Letters to the Editor

Clustering and frequency of mutations in the retinal guanylate cyclase (*GUCY2D*) gene in patients with dominant cone-rod dystrophies

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EDITOR—Guanylate cyclase (retGC-1) is a key enzyme in the recovery phase of phototransduction in both cone and rod photoreceptor cells.¹ Upon excitation by a photon of light, an enzymatic cascade of events occurs which leads to the hydrolysis of cGMP and the closure of the cGMP gated cation channels. This results in hyperpolarisation of the plasma membrane and the generation of a signal higher up in the visual pathway. Upon closure of the ion channels, the cytosolic levels of Ca²⁺ decrease because export by the Na⁺, K⁺, Ca²⁺ exchanger continues. This reduced Ca²⁺ concentration results in the activation of retGC by activating proteins (GCAPs) and the increased conversion of GTP to cGMP, thus restoring the level of cGMP in the photoreceptors to their dark level.

Mutations in *GUCY2D*, the gene encoding retGC-1, are a cause of Leber congenital amaurosis (LCA1), a recessive condition which manifests itself either at birth or during the first few months of life as total or near total blindness.^{2,3} Recently, we identified mutations in *GUCY2D* in four British families with autosomal dominant cone-rod dystrophy (ADCORD).⁴ Subsequent to this, mutations in this gene were shown to be responsible for ADCORD in a French,⁵ a Swiss,⁶ and a Norwegian⁷ family. In all seven families, the mutations are either in the same or in adjacent codons in a highly conserved region of the protein. In our four families and in the Swiss and Norwegian families, mutations were found in either codon 837 or 838,^{4,6,7} whereas codons 837-839 each encode for an amino acid substitution in the French family.⁵

In order to determine whether ADCORD arising from mutations in *GUCY2D* are restricted to these codons and how important these mutations are to autosomal retinal disease in general, we have screened an additional group of unrelated patients diagnosed with autosomal dominant macular dystrophy or autosomal dominant cone or cone-rod dystrophy.

Methods

MUTATION SCREENING

The coding exons of *GUCY2D* were amplified using the intronic primers and annealing temperatures essentially as described previously^{2,4}

and subjected to heteroduplex analysis.⁸ All fragments exhibiting band shifts were directly sequenced using the PRISMTM Ready Reaction Sequencing Kit (Perkin Elmer PE Biosystems), and the products were visualised on an ABI Model 373 DNA sequencer.

HAPLOTYPE ANALYSIS

One of each primer pair was end labelled with 10 µCi of [γ -³²P]ATP using polynucleotidyl kinase for 30 minutes at 37°C, followed by 10 minutes at 65°C. PCR was carried out using 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP mix, KCl buffer, 0.05 U/ml *Taq* polymerase (Bioline), 0.1 mmol/l of each primer, and 0.1-0.2 µg of genomic DNA. The amplification protocol was 94°C for three minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. The resulting products were visualised on a 6% polyacrylamide/urea denaturing gel. The gel was dried down at 80°C under vacuum and autoradiographed over x ray film overnight. The DISLAMB program⁹ was used to obtain an estimate of linkage disequilibrium.

Results

A group of 40 patients, 27 with autosomal dominant macular dystrophy and 13 with autosomal dominant cone or cone-rod dystrophy, was screened for mutations in all exons of *GUCY2D*. This group was drawn from the same panel that was used in our original study⁴ and is composed of unrelated patients with autosomal dominant macular dystrophies or cone or cone-rod dystrophies attending a Medical Retina Clinic at Moorfields Eye Hospital, London, UK. From this screen, three additional probands with mutations in *GUCY2D* were identified. Of these, two have the identical R838C substitution to that previously reported⁴ and one has a novel G2586A transition in codon 838, resulting in an R838H substitution (fig 1). In addition, a re-examination of our CORD6 family has shown a second mutation, a C2585A transversion again in codon 838 that results in the substitution of arginine by serine (fig 1). This mutation is in the adjacent codon to the originally reported E837D substitution.⁴ This is therefore a second example of a *GUCY2D* disease

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Figure 1 Sequence of exon 13 of *retGC1*. Heterozygous mutations in adjacent codons of the original CORD6 family to give the Glu837Asp and Arg838Ser substitutions, and in family 8 to give the Arg838His substitution are shown.

allele carrying multiple mutations. In total, five of our families carry a C to T change in codon 838, one family has a G to A change in codon 838, and one family has a double mutation in codons 837 and 838. All these mutations were confirmed by restriction enzyme digestion, since all cause the loss of a *HhaI* site. None of these changes were observed in 50 ethnically matched controls. In each case, the diagnosis was confirmed as cone-rod dystrophy⁴⁻¹⁰ (D Bessant, personal communication). Excluding the original CORD6 family, the 90 unrelated patients screened in this and the previous study therefore yielded a total of six ADCORD patients with mutations in codon 838 of the *GUCY2D* gene. The above mutations, together with all previously reported mutations,⁴⁻⁷ are summarised in table 1.

Haplotype analysis was used to investigate whether there is evidence for relatedness among the five families with the R838C substitution (table 2). In order to determine the

Table 1 Dominant cone-rod mutations in the *GUCY2D* gene

Families/probands	Mutation	Amino acid substitution
1*	G2584C	E837D
	C2585A	R838S
2-4†	C2585T	R838C
5-6‡	C2585T	R838C
7§	C2585T	R838C
8‡	G2586A	R838H
9¶	G2586A	R838H
10**	G2584C	E837D
	C2585T	R838C
	C2589T	T839M

*Original CORD6 family.
†Kelsell *et al.*⁴
‡This study.
§Van Ghelue *et al.*⁷
¶Weigell-Weber *et al.*⁶
**Perrault *et al.*⁵

haplotype of the disease chromosome, additional family members were sought. However, family 5 could not be extended beyond the original proband; the disease associated alleles for markers D17S1881 and D17S1852 could not therefore be fully resolved. All families show some commonality for marker alleles adjacent to the *GUCY2D* gene; families 2, 3, 5, and 6 share allele 5 at D17S960, families 2, 4, 6, and possibly 5 share allele 2 at D17S1796, and families 3 to 6 share allele 5 at D17S1881. However, although family 3 shares the same allele as families 4, 5, and 6 at D17S1881, it is unlikely that this is part of a founder haplotype since it would require a double crossover within a very short map interval. An estimate of the likelihood of linkage disequilibrium was obtained from the DISLAMB program⁹ by using allele frequencies obtained from 20 unrelated “married in” subjects in the families. This is significant at the 5% probability level only for D17S960; the lower estimates of λ and p for the other markers reflect in part the common occurrence of the disease associated alleles in the “married in” subjects.

During our extensive sequence analysis of the *GUCY2D* gene, a number of single nucleotide polymorphisms (SNPs) were identified as follows: a silent C220A transversion in exon 2, coding G227A (A52S) and G227T (A52T) changes in exon 2 (the G227T transversion has been previously reported as a possible sequence polymorphism²), a silent G2182A transition in exon 10, a coding T2418A (L783H) transversion in exon 12, a silent G2589A transition in exon 13, a G to A transition in intron 17, and a T insertion in intron 19. Unfortunately, in each of our R838C disease families, the more common nucleotide was present at each position. These SNPs do not therefore help to resolve the ancestry of the R838C mutations.

Discussion

In this and our previous study,⁴ the panel of patients with autosomal dominant disease was drawn at random from unrelated subjects who had received the diagnosis of cone-rod, cone, or macular dystrophy. Our previous study examined 50 members of this panel and identified three probands with an R838C mutation in the *GUCY2D* gene. In this follow up study of

Table 2 Microsatellite markers in the vicinity of the *GUCY2D* gene

	Intermarker distance (cM)	Association with disease		Haplotypes or genotypes in families or probands				
		λ	<i>p</i>	2	3	4	5	6
D17S796	0.13	0	0.5	2 (0.25)	5 (0.30)	2	25	1 (0.23)
D17S938		0.36	0.25	7 (0.06)	1 (0.31)	1	1,3 (0.03)	7
D17S960	0.2	0.57	0.03	5 (0.28)	5	3 (0.21)	5	5
<i>GUCY2D</i>	0	—	—	R838C	R838C	R838C	R838C	R838C
D17S1796	0	0	0.5	2 (0.60)	4 (0.30)	2	2,5 (0.10)	2
D17S1881	0	0.53	0.07	6 (0.17), 8 (0.10)	5 (0.34)	5	5	5
D17S1852	2.2		ND	7	612	910	67	78

The family numbers are identical to those in table 1. The numbers in parentheses are the frequencies of each allele on 40 chromosomes obtained from unrelated, "married in" subjects in the families. The statistic λ gives an estimate of linkage disequilibrium as determined by the DISLAMB program.⁹ Only D17S960 shows significant disease association.

a further 40 patients, three additional patients with mutations in this codon have been identified, two with an R838C substitution and one with an R838H substitution.

The clinical phenotypes in the families with single (R838C or R838H) and double (E837D, R838S) mutations have been reported in detail elsewhere.^{4 10 11} In summary, the cone-rod dystrophy exhibited by the single mutation patients is less severe than that in the original CORD6 family with the double mutation, with mild variation in disease severity in the R838C families. In all cases, photophobia with decreased visual acuity and loss of colour vision is present from early childhood. However, during the early phases of the disorder when visual acuity is still good, a marked reduction in visual function in bright light is characteristically present. Fundoscopic abnormalities are confined to the central macula with increasing central atrophy with age. Electrophysiological testing showed a marked loss of cone function with only minimal rod involvement in the single mutation families. This contrasts with expression in the CORD6 family where moderate to severe rod involvement is present.¹¹ Different mutations in this region of the *GUCY2D* gene can result therefore in differing severities of cone-rod dystrophy, especially with regard to the involvement of the scotopic system.

Pooling across our two studies, a conservative estimate of the overall frequency of mutations in codon 838 of *GUCY2D* among autosomal dominant patients with macular, cone, or cone-rod dystrophy is therefore 6.7%, although this rises to 23% if only the three new mutations found among the 13 cone and cone-rod dystrophy patients examined in this study are considered. It is important to emphasise that these two frequencies are estimates of the relative contribution that mutations in this codon make to the total frequency of autosomal dominant cone-rod disease in the population and that this conclusion is valid irrespective of the presence or absence of a founder effect for the R838C mutations. Whether such a founder effect is present is unclear from the present data. There is evidence for linkage disequilibrium between the disease allele and one of the flanking markers (D17S960) although,

since the disease associated allele is relatively common (28%), this renders the test of association less powerful, and the situation is not further resolved by a number of SNPs scattered through the *GUCY2D* gene, since none was informative in our five families. Where a founder effect has been clearly established, for example for Sorsby's fundus dystrophy,¹² a highly significant disease associated haplotype covering 3 cM of the chromosomal region surrounding the disease gene was present. In contrast, the disease associated haplotype for the R838C mutations covers <0.2 cM. This indicates that either the R838C mutations have arisen separately from each other or that a single mutation occurred in a much more distant ancestor than the common mutation for Sorsby's fundus dystrophy, with a consequent wider distribution in the population. Furthermore and again irrespective of the presence or absence of a common ancestor for our R838C families, the occurrence of the R838C mutation in a presumably unrelated Norwegian family,⁷ the R838H mutations in one of our British families and in a Swiss family,⁶ and the multiple mutations in codon 838 and adjacent codons in the original CORD6 family,⁴ as well as in a French family,⁵ all identify this codon as particularly mutation prone.

Two other dominantly inherited diseases have been associated with mutation prone regions: recurring C to T and G to A transitions were found in adjacent nucleotides within the *MYH7* gene in hypertrophic cardiomyopathy¹³ and recurring G to A transitions and G to C transversions were found at the same nucleotide within the *FGFR3* gene in achondroplasia.¹⁴ The recurring DNA transitions at these two loci are situated at CpG dinucleotides and a study of nucleotide substitution rates¹⁵ has confirmed the high mutability of CpG sequences. The spontaneous deamination of methylated cytosine, its relatively slow repair in mammalian cells, and the production of an intermediate susceptible to deamination in the enzymatic process by which cytosine itself is methylated, are all mechanisms which make CpG sequences preferential targets for spontaneous mutation.^{16 17} It is perhaps significant therefore that the C to T transitions and C to A transversions found in codons 838 and

Codons	Amino acid substitutions
836 837 838 839 840	
CGG GAG CGC ACG GAG	
... ..C T... ..T. ...	E837D, R838C, T839M
... ..C A... ..	E837D, R838S
... ..T... ..	R838C
... ..A... ..	R838H

Figure 2 Nucleotide and amino acid substitutions in exon 13 of *GUCY2D* associated with autosomal dominant cone-rod dystrophy.

839 of the *GUCY2D* gene all occur within a CpG dinucleotide (fig 2). What remains unclear is the mechanism responsible for the generation of multiple mutations in this region of exon 13 of the *GUCY2D* gene.

Recessive mutations in *GUCY2D* are a relatively common cause of LCA. However, the widespread distribution of LCA mutations (including missense, frameshift, and splice site changes) throughout the gene¹⁸ contrasts with the clustering of ADCORD mutations to codons 837, 838, and 839 encoded by exon 13. To date, no LCA mutations have been localised to exon 13. The causative ADCORD mutations may, however, be even more restricted since the E837D substitution present in patients with double (the original CORD6 family) and triple mutations⁵ would appear by itself to have little effect on enzyme activity in vitro.¹⁹ In contrast, the changes in codon 838, R838H, R838C, or R838S, have all been shown to alter the sensitivity of the protein to Ca²⁺ inhibition via interactions with GCAPs.^{19–21} Substitution at this site may be the critical change therefore in all cases so far reported, in causing ADCORD rather than recessive LCA. The effect of this dominant mutation is a change in function (altered Ca²⁺ sensitivity) whereas the recessive *LCA1* mutations may represent loss of activity.²²

There have been reports of other retinal dystrophies mapping to regions of chromosome 17p which overlap with the position of the *GUCY2D* gene. These include two dominant cone dystrophies and dominant central areolar choroidal dystrophy, diseases which exhibit degeneration primarily of the cone-rich macular region only.^{23–25} As yet, there have been no reports of *GUCY2D* mutations in these disorders, despite screening this gene in patients with central areolar choroidal dystrophy.²⁶

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A G339R mutation in the *CTNS* gene is a common cause of nephropathic cystinosis in the south western Ontario Amish Mennonite population

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Table 1 Primers for amplifying *CTNS* exons and testing for the 65 kb deletion

Exon	Direction	Sequence
3	Forward	5'- CAG ATT GTC TAC AGG GAG CT -3'
	Reverse	5'- CTT GGC AAC AAA CAG ATC AG -3'
4	Forward	5'- CTG ACC CAG TGC CTC ATG TC -3'
	Reverse	5'- GAG CTG AGC ACA GCG CCA -3'
5	Forward	5'- TCC AGC TTC TCA GCA GTA AT -3'
	Reverse	5'- ACC TAG CAT TTC CCT ACC C -3'
6	Forward	5'- GCG GGG TCC TCG GTA ACT G -3'
	Reverse	5'- CAG CAC GGC CCC CTT CT -3'
7	Forward	5'- AGT CTC CTT CAG AAG CCC AG -3'
	Reverse	5'- GGC AGA CAG AAG GGT AGA GG -3'
8	Forward	5'- CCC TGC CCT GTC TTG TCC -3'
	Reverse	5'- CAG AGA TGT AGG GCA GGC AA -3'
9	Forward	5'- CAT CTC TGC CCA CAT GGC GT -3'
	Reverse	5'- GCT CTG CCG TGT CTT CTG TC -3'
10	Forward	5'- GGC CTC TGT GTG GGT CC -3'
	Reverse	5'- GGC CAT GTA GCT CTC ACC TC -3'
11	Forward	5'- GCC CTC CGT CTG TCT GTC CG -3'
	Reverse	5'- GCC CGA TGC CCC AGC -3'
12	Forward	5'- TCG GAG ACC CAA CCA AGT TT -3'
	Reverse	5'- TGG CCC CAG GAG CAG AGT GG -3'
LDM-1*	Forward	5'- CCG GAG TCT ACA GGG CAC AG -3'
	Reverse	5'- GGC CAT GTA GCT CTC ACC TC -3'
D17S829*	Forward	5'- CTA GGG GAG CTG GTT AGC AT -3'
	Reverse	5'- TGT AAG ACT GAG GCT GGA GC -3'

*Sequences from Anikster *et al.*⁴

EDITOR—Nephropathic cystinosis (MIM 219800) is a rare autosomal recessively inherited lysosomal storage disorder with a newborn incidence of about 1 in 100 000–200 000 in the general population (OMIM). Cystine accumulates in lysosomes because of dysfunctional cystinosis mediated transport of cystine out of lysosomes. The accumulation of cystine results in damage to several organs with renal damage being the most pronounced in the first decade of life. Patients with cystinosis experience both tubular dysfunction (renal Fanconi syndrome) and glomerular deterioration. Renal Fanconi syndrome usually occurs within the first year of life with glomerular deterioration progressing throughout the first decade of life resulting in end stage renal failure.¹

The *CTNS* gene was mapped to chromosome 17p13 and subsequently isolated and characterised to have 12 exons spanning 23 kb of genomic DNA.^{2–3} The most common mutation that causes cystinosis is a large deletion that encompasses exons 1–10.⁴ Originally, this deletion was described as 65 kb long but the size has been recently refined to 57 257 bases.⁵ Forty four percent of 108 American based patients with nephropathic cystinosis were homozygous for this deletion.⁶

At least seven children in the Old Order Amish population in south western Ontario, Canada have been diagnosed with nephropathic cystinosis. This population is a culturally isolated population founded in 1824 by emigrants from Bavaria and Alsace-Lorraine.

