRSS3 (wild type or variants) were plated on non-selective (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.

ACKNOWLEDGEMENTS

The first two authors contributed equally to this work. This work was supported by grants from the National Research Laboratory programme (M1-0104-00-0007) of the Ministry of Science and Technology and the BK21 programme of the Ministry of Education, Korea.

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Heteroplasmy for the 1555A>G mutation in the mitochondrial 12S rRNA gene in six Spanish families with non-syndromic hearing loss

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J Med Genet 2003;**40**:632–636

Hearing impairment is the most prevalent sensory disorder and genetic causes are thought to be responsible for over 60% of the cases in developed countries.[1] Inherited hearing impairment is highly heterogeneous from both the clinical and genetic points of view.[1 2] It varies in age of onset, severity, and audiological characteristics, and it can be associated or not with other clinical features (syndromic or non-syndromic hearing impairment). Genetic transmission includes autosomal (dominant and recessive), X linked, and maternal inheritance patterns. This unparalleled heterogeneity is well illustrated by the fact that over 70 loci in the nuclear genome have been reported to be involved in non-syndromic hearing impairment, and about 30 genes have been isolated from their critical intervals.[3] Furthermore, a number of different mutations in several genes of the mitochondrial genome are responsible for syndromic and non-syndromic forms of hearing loss.[4 5]

Mutations responsible for maternally inherited non-syndromic hearing loss are so far confined to only two genes in the mitochondrial genome. These include mutations 7510T>C[6] and 7511T>C[7] in the tRNA$^{Ser(UCN)}$ gene, and 1095T>C[8] and 1555A>G[9] in the gene for the 12S rRNA. This last mutation is responsible for a dual phenotype, since it also confers increased susceptibility to the ototoxic action of aminoglycoside antibiotics.[9] Most of these mutations have been reported in a small number of families from several countries, with the exception of 1555A>G, which seems to be more frequent than the others,[10–13] although its real prevalence remains to be determined in most populations. Remarkably, in Spain it accounts for about 15-20% of all familial cases of non-syndromic hearing loss, irrespective of their mode of inheritance and age of onset[14 15] (our unpublished results). In a majority of these patients, the hearing loss is not attributable to aminoglycoside ototoxicity. A phylogenetic analysis of mitochondrial DNA (mtDNA) haplogroups, performed on 50 unrelated Spanish families, showed that the 1555A>G mutation could be caused by over 30 independent mutational events, occurring in mtDNA haplogroups which are common in all European populations.[16] These data indicate that the high detection rate of this mutation in Spain is not the result of a single major founder event, at least with regard to the mitochondrial genome. Given the high prevalence of the 1555A>G mutation in Spain, and the possibility of preventing aminoglycoside ototoxicity in mutation carriers, its detection has become a priority in routine genetic testing. In contrast to other mutations in the mtDNA, which are frequently heteroplasmic,[4 5] the 1555A>G mutation has been found in homoplasmy in all but one of the families reported so far.[17] In that study, the mutation was found in heteroplasmy in three subjects, with the proportion of mutant copies (the "mutation load") ranging from 85-94%. Here we report the genetic and clinical characterisation of six novel unrelated Spanish families segregating the 1555A>G mutation in heteroplasmy, with a wide range of percentages of mutant copies in a total of 19 subjects.

Key points

- Mutation 1555A>G in the 12S rRNA gene of the mitochondrial genome is responsible for non-syndromic hearing loss, as well as for increased susceptibility to the ototoxicity of aminoglycoside antibiotics.
- In almost all the cases reported so far the mutation was found in homoplasmy. Here we report the clinical and genetic characterisation of six Spanish families with sensorineural hearing loss, totalling 19 subjects with heteroplasmy for 1555A>G.
- The proportion of mutant copies ranged from 3.75-96.60%. Subjects carrying less than 20% of mutant copies were asymptomatic or had a mild hearing loss, whereas heteroplasmic subjects with over 52% of mutant copies suffered from moderate to severe hearing loss.
- Taking the six families together, there is a correlation of the mutation load with the severity of the hearing loss. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another.
- Our study illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding the 1555A>G mutation.

METHODS

Subjects

Familial cases of non-syndromic hearing loss were collected with the only criterion of having at least two affected subjects. Our collection procedure did not cause any other bias, such as preferential selection of large pedigrees or compatibility with maternal inheritance. A total of 649 unrelated Spanish families were enrolled in the study. After getting informed consent, peripheral blood samples were obtained from all participating family members, and DNA extraction was performed by standard procedures.

Mutation detection

Screening for the 1555A>G mutation was carried out by PCR amplification of a 339 bp DNA fragment containing the mutation site, followed by digestion with restriction endonuclease *Hae*III, as described previously.[14] In the wild type allele, digestion results in two fragments of 216 bp and 123 bp. The mutation specifically creates a novel restriction site, and so digestion results in three fragments (216 bp, 93 bp, and 30 bp).