Further immigration occurred from the same regions and more recently from the United States.

Within this Amish community, we have recently diagnosed four children with cystinosis, two sibs in two families not known to be related. In each family the parents are consanguineous. The proband presented at 14 months of age with protracted vomiting and dehydration, polyuria, polydipsia, and failure to thrive. On initial investigations he had a metabolic acidosis, hypophosphataemia, hypokalaemia, and glucosuria. Slit lamp examination of his eyes showed corneal crystals. Urine amino acid analysis indicated a generalised amino aciduria. At presentation he had no evidence of renal insufficiency. A leucocyte cystine level done before the initiation of treatment was 1.99 nmol 1/2 cystine/mg protein. Patients with untreated cystinosis usually have greater than 2.0 nmol 1/2 cystine/mg protein (Dr J A Schneider, San Diego). His younger sister was diagnosed at 8 months when she presented with a similar clinical history and a leucocyte cystine of 1.19 nmol 1/2 cystine/mg protein before the initiation of treatment. Leucocyte cystine concentrations were measured at the Cystine Determination Laboratory, UCSD, La Jolla, CA using the cystine binding protein assay.

DNA was isolated from blood specimens that were obtained after receiving consent from the parents. Mutations were identified in the *CTNS* gene by PCR amplification and direct sequencing (ABI PRISM Model 377 sequencer) of exons 3–10 and PCR amplification for detection of the 57 257 base deletion using flanking primers as listed in table 1.

A mutation, 1354 G→A, was identified in exon 12. This mutation results in the loss of an *AvaI* restriction site. The proband was homozygous for the 1354 G→A mutation as shown in fig 1 and no other mutations were identified. This mutation results in a glycine 339 to arginine amino acid change in a transmembrane region of cystinosis. All four cystinosis patients from the two families were homozygous for this mutation and an unaffected sister was heterozygous.

The G339R mutation has been previously identified in one allele in a compound heterozygous patient of Italian ancestry.⁶ Further evidence that this mutation is pathogenetic is that glycine 339 is an amino acid which is conserved between *C elegans* and humans in cystinosis.⁶ In our patients, homozygosity for the G339R mutation seems to be associated

Figure 1 Part of the DNA sequence of exon 12 showing that the patient is homozygous for the 1354 G→A mutation. The sequence is in the reverse direction with the normal sequence across this region being GAAGACCCCGAGTC.

with a relatively low concentration of leucocyte cystine.

Germany is likely to be the country of origin for the common 57 257 base deletion in the *CTNS* gene.⁵ The Amish Mennonite population originated in Germany but appears to have the G339R mutation exclusively rather than the 57 257 base deletion. This may reflect a founder effect but there are no data to indicate from whom or when the founder allele originated. Cystinosis does not appear to be present in the Amish population of Pennsylvania, suggesting that the mutation may have originated in a founder who emigrated to south western Ontario directly from Europe. A study of other populations that are related to the population from which this Amish community is derived would be helpful in this regard.

There are no data on the incidence of cystinosis or the prevalence of the G339R allele in the south western Ontario Amish Mennonite

community. Our awareness of seven cases in the past 10 years suggests an incidence far greater than that of the general population.

There is evidence that the earlier that cysteamine therapy is started the less cystine accumulates in tissues. Markello *et al*⁷ showed that the treatment of children with cystinosis with cysteamine before the onset of end stage renal disease resulted in a delay in the need for renal replacement therapy when compared to children not treated or not compliant with therapy. Early therapy has also been shown to prevent hypothyroidism⁸ and the accumulation of cystine in muscle.⁹

If this Amish Mennonite community wishes, the determination of the frequency of the G339R allele within the population using the *Ava*I restriction site would enable the prediction of the population incidence of cystinosis. This incidence may be high enough to justify targeted newborn screening and early institution of management.

Electronic database information: Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for cystinosis (MIM 219800)).

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De novo terminal deletion of chromosome 15q26.1 characterised by comparative genomic hybridisation and FISH with locus specific probes

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EDITOR—Reports of patients with terminal de novo deletions of chromosome 15q26 are rare. Excluding cases of ring chromosome 15 formation with different sized deleted chromosomal segments, only seven cases with solely distal deletions of 15q have been published.¹⁻⁷ All other cases resulted from unbalanced reciprocal translocations involving different chromosomes and are therefore not comparable with de novo terminal deletions as described in our case.

With two exceptions, all de novo cases had interstitial deletions between chromosomal bands 15q21-q25. Only the patients described by Roback *et al*⁶ and Siebler *et al*⁶ had terminal deletions of 15q26.1. The deletions in these patients were not investigated by FISH, but molecular genetic techniques showed the loss of one copy of the insulin-like growth factor 1 receptor gene. IGF1R is a tyrosine kinase containing transmembrane protein that plays an important role in cell growth control. It has been assumed that monozygosity for this gene, which maps to distal 15q26, will directly disturb this pathway and inhibit normal growth of patients.⁸

Today, in addition to classical cytogenetic banding methods, FISH techniques including comparative genomic hybridisation (CGH) can be used to provide a powerful tool to characterise chromosomal aberrations. In this study, we present the molecular cytogenetic findings and the detailed clinical phenotype of a girl with deletion 15q26.1 and compare these with other published cases. Our patient described here is, to the best of our knowledge, the second patient with a de novo terminal deletion at 15q26.1 and the first one well characterised by molecular cytogenetic techniques.

Case report

The female infant was the first child of healthy, unrelated parents. An ultrasound examination at 15 weeks of gestation showed intrauterine growth retardation. At 39 weeks of gestation a caesarean section became necessary because of fetal heart rate deceleration. The Apgar scores were 6, 8, and 10 at one, five, and 10 minutes, respectively. Her birth weight was 1980 g (<3rd centile) with a length of 42 cm (<3rd centile) and a head circumference of 30 cm (<3rd centile). The first chromosome analysis after birth in an outside laboratory showed a normal female karyotype. The girl had minor anomalies including micrognathia, low set ears, a broad nasal bridge, and a short neck (fig 1). Furthermore, there was a blood pressure difference between the upper and the lower

extremities. Cardiac examination including cardiac catheterisation exhibited a complex heart defect with ventricular septal defect (VSD), atrial septal defect (ASD), preductal coarctation of the aorta, patent ductus arteriosus, and arteria lusoria. This complex congenital heart disease was corrected by several surgical interventions up to the age of 3 months. Laboratory findings including IGF1 and a screening for congenital infection were normal except for a transient hypothyroidism owing to maternal hypothyroidism. Renal ultrasonography showed a slight ectasia of the left renal pelvis from the age of 7 months. Neurological examination showed developmental delay but no other pathological findings. At the age of 15 months the infant could roll over but could not sit without support. Furthermore, she had severe feeding problems with gastro-oesophageal reflux and vomiting. Because of increasing vomiting and a lack of weight gain, a gastrostomy feeding tube had to be inserted. For the whole period of time the girl continued to have poor development and severe failure to thrive. At the age of 16 months her weight was 5300 g (<<3rd centile), length was 62 cm (<<3rd centile), and head circumference was 39 cm (<<3rd centile).

Figure 1 The patient at the age of 19 months.

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Material and methods

Blood samples from the patient and her parents were drawn after informed consent. High resolution chromosome analyses from peripheral blood lymphocytes of the patient and both parents were performed using standard techniques. Preparations were GTG banded and karyotyped using the Ikaros system (Metasystems, Altlußheim, Germany).

Whole chromosome painting (WCP) was initiated using the probe for chromosome 15 (VYSIS). YAC clones for chromosome 15 were selected from the CEPH mega-YAC library and obtained through the Positional Cloning Centre at the Max-Planck Institute of Molecular Genetics (Berlin, Germany). YAC DNA was amplified and labelled by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) with minor modifications.⁹ YAC-FISH was performed according to standard protocols. Hybridisation of commercial probes for the subtelomeric region of chromosome 15q (TelVysion 15q, VYSIS) and the all human telomeres probe (ONCOR) were according to the manufacturers' instructions. All probes used were directly labelled with fluorochromes.

Genomic DNA of the patient was investigated by comparative genomic hybridisation using normal male reference DNA as a control. DNA was isolated using standard methods. Briefly, genomic DNA samples were differently labelled by nick translation with SpectrumGreen®-dUTP (VYSIS, test DNA) and SpectrumOrange®-dUTP (VYSIS, reference DNA). For each hybridisation, 200 ng of labelled test DNA, 200 ng reference DNA, and 12.5 µg Cot-1 DNA were coprecipitated, resuspended in 14 µl hybridisation mix containing 50% formamide, 2 × SSC, and 10% dextran sulphate, denatured at 70°C for five minutes, and hybridised to denatured normal male metaphase spreads. Slides were incubated at 37°C in a moist chamber for two days. Post-hybridisation washes were performed as described previously.¹⁰ Images of the hybridised metaphases were evaluated using an epifluorescence microscope (Axiophot, ZEISS, Germany) fitted with different single band pass filter sets for DAPI, SpectrumGreen®, and SpectrumOrange® fluorescence. The microscope is equipped with a cooled CCD camera (Hamamatsu) for image acquisition. Image analysis and karyotyping (CGH) was performed using the ISIS analysis system (Metasystems, Germany). Diagnostic thresholds used for the identification of chromosomal under-representations (deletions) and over-representations (duplications) were 0.85 and 1.17.¹¹

Microsatellite markers on chromosome 15q were analysed in the patient and her parents. Marker loci were chosen from the Génethon final linkage map and from the Marshfield comprehensive human genetic maps.^{12,13} Markers were amplified by PCR in a final reaction volume of 10 µl containing 10 mmol/l Tris, 1.5 mmol/l MgCl₂, 100 µmol/l each dNTP, 0.4 U polymerase (Applied Biosystems), 7 pmol of each primer, and 20 ng of genomic DNA. One

of the primers was end labelled with fluorescent dye. DNA amplification was carried out in an MJ Research PTC-225 thermal cycler. Reactions were electrophoresed on an ABI PRISM 377 automatic DNA sequencer (Applied Biosystems). Data were analysed using the computer programs Genescan v3.0 and Genotyper v2.5 (Applied Biosystems).

Results

Cytogenetic studies from the peripheral blood lymphocytes of the patient at the age of 9 months showed a female karyotype with a small deletion in the long arm of chromosome 15 at the 500-600 band level (fig 2).¹⁴ After conventional cytogenetics, the extent of the deletion was assumed to be from band 15q25-26 to the distal end of the chromosome, but it was impossible to decide whether the deletion was interstitial or terminal. Maternal and paternal karyotypes were normal at the same resolution level.

For further characterisation of the deletion, CGH was performed using total DNA from the patient as a probe. The averaged ratio profile analysis clearly indicated a terminal deletion (dim) of the chromosomal region 15q26 (fig 2). No other chromosome showed any ratio profile imbalance.

This result was in agreement with the FISH analysis using a chromosome 15 specific whole chromosome paint (VYSIS) showing homogeneous painting of the whole deleted chromosome 15 without any hint of a translocation of the missing chromosome 15 material to any other chromosome (data not shown).

To define the proximal and distal boundaries of the deletion, FISH with different YAC clones was performed. Two of five YAC clones localised in chromosome band 15q25 (81-84 cM, table 1) showed signals on both chromosomes 15 on metaphase preparations of the patient (fig 3). Three YAC clones, 963d03, 895h10, and 882h08, localised distal to chromosome band 15q25 (98-110 cM), were missing from the patient's deleted chromosome 15 (fig 3).

To delineate this chromosomal abnormality further, FISH with a probe hybridising to unique telomeric DNA sequences of chromosome 15q (TelVysion 15q, VYSIS) was performed. The investigation showed that a signal of this 100 kb sized probe for chromosome 15q is missing on the deleted chromosome 15 (fig 3). In contrast, FISH with an all telomeric probe (ONCOR) detecting the highly repeated (TTAGGG)_n sequences located at the telomeres of all human chromosomes showed telomeric signals on both the normal and the deleted chromosome 15 as well as on all other chromosomes (fig 3). Thus the patient's karyotype can be summarised as: 46,XX, del(15)(q26.1).ish del(15)(D15S130-, D15S207/D15S157-, D15S120/D15S203-, D15S936-).

In order to complement the FISH data and to substantiate the loss of the *IGF1R* gene locus, a microsatellite analysis was performed. Twelve polymorphic markers from chromosome 15q were analysed (table 1). All the

Figure 2 Ideogram¹⁴ of the human chromosome 15 (A) and the patient's chromosomes 15 (B) after GTG banding. The normal chromosome 15 is to the left of the deleted chromosome 15. (C) Averaged CGH ratio profile of 12 measured chromosomes 15 of the patient.

markers but those at D15S152, D15S1014, and D15S120 were informative for the family. Segregation of two different alleles clearly showed that the patient carries two copies of chromosome 15q proximal to D15S652 (table 1). Hence, the proximal boundary of the deletion is in the 10 cM interval between D15S652 and D15S130, so the deletion lies between D15S652 and the telomere. This finding is in accordance with the proximal boundary of the deletion defined by YAC hybridisation (table 1). Unfortunately, there is no true telomeric marker available on chromosome 15q, and the distance between the most distal marker at D15S642 and the telomere remains unclear. Additionally, it could be determined that the

aberrant chromosome 15 was of paternal origin. The *IGF1R* gene is located close to D15S120 as shown by radiation hybrid mapping between D15S107 and D15S87.^{15 16} These two markers are within the deleted region of our patient who therefore exhibits monozygosity for the *IGF1R* gene.