Quantification of the mutation load

The proportion of mutant copies was quantified by detection of fluorescently labelled PCR products separated by capillary electrophoresis. A 359 bp DNA fragment was amplified with hot start FastStart *Taq* DNA polymerase (Roche) using 75 ng of DNA from the subject as template. Primer sequences were: upper primer 5′-AGACGTTAGGTCAAGGTG-3′; lower primer, 5′-GTTTAGCTCAGAGCGGTC-3′. The upper primer was fluorescently labelled at 5′ with TET. PCR was carried out in a final volume of 15 µl with the following conditions: an enzyme activation step, at 95°C for six minutes; 10 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 30 seconds; 13 cycles of denaturation at 89°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 30 seconds; plus a final extension step at 72°C for 10 minutes. Subsequently, 8 µl of the PCR product were digested at 37°C for two hours with a large excess (10 units) of restriction endonuclease *Alw*26I (Fermentas), in a final volume of 10 µl. Three microlitre aliquots of digestion were subjected to capillary electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the recommendations of the manufacturer. By using this technique, two fluorescent products can be detected in a given sample, wild type (127 bp) and/or mutant (359 bp, since the 1555A>G mutation destroys the restriction site for *Alw*26I). In heteroplasmic subjects, the proportion of mutant copies was estimated from the peak areas of the fragments, in quantification experiments from three independent PCR amplifications. The quantification results were consistent with those previously observed in agarose gels after *Hae*III digestion.

RESULTS AND DISCUSSION

At least one subject from each of the 649 collected families was tested for the presence of the mitochondrial 1555A>G mutation, the result being positive in 105 families (16%). In the positive cases, a search for mutation carriers was performed on all the remaining participating relatives. This screening showed heteroplasmy for the mutation in 19 subjects from six unrelated families, which also included 12 subjects with the mutation in homoplasmy (fig 1).

All of the mutation carriers in the families with heteroplasmy were studied clinically. There were records of treatment with aminoglycoside antibiotics in only two subjects (S138 I.2 and S160 I.2, streptomycin). Other environmental factors were excluded as causes of hearing loss in all the subjects. No syndromic features were found. Conductive hearing loss was ruled out by otoscopic examination, tympanometry with acoustic reflex testing, and use of the tuning fork tests. Pure tone audiometry, testing for air and bone conduction, confirmed that the hearing loss was bilateral and sensorineural in all affected subjects. Audiograms for air conduction are shown in fig 1. There were no vestibular symptoms except in patient S141 I.2, who reported episodes of positional vertigo. Patients S141 I.2, S141 II.3, and S338 II.1 reported bilateral tinnitus.

As expected, the pattern of transmission of the 1555A>G mutation was consistent with maternal inheritance in all the families (fig 1). The proportion of mutant copies was determined in the 19 heteroplasmic subjects and ranged from 3.75-96.60% (fig 1). The mutation load in the offspring of a heteroplasmic mother was highly variable. For instance, subject S338 I.2 (52.14% of mutant copies) has two sons with the mutant allele in homoplasmy within our detection limits, and another son with values close to homoplasmy for the wild type allele (3.84% of mutant copies). A wide variation is also observed in the offspring of subject S138 I.2 (fig 1). In addition, pedigrees S160 and S068 have some relevant characteristics. Subject S160 I.2, homoplasmic for the mutant allele within our detection limits, has two heteroplasmic daughters (94.74% and 96.15% of mutant copies, respectively). This result indicates that subject S160 I.2 keeps some wild type copies at least in the germline. In peripheral blood, the wild type allele would have been lost or would be in a proportion small enough to go undetected. As regards pedigree S068, heteroplasmic subject II.5 has two sibs (a brother and sister) who are apparently homoplasmic for the wild type allele. However, both her brother (II.1) and a son of her sister (III.1) are affected by bilateral sensorineural hearing loss, more severe in the high frequencies. Two hypotheses may explain these data. First, I.2, the mother of subjects II.1, II.3, and II.5, would have carried the 1555A>G mutation in heteroplasmy. The mutation would remain in II.5, but would have been lost in II.1 and II.3. If this were the case, the hearing loss in subjects II.1 and III.1 would have a cause different from the 1555A>G mutation, which is a plausible explanation given the genetic heterogeneity of non-syndromic hearing loss. It should be taken into account, however, that the characteristics of the hearing loss in subjects II.1 and III.1 closely resemble those of other members of the family, but it is also true that high frequency hearing loss is the most common type. The second hypothesis concerns the possibility that peripheral blood from subjects II.1, II.3, and III.1 contained a very small, undetected, proportion of mutant copies (apparent homoplasmy) or none at all (real homoplasmy). However, the mutation load in the inner ear would be large enough to be pathogenic in subjects II.1 and III.1. Were this the case, its implications would be relevant for genetic diagnosis (see below).

We investigated the effect of heteroplasmy on the severity of the hearing loss. Subjects carrying less than 20% of mutant copies were asymptomatic (S138 II.1 and II.2, S297 III.2, S338 II.3 and II.4), or had a mild hearing loss (subject S297 IV.1, with a U shaped audiogram). Conversely, the remaining 13 subjects, with percentages of mutant copies between 52.14% and 96.60%, suffered from hearing loss. Two of them, monozygotic twins from family S141 (II.1 and II.2) with 70.93% and 64.48% of mutant copies, respectively, had a mild hearing loss for high frequencies. Five others (S068 II.5 and S138 II.3, II.4, II.5, and II.6), with percentages of mutant copies between 69.78% and 96.60%, had normal hearing for low and middle frequencies, but suffered from moderate or severe hearing loss for high frequencies. In the remaining six cases, the audiogram shape was sloping, affecting both middle and high frequencies (S141 II.3, S160 II.1, and S338 I.2; mutant copies ranging from 52.14% to 94.74%) or all the frequencies (S138 I.2, S141 I.2, and S160 II.3; mutant copies ranging from 61.03% to 96.15%). In this last group, subject S138 I.2 had a history of treatment with streptomycin. The study of 10 subjects from families S068, S160, and S338, carrying the mutation in homoplasmy, showed that eight of them had hearing losses which were more severe than those of their heteroplasmic relatives (only one homoplasmic subject, S160 I.2, had a history of treatment with streptomycin). The two remaining cases (S160 II.5 and II.6) were asymptomatic, but it should be considered that they are younger than their four affected sibs, and may be below the age of onset.

A statistical analysis of our data for all the six families showed significant correlation of the mutation load with the hearing thresholds, for all the frequencies (125-8000 Hz range) (fig 2A), and for only the high frequencies (2000-8000 Hz range) (fig 2B). However, these results must be interpreted cautiously, as indicated by intrafamilial analysis. In three families (S068, S160, S338), the severity of the hearing loss clearly correlates with the mutation load. Regarding family S141, the proportion of mutant copies influences the severity, but this seems to be modulated also by age. Conversely, no apparent correlation is observed between the severity of the hearing loss and the mutation load in four sibs from family S138 with mutant copies ranging from 69.78% to 96.60% (II.3-II.6).

In subjects with only high frequency hearing loss, it was difficult to ascertain their age of onset, since frequently they were not aware of their hearing loss. However, a majority of cases with the mutation in heteroplasmy reported that the hearing loss first manifested in adulthood (between 17 and 50 years of age). In contrast, in eight out of 10 subjects carrying