Discussion

Terminal deletions of chromosome 15q are rare events or are seldom diagnosed. Only a few cases of de novo distal deletions of chromosome 15q without ring formation have been described and the vast majority have been characterised by standard banding only yielding breakpoints in the range from 15q24 to 15q26. We describe here a new case of terminal deletion 15q26. Even with high resolution chromosome analysis, it was difficult to determine the exact size of the deletion. Therefore, we used different molecular cytogenetic approaches like CGH and FISH with YAC clones and commercially available telomeric probes to refine the deleted chromosome region to chromosome band 15q26. However, even with the molecular cytogenetic investigation, it was impossible to differentiate between an interstitial versus terminal deletion. The result of the FISH analysis with the YAC from the subtelomere of 15q (Telvision, D15S936) clearly showed a deletion on the aberrant 15 while a signal could be detected on both chromosomes 15 with the all telomeric repetitive probe (TTAGGG)n.

Therefore, it cannot be shown whether the telomeric sequence (TTAGGG)_n at the distal end of the deleted chromosome 15 was from the paternal chromosome, or whether it derived from another chromosome by translocation.

Table 1 Detection of chromosome 15q loci by FISH and microsatellite analysis

STS	cM*	Probe (YAC clone)	Method†	Normal chromosome 15	Derivative chromosome 15
D15S153	62.1	—	MS	+	+
D15S114	72.3	—	MS	+	+
D15S152	78.6	—	MS	NI	NI
D15S199	81.9	913e02	FISH	+	+
D15S979	82.4	—	MS	+	+
D15S1045	84.7	859c06	FISH	+	+
D15S127	84.8	—	MS	+	+
D15S963	85.8	—	MS	+	+
D15S652	88.0	—	MS	+	+
D15S130	98.0	963d03	FISH	+	—
D15S130	98.0	—	MS	+	—
D15S207/	100.8	895h10	FISH	+	—
D15S157	103.5	—	—	—	—
D15S1014	103.5	—	MS	NI	NI
D15S120/	109.6	882h08	FISH	+	—
D15S203	109.6	—	—	—	—
D15S120	109.6	—	MS	NI	NI
D15S966	110.2	—	MS	+	—
D15S642	(119.8)	—	MS	+	—
D15S936	?	TelVysion 15q	FISH	+	—
Telomere	?	All telomeric probe	FISH	+	+

*Genetic localisation according to Dib *et al.*¹² The distance between D15S966 and D15S642 was obtained from Broman *et al.*¹³

†Loci were studied either by FISH with YAC clones or by analysis of microsatellites (MS).

+, allele detected; —, allele missing; NI, not informative.

Figure 3 FISH images of YAC clones and commercially available probes hybridised to the patient's chromosomes. (A) Fluorescence signals after hybridisation of the YAC clones 859c06 and 963d03. There is no signal for the latter clone in the patient's deleted chromosome 15. Both signals are seen in the linear orientation in the normal chromosome 15 (see B, magnification). (C) The subtelomeric TelVysion probe for chromosome 15q is also missing in the deleted chromosome 15. (D) A normal signal is seen for the all human telomeres probe detecting the highly repeated DNA (TTAGGG)_n sequences located at the telomeres of all human chromosomes.

New studies on terminal deletions also suggest that de novo telomere addition could occur either mediated by telomerase or by recombination based mechanisms.¹⁷ In addition to the characterisation of the size of the deletion by in situ hybridisation, the deleted interval was determined by the analysis of microsatellites. These studies showed that the de novo deleted chromosome 15 was of paternal origin. This result is consistent with the paternal origin in the case described by Roback *et al.*⁵

Most patients with deletions of distal 15q have intrauterine growth retardation (IUGR), microcephaly, abnormal face and ears, micrognathia, a high arched palate, renal abnormalities, lung hypoplasia, failure to thrive, developmental delay, and mental retardation.⁵ Apart from unbalanced chromosome translocations involving distal 15q and ring chromosome 15 syndromes, there are only seven previously described patients with de novo deletions of the distal long arm of chromosome 15.¹⁻⁷ Most of these patients had interstitial deletions with different breakpoints indicating that the phenotypic discordance observed probably results from differences in the size and localisation of the deleted material.

Similarly to patients with distal deletion of 15q, many patients with ring chromosome 15 syndrome showed symptoms like IUGR, mental retardation, and microcephaly, but they

more frequently had a triangular face, hypertelorism, café au lait spots, cryptorchidism, cardiac anomalies, and brachydactyly.¹⁸

To the best of our knowledge there are only two comparable cases to our patient with a deletion of 15q26.1 (table 2) that have been investigated by molecular genetic techniques.^{5,6,18} These patients and our patient share intrauterine growth retardation, poor growth and development, and minor anomalies of the face. The female child described by Siebler *et al.*⁶ also had a triangular face and brachydactyly and exhibited characteristics of patients with ring chromosome 15 syndrome and deletion of 15q26.1. Renal malformations were only reported in the case of Roback *et al.*⁵ and our case. The patient of Roback *et al.* also had lung hypoplasia, while our patient suffered from a complex heart defect. Feeding difficulties, as in our patient, were reported in four cases out of seven.

Only a couple of genes have been mapped to date in the distal part of chromosome 15, one of which is *IGF1R* (OMIM, <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/getmap?chromosome=15q26>). It has been proposed that haploinsufficiency of the *IGF1R* gene, which has been assigned to 15q25-q26,¹⁹ may play a role in the growth deficiency seen in patients with distal deletions of 15q25-26. Roback *et al.*⁵ refined the mapping of *IGF1R* distal to 15q26.1 by deletion mapping. These findings were corroborated by Southern blot analysis of two patients with deletions of 15q26.1.⁶ The *IGF1R* gene locus lies physically between the STS markers D15S107 and D15S87.¹⁶ Therefore, *IGF1R* is also deleted in our patient who displayed extreme pre- and postnatal growth retardation.

Peoples *et al.*¹⁶ investigated five children with de novo ring chromosomes 15 with breakpoints in 15q26.3 showing monozygosity of the *IGF1R* gene in three of them. These three children had significantly more severe growth retardation in the first few years of life than one patient who retained the *IGF1R* gene on the ring chromosome. These data support a correlation between monozygosity for the *IGF1R* gene and severe growth retardation in early childhood, while patients who have retained two copies of the *IGF1R* gene show milder growth retardation.²⁰

In vitro studies of fibroblasts of the two patients described by Siebler *et al.*⁶ showed that IGF1 receptor expression was decreased, while there was no evidence for impairment of the response to IGF1. Thus, Siebler *et al.*⁶ suggested that the growth retardation might not be related to monozygosity for *IGF1R*. However, the authors conceded that extrapolation from findings in skin fibroblasts to the situation in vivo is difficult.

De Lacerda *et al.*²¹ were the first to describe in vitro and in vivo studies of a patient with ring chromosome 15 syndrome and monozygosity for *IGF1R*. The female child showed prenatal and severe postnatal growth failure, a slightly triangular face, high arched palate, café au lait spots, and delayed psychomotor development. The patient's fibroblasts exhibited growth

response in vitro to the addition of IGF1, similar to that of control fibroblasts. In contrast, the treatment of the child with short term recombinant human IGF1 (rhIGF1) caused no significant reduction in urinary urea nitrogen excretion, only 60% increase in calcium excretion, and no significant decrease in the GH secretion. Therefore, the authors suggested that the growth retardation could be the result of the absence of one *IGF1R* allele because of in vivo resistance to IGF1.

Studies on the effects of IGF1R in the cardiovascular system may support this assumption. These data showed evidence that IGF1 is an essential regulator of developmental growth and plays an important role in cardiovascular development.²² A variety of growth factors upregulate IGF1R on vascular smooth muscle cells and the data support the concept that IGF1R number per cell is an important factor for cellular growth response.

Therefore, monozygosity for *IGF1R* would be the best explanation for the complex heart defect seen in our patient. Thus, in addition to severe growth retardation, monozygosity for *IGF1R* might be a risk factor for the development of complex heart defects.

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Interstitial deletion of chromosome 11 (q22.3-q23.2) in a boy with mild developmental delay

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EDITOR—Deletions of the terminal region of the long arm of chromosome 11 (bands 11q23.3-11q24) are associated with a clinically recognisable phenotype, also called Jacobsen syndrome (JS).¹ Reports on more proximal 11q

deletions are rare. This is the second report describing a de novo interstitial deletion of the 11q22.3-q23.2 region. The first described a de novo interstitial deletion of the 11q22.3-q23.2 region in a mildly retarded male with minor

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dysmorphic signs (high and narrow palate, low set, dysplastic ears, small hands and feet, and slender fingers) and epileptic seizures.² However, no FISH studies were performed in this patient.

In this report we describe a small *de novo* interstitial deletion in the long arm of chromosome 11 (bands q22.3-q23.2) in a 2 year 8 month old boy with mild developmental delay and without major associated dysmorphic features or a clinically recognisable phenotype.

Case report

The proband, a boy, is the second and youngest child of healthy, non-consanguineous parents. His 5 year old sister is normal. Pregnancy and delivery, at 39 weeks, were normal. Birth weight was 3130 g, length 49 cm, and head circumference 33.5 cm. Clinical examination in the neonatal period was normal, apart from mild axial hypotonia. Motor development was slightly retarded and he walked without support at the age of 17 months.

Now, at the age of 2 years 8 months, psychomotor development is borderline normal (2 years 2 months to 2 years 4 months on the Bayley Developmental scale). Social contact is adequate but expressive language is mildly retarded at a developmental level of 2 years. Height is 89.5 cm (10th centile), weight 12.5 kg (10th centile), and head circumference 48 cm (3rd centile for age). Except for the relative microcephaly and mild trigonocephaly, craniofacial dysmorphism is mild and non-specific, including a somewhat large mouth with a thin upper lip and everted lower lip and rather large and everted ears. Both thumbs are proximally implanted. Further clinical and neurological examinations were normal. Additional examinations including MRI scan of the brain, metabolic screening, and ophthalmological examinations were normal.

Cytogenetic studies were performed using PHA stimulated lymphocytes according to standard cytogenetic procedures. G banded chromosome analysis showed an interstitial deletion of the long arm of chromosome 11 (q22.2-q23.1) (fig 1). The karyotype was 46,XY,del(11)(pter→q22.3::q23.1→qter). The parental karyotypes were normal.

FISH with chromosome 11 specific paint probe (Cambio) showed no translocation of chromosome 11 material (fig 2A). FISH analysis was performed with five YAC probes (878C12, 876G04, 801E11, 755B11, and 742F09), BAC442e11, and three cosmid probes that map to the 11q22-11q23 region. BAC442e11(RPC11 human BAC library, Roswell Park Cancer Institute) has been recently reported and spans the t(11;22) breakpoint on chromosome 11.³ Cosmid probes 4746 and 4748 cover the *MLL* gene region and 2072c1 is a subtelomeric probe (table 1).⁴

FISH results defined the extent of the deletion (from q22.3 and q23.2). The proximal boundary of the deleted region is between D11S1762/D11S1339 and D11S1167 because FISH with YAC878C12 gave a signal on the deleted chromosome, whereas the terminal

Figure 1 Partial G banded karyotype showing the normal chromosome 11 (on the left) and the deleted chromosome 11 (on the right). An ideogram of chromosome 11 is also shown. The deleted region is indicated by brackets.

boundary is placed proximal to the *MLL* locus (fig 2B, C, D, E, F). Chromosomes were viewed with a Zeiss Axioplan epifluorescence microscope. For digital image analysis the Cytovision System (Applied Imaging) was used.

Discussion

Chromosomal region 11q22-q23 is apparently prone to instability (recombination, breakage, or rearrangement). The breakpoints of the classical constitutional t(11;22) and the breakpoints in the majority of cases with terminal 11q deletions and derivative chromosomes 11 are located in this region. This region is often involved in multiple tumour associated rearrangements of chromosome 11 and distally lies the *MLL* gene region that is frequently rearranged in haematopoietic malignant disorders.⁵ On the telomeric side of *MLL* is the fragile site FRA11B and also the Jacobsen syndrome breakpoints (11q23.3-11q24.2).^{1,3} Consequently the region could be considered as a hot spot of chromosomal recombination and breakage.

In this case, the deletion is smaller than the previously reported deletions on 11q, for example, deletions critical for the diagnosis of Jacobsen syndrome (MIM 147791)¹ or larger deletions involving the 11q22-q23→11qter region.⁶⁻⁹

The fragile site at 11q23.3 (FRA11B) is linked to some Jacobsen syndrome breakpoints (10% of the cases) but the majority are located distal to FRA11B. It was proposed that JS is not a single disease but a collection of different genetic disorders with overlapping phenotypes. The phenotypic variability observed is because of the variation of breakpoints and the different genes involved.^{10,11} Thus, the 11q22.3-q32.2 deletions in the present patient could be considered as a part of the spectrum of 11q deletions resulting from a similar mechanism, with the more distal deletions resulting in Jacobsen syndrome and the more proximal resulting in a milder phenotype.

In the present case, Bac442e11, which spans the t(11;22) breakpoint in 11q23, was deleted. This BAC clone is related to a palindromic AT

rich region.¹² The mechanism of formation of the deletion in the reported case could be consistent with the one proposed by Akgun *et al.*¹³ They proposed that in mammals palindromic DNA sequences can lead to the formation of unstable DNA structures, such as single stranded hairpin and double stranded cruciform structures, and they hypothesised that a small disruption of symmetry in the palindrome could

stabilise the locus. To explain their results, they proposed two different models that could explain the formation of deletions and translocations. According to one model, replication slippage could result in two sided palindrome deletions spanning the tip of the hairpin and create a product with a deletion in the palindrome. This mechanism could explain the deletion in the present patient. According to the

Figure 2 (A) Hybridisation with YAC 801E11 and YAC 755B11. (B) Hybridisation with YAC 878C12. (C) Hybridisation with YAC 876G04 and 742F09. (D) Hybridisation with cos 4746 and cos 4748. (E) Hybridisation with BAC 442e11. (F) Hybridisation with cosmid 2072c1. An explanation for the mechanism of the deletion has been included.

Table 1 FISH data

Probe	STS markers covered	Result	Cytogenetic position
YAC878C12	D11S1762/D11S1339	- D11S1167 +	q22.2
YAC876G04	D11S817	- Y734D08L -	q22.3
YAC801E11	D11S384	- D11S1897 -	q22.3
YAC755B11	D11S1960	- Y296F0FR -	q23.1
BAC442e11	D11S1340	- D11S4516 -	q23.2
Cos4746	MLL	+	q23.3
Cos4748	MLL	+	q23.3
YAC742F09	D11S461/D11S939	- HLR2 +	q23.3
Cos2072c1	(Subtelomere)	+	q25

(+) Hybridisation to both chromosomes 11. (-) Hybridisation only to normal chromosome 11.

second model, a single strand nick in the tip of the hairpin could result in a double strand break and then lead to illegitimate recombination. The second model could be consistent with the formation of the t(11;22).¹³

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Microdeletion in the *FMR-1* gene: an apparent null allele using routine clinical PCR amplification

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EDITOR—Fragile X syndrome is the most common chromosomal cause of inherited mental retardation. At the chromosome level, this syndrome is characterised by the presence of a fragile site at Xq27.3.¹ The incidence of this disorder is approximately 1 in 4000 and 1 in 7000 in males and females, respectively.^{2,3} In most cases, the mutation responsible for fragile X syndrome is a CGG repeat expansion in the 5' untranslated region (UTR) of exon 1 of the *FMR-1* gene. People in the normal population have six to approximately 50 repeats.^{4,5} Those with 50 to 200 repeats correspond to the premutation class. Repeats in this class are meiotically unstable and can expand to a full mutation.⁴ The premutation class encompasses the "grey area" of 45-55 CGG repeats for which there is a variable risk of repeat expansion.⁶ Subjects with a full mutation have repeat lengths in excess of 200, which are associated with hypermethylation of the CpG island immediately upstream of the *FMR-1* gene.⁷⁻⁹ This methylation correlates with transcriptional suppression of the *FMR-1* gene, while the repeat expansion has been suggested to cause translational suppression by impeding the migration of the 40S ribosomal subunit

along the 5' UTR of the *FMR-1* gene transcript.⁹⁻¹¹

Fragile X syndrome has also been found to occur in a few patients without CGG repeat expansions. These mutation events fall into two classes, intragenic point mutations^{12,13} and deletion events.¹⁴⁻²² Of the latter class, five patients with microdeletions in the 5' UTR of the *FMR-1* gene transcript have been described.^{23,24}

We report here a patient referred for fragile X testing who was found to carry an apparent null allele by PCR amplification of the CGG repeat region of the *FMR-1* gene. This patient was analysed further using a combination of primers flanking the CGG repeat region, together with FMRP studies, in order to characterise the nature of the molecular defect underlying this apparent null allele.