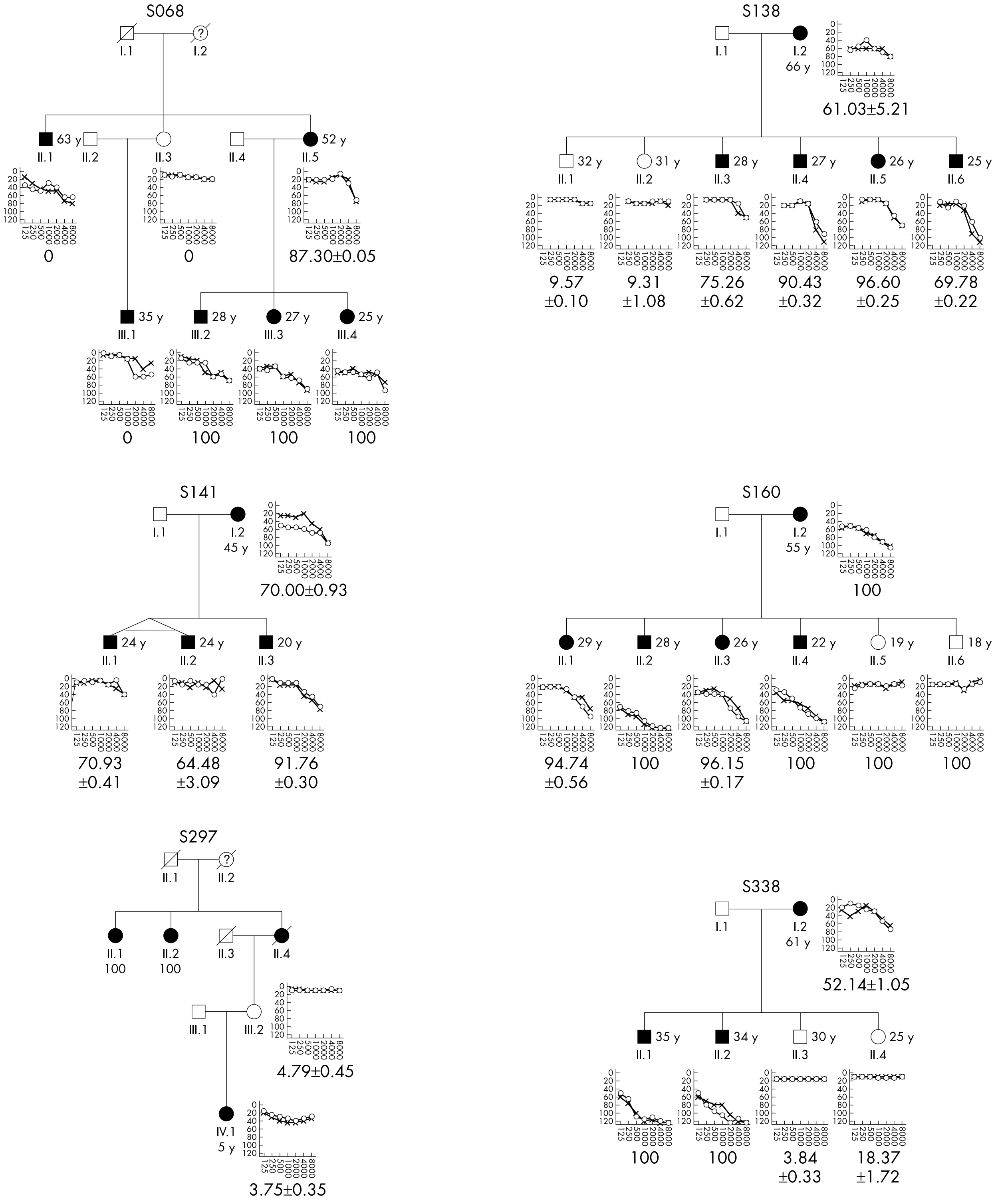

Figure 1 Pedigrees of the six Spanish families segregating the 1555A>G mutation in heteroplasmy. A question mark inside a symbol is used to represent subjects whose clinical status could not be ascertained. Age (in years) and audiograms are shown below or to the right of subject symbols. Hearing level (in dB) is plotted versus sound frequency (in Hz). Since the hearing loss was sensorineural in all cases, only results for air conduction are depicted. Circles, right ear; crosses, left ear. For each subject, the proportion (%) of mutant copies (mean of three independent experiments (standard deviation)), estimated from DNA from peripheral blood, is indicated below the audiogram.

the mutation in homoplasmy, onset was in early childhood (between 1 and 5 years of age).

The study of the genotype-phenotype correlation in subjects carrying the 1555A>G mutation in homoplasmy, which are the vast majority of the cases reported so far, has shown considerable heterogeneity in age of onset, evolution, severity, and other audiological features of the hearing loss resulting from this mutation.[4 5] This variability has been attributed to the influence of both environmental and genetic factors. Undoubtedly, aminoglycoside antibiotics induce a severe worsening of the hearing loss in mutation carriers. In addition, there is in vitro evidence of the influence of the nuclear background

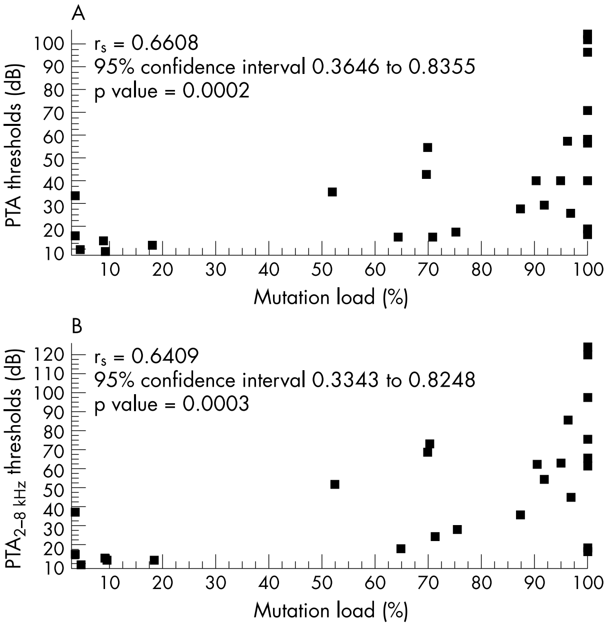

Figure 2 Statistical correlation analysis of mutation load (proportion of mutant allele) and hearing thresholds. Data from a total of 27 subjects from the six families were included in the analysis (the two subjects with a record of treatment with aminoglycoside antibiotics were excluded). Spearman rank correlation tests were performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, California, USA). r_s, Spearman correlation coefficient. (A) Pure tone average (PTA) of hearing thresholds for all the frequencies (125-8000 Hz range) versus mutation load. (B) Pure tone average (PTA) of hearing thresholds for the high frequencies (2-8 kHz range) versus mutation load.

in modulating the phenotype caused by the 1555A>G mutation.[18 19] Also, the hypothesis of the existence of nuclear genes acting as modifiers of mitochondrial hearing loss has recently received strong support.[20–23] The existence of a not negligible percentage of cases with heteroplasmy (5.7% in our sample of 105 families with the 1555A>G mutation) adds more complexity to the picture. Our study of a set of 19 heteroplasmic subjects illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding this mutation. First, among our heteroplasmic cases, most of the subjects carrying less than 20% of mutant copies were asymptomatic, whereas all of the subjects with mutation loads higher than 52% suffered from hearing loss. This suggests that there is a threshold in mutation load for manifestation of clinical symptoms. However, it is also known that there exist subjects homoplasmic for 1555A>G, who are asymptomatic (for example, subjects S160 II.5 and II.6). Second, statistical analysis of our data indicates a significant correlation of the the severity of the hearing loss with the mutation load when considering the six families altogether. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another (family S138). This situation may be because of intrafamilial differences in the nuclear background modulating the phenotype, and/or individual variability in mutation load in peripheral blood and inner ear. In fact, it has been reported that the level of heteroplasmy for a given mutation can vary among different tissues within the same person.[24]

The conclusions of our study are relevant for genetic diagnosis of mitochondrial mutations that are responsible for non-syndromic hearing loss. The estimations of mutation load obtained from mitochondrial DNA from peripheral blood may not always reflect accurately the real situation in the inner ear. In extreme cases, the mutation load may be pathogenic in the inner ear and remain undetectable in blood. Therefore, in large families with several affected subjects and a clear maternal inheritance of the disorder, several probands from different branches in the pedigree should be tested before excluding the presence of the mutation. This issue may be critical for prevention of aminoglycoside ototoxicity in subjects whose carrier status would go unnoticed.

Although the last few years have witnessed great advances in the understanding of mitochondrial pathogenesis, there are many important issues that remain unsolved, such as the basis of tissue specificity and the mechanisms by which a heteroplasmic mutation segregates and is fixed. Further investigation of these matters is needed to improve genetic counselling regarding the 1555A>G mutation.

ACKNOWLEDGEMENTS

We thank the patients and their relatives for their kind cooperation with this study, and FIAPAS for their enthusiastic support of this research. FJdC and MV were recipients of fellowships from the Comunidad de Madrid. MRB and JG were recipients of fellowships from Fundación ONCE and Fundación Marqués de Valdecilla, respectively. This work was supported by grants from the European Community (QLG2-CT-1999-00988), CAICYT of Spanish Ministerio de Ciencia y Tecnología (SAF99-0025), and Spanish Fondo de Investigaciones Sanitarias (FIS 00/0244).

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Uniparental disomy of chromosome 13q causing homozygosity for the 35delG mutation in the gene encoding connexin26 (*GJB2*) results in prelingual hearing impairment in two unrelated Spanish patients

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J Med Genet 2003;**40**:636–639

Inherited hearing impairment is a highly heterogeneous group of disorders with an overall incidence of about 1 in 2000 newborns.[1] In approximately 70% of cases, the auditory impairment is not associated with other clinical features, that is, it is non-syndromic. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait.[1] To date, 31 different DFNB loci for autosomal recessive non-syndromic hearing loss have been reported, and 16 genes have been identified.[2] Among these loci, DFNB1 in 13q12 stands out because of its complexity and clinical relevance. It contains the gene *GJB2*, which encodes connexin26, a component of intercellular gap junctions. Mutations in the *GJB2* gene are responsible for up to 50% of all cases of autosomal recessive hearing impairment in most of the populations tested so far, with a frequent mutation (35delG) accounting for up to 86% of the *GJB2* mutant alleles in white populations.[3 4] However, not all the DFNB1 mutations affect the *GJB2* gene. Recently, several research teams found a deletion in the 13q12 region which is frequently inherited in double heterozygosity with mutant *GJB2* alleles in affected subjects,[5–7] but it was also found in homozygosity.[6 7] Molecular characterisation of this deletion, termed del(*GJB6*-D13S1830), showed that it encompasses 342 kb and it does not affect the *GJB2* gene, but it truncates the gene encoding connexin30 (*GJB6*), another gap junction protein expressed in the inner ear.[6] The existence of this deletion was first suspected by the finding of inconsistencies in the segregation of genetic markers distal to *GJB2*.

Key points

- Mutations in the gene encoding the gap junction protein connexin26 (DFNB1 locus on 13q12) are responsible for up to 50% of all cases of autosomal recessive hearing impairment in most populations, the 35delG mutation being the most frequent in white populations.
- Here we report two unrelated cases of homozygotes for 35delG whose biological fathers were not carriers of the mutation. The study of the segregation of polymorphic genetic markers showed uniparental (maternal) disomy of chromosome 13, causing homozygosity for the mutation. In both cases, the disomic maternal gamete may have resulted from non-disjunction of chromosome 13 in meiosis II.
- These two cases represent the first description of UPD(13) with an abnormal phenotype, and they are also the first cases of UPD resulting in non-syndromic hearing impairment.