Case report

The proband was born to healthy, non-consanguineous parents at 40 weeks of gestation. There was no significant family history. He weighed 4500 g (>90th centile), head circumference was 37.5 cm (>90th centile), and length was 57.5 cm (>90th centile). There

was aspiration of meconium at delivery necessitating assessment in the neonatal unit. He appeared well initially but on the following day was noted to be irritable and hypotonic with an abnormal Moro reflex. A cranial ultrasound scan was normal. He required an inguinal hernia repair at a few weeks of age. His early development was felt to be normal. He had gastro-oesophageal reflux diagnosed at 8 months and was treated with ranitidine. He had mild plagiocephaly and a torticollis that required surgical correction at 18 months. He had persistent problems with drooling of saliva and tends to have an open mouthed expression. In the second year of life he had problems with recurrent ear infections requiring insertion of grommets and adenoidectomy. An assessment at the age of 3 years showed his speech and language development to be significantly delayed. His parents felt his comprehension was limited and he had difficulty retaining information. The delay had been noted earlier but had been attributed to his recurrent ear infections. Full assessment at that time showed that he had developmental delay in all areas. He had some behavioural problems with trichotillomania and obsessive traits. He did not play well with other children.

On examination by a clinical geneticist, the proband was found not have any phenotypic features suggestive of fragile X syndrome, although he did have early features of joint laxity. His head circumference was on the 50th-90th centile, his height on the 75th centile, and weight on the 50th centile. He had mild clinodactyly and fetal pads. He had mild facial asymmetry and a deep crease between his first and second toes. Examination was otherwise unremarkable. The case was referred to the laboratory for fragile X screening.

Materials and methods

CYTOGENETIC AND DNA ANALYSIS

Cytogenetic analysis of a folate deprived culture of lymphocytes was performed as previously described.²⁵ An estimation of the length of the CGG repeats, together with an analysis of the methylation status of the CpG island of the *FMR-1* gene, were performed by PCR amplification and Southern blot analysis, respectively. In the case of the latter, 5 µg of genomic DNA was digested with *EcoRI* and *NruI*, electrophoretically separated, blotted onto a positively charged nylon membrane, and hybridised with approximately 10-20 ng of probe StB12.3, as described previously.²⁶ The hybridisation solution contained herring sperm DNA at 75 µg/ml to prevent non-specific binding of the probe. The blots were washed finally in 0.2 × SSC plus 0.1% SDS at 60°C. DNA controls included a normal male, a male with a full mutation (expanded CGG repeat with hypermethylation of the CpG island), a female with a premutation, and a normal female control. A radioactively labelled 1 kb ladder was included for sizing purposes.

PCR amplification of the CGG repeat region of the *FMR-1* gene using primers FMRA and FMRB was carried out in 15 µl reactions. Each

reaction comprised 10% DMSO, 50% w/v glycerol, 60 pmol of each primer, 0.4 U of *Taq* DNA polymerase, 1 × PCR buffer with 0.32 mmol/l of dCTP, dATP, dTTP, and 1.5 mmol/l deaza GTP, 0.25 µl of 10 µCi µl α³²P dCTP, and 0.6 mg/ml genomic DNA. Non-radioactive PCR amplification using primers FMR1 and FMR2 was carried out using the GC rich kit of Roche Diagnostics Ltd according to the manufacturer's instructions. The sequences of the primers used in the amplification reactions were FMRA (5'-GACGGAGGCGCCCGTGCCAGG-3'), FMRB (5'-TCCTCCATCTTCTCTTCAGCCT-3'), FMR1 (5'-ATAACCGGATGCA TTTGAT-3'), and FMR2 (5'-AGGC CCTAGCGCCTATCGAAATGAGAGA-3'). Primers FMR1, FMRA, FMRB, and FMR2 were designed using the *FMR-1* gene sequence deposited in GenBank (Accession number X61378), with their 5' ends at base positions 2271, 2684, 2844, and 3106, respectively. The PCR cycling conditions comprised 95°C for two minutes followed by 30 cycles of 97°C for 30 seconds, 55°C for one minute, and 72°C for one minute. The reactions were held at 4°C following a final extension of 72°C for 10 minutes. Amplification products were electrophoresed in a 1% agarose gel, together with a 100 bp DNA ladder. In the case of radioactive amplification, the products were electrophoresed in a denaturing sequencing gel using a radioactively labelled M13 sequencing ladder for sizing purposes.

Amplification products were purified for sequencing using a PCR purification kit (Roche Diagnostics). Each amplicon was sequenced using the forward and reverse amplifying primers and an Applied Biosystems (ABI) sequencing kit. DNA was recovered by ethanol precipitation and subsequently washed in 70% ethanol before the addition of denaturation buffer and loading in an ABI PRISMTM 377 DNA sequencer. The electropherograms were subsequently assembled using SeqMan DNA software.

PROTEIN ANALYSIS

An EBV transformed B lymphoblastoid cell line was established from a peripheral blood sample of the proband. FMRP and eIF4e levels were determined in whole cell lysates in a slot-blot based assay, using purified flag tagged murine Fmrp²⁷ and purified human eIF4e²⁸ as standards. Sample proteins and standards were applied to nitrocellulose membranes with a Bio-Rad slot blot apparatus. Using standard protocols, FMRP and eIF4e were detected with mouse monoclonal primary antibodies mAb 1C3 for FMRP, kindly provided by Jean-Louis Mandel,²⁹ and anti-eIF4e (Transduction Laboratories) and HRP conjugated goat anti-mouse secondary antibody (Kirkegaard and Perry Laboratories). Signals were generated by Enhanced Chemi Luminescence (Amersham) and detected by exposure to Hyperfilm (Amersham). Signal intensities were quantified by analysis of digital scans using the program NIH Image 1.62b7f to plot signal profiles. Areas under the plot profile were calculated and used as signal intensities after subtracting out signals

Figure 1 DNA analysis of the CGG repeat region of the FMR-1 gene. (A) EcoRI plus NruI digested genomic DNA from a normal male (lane 1), a male with a full mutation (lane 2), a normal female (lane 3), and the proband (lane 4) was probed with StB12.3. The 1 kb ladder is shown in lane 5, with the lengths of the unmethylated and methylated alleles in a normal subject indicated on the right hand side of the panel. (B) PCR amplification products encompassing the CGG repeat region of the FMR-1 gene are shown. The proband and normal males are represented by the filled and open symbols, respectively, while negative PCR controls are indicated by the letter N. The radioactively labelled products corresponding to PCR amplification using primers FMRA and FMRB were electrophoresed in a denaturing sequencing gel with a labelled M13 sequencing ladder, while the other amplification products were separated in 1% agarose gels with 100 bp ladders. (C) Electropherogram of the sequence of the proband's FMR-1 gene encompassing the ATG initiation codon (indicated by a horizontal bar). The sequence is shown in the 3' to 5' direction. The vertical arrow indicates an arbitrary start site for the sequence presented in (D). (D) Partial sequence of the FMR-1 gene (GenBank accession number X61378) indicating the GAAGA direct repeats (in bold type and numbered horizontal arrows) and the ATG initiation codon (underlined). The location of the FMRA and FMRB primers are shown as horizontal arrows, together with their position with respect to the transcription start site. The nucleotide sequence derived from the electropherogram is shown starting at an arbitrary site, indicated by an arrow.

Figure 2 FMRP analysis. (A) Western blot analyses of FMRP expressed by the proband and a normal male are shown in lanes 1 and 2, respectively. Molecular weight standards (expressed in kDa) are indicated on the left hand side of the panel. (B) Immunohistochemical staining of FMRP in lymphocytes of the proband (I), the proband's carrier mother (II), a negative control (III), and a positive control (IV). FMRP staining is seen in the cytoplasm, with the nuclei stained with Nuclear Fast Red.

from the background and from the secondary antibody controls as appropriate. Standard curves were generated using data from the purified proteins, which then allowed the quantitation of protein levels in the samples. Quantitation data was calculated as the molar ratio of FMRP:eIF4e. Purified FMRP was obtained from Keith Wilkinson and eIF4e was from Curt Hagedorn, both of Emory University.

In the case of western blot studies, total proteins were isolated from EBV transformed B lymphoblasts of the proband, as well as from a normal male control. The proteins were electrophoresed in a 7.5% non-denaturing polyacrylamide gel, and transferred to nitrocellulose and hybridised using mAb 1C3 as described above.

In the case of immunohistochemical staining of FMRP from blood smears, a modification of the method of Willemsen *et al*³⁰ was used. Blood smears were counterstained with Nuclear Fast Red and 100 lymphocytes were examined for each person, together with positive and negative control blood samples. Less than 42% of lymphocytes are FMRP positive in affected males, whereas for carrier females this figure is 83%; the specificity of this assay is 100% for males and 41% for females.³¹

Results

Cytogenetic analysis of the proband's chromosomes indicated an apparently normal 46,XY karyotype. Southern blot analysis showed a positively hybridising 2.8 kb DNA fragment, suggesting a normal sized CGG repeat length in the *FMR-1* gene (fig 1A). PCR amplification of this locus using previously published primers FMRA and FMRB³² yielded no product

from the proband's genomic DNA. However, amplification products were obtained using primers FMRA and FMR2 (643 bp) and FMR1 and FMR2 (1 kb, fig 1B). The latter product was sequenced and showed a deletion of a 5 bp direct repeat, GAAGA, either immediately upstream, or encompassing the first base, of the ATG initiation codon of the *FMR-1* gene (fig 1C, D). The mother of the proband was found to be heterozygous for this deletion event (data not shown). The deletion leaves the ATG codon unchanged and in phase with the remaining open reading frame of the *FMR-1* gene.

FMRP quantitation, western blot analysis, and immunohistochemical studies were undertaken using the patient's lymphoblasts to determine the effect of the deletion event on translation initiation (fig 2). In order to assess the level of FMRP in the patient's lymphoblasts, quantitation studies were undertaken using the protein eIF4e as an internal control. The latter protein is the cap binding protein in eukaryotic translation initiation³³ and is the rate limiting factor in translation initiation.³⁴⁻³⁶ FMRP levels were normalised with respect to eIF4e levels as a loading control. In seven cell lines from males with normal CGG allele lengths, the mean molar ratio of FMRP:eIF4e is 0.218 (standard deviation of 0.009). In the case of the cells from the proband, the molar ratio was 0.214, and thus the level of FMRP is not reduced compared to normal cell lines. In the case of the western blot analysis, normal sized FMRP was detected (fig 2A). Immunohistochemical staining of lymphocytes from the proband and his carrier mother showed FMRP staining in 80% and 98% of 100 lymphocytes examined, respectively (fig 2B).

Discussion

The proband reported here carries an apparent null allele with respect to the primer pair FMRA and FMRB, which are used routinely for amplifying the CGG repeat tract of the *FMR-1* gene. This case suggests that caution should be exercised regarding predictive testing for fragile X syndrome that relies solely on PCR amplification of the *FMR-1* gene using one primer pair only. This reliance has been suggested as a first level predictive screen for fragile X syndrome in the general population.³⁷ The need for caution with respect to single PCR amplifications of trinucleotide repeats has also been described with regard to predictive testing for the Huntington's disease (HD) gene.³⁸ Our data underline the need for complementing PCR analysis with Southern blotting or, at minimum, PCR amplification of the CGG repeat region with two primer pairs.

Direct sequencing of amplification products using primers that map further upstream and downstream of FMRA and FMRB identified a 5 bp microdeletion near, or encompassing, the initiation codon of the *FMR-1* gene. It appears that this deletion affects the annealing of the FMRB primer leading to inefficient amplification using this primer. The proband represents one of only a few cases that have been reported to have microdeletions in the *FMR-1* gene.^{23, 24} In these other cases, which were found in subjects with fragile X syndrome, the microdeletions ranged from 116 bp to 567 bp and were located in the 5' UTR of the *FMR-1* gene. The deletions were expected to lead to a lack of the *FMR-1* gene product, which was confirmed in some patients.²³ A mispairing model for the generation of a 486 bp deletion was described by Schmucker *et al.*,²⁴ which involved chi-like elements flanked by direct tandem repeats. In the case reported here, end joining, strand slippage, or indeed homologous recombination are possible molecular mechanisms that could account for the 5 bp deletion event.

Changes in the sequence of DNA upstream of an initiation codon can dramatically influence translation efficiency.³⁹ Fragile X males with a full mutation have complete absence of FMRP. However, in the case described here, FMRP was detected of apparently normal size and at normal levels in the lymphocytes of the proband.

This study leads to the suggestion that the proband does not have fragile X syndrome and that the 5 bp deletion in this patient's *FMR-1* gene is not causative of his phenotype. The FMRP detected in this patient appears to be qualitatively and quantitatively normal. Therefore, the comprehensive screening of genes implicated in disorders that are similar to fragile X syndrome may help resolve the cause of this patient's phenotype.

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- We report here a case that was referred for testing for fragile X syndrome. The patient was found to carry an apparent null allele by routine clinical PCR, but with CGG repeats that fall within the normal range.
- DNA sequencing showed that the patient carried a microdeletion of a 5 bp direct repeat immediately upstream, or encompassing, the translation initiation codon of the *FMR-1* gene.
- Protein studies indicated that the patient expressed the protein product of the *FMR-1* gene (FMRP), and that this expression was at near normal levels in the patient's lymphoblasts.

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Non-invasive evaluation of arterial involvement in patients affected with Fabry disease

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EDITOR—Fabry disease (FD) (OMIM 301500) is an X linked recessive disease resulting from deficiency of the lysosomal hydrolase α -galactosidase A.¹ The enzymatic defect leads to the widespread deposition of uncleaved neutral glycosphingolipids in the plasma and lysosomes, especially in vascular endothelial and smooth muscle cells. Initial clinical signs include skin lesions (angiokeratoma), excruciating acral pain, and benign corneal opacities. Progressive glycosphingolipid deposition in the microvasculature of hemizygous males subsequently leads to failure of target organs and to ischaemic complications involving the kidneys, heart, and brain.^{2,3} Much interest is currently shown in emerging therapies for FD and recent studies have reported that genetic engineering has removed many of the obstacles to the clinical use of enzyme replacement and that infusions of purified α -galactosidase A are safe and biochemically active.^{4,5} However, clinical and laboratory indicators of benefit are lacking, given the slow course of the disease. This emphasises the need for non-invasive surrogate endpoints to delineate target organ damage and to monitor the efficacy of enzyme replacement therapies.