Here we report another inconsistency in the segregation of markers in the 13q12 region in two unrelated cases of subjects with prelingual hearing impairment. In these two cases, uniparental disomy of chromosome 13 caused homozygosity for the 35delG mutation in the *GJB2* gene, resulting in the hearing defect.

MATERIALS AND METHODS

Informed consent was obtained from all the subjects that were enrolled in this study. Peripheral blood samples were obtained, and DNA extraction was performed by standard procedures.

Screening for the 35delG mutation was carried out by PCR amplification of a 122 bp DNA fragment including the first 34 codons of the *GJB2* gene, using the following primers: forward, 5′-CAAACCGCCCAGAGTAGAAG-3′; reverse, 5′-CATAATGCGAAAAATGAAGAGG-3′. The forward primer was 5′ end labelled with a fluorescent dye (TET, 6-FAM, or HEX, which allows us to mix three samples per tube in the separation procedure). The PCR reaction was carried out in a total volume of 15 µl including 20-40 ng of genomic DNA from the patient, 10 pmol of each primer, 2.5 nmol of each dNTP, $MgCl_2$ at a final concentration of 1.5 mmol/l, and 0.75 units of Ampli*Taq* Gold DNA polymerase (Perkin-Elmer), in the buffer provided by the manufacturer. PCR was performed using standard conditions with an annealing temperature of 59°C. Protruding tails of adenine nucleotides, which are added by the Ampli*Taq* Gold DNA polymerase to the 3′ ends of the DNA product during the PCR, were eliminated by treatment with T4 DNA polymerase (Roche), under the conditions recommended by the manufacturer. Then, samples were resolved by capillary electrophoresis in an Abi Prism 310 Genetic Analyzer (Applied Biosystems). The rationale of this procedure is to detect the loss of one nucleotide in the mutant allele. Then, the presence of the mutation was confirmed by DNA sequencing of a second PCR product.

Primers and PCR conditions for the amplification of the microsatellite markers used in this study have been previously reported.[8 9]

RESULTS

During the routine screening of subjects with non-syndromic prelingual hearing impairment for the 35delG mutation, we found two unrelated cases, E112-3 and E232-3, who were homozygous for 35delG, and had a relevant characteristic in common. In both cases, the mother of the patient carried the mutation, but the father did not. Both cases were sporadic, not having any other affected relative. In family E112, there was also a brother with normal hearing who was a carrier of the 35delG mutation (fig 1). Both patients and their participating relatives were genotyped for seven microsatellite markers flanking the *GJB2* gene within an approximate 2 cM interval. These included D13S175, D13S1275, and D13S292,[8] and D13S1830, D13S1831, D13S1832, and D13S1835.[9] The marker order is indicated in fig 1. Subject E112-3 was homozygous for all these markers except for the most distal (D13S292). In addition, haplotype analysis showed that the subject had not inherited any allele from his father for five of these markers (fig 1). His brother had inherited the 35delG mutation from their mother, and there was no segregation inconsistency in the alleles he had received from his parents, as expected. Subject E232-3 was homozygous for the seven markers, and she did not share any allele with her father for six of them, as shown by haplotype analysis (fig 1). False paternity was investigated by genotyping the patients and their parents for a series of highly polymorphic microsatellite markers in other chromosomes. Ten markers were completely informative, namely D1S220, D1S234, D1S425, D7S2420, D7S2459, D14S288, D15S153, D15S205, D16S404, and D21S1252.[8] In both cases E112 and E232, the non-maternal alleles of all these markers in the child fitted those of the alleged father, the residual probability of a false paternity being 10^{-7}. This suggested that the anomalous inheritance would be confined to chromosome 13.

Therefore we genotyped the patients and their parents for a set of 13 additional markers from the whole long arm of chromosome 13, evenly distributed at intervals of about 10 cM (fig 1). In patient E112-3, there were two markers with homozygosity for an exclusively maternal allele (D13S217 and D13S265). There was heterozygosity for all the other markers. For six of them, the patient did not share any allele with his father (maternal heterodisomy). For the remaining five markers, although the patient did share at least one allele with his father, his genotypes were also consistent with maternal heterodisomy. As regards patient E232-3, there were four markers with homozygosity for a exclusively maternal allele (D13S156, D13S265, D13S158, and D13S1265). There was also homozygosity for two other markers (D13S173 and D13S285) for which the patient did share an allele with her father, but the genotypes were also consistent with maternal disomy. There was heterozygosity for the remaining seven markers, all the genotypes being consistent with maternal heterodisomy (for two of these markers, the patient did not share any allele with her father).