Methods and results

In the present study, we determined intima-media thickness (IMT) at the site of the radial artery, a distal, muscular, medium sized artery, in a cohort of 21 hemizygous male FD patients, with a mean age of 32 years (SD 13, range 13-56 years), compared with 21 age and sex matched normal controls. All patients were diagnosed with FD by the presence of both clinical signs and a markedly decreased α -galactosidase A activity in leucocytes (<4 nmol/h/mg protein, normal values 25-55 nmol/h/mg protein). No patient had end stage renal disease. Measurements of the radial artery parameters were obtained with a high precision echotracking device (NIUS 02, SMH, Bienne, Switzerland) as previously described.^{6,7} Briefly, the radiofrequency signal was visualised and the peaks corresponding to the blood-intima and media-adventitia interface were electronically tagged and followed over several cardiac cycles. Internal diameter and wall thickness were then measured with a precision of about 10 μ m. Four to six measurements were averaged.^{6,7} Radial artery IMT was measured 2 cm upstream from the wrist.

Compared to controls, FD patients had considerably higher IMT values at the site of the radial artery (fig 1). IMT was twice as high in

Figure 1 Correlation between radial artery intima-media thickness and age in patients with Fabry disease (circles) and in control subjects (triangles). Correlations are significant ($p < 0.001$) in both populations and slopes differ significantly (59 (SD 14) v 25 (SD 4) μm per 10 years, $p < 0.001$).

FD patients than in controls, even after adjustment for body surface area, age, and mean blood pressure ($p < 0.001$). Radial artery IMT

increased significantly with age in each group. However the slope was 2.3-fold higher in FD patients than in controls ($p < 0.001$) (fig 1).

Discussion

In the present study, we describe evidence of a major, accelerated hypertrophy of the wall of a medium sized artery in a cohort of patients with FD. The magnitude of the difference in radial artery IMT was very large, with virtually no overlap between FD patients and controls. With age, the radial artery wall thickening was 2.3-fold faster in FD patients than in controls. The high definition echotracking system used in the present study has been previously validated in large subsets of patients with various diseases, and its accuracy and reproducibility are well accepted.^{6 7}

The most commonly proposed explanation for the pathogenesis of cardiovascular lesions in FD patients is the slow deposition of uncleaved neutral glycosphingolipids within the arterial and cardiac tissues. However, the hypothesis of

Figure 2 Bidimensional scans and radiofrequency signals (RF) of the right radial artery from a control (A) and a patient with Fabry disease (B). Lumen and posterior wall contours have been emphasised. Intima-media thickness (IMT) was measured from the distance between the RF peaks corresponding to the blood-intima and media-adventitia interfaces. Note the irregularity and the prominent thickening of the arterial wall in the Fabry patient.

a sole lysosomal accumulation of sphingolipids is somewhat simplistic since in the most advanced reported cases of left ventricle hypertrophy in FD patients, the amount of uncleaved glycosphingolipids found in the cardiac tissue did not exceed 1.6% of tissue weight (10–20 mg/g wet weight).⁸ Other mechanisms are thus probably involved. First, although accumulation of globotriaosylceramide is the main mechanism in FD, the metabolism of other glycosphingolipids may also be dysregulated.⁹ Among them, lactosylceramide, which mimics the biological function of cytokines, growth factors, and stress signalling molecules^{10,11} and accumulates in vascular tissues of FD patients,^{8,9} could act as a second messenger and potentiate the hypertrophy of the arterial wall. Second, the smaller internal diameter of the radial artery in FD patients may be the result not only of wall hypertrophy encroaching the lumen (fig 2), but also endothelial dysfunction. Deposition of glycosphingolipids occurs predominantly in the lysosomes of endothelial and smooth muscle cells, with consequent cellular dysfunction.³ An altered endothelium dependent relaxation of arterial smooth muscle could occur at the site of the radial artery or downstream, in arterioles, influencing the tonic flow dependent vasodilatation. The mechanism of flow dilatation is known to occur physiologically at the site of the radial and brachial arteries,¹² and has been related to changes in basal and stimulated nitric oxide (NO) release.¹² Finally, both in the media and intima, smooth muscle cells with glycosphingolipid inclusions secrete important quantities of extracellular matrix, notably elastic fibres.⁸ Proliferation of smooth muscle cells and extracellular matrix deposition may thus contribute to the hypertrophy of the radial artery observed in FD patients.

In conclusion, this study presents the first non-invasive demonstration of a major increase in arterial wall thickness at the site of the radial artery in a cohort of patients with confirmed FD. The assessment of the involvement of the large arteries, through non-invasive procedures, could prove useful in monitoring new therapies for FD in providing an intermediate phenotype or a surrogate marker. However, the prognostic significance of the radial artery wall hypertrophy and its ability to regress with

emerging treatments, such as enzyme replacement^{4,5,13,14} or gene therapy,¹⁵ remains to be determined during follow up studies.

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Variation of iron loading expression in C282Y homozygous haemochromatosis probands and sib pairs

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EDITOR—Hereditary haemochromatosis (HH), a common autosomal recessive disease of iron metabolism, is more prevalent among populations of northern Europe with an affected rate of 1 in 200 to 400 and a carrier frequency of around 1 in 10.^{1,2} The disease is characterised by progressive iron overload, and the clinical onset usually appears after middle age. The phenotypic manifestations of HH are variable, and the severity of the disease is related to the iron loading; the most common symptoms of iron overload are fatigue, lethargy, arthropathy, and skin pigmentation, often along with more serious organ damage including cirrhosis, diabetes mellitus, myocardopathy, and endocrine dysfunction. The assessment of iron loading is currently based on the levels of biochemical iron markers such as transferrin saturation percentage, serum ferritin, and serum iron concentrations. However, the diagnosis of haemochromatosis can now be confirmed using direct HFE mutation testing. The prognosis depends on early diagnosis and therapeutic venesections. Thus, population screening would allow early diagnosis during the asymptomatic phase and prophylactic treatment by repeated venesection to prevent the irreversible damage of iron overload,³ but predictive diagnosis requires a well established phenotype-genotype correlation.

The identification of the haemochromatosis gene,⁴ now referred to as *HFE*,⁵ enables the performance of direct genetic testing for diagnosis. The role of the HFE protein in iron metabolism has not yet been clearly established, but it seems that the complex of HFE with β 2-microglobulin interacts with the transferrin receptor (TfR) on the cell surface, which decreases the affinity of TfR for transferrin.⁶⁻⁹ Some mutations characterised in the *HFE* gene and leading to a functional defect have been correlated with HH. Two missense mutations, 845G→A (C282Y) accounting for 80-90% of HH chromosomes,^{4,10-13} and 187C→G, (H63D) representing 40-70% of non-C282Y HH chromosomes,^{4,12-15} leading to the absence and decrease of HFE activity, respectively, have been described.^{16,17} Another variant, 193A→T, leading to the missense substitution S65C, has been reported to be increased in HH chromosomes, accounting for 7.2% of HH chromosomes, neither 845A (C282Y) nor 187G (H63D).¹⁸

The considerable heterogeneity of iron loading observed in HH patients has been correlated with their genotype. Several studies have confirmed that HH patients homozygous for

the C282Y mutation are associated with a more severe form of the disease than those carrying other genotypes (H63D/H63D, C282Y/H63D, C282Y/S65C).^{12,13,19} Phenotype-genotype correlation studies have shown discrepancies. Some reports have mentioned patients diagnosed with haemochromatosis who did not carry known HFE mutations on both chromosomes, accounting for up to 21% of the HH population.^{4,12,13} Thus, the aetiology of the iron loading in these patients remains unclear. Non-HFE related patient cases may have been included in these HH subjects because of misdiagnosis owing to secondary iron overload or atypical juvenile haemochromatosis linked to chromosome 1q²⁰⁻²¹; this point still needs to be clarified. In addition, despite the prominent role of the C282Y mutation in HH, population screening indicates that 17.6% of homozygotes for C282Y were asymptomatic patients.²² Thus, C282Y penetrance confronts one with a problem and requires more investigation.

In the present study, we assessed the biochemical expression of iron loading in *HFE* C282Y homozygotes. We thus examined the parameters indicative of iron loading in a series of probands homozygous for the C282Y mutation. Then we conducted a family case study of *HFE* identical sibs enrolled because one of them had HH. The whole study showed a variable biochemical expression of iron overload related to the patients' age and sex, which was not correlated in subjects with an identical inherited genotype at the *HFE* locus.

Patients and methods

SUBJECTS

A series of 545 unrelated probands, all homozygous for C282Y, showing various symptoms of clinical haemochromatosis and referred from clinicians to our blood centre for treatment by venesection, was included in this study. Before treatment the diagnosis was confirmed by their iron status markers, and all of them showed at least two of the following criteria: (1) transferrin saturation higher than 60% in males and 50% in females, (2) serum ferritin concentration exceeding 400 μ g/l in males and 300 μ g/l in females, and (3) serum iron above 20 μ mol/l. These iron status markers were measured by standard techniques.

SIB PAIR STUDY

As part of genetic counselling a family study was conducted. Partners and sibs of C282Y HH probands were screened for *HFE* mutations and biochemical iron markers. The study

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Table 1 Iron status in 545 probands homozygous for the C282Y mutation

Sex	Age	No	Transferrin saturation (%)	Serum ferritin (µg/l)	Serum iron (µmol/l)
F	<30	11	68 (11.8) [55–82]	262 (104) [108–398]	37 (5.6) [28–42]
M	<30	22	79.6 (16.7) [51–98] NS	598 (506) [83–2000] NS	38.4 (8.8) [21–49] NS
F	<40	30	79 (14.7) [57–99]	663 (944) [69–4000]	37 (11.3) [14–62]
M	<40	105	78.2 (15.5) [34–100] NS	1341 (1078) [240–4800] p=0.02	38.8 (7.8) [22–69] NS
F	<50	50	76.6 (14.6) [54–95]	588 (764) [36–3300]	33.2 (6.6) [22–53]
M	<50	117	82.5 (12.1) [44–100] p=0.05	1822 (1672) [95–8890] p=0.0003	38.4 (6.3) [22–51] p=0.055
F	<60	52	70 (16.9) [27–96]	847 (657) [77–2500]	31.8 (6.4) [22–48]
M	<60	71	81.8 (12) [47–97] p=0.001	2133 (1601) [450–5368] p=7.10 ⁻⁶	44.7 (25.2) [27–57] p=0.01
F	>60	54	75.8 (18.4) [37–98]	1649 (1525) [195–6680]	36.3 (8.4) [14–55]
M	>60	33	79.6 (18) [37–95] NS	2349 (2059) [215–8800] p=0.033	38.8 (6) [28–49] NS

Transferrin saturation, serum ferritin, and serum iron values are expressed as means (SD) and range (NS = not significant).

group consisted of 53 subjects from 24 unrelated HH families in whom at least one sib had exhibited symptoms and an increased total body iron loading, indicative of HH. This allowed the examination of 18 same sex sib pairs, homozygous for C282Y, and composed of 13 male pairs and five female pairs; eight others were opposite sex pairs. At the time of the biochemical diagnosis, all these sibs were 32 to 64 years old. The mean difference in age within a set of sibs was 5.6 years.

HFE MUTATION ANALYSIS

DNA was extracted from peripheral blood leucocytes. C282Y, H63Ds, and S65C substitutions were analysed as previously described.¹⁸

STATISTICAL ANALYSIS

Measurements of transferrin saturation, serum ferritin, and serum iron are expressed as means (SD) and range is indicated. Comparisons between groups of subjects were made with Student's *t* test and correlation between iron parameters was assessed. The relationship between the iron parameters and the age of patients was studied separately in males and females using linear regression analysis.

Results

A total of 545 unrelated subjects including 197 females and 348 males, all diagnosed with HH and homozygous for the C282Y mutation, were studied. Thus, the male to female sex ratio was 1.7:1 showing a reduced penetrance of the C282Y mutation in females compared to males. The difference in age at onset was recorded according to the sex of probands. The mean age was 44.1 (SD 10.9) and 49.8 (SD 12.5) in males and females, respectively. The difference in mean age at onset calculated between the males and the females using Student's *t* test was significant ($t=4.57$, $p=3.3 \times 10^{-6}$) showing that, at onset, females were significantly older than males. Approximately

Figure 1 Distribution and correlation of serum ferritin concentration in 348 males ($r=0.29$) and 197 females ($r=0.23$) according to age. The equation of the fitted regression line was $Y=39.9X-43.4$ in males (light line) and $Y=21.1X-217.2$ in females (dark line).

70% of the males were diagnosed with HH before the age of 50, whereas only 48.6% of females were; 90% of the males and 76.5% of the females were diagnosed with HH before the age of 60 (table 1). These results show that the biochemical expression of haemochromatosis strongly depended on both sex and age in C282Y homozygotes. Iron marker values ranged between normal and significantly increased compared with control values and regardless of the proband's sex and age. However, transferrin saturation, serum ferritin, and serum iron values as a whole were significantly higher in males than in females ($p=1.2 \times 10^{-3}$, 6.7×10^{-8} , 9×10^{-3} , respectively).

According to the age range, above 30 years old serum ferritin and serum iron concentrations were significantly higher in males than in females; transferrin saturation was significantly increased with age only after 40 years in males compared with females (table 1). A correlation analysis showed that, whereas transferrin saturation and serum iron remained stable with age in both sexes, there was a progressive increase of serum ferritin concentration with age in males ($r=0.29$) and females ($r=0.23$) (fig 1); it increased from 262 to 1355 µg/l in females and from 598 to 2349 µg/l in males aged from 30 to over 60 years of age. The linear regression analysis indicated a significant mean annual progression of serum ferritin of 39.9 µg/l ($p<10^{-3}$) in males and 21.1 µg/l ($p=10^{-2}$) in females.

The biochemical data of families were reviewed following the discovery of one sib pair HFE identical by descent, in which one sib exhibited total body iron overload and was clinically diagnosed as HH. Sex matched sibs, homozygous for the C282Y mutation, were reviewed to determine the degree of iron loading in the other sib through transferrin saturation percentage and serum ferritin and serum iron concentrations. Opposite sex sibs were not compared because iron overload is known to be higher in male subjects compared to females. Therefore, 18 C282Y homozygous same sex sib pairs were examined; this showed that transferrin saturation ranged between 39 and 98%, serum ferritin between 159 and 4900 µg/l, and serum iron from 12.5 to 48 µmol/l.

Table 2 Comparison of 18 same sex sib pairs homozygous for the C282Y mutation

	Transferrin saturation (%)	Serum ferritin (µg/l)	Serum iron (µmol/l)
Correlation	r=0.47 p=0.07	r=0.10 NS	r=0.23 NS
Mean difference	14 [0–35] t=5.1 p=0.00017	1026 [90–4243] t=3.12 p=0.0065	7.5 [0–20] t=6.1 p=0.00002

Transferrin saturation, serum ferritin, and serum iron means in probands were 79.4% (SD 13), 1382 µg/l (SD 1348), 39.5 µmol/l (SD 6.4), respectively, and in sib cases 74.2% (SD 19), 1069 µg/l (SD 1166), and 38.1 µmol/l (SD 8.1), respectively; these results were not significantly different. The concordance of these parameters between sib pairs was also assessed. There was no correlation of serum ferritin or serum iron levels between the HH diagnosed sibs and other sibs, while transferrin saturation level tended to be correlated, but remained non-significant (p=0.07); the mean differences in transferrin saturation, serum ferritin, and serum iron values in sib pairs were all significant (table 2). In addition, no significant correlation was found between the oldest and the youngest sibs for the three iron markers when all same sex pairs were considered, indicating that, in this case, the lack of correlation was not related to the age of the subjects. This intrafamilial study showed a variable level of iron overload for subjects with *HFE* genotype identical by descent. In addition, among the 18 same sex sibs, two, six, and one sibs were in the normal range of values for transferrin saturation percentage (<43%), serum ferritin concentration (<300 µg/l), and serum iron concentration (<20 µmol/l), respectively.