DISCUSSION

Altogether, our data indicate that the anomalous segregations of the 35delG mutation in the two cases reported here are the result of uniparental disomy (UPD) of chromosome 13. UPD is defined as the inheritance of both homologues of a pair of chromosomes from only one parent.[10–12] This includes isodisomy (two copies of the same parental chromosome), heterodisomy (one copy of each homologue from the same parent), or a mixture of both.[11 12] In case E112-3, maternal isodisomy seems to be confined to 13q11-q12, with maternal heterodisomy in the rest of the long arm. Conversely, in case E232-3, maternal isodisomy alternates with maternal heterodisomy along 13q. Depending on the affected chromosome and on the resulting homozygosities, UPD can produce no clinical manifestations or a diversity of abnormal phenotypes. In our two cases, maternal UPD of chromosome 13 results in homozygosity for the 35delG mutation, which causes profound, prelingual non-syndromic hearing impairment. Similar cases of uniparental disomy creating homozygosity for autosomal recessively inherited mutations have been reported in over 20 disorders.[12 13] No other clinical signs or symptoms were observed in our two patients, who were aged 15 years (E112-3) and 2 years (E232-3) at the time of examination. In the last few years, several cases of either maternal or paternal UPD of chromosome 13 (UPD13) have been reported.[14–20] All of these cases were phenotypically normal, indicating that there are no maternally imprinted genes in chromosome 13.[14 16] Our data further support this conclusion.

Mechanisms leading to UPD include (1) gamete complementation, when the zygote arises from the union of a disomic gamete and a nullisomic gamete; (2) trisomy rescue, when a trisomic zygote loses one chromosome in an early mitotic division (one third of the cases result in UPD); (3) monosomy rescue, by mitotic duplication of the monosomic chromosome, which leads to isodisomy for the whole chromosome; and (4) postzygotic errors, when in a normal zygote one chromosome is lost and it is replaced by duplication of its homologue (isodisomy for the whole chromosome).[11 21] In our two cases of UPD(13), regions of maternal isodisomy and heterodisomy are observed, which excludes monosomy rescue and postzygotic errors. Although we cannot distinguish which of the two remaining mechanisms (gamete complementation or trisomy rescue) led to UPD(13) in the cases reported here, we can conclude that in both cases a disomic maternal gamete was involved. Formation of abnormal gametes is the result of meiotic non-disjunction events. When non-disjunction errors occur in meiosis I, they result in heterozygosity for centromeric genetic markers (primary heterodisomy), whereas if they occur in meiosis II, they result in homozygosity for the