Moreover, in a family of five *HFE* identical sibs homozygous for the C282Y mutation, ranging in age from 53 to 61 years old, the sibs

displayed variable biochemical expression of iron loading (fig 2). One 60 year old female had no significant increase of any of the iron parameters. Her two sisters (mother of four and two children, respectively) and two brothers, who were C282Y homozygotes, were affected with HH and showed iron overload according to their iron status parameters.

Another case of discrepancy between genotype and phenotype was discovered in a female homozygous for the C282Y mutation; she had five children. Her husband was genotyped to evaluate the potential risk for the children of having HH. He was found to be homozygous for C282Y and his biochemical iron status at 61 years of age did not show any sign of iron loading (fig 3). Their son, 35 years old and C282Y homozygous, showed signs of iron overloading whereas of their four daughters, aged 30 to 40 years old, only the oldest showed raised transferrin saturation and serum iron concentration.

Discussion

The present study reports on the relationship between the biochemical expression of iron loading and the homozygous genotype for the C282Y mutation. Iron loading was first examined in a series of 545 probands homozygous for the C282Y mutation. The iron loading was significantly lower in females than in males whatever the parameter investigated; moreover the study confirmed the reduced penetrance of C282Y in females with a male to female sex ratio of 1.7:1 in probands with clinical HH. The biochemical expression of HH, lower in females than in males, indicated that some C282Y homozygous females may not develop signs of iron overload. In a family study, one case of a female identical by descent to four sibs homozygous for C282Y and diagnosed with HH did not reach the threshold values for iron

Figure 2 A familial case of a female (II.2) homozygous for the C282Y mutation without haemochromatosis. The genotypes are given in order C282Y, H63D, and S65C. + indicates the presence of the mutant allele and – the presence of the wild type allele.

Figure 3 A male case (I.1) homozygous for the C282Y mutation without reaching iron status level for haemochromatosis. The genotypes are given in order C282Y, H63D, and S65C. + indicates the presence of the mutant allele and – the presence of the wild type allele.

overload expression defined for haemochromatosis, which indicated that the C282Y mutation did not show complete penetrance in females.

In this series, iron marker values ranged between normal and significantly increased; transferrin saturation percentage seemed to be the best parameter to predict haemochromatosis in young C282Y homozygous subjects whereas serum ferritin, the only value to increase progressively, was proved better to show overloading extent. This study thus confirmed that the extent of iron loading in haemochromatosis C282Y homozygotes is directly related to the age and sex of probands.^{23–25} However, when considering age range, large variations in iron status values were observed in subjects homozygous for C282Y mutation; serum ferritin showed the largest variation. We thus checked for intrafamilial variation of iron markers in sib pairs homozygous for the C282Y mutation. The lack of correlation between sibs and the significant differences of the iron marker values between sib pairs clearly showed a variable biochemical expression of iron overload in sibs with genotype identical by descent at the *HFE* locus. Although the expression of the disease is strongly influenced by the C282Y mutation, an intrafamilial study of subjects also confirmed that an identical genotype for the *HFE* gene can show variable iron loading; thus, intrafamilial variations in iron loading do not only represent a variation in the genetic expression of the *HFE* gene. The cases of non-expressing C282Y homozygous males aged over 60 years old, and displaying iron parameters in the normal range, as reported here and by others,²⁶ showed that the biochemical expression of HH is under the influence of other factor(s). In the families studied, transferrin saturation, serum ferritin, and serum iron variations could result from environmental and non-genetic factors or other genetic factors than those examined. Recent population studies have shown that,

compared with non-carriers, any genotype group defined as carriers of a *HFE* mutation had higher mean serum iron concentration and mean transferrin saturation, whereas only C282Y homozygotes showed higher serum ferritin concentration²³; they also highlighted that some C282Y heterozygotes could be diagnosed as HH.²² Thus, despite a good correlation between *HFE* defined genotypes and phenotype in population studies, *HFE* genotyping does not show a clear level of iron loading in subjects and may have implications in genetic counselling.

- Hereditary haemochromatosis (HH) is a common autosomal recessive disease characterised by progressive iron overload. The identification of the *HFE* gene and mutations involved in haemochromatosis allows direct genetic testing for diagnosis. However, correlations between phenotype and *HFE* genotypes showed discrepancies and mutation penetrance raises questions.
- We examined the iron loading status in 545 probands and 18 same sex sibs, all homozygous for the C282Y mutation.
- Our data support transferrin saturation percentage and serum ferritin concentration as the best biochemical iron marker for HH phenotype in young subjects and extent of overload in these patients, respectively. The results also confirm a clear correlation of the iron loading level with age and sex of the patients. However, the lack of correlation of the iron marker status between pairs of sibs, homozygous for C282Y identical by descent, indicated a variable phenotypic expression of iron loading independent of *HFE* genotype.

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Haptoglobin genotype as a risk factor for postmenopausal osteoporosis

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EDITOR—Some epidemiological and experimental data have shown a correlation between iron metabolism and calcium, phosphate, and magnesium turnover.^{1,2} In particular, previous reports have shown that iron availability can play a fundamental role in bone metabolism and that iron depletion can lead to bone demineralisation. For example, in patients who underwent gastrectomy³⁻⁵ or in rats treated similarly,⁶ osteoporosis was accompanied by laboratory and clinical signs of iron deficiency and was prevented by the administration of fructo-oligosaccharides, a substance that promotes iron absorption from the gut. In oophorectomised rats (a condition mimicking the oestrogen levels commonly found in the menopause), a wide range of cells, including osteoblasts, displayed a reduced number of transferrin receptors and hence a reduced iron uptake.⁷ In humans, it has been assessed that out of 14 nutrients tested (including calcium), iron was the best positive predictor of BMD in the femoral neck,⁸ and furthermore a negative correlation between ascorbic acid content of the diet and osteoporosis has been found^{9,10}; it is notable that ascorbic acid in the diet affects iron absorption increasing it by a factor of 2-3. A severe nutritional iron deficiency anaemia provokes significant alterations in the metabolism of calcium, phosphorus, and magnesium

in rats with a noticeable degree of bone demineralisation, even in the presence of normal serum levels of calcium, phosphorus, and magnesium.¹

On the basis of the above evidence, we searched for a genetic marker of iron disposal (haptoglobin genotype) as a risk factor for postmenopausal osteoporosis.

Only about 5% of daily iron turnover comes from intestinal absorption, most of it coming from haemoglobin turnover, which requires three proteins, haemopexin, haptoglobin, and haem oxygenase. We focused our attention on haptoglobin since it is the only one with a well known polymorphism.

Haptoglobin (HP) is a serum α 2 glycoprotein that exists as a tetramer, composed of two smaller identical alpha (α) and two larger identical beta (β) chains. At present, three main different genotypes of haptoglobin in normal adult plasma have been identified. Differences among the three haptoglobin genotypes are given by light alpha subunit structures: type 1.1, type 2.2, which has homozygous α 1 (9 kDa) and α 2 (18 kDa) subunits, and type 2.1, which has heterozygous α 1 and α 2 subunits, with a shared β subunit in all three genotypes (38 kDa). The β chain is a glycoprotein which does not exhibit polymorphism but only some rare variants.

Figure 1 Electrophoretic runs of haptoglobin: type 1 appears as a single band furthest from the origin, while types 2.1 (200 kDa) and 2.2 (400 kDa) appear as a series of bands nearer to the origin.

The main function of haptoglobin is to bind free haemoglobin in a stable complex, which is later cleared from the plasma by the liver reticuloendothelial system. Haemoglobin binding capacity depends on the genetic haptoglobin type, on the amount of haptoglobin, and on the number of polymers.^{11 12}

Functional differences between haptoglobin genotypes have been described¹²; type 1.1 has the highest haemoglobin carrying ability, while type 2.2 is almost unable to carry it because the Hb binding site is buried by the polymerisation process.

In European populations, the genotype distribution is as follows: about 16% has genotype 1.1, about 48% genotype 2.1, and the remaining 36% genotype 2.2.¹³ Several authors have studied the haptoglobin haplotype frequency in different populations and different pathologies,^{13 14} and various haptoglobin genotypes have also been correlated with the serum iron.¹² In spite of the large number of published reports on the topic, no study has been performed to investigate a possible difference in the incidence of haptoglobin genotypes in osteoporotic patients and non-osteoporotic subjects.

In order to investigate the possible correlations between postmenopausal osteoporosis and frequencies of haptoglobin genotypes, we studied a group of women affected by postmenopausal osteoporosis and a control group of non-osteoporotic postmenopausal women.

Methods

The osteoporotic group consisted of 135 subjects (age range 40–73 years, postmenopausal age range 6 months–26 years) and the osteoporosis was diagnosed using the Double Emission X ray Absorptiometry (DXA) technique

with a Hologic QDR4500 densitometer (Hologic Inc, Waltham, MA, USA). In particular, we considered as osteoporotic patients with a T score value of 2.5 SD or less, according to WHO (WHO Technical Report Series No 843 “Assessment of fracture risk and the application to screening for postmenopausal osteoporosis”, 1994). Secondary osteoporosis was excluded by history, physical examination, and measurement of calcium, phosphorus, and bone alkaline phosphatase (BAP) in the blood.

The control group consisted of 65 non-osteoporotic women (age range 47–76 years, postmenopausal age range 6 months–33 years) (T score >–1 SD).

The HP genotypes of patients and controls were analysed with SDS-PAGE electrophoresis (fig 1). Data were analysed by the χ^2 test using the Statistical Analysis System (SAS Institute Inc). The odds ratio and the corresponding confidence interval were also calculated for genotype 1.1 versus genotype 2.2 and 2.1. To avoid possible selection bias, we compared the patients and the control group for age, postmenopausal age, body mass index (BMI), and T score (table 1). The controls were, on average, older than the patients ($p=0.01$) with a longer postmenopausal period ($p=0.05$).

Results

The frequencies of the three haptoglobin genotypes are 32.6% for 2.2, 55.5% for 2.1, and 11.9% for 1.1 in the patient group, while in the control group they are 47.7%, 50.8%, and 1.5%, respectively, with significant differences between the two groups ($p=0.0076$, χ^2 test). The odds ratio between genotype 1.1 and genotype 2.2 was 12 (confidence interval = 1.34–106.7). The odds ratio between genotype 1.1 and genotype 2.1 was 7.04 (confidence interval = 1.21–2.9).

Discussion

It is well known that advancing age, a prolonged period of amenorrhoea, and low BMI are risk factors for osteoporosis. Any possible bias in selection of subjects was excluded as the controls were on average significantly older and the BMI of the two groups was not significantly different.

Our data show that the presence of haptoglobin genotype 1.1 is an important risk factor for postmenopausal osteoporosis. The functional differences between haptoglobin genotypes, namely the fact that type 1.1 has the highest haemoglobin carrying ability and, hence, the highest elimination rate through the liver, while type 2.2 is almost unable to carry it to the liver, can account on a molecular basis for the increased risk for osteoporosis linked to the presence of haptoglobin genotype 1.1. In normal subjects, the amount of iron stores (namely ferritin) is significantly correlated with the HP genotype.¹²

The daily requirement of iron intake to keep the body iron store stable is therefore strongly dependent on the ability of the organism to store iron (HP 2.2) or to waste it (HP 1.1) through the liver.

Table 1 Characteristics of the patients compared to the controls (age, postmenopausal age, BMI, T score). Shown are the mean values, the standard deviation (SD), and the result of Student's *t* test

	Patients (n=135)		Controls (n=65)		<i>p</i>
	Mean	SD	Mean	SD	
Age	57.7	5.4	60	7.2	0.01
Postmenopausal age	8.7	6.5	10.7	7.9	0.05
BMI	24	13.9	24.6	4.2	NS
<i>t</i> score	–3.16	0.55	–0.76	0.56	<0.0001

The finding that HP genotype may play an important role as a risk factor for osteoporosis may be useful in clinical practice to identify in advance the women who will probably develop postmenopausal osteoporosis and so allow primary prevention of the disease and reduce the social cost of its consequences.

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GDNF as a candidate modifier in a type 1 neurofibromatosis (NF1) enteric phenotype

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EDITOR—Neurofibromatosis type 1 (NF1) is a common human disorder (1/3500 live births) with neuroectodermal involvement primarily resulting in dermatological manifestations of café au lait spots, cutaneous/subcutaneous neurofibromas, and freckling of major skin folds.¹ Owing to diagnostic uncertainties, especially in young patients, an international scoring system has been discussed and agreed upon.² Half of the cases result from new mutations, while others show an autosomal dominant mode of inheritance. The encoded product, referred to as neurofibromin, is a member of the so called GTPase activating proteins (GAPs), and is an upstream down-regulator of the RAS(p21)/RAF/MAPkinase³ and RAS/RAL⁴ signalling pathways. Although locus homogeneity is a hallmark of this condition, phenotypic heterogeneity has been exemplified by a wide spectrum of diversity ranging from malformation or malignant variants to virtually benign dermatological changes.¹ In particular, and among the many causes of gastrointestinal involvement in NF1 patients, the association with intrinsic intestinal dysmotility, resulting from intestinal neuronal dysplasia type B (IND B)^{5–6} or aganglionic megacolon (Hirschsprung's disease, HSCR),⁷ has been documented and is now well established.

Interestingly, a substantial fraction of the phenotypic variability seen in NF1 patients

might be governed by non-allelic, trait specific, “modifying” loci.⁸ Although the action of such modifying loci has been primarily shown in the number of café au lait spots or the number of cutaneous/subcutaneous neurofibromas, it can be speculated that such a genetic phenomenon might also be operative in other phenotypic traits, especially in individual or familial cases with an enteric phenotype.

The female proband from the family analysed here (fig 1A) had minor cutaneous manifestations of NF1, dysmorphic facial features (midface hypoplasia), congenital heart disease (ventricular septal defect, coarctation of the aorta), and congenital megacolon. She subsequently underwent a Duhamel abdominoperineal pull through and pathological examination of the whole colectomy specimen pointed to IND B (fig 2), because of findings of (1) abnormal submucosal plexuses showing focal hyperplasia (in terms of density and sizes), (2) occasional giant ganglia harbouring >10 neurones, and (3) nerve cell buds along afferent nerves.⁹ The older sister also had NF1 and congenital megacolon, while a brother was totally unaffected. NF1 was inherited from the mother and maternal grandmother, who both had a mainly cutaneous form of the condition. The father was healthy.¹⁰

Although IND B may segregate as a monogenic disorder, no specific locus has been

Figure 1 (A) Family pedigree. A miscarriage, which occurred between II.2 and II.3, is not represented here. CAL=café au lait spots, NFs=neurofibromas. A cytogenetically balanced reciprocal $t(15;16)(q26.3;q12.1)$ translocation is shared by I.1, II.1, II.2, and II.3,¹⁰ as indicated by asterisks. (B) NF1 and GDNF restriction. DNA from the proband and relatives was screened for the NF1 lesion with PCR primers and HphI enzyme cleavage as previously stated¹ and for the GDNF mutation using the upper primer GDNFEx2F (5'-CAAATATGCCAGAGGATTATC-3') and lower primer GDNFEx2R (5'-TATTTTGTCTGACGTTGTCTC-3') with cycling conditions of 5' at 94°C and 30 rounds of 20 seconds at 94°C, 20 seconds at 55°C, and 30 seconds at 72°C followed by restriction with HinfI endonuclease. Restriction products were size fractionated through a 8% polyacrylamide gel, stained with ethidium bromide, and visualised by ultraviolet transillumination. NF1 exon 16 and GDNF exon 2 amplicons are shown unrestricted and restricted for I.1 (lanes 1 and 2) and I.2 (lanes 3 and 4) and restricted only for II.1, II.2, and II.3 (lanes 5, 6, and 7). Arrowheads indicate theoretical fragment sizes in base pairs (bp). HphI restriction of the 552 bp wild type NF1 amplicon generates a 532 bp fragment, whereas restriction of the 559 bp mutated amplicon generates a 457 bp fragment. On the other hand, restriction with HinfI of the 326 bp wild type GDNF amplicon generates a 204 bp and a 122 bp fragment but leaves the (326 bp) mutated amplicon unaltered. Only II.1 and II.3, sharing both the paternally derived GDNF lesion and the maternally inherited NF1 mutation, have megacolon. Note the presence of heteroduplexes in NF1 heterozygotes (lanes 3-5 and 7).

linked to this recognised Mendelian entity (MIM 601223). However, since ~30% of IND B patients have accompanying aganglionosis, that is, HSCR,⁹ the *RET* proto-oncogene,¹¹⁻¹³ the genes encoding endothelin receptor B (*EDNRB*),¹⁴⁻¹⁶ or its ligand endothelin 3 (*EDN3*)¹⁷ are candidate genes for isolated IND B.¹⁸ Here, none of these genes was found to be mutated, suggesting that the NF1-IND B combination observed here was indeed an integral NF1 variant.¹⁰

However, the scarcity of this specific variant and the small family sizes has hindered the identification of modifying loci by phenotype and pedigree based analyses.⁸ The initial observation of an out of frame insertion within NF1 exon 16 (2424-2425insCCTTCAC, fig 1B) favoured a null lesion, that is, it did not support causal genotype-phenotype correlation. The fact that only those relatives with the NF1 mutation and a cytogenetically balanced reciprocal translocation $t(15;16)(q26.3;q12.1)$ (fig 1A) had intrinsic intestinal dysmotility suggested that a modifier gene might have been altered at one of the translocation breakpoints.¹⁰ However, none of the breakpoint regions is known to harbour a gene involved in the development of the parasympathetic ganglion cells of the digestive tract and this family was investigated further for candidate genetic modifiers lying elsewhere in the genome.