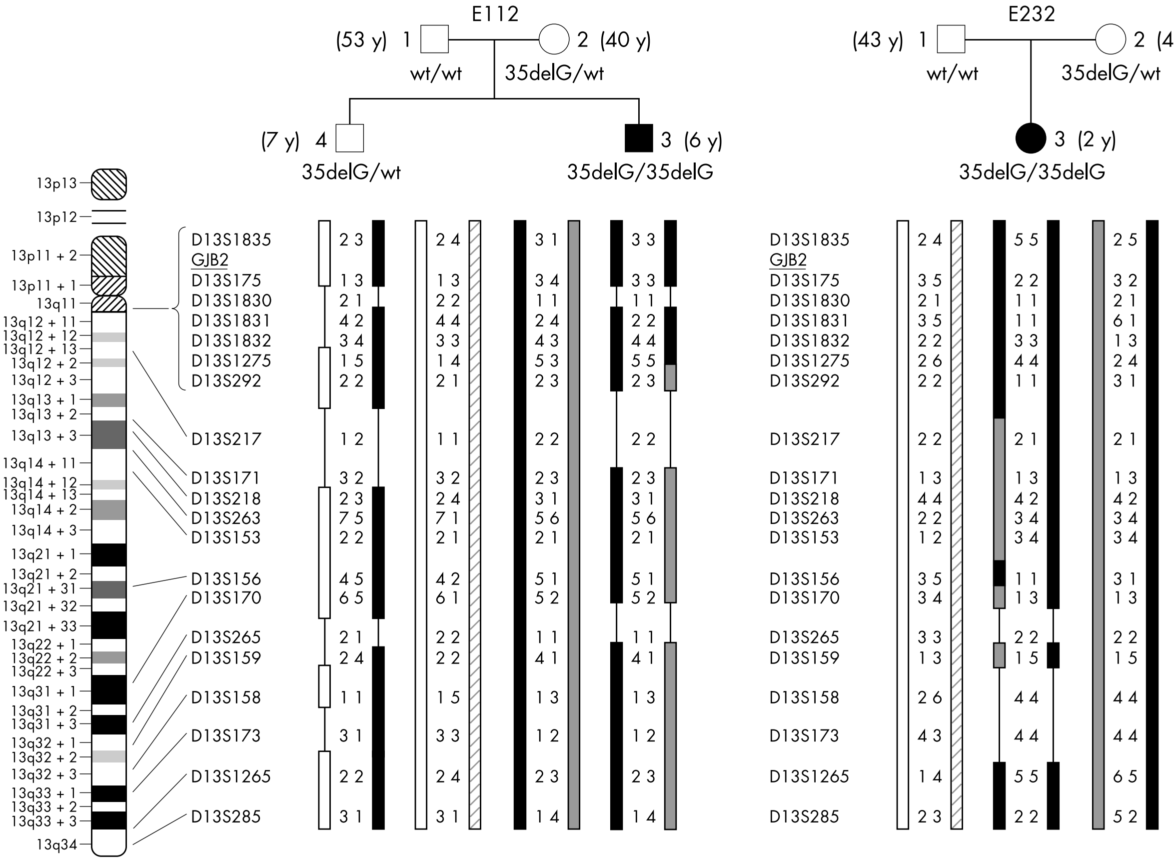

Figure 1 Pedigrees of families E112 and E232, showing the genotypes for a set of genetic markers from chromosome 13. Ages in years (y) are indicated between parentheses. The carrier status for the 35delG mutation in the *GJB2* gene is shown below each subject symbol; wt, wild type allele. The location of the genetic markers, as well as that of the *GJB2* gene, is indicated to the right of a schematic drawing of chromosome 13. Currently, the most centromeric sequence contig from chromosome 13 is NT_009917.11 (NCBI database, http://www.ncbi.nlm.nih.gov). In this contig, marker D13S1835 is approximately 1.55 Mb from the centromeric end. Alleles are represented by numbers. Vertical bars represent chromosomes. Regions of non-informativeness (that is, when it was not possible to determine unambiguously the parental chromosome from which an allele in a child was being inherited) are indicated by thin lines.

centromeric markers (primary isodisomy). Recombination events can introduce regions of homozygosity in a situation of primary heterodisomy (secondary isodisomy) and, conversely, they can introduce regions of heterozygosity in a situation of primary isodisomy (secondary heterodisomy).[22] In both E112-3 and E232-3, there are regions of primary isodisomy for centromeric markers, whereas secondary heterodisomy is observed in other regions. This suggests that the non-disjunction event leading to the formation of the disomic maternal gamete took place in meiosis II. The formation of abnormal gametes leading to UPD is frequently found in association with chromosomal rearrangements. In fact, all of the published cases of UPD(13) were associated with Robertsonian translocations or isochromosomes.[14–20] However, the karyotypes of both patients E112-3 and E232-3 were normal, as well as that of subject E232-1 (father of E232-3) (data not shown). No karyotyping data from the other parents could be obtained.

The two cases reported here are the first cases of UPD(13) with an abnormal phenotype, and they also represent the first cases of UPD resulting in non-syndromic hearing impairment. They were found among a total of 115 unrelated affected subjects who were homozygous for the 35delG mutation (1.7%). Given the high incidence of inherited hearing impairment, this frequency should be kept in mind when performing large screenings of patients for recessively inherited mutations, and UPD should be considered a possibility when anomalous segregation patterns are found in routine genetic testing.

ACKNOWLEDGEMENTS

We thank the members of families E112 and E232 for their kind cooperation in this study, and FIAPAS for their enthusiastic support of this research. AA and MV were recipients of fellowships from the Fondo de Investigaciones Sanitarias (BEFI) and from Comunidad de Madrid, respectively. This work was supported by grants from the European Community (QLG2-CT-1999-00988) (to FM), CAICYT of Spanish Ministerio de Ciencia y Tecnología (SAF99-0025) (to FM), and Fondo de Investigaciones Sanitarias (FIS 00/0244) (to IdC).

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ECHO

Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy

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Background and aims: The aim of this study was to investigate the genetic aetiology of intrahepatic cholestasis of pregnancy (ICP) and the impact of known cholestasis genes (*BSEP*, *FIC1*, and *MDR3*) on the development of this disease.

Patients and methods: Sixty nine Finnish ICP patients were prospectively interviewed for a family history of ICP, and clinical features were compared in patients with familial ICP (patients with a positive family history, n=11) and sporadic patients (patients with no known family history of ICP, n=58). For molecular genetic analysis, 16 individuals from two independently ascertained Finnish ICP families were genotyped for the flanking markers for *BSEP*, *FIC1*, and *MDR3*.

Results: The pedigree structures in 16% (11/69) of patients suggested dominant inheritance. Patients with familial ICP had higher serum aminotransferase levels and a higher recurrence risk (92% v 40%). Both segregation of haplotypes and multipoint linkage analysis excluded *BSEP*, *FIC1*, and *MDR3* genes in the studied pedigrees. Additionally, the *MDR3* gene, previously shown to harbour mutations in ICP patients, was negative for mutations when sequenced in four affected individuals from the two families.

Conclusions: These results support the hypothesis that the aetiology of ICP is heterogeneous and that ICP is due to a genetic predisposition in a proportion of patients. The results of molecular genetic analysis further suggest that the previously identified three cholestasis genes are not likely to be implicated in these Finnish ICP families with dominant inheritance.

▲ *Gut* 2003;**52**:1025–1029