As changes in *GDNF*¹⁹⁻²² and the neurturin gene (*NRTN*)²³ suggested polygenic causation

of anomalous neural crest cell derived structures, especially parasympathetic enteric neurones, these genes were good candidate modifiers. For this reason, *GDNF* and *NRTN* gene exons were analysed for DNA changes by SSCP and sequence analysis, as previously reported.¹⁹⁻²³ While *NRTN* showed no sequence variation (data not shown), a missense mutation was found within *GDNF* exon 2, changing codon 93 from arginine (CGG) to tryptophan (TGG) (R93W, fig 1B). The R93W mutation could be regarded as potentially pathogenic, since (1) it resulted in a non-conservative amino acid change at an evolutionarily conserved residue in the direct vicinity of a putative propeptide cleavage site and transforming growth factor beta (TGFB) related cysteine rich motifs,²⁴ (2) it was found in other patients with distinct neural crest cell related anomalies such as HSCR (in addition to mutations of *RET*)²¹⁻²² or the CCHS-HSCR association,¹⁹ and (3) it was conspicuously absent from a large number of control DNAs.²¹⁻²⁵ Of interest, this is the first *GDNF* mutation seen in a patient with IND B without aganglionosis. The recurrence pattern of this mutation is probably explained by its relation to a CpG dinucleotide mutational hot spot.²⁶

GDNF is probably the most powerful neurotrophin identified to date. It is the first identified member of a family of growth factors distantly related to TGFBs²⁴ (see Bohn²⁷ for a recent review and Ramer *et al*²⁸ for an update on neurotrophic effects), whose action is mediated by binding to a multicomponent system composed of RET receptor tyrosine kinase (RTK)²⁹ and glycosyl-phosphatidylinositol (GPI) linked cell surface adapter proteins (GFRA1, GFRA2).³⁰⁻³¹ Signalling through RET can trigger phosphatidylinositol-3 kinase (PI3K), leading to activation of either members of the RHO family of GTPases (RHO, RAC, and CDC42) with ensuing rearrangements of the actin cytoskeleton and axon outgrowth, or protein kinase B (PKB) mediated effects on metabolism or gene transcription. Interestingly, in both these pathways, PI3K requires functional RAS.³² In addition, stimulation of RET leads to SHC-GRB2-SOS complex formation and RAS activation, the RAF and RAL families of GTPases acting as downstream effectors³³ (fig 3). Although it is difficult to predict the exact outcome of the combination of mutations reported here for the subcellular signalling network, it can be speculated that certain pathways are markedly impaired while others are only mildly affected, especially since the NF1/*GDNF* double heterozygote infants depicted here have severe developmental alterations but only minor symptoms related to deregulated cell growth.¹⁰ Of special interest is that one might have expected functional recovery of the NF1 mutation by the *GDNF* lesion, since NF1 disruption generates activated, GTP bound RAS, whereas low *GDNF* maintains RAS in the GDP bound form. A possible rationale comes from the observation that RAF1 is able to induce growth arrest and differentiation of discrete human carcinoma

Figure 2 Intestinal neuronal dysplasia. The colectomy specimen was fixed in Bouin's reagent for 12 hours. Transverse sections were cut every centimetre, from both normal and pathological segments, and were routinely processed for histology. Four millimetre serial paraffin embedded sections were stained with haematoxylin-eosin-saffron (HES) for standard light microscopy. Immunodetection of protein S100, a marker of Schwann cells, was used to outline nerve structures. After microwaving for antigen retrieval, paraffin embedded sections were incubated with Dako Z311 anti-S100 antibody (diluted 1/800) in a Ventana ES 320 automated immunostainer, using appropriate immunoperoxidase LSAB detection and revelation kits (Ventana, Tucson, AZ). Sections from normal and pathological specimens were analysed for the presence and density of intramural plexuses, the thickness of nerve trunks, and the features of ganglion cells. (A) Hyperplasia of nerve structures in the lamina propria and muscularis mucosae. Numerous nerve fibres are stained with anti-S100 antibody. Four submucosal plexuses (arrows) contain numerous immunonegative ganglion cells (immunoperoxidase staining). (B) Giant submucosal ganglion containing ≥ 12 distinctive neurones, as shown by characteristic vesicular nuclei and abundant amphophilic cytoplasm (HES).

cell lines, with downregulation of endogenous *RET*^{34, 35} and resistance to activated exogenous *RET* alleles.³⁶ The bona fide enteric phenotype observed here is indeed consistent with a feedback loop of RAS/RAF downstream effectors upon RET, or at least with a relatively complex interplay of factors influencing cell growth and differentiation. An interesting alternative hypothesis comes from the so called downstream model in which RAS is regarded as a regulator of neurofibromin, the process of RAS conversion from the GTP bound to the GDP bound form inducing neurofibromin to transmit signal through its influences on microtubule organisation.³⁷ In that model, both low GDNF and low neurofibromin concur to disrupt the downstream effects of neurofibromin upon cell proliferation or differentiation.

Murine models were generated for both *NF1* and *GDNF* through disruption by homologous recombination in embryonic stem cells. Mice lacking *Gdnf* (*Gdnf*^{-/-})³⁸⁻⁴⁰ had total renal agenesis, resulting from defective induction of the ureteric bud and absent enteric neurones, also consistent with additional manifestations of pyloric stenosis, duodenal dilatation, and congenital megacolon. These models are highly reminiscent of *Ret* deficient mice (*Ret*^{-/-}), providing a functional confirmation that GDNF is a ligand of RET. Heterozygotes (*Gdnf*^{+/-}) were indiscernible from wild type litter mates. However, heterozygous *Nf1* mutants (*Nf1*^{+/-}) do not replicate the human disorder (in particular, they do not develop obvious neurofibromas or pigmentation defects).^{41, 42} Conversely, these mice are prone to age related tumours, in addition to malignancies reminiscent of human NF1 (especially pheochromocytomas and myeloid leukaemia).⁴² Interestingly, homozygotes (*Nf1*^{-/-}) die in utero from severe cardiac malformation, especially involving the neural crest cell derived conotruncus,^{41, 42} and show hyperplasia of the pre- and paravertebral sympathetic ganglia,⁴¹ indicating that the phenotype is both dosage sensitive, as in *GDNF*, and malformative rather than tumourous, eventually confirming the important role of neurofibromin during development. These models and the family presented here suggest murine *Nf1*^{+/-} x *Gdnf*^{+/-} intercrosses for the phenotypic analysis of double mutants as an ultimate demonstration of a modifier gene effect.⁴³

Modifying genes are not just hypothetical and HSCR families have provided particularly fruitful material for eliciting such entities. Two hitherto anonymous loci have been indicted in polygenic inheritance of HSCR. The first such example was illustrated by a large inbred Menonite HSCR pedigree that segregated a missense mutation in *EDNRB*⁴⁴ and otherwise showed linkage disequilibrium with marker alleles mapped to 21q22. This finding was highly suggestive of a HSCR genetic modifier linked to this chromosomal region, which might elsewhere account for the high HSCR prevalence among trisomy 21 patients. More recently, genome wide non-parametric linkage was performed on a panel of HSCR pedigrees which selected a particular subgroup in the sense that these were either unlinked to *RET* or showed positive linkage but with no sequence alteration identified at that locus. From these families, significant linkage to a locus in 9q31 was found, suggesting that this genetic region contains a gene whose variation entails a specific susceptibility to HSCR, with or without concomitant linkage to *RET*.⁴⁵ More straightforward evidence for a HSCR modifier is exemplified by the genes encoding glial cell line derived neurotrophic factor (*GDNF*)²⁰⁻²² and, more recently, neurturin (*NRTN*),²³ two highly homologous natural ligands of the RET tyrosine kinase receptor protein. Indeed, since *GDNF* and *NRTN* were found to be mutated in families also segregating well characterised *RET* alleles, it was postulated that alterations of these genes were not sufficient in themselves to

Figure 3 *NF1* (neurofibromin) and GDNF signalling partnership. Epistatic interaction of *NF1* and GDNF is sustained by the signalling partnership of their respective products, neurofibromin and GDNF. Whereas alteration of GDNF is expected to balance the lack of inhibition of RAS owing to low neurofibromin, the effects on the PI3K dependent pathways are liable to aggravate the negative feedback loop of RAS/RAF on RET and RTK activity (adapted from van Weering and Bos³³ and others).

cause HSCR, but that they probably contributed to the severity of the phenotype or to higher penetrance of the *RET* mutations.

Although molecular evidence for modification proper in the pathogenesis of human *NF1* has not been provided to date, the possibility of epistatic interaction of the *NF1* (*Nf1*) gene with other discrete loci was recently illustrated both in man and in a murine model for human *NF1*.

Human pedigrees with hereditary non-polyposis colorectal cancer (HNPCC) have been reported in which children homozygous (doubly heterozygous) for an *MLH1* mutation were shown to develop extracolonic malignancies of early onset and de novo *NF1*.^{46–47} These observations suggest that the *NF1* gene is prone to common replication errors during mitosis and/or meiosis, and that *MLH1* plays a particular role in monitoring these types of DNA lesions. These very important observations point to mismatch repair (MMR) genes as possible targets during *NF1* tumour advancement and shed new light onto the rather unexpected microsatellite instability observed in *NF1* derived tumours.⁴⁸

In mice, different combinations of mutations of *Nf1* and/or *Trp53* (homologous to *TP53*) were generated through intercrosses.^{49–50} The first remarkable observation was that, unlike their *Nf1*^{+/–} or *Nf1*^{–/–} congeners, mice that were

haploinsufficient for both *Nf1* and *Trp53* developed benign or malignant peripheral nerve sheath tumours (MPNSTs) and malignant Triton tumours reminiscent of human *NF1*. In addition, permanent cell lines established from some of these mice showed a pattern of gene expression consistent with immortalisation of pluripotent neural crest stem cells, whether the original tumours were of seemingly mesodermal or of conspicuous neural crest origin.⁵⁰ These data provide further evidence of linkage of RAS with the cell cycle machinery, especially with the pathways that are linked with the activation of *TP53*, and confirm the commonality of neural crest involvement in *NF1* pathogenesis. In addition, these intercrosses clearly support the hypothesis that functional protein-protein interaction is a strong substratum for epistasis or modification.

In the family presented here, especially in the two infants who are doubly heterozygous for the *NF1*/*GDNF* lesions, it is questionable whether *GDNF* modification accounts for wider involvement of neural crest cell derivatives, such as midface hypoplasia (through disruption of the cranial crest mesectoderm) or conotruncal heart disease (VSD and coarctation of the aorta), also observed in the proband. Whatever the case, our findings seemingly confirm the previous speculation

that genes whose products interact functionally with RAS are potential NF1 modifiers.⁴³ Once identified, these modifiers will provide tools to unravel interactions with the subcellular signalling network in NF1 patients and may lay the basis for new therapeutic approaches.

- A family with neurofibromatosis type 1 (NF1, MIM 162200) and congenital megacolon (intestinal neuronal dysplasia, IND B) was investigated for possible genetic modifiers. A germline mutation in the *NF1* gene, c.2424InsCCTTCAC, and a germline *GDNF* variant R93W were found in this family.
- In this kindred, only members with both the paternally derived *GDNF* R93W and the maternally inherited *NF1* mutation had megacolon.
- Such epistatic interaction between *NF1* and *GDNF* is in keeping with functional cross talk of the RET and RAS pathways in a complex subcellular signalling network.

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Six novel mutations in the *PRF1* gene in children with haemophagocytic lymphohistiocytosis

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EDITOR—The histiocytoses represent a heterogeneous group of disorders including both hereditary and sporadic forms. The familial form of haemophagocytic lymphohistiocytosis (HLH) was originally described by Farquhar and Claireaux¹ in 1952. The main features of this disease are fever, hepatosplenomegaly, cytopenia, hypertriglyceridaemia, hypofibrinogenaemia, and central nervous system involvement.^{2,3} Haemophagocytosis is observed at presentation or later during the course of the disease in most patients. In 1991, the Histiocyte Society defined its diagnostic criteria⁴; however, the differential diagnosis of HLH from other disorders may remain problematical, especially in patients without familial recurrence. Linkage of the disease gene to an approximately 7.8 cM region between markers D9S1867 and D9S1790 at 9q21.3-22 was identified by homozygosity mapping in four inbred families with HLH of Pakistani descent.⁵ Also, linkage analysis of a group of 17 families with HLH indicated mapping of a locus linked to HLH to the proximal region of the long arm of chromosome 10 in the 10q21-22 region in 10 families but not in the remaining seven, providing evidence for genetic heterogeneity of this condition.⁶⁻⁹ While no further cases of HLH linked to the 9q21.3-22 locus have been reported, recently Stepp *et al*¹⁰ identified nine different mutations, three nonsense and six missense, in the two coding exons of the perforin 1 gene (*PRF1*) in a group of eight unrelated patients, providing the first evidence for a disease related to

PRF1.¹⁰ Perforin is an important mediator of lymphocyte cytotoxicity in a pathway independent from the Fas mediated apoptotic machinery. Thus, *PRF1* mutations may affect cellular cytotoxicity, resulting in impaired antiviral defence and dysregulation of the apoptotic mechanisms involved in regulation of the immune response.¹¹

We report six novel mutations and also confirm three additional mutations which had been previously reported. They were observed in 10 patients of Italian, Turkish, and Ghanaian origin.

Materials and methods

We studied 10 families in which the index case fulfilled the diagnostic criteria for HLH⁴ and a careful family history was collected. Consanguinity was investigated and when not evident the parents were asked to obtain further information including the birth place of their ancestors. Clinical data were obtained from the attending physicians and from thorough evaluation of records.

Natural killer activity was determined in one of the two reference laboratories (Dr Rita Maccario, Pavia, Italy and Professor Marion Schneider, Ulm, Germany) as previously reported.^{12,13} Molecular analyses were performed as reported by Stepp *et al*,¹⁰ sequencing exons 2 and 3 of the *PRF1* gene. The sequences obtained were compared to the reported gene structure (gene number 190339 NCBI) using the BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST>). In order to

Table 1 Details of mutations in the perforin 1 gene observed in 10 patients with HLH

Case	Ethnic origin	*/**	Nucleotide	Exon	Mutation	Predicted effect	Domain
1	Ghana	**	50	2	del t‡	L 17 Fs and stop	
2	Italy	**	283	2	t>c	W 94 R	
3	Italy	**	657	3	c>a	Y 219 stop	2nd TM
4	Italy	**	658	3	g>a	G 220 S	2nd TM
5	Italy	*	662	3	c>t	T221I	2nd TM
		*	673	3	c>t‡	R225W	2nd TM
6	Italy	*	694	3	c>t	R 232 C	
		*	1182	3	ins t	G 394 Fs and stop	EGF-like
7	Turkey	**	1122	3	g>a‡	W 374 stop	EGF-like
8	Turkey	**	1122	3	g>a	W 374 stop	EGF-like
9†	Turkey	**	1122	3	g>a	W374 stop	EGF-like
10	Turkey	**	1122	3	g>a	W374 stop	EGF-like

*/** = heterozygous/homozygous mutation.

†The same mutation was also observed in his brother with an identical clinical picture.

‡These mutations have been previously described by Stepp *et al.*¹⁰

2nd TM = second transmembrane domain.

EGF-like = epidermal growth factor-like domain.

Fs = frameshift.

confirm the mutations found, the parents were also tested in all but three families, in which the mutations were confirmed by repeated experiments or by identification of the same mutation in the affected sib. In families in which consanguinity was considered possible on the basis of available information, polymorphic markers (D10S537, D10S676) were tested to confirm this.

STATISTICAL ANALYSIS

The median age at diagnosis and quartiles were calculated for each group of children. The cumulative probability of diagnosis free survival was computed by means of Kaplan Meier estimation. Incidence rates expressed as events per person month were calculated for each group. Time to diagnosis distributions were compared between mutated and non-mutated subjects by means of the log rank test.

Results

We have identified six novel mutations in the *PRF1* gene; three additional mutations that we observed had been previously reported by Stepp *et al.*¹⁰ Two novel mutations (C657A in case 3 and 1182 ins T in case 6) (table 1) introduced a stop codon in the sequence which resulted in a truncated protein. The other novel mutations (T283C in case 2, G658A in case 4, C662T in case 5, and C694T in case 6) caused an amino acid change. The mutations we observed are scattered along exons 2 and 3 without any obvious clustering. Four mutations were located in the second transmembrane domain while two occurred within or close to the EGF-like domain of the protein.¹⁴ It is remarkable that all the four patients of Turkish origin had the same mutation, G1122A. In 21 additional caucasian patients with HLH a mutation was not found. In particular, none of the patients of German origin from this group harboured the mutation⁹ (zur Stadt *et al*, manuscript in preparation).

Parental consanguinity was documented in five families and was very likely in three additional families, in which the ancestors originated from the same small villages or geographical regions. These patients (cases 1, 2, and 4, tables 1 and 2) were homozygous for loci D10S537 and D10S676, thus supporting

parental consanguinity. Thus, eight of the 10 patients had related (or very probably related) parents. In 231 cases enrolled in the International HLH Registry³ (M Aricò, unpublished data) with information on this, 56 (23.2%) had related parents. Among the 10 patients with *PRF1* mutations, seven had one or more sibs. Of a total of 21 sibs, six were affected. Four of 10 patients had one or more affected sib.

The clinical and laboratory features of the 10 patients with *PRF1* mutations fit the diagnostic criteria for HLH (fever, splenomegaly, cytopenia, hypertriglyceridaemia or hypofibrinogenemia, and haemophagocytosis).⁴ Additional features, like CNS alterations, skin rash, lymphadenomegaly, and oedema, were also present in some cases (table 2). Eight of the 10 patients with *PRF1* defects developed HLH by the third month of life (median 2.2 months). This age was lower than that of our additional 21 patients in whom no *PRF1* mutations were found (median 5 months, range 0.5–86). In 209 cases enrolled in the Registry with information on this, the median age at diagnosis was 5.3 months (range 0–254 months) ($p=0.05$); 32% were diagnosed within three months and 80% within two years. One of our patients with *PRF1* mutations remained asymptomatic until the age of 6 years when he developed full blown HLH. Among the Registry patients, 17 (8.1%) presented when older than 5 years, with an estimated risk of being diagnosed when older than 5 years of 8.1% (SE 1.8).

Although difficult to quantify, all patients had a very severe presentation and clinical course. They had to be aggressively treated and showed early relapse after disease control was initially achieved. All six patients who underwent BMT remain asymptomatic.

Discussion

We have described six novel mutations in the *PRF1* gene in children with HLH; two introduced a premature stop codon in the sequence which resulted in a truncated protein, while the other four caused an amino acid change. Caution should be exercised in interpreting a missense mutation which causes an amino acid change to be responsible for the disease and is not just a population polymorphism. In our cases, these mutations modified a conserved amino acid and were never found in other subjects tested. These mutations were scattered along exons 2 and 3 without any obvious clustering, in keeping with the previous report by Stepp *et al.*¹⁰

The same mutation, G1122A, was observed in the four patients of Turkish origin. This mutation had been observed twice by Stepp *et al.*¹⁰ in patients of unspecified origin and was also reported in patients of Turkish origin.¹⁵ Altogether these data indicate that, at least in a subset of patients of Turkish origin with HLH, a founder effect is possible. Further analysis of affected children from the same geographical region should be undertaken to confirm this. No founder effect can be hypothesised in patients of Italian origin.

Table 2 Presenting features and treatment outcome in 10 patients with HLH and PRF1 gene mutations

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
No of sibs/affected	0/0	1/0	3/2	2/0	0/0	0/0	3/1	4/1	3/2	5/0
Ethnic origin	Ghana	Italy	Italy	Italy	Italy	Italy	Turkey	Turkey	Turkey	Turkey
Consanguinity	P	P	+	P	-	-	+	+	+	+
Sex	M	M	M	F	F	M	M	F	M	M
Age at diagnosis	2 mth	3 mth	1.5 mth	1.5 mth	6 mth	6 y	3 mth	2.5 mth	2 mth	2 mth
Fever	+	+	+	+	+	+	+	+	+	+
Hepatosplenomegaly	+	+	+	+	+	+	+	+	+	+
Skin rash	-	-	-	-	-	-	-	-	-	-
Lymphadenomegaly	-	-	-	-	-	-	-	-	-	-
CNS alterations	-	-	-	+	+	-	-	+	-	-
Oedema	-	-	-	+	+	-	+	+	-	ND
Jaundice	-	-	-	-	+	-	-	-	+	-
Associated infection	-	<i>E. coli</i> , EBV	-	-	URI	EBV	CMV	URI	CMV	-
Haemoglobin (g/dl)	5.5	6.4	7.8	9.6	8.4	5.2	8.2	4.4	9.6	5.3
Platelets ($\times 1000/\text{mm}^3$)	15	10	46	35	58	12	42	12	14	52
Neutropenia	+	+	-	+	+	+	+	+	+	+
Hypertiglyceridaemia	+	+	+	+	+	+	+	+	+	+
Hypofibrinogenaemia	+	+	+	-	+	+	+	+	+	+
CSF pleiocytosis	+	+	+	+	-	ND	+	+	ND	-
Absent NK activity	+	+	+	+	+	+	+	+	+	+
Response to therapy	+	+	+	+	+	+	+	+	+	+
Early reactivation	+	+	+	-	+	-	+	+	+	+
Present status	Asymptomatic 4+ mth after BMT	Asymptomatic 4+ mth after BMT	Asymptomatic 13+ mth after BMT	NA Dead of infection day +21	Dead of disease at 4 y	Dead of disease day +15	Asymptomatic 60 mth after BMT	Alive without disease but severe neurological impairment 56 mth after BMT	Dead of disease +	Asymptomatic 96 mth after BMT

Items in bold are among the diagnostic criteria for HLH.

P: possible; URI: upper respiratory infection; NA: not applicable; ND: not determined; ↓: decreased.

In this small series of patients with HLH and *PRF1* mutations, each patient presented the symptoms which form the diagnostic criteria for HLH, as well as some of the less frequent abnormalities (table 2). Comparison with the additional 21 patients in whom *PRF1* mutations were not found confirms that no striking difference based on clinical grounds is evident between the two groups. The presence of an associated infection emphasises the triggering role of common pathogens and confirms that infection associated with HLH is common in patients with *PRF1* mutations. All these mutations are very likely to cause a severe impairment of perforin function and in fact NK activity was severely impaired or absent in all of these patients.

Delayed onset of HLH, beyond five years, was reported in 8% of the Registry patients and was also documented in one of our patients with *PRF1* mutations (case 6), who remained asymptomatic until the age of 6 years. Since patients of relatively older age, although fitting the diagnostic criteria, have often been thought to be potentially misdiagnosed, this information is relevant in that it confirms that, at least in a minority of cases, HLH should be suspected even beyond the usual age range.¹⁶ Whether HLH resulting from *PRF1* mutation may present during adulthood remains an issue to be addressed.

All 10 patients with *PRF1* mutations had a very severe presentation and clinical course. In some cases, HLH, either apparently sporadic or familial, may present with an incomplete picture and/or a mild course, including repeated episodes of remission, which may be controlled with minimal or intermittent treatment, and may even undergo spontaneous remission at least for a certain time, occasionally up to some years. This was not the case in our patients, all of whom had to be treated aggressively, showed early relapse after control of the disease was initially achieved, and were considered candidates for early BMT. All six patients who underwent BMT remain asymptomatic, confirming the unique potential of BMT for long lasting remission and even cure in HLH patients with *PRF1* mutations.¹⁷⁻¹⁹

Our findings underline the need to redefine the diagnostic approach to HLH in children. In particular, evaluation of NK activity, which was severely impaired in all but one (low-normal) case with *PRF1* mutations, should be included in the clinical diagnostic work up of HLH.

In conclusion, our data confirm that *PRF1* mutations can occur throughout the coding region of exons 2 and 3 and suggest a founder effect for HLH in Turkey but not in Italy. HLH resulting from *PRF1* mutation usually presents in infancy but occasionally may occur in older patients. Identification of a genetic defect in patients with HLH has diagnostic, prognostic, and therapeutic implications and should be pursued whenever possible. Despite frequent concordance of the age at onset within each family, asymptomatic sibs (including potential stem cells donors) cannot be safely defined as unaffected, unless their genetic status for HLH is assessed. Lack of this information may risk

BMT from an affected donor in a presymptomatic phase.¹⁸ Identification of *PRF1* gene mutations allows diagnostic confirmation, correct genotype determination in the family, confirmed indication for BMT even from alternative donors, proper genetic counselling, and prenatal diagnosis. A detailed genotype-phenotype correlation cannot be performed until a much larger number of patients with and without *PRF1* mutations are identified.

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A novel mutation in the endothelin B receptor gene in a patient with Shah-Waardenburg syndrome and Down syndrome

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EDITOR—A case of Down syndrome, total gut Hirschsprung disease (HSCR), and segmental hypopigmentation is described in a neonate presenting with bowel obstruction. In addition to having trisomy 21, this patient was homozygous for a novel mutation in the endothelin B receptor (*EDNRB*) gene.

A term female infant with karyotype 47,XX,+21 presented on day 3 of life with bowel obstruction. She was of Somali origin and had large areas of segmental hypopigmentation affecting the left side of the face and trunk, the left upper limb, including the hair follicles, and had white scalp hair. At laparotomy she had an annular pancreas, duodenal web, and inspissated meconium in the ileum and colon, for which she underwent a duodenoduodenostomy. Histology of the rectal biopsy and appendix was inconclusive at this stage. Intestinal obstruction persisted and on day 20 she underwent a further laparotomy,

which showed breakdown of the original anastomosis. Intraoperative frozen sections showed complete aganglionosis throughout the entire large and small bowel, sparing only the stomach and oesophagus; this is incompatible with life. An ileostomy was fashioned, intensive care was withdrawn, and the baby died the following morning. Necropsy confirmed total bowel aganglionosis. Her parents are not known to be consanguineous and there is no history of pigmentary disturbance or bowel disease in either them or her five sibs. Family genetic studies and clinical photographs were declined; a hearing assessment was precluded by her being ventilated and sedated for the duration of her life.

Shah-Waardenburg syndrome describes the association of HSCR with Waardenburg syndrome, and consists of deafness, pigmentary disturbance, and aganglionic megacolon. It is the result of defective development of two neural

Figure 1 Sequence analysis showing nucleotides corresponding to codon 186 of the *EDNRB* gene. A DNA sample from the infant was used as a template in a PCR reaction in order to amplify exon 2 of the *EDNRB* gene. The resulting product was subsequently sequenced using standard methods. The electropherogram shows the presence of the Gly/Arg mutation at codon 186. The mutated nucleotide at codon 186 (A*GA) is marked with (*) to distinguish it from the wild type sequence (GGA).

crest derived cell lineages: epidermal melanocytes and enterocytes.

A number of susceptibility genes for HSCR alone have been identified from the 5% of HSCR cases in whom there is an associated chromosomal or hereditary disorder and from HSCR affected kindreds.¹ Susceptibility to Shah-Waardenburg syndrome is conferred by mutations in three genes, the endothelin B receptor (*EDNRB*) gene at 13q22, its ligand the endothelin-3 gene (*EDN3*) at 20q13.2-13.3,² and in the *SOX10* gene at 22q13.³

All exons of the *EDNRB* gene were amplified by polymerase chain reaction (PCR), and PCR products were sequenced using standard methods on a ABI PRISM 377 DNA sequencer.^{2,4} This infant appeared to be homozygous for a novel missense mutation in exon 2 (codon 186, GGA-AGA) of the *EDNRB* gene, a mutation that leads to the substitution of glycine with arginine (fig 1). As the family declined further genetic studies, it is not possible to rule out hemizyosity or disomy in this patient.

The *EDNRB* gene codes for a G protein coupled transmembrane receptor protein which is necessary for the development of enteric neurones and epidermal melanocytes. The receptor ligand is endothelin-3, and mutations in this axis in both rodent models and

humans result in a phenotypic spectrum comprising HSCR and pigmentary abnormalities.^{5,6} The Gly186Arg mutation is located in the third transmembrane domain of the endothelin-B receptor and disrupts receptor function, suggested by the finding that several other mutations in the transmembrane domains of the protein are known to cause a phenotype of aganglionosis and hypopigmentation; the human manifestations are the spectrum of Shah-Waardenburg phenotypes.⁷ The exact position of the mutation in the homozygous state is likely to produce the pleiotropic features observed in these patients.

There are case reports of patients with Down syndrome in association with both HSCR and/or Shah-Waardenburg determining genes.^{8,9} However, this patient had the coexistence of Down syndrome and a novel homozygous mutation of the *EDNRB* gene. This case emphasises that although HSCR has a well recognised association with Down syndrome, other causes of HSCR should be considered. Mutation analysis of known susceptibility genes might be helpful in cases of long segment HSCR, especially in those patients with pigmentary abnormalities and those with a positive family history of bowel dysfunction.

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Standing Committee on Human Cytogenetic Nomenclature 2001-2006

Elections for the Standing Committee on Human Cytogenetic Nomenclature were held at the 10th International Congress of Human Genetics in Vienna, Austria, on 16 June 2001. The following members were elected for the period 2001-2006: Niels Tommerup (Denmark) (Chairman), Lynda Campbell (Australia), Christine Harrison (UK), David Ledbetter (USA), Albert Schinzel (Switzerland), Lisa Shaffer (USA), Angela Vianna-Morgante (Brazil). Issues regarding human cytogenetic nomenclature can be addressed to any member of the Committee.