

Letters to the Editor

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Mutation analysis of the methyl-CpG binding protein 2 gene (*MECP2*) in patients with Rett syndrome

EDITOR—Rett syndrome (RTT, MIM 312760) is a neurodevelopmental disorder characterised by normal early psychomotor development followed by a period of regression, the loss of acquired purposeful manual and speech skills, hand wringing, gait disturbance, and growth retardation.¹ As RTT occurs exclusively in females and almost all patients with RTT are sporadic, it has been proposed that RTT is caused by an X linked dominant mutation with lethality in hemizygous males.¹ Recently, DNA mutations in the methyl-CpG binding protein 2 gene (*MECP2*), mapped to Xq28, have been detected in some patients with RTT.^{2,3} We carried out a mutation analysis in 40 Japanese patients with RTT to confirm that *MECP2* is the gene responsible for RTT and to detect common mutations in *MECP2*.

All patients screened in this study were sporadic cases, 38 patients with clinically typical phenotypes of RTT and two patients with preserved speech variant of RTT.⁴ Genomic DNA was extracted from the peripheral blood of 40 patients with RTT, their parents, and 105 healthy Japanese women. Primer pairs for polymerase chain reaction (PCR) amplification, designed using the genomic sequence of *MECP2* (Gen Bank accession number, MeCP2 locus, AF030876, AJ132917), and the sizes of the products are shown in table 1. PCR amplification was performed in a final volume of 25 µl with PCR buffer, dNTPs, *Taq* polymerase, and each primer set. PCR conditions were: initial denaturing at 94°C for three minutes followed by 35 cycles of denaturing at 94°C, annealing at 56°C, and extension at 72°C for one minute each, and final extension for five minutes. To detect sequence variations, the products of PCR were analysed by electrophoresis on 6.5% polyacrylamide gels containing 0–100% linearly increasing denaturing agents at 60°C using a Bio-RAD D GENETM system and by direct DNA sequencing.

We found 15 different DNA mutations of *MECP2* in 36 (90%) of 40 patients with RTT (table 2). These mutations were not found in parents of patients or 105 healthy Japanese women, indicative of non-polymorphic variations. Three nonsense mutations, R168X, R270X, and R294X, were common in patients with RTT and have been identified in 12 (33.3%) of 36 patients with RTT (fig 1, table 2). In addition, four missense mutations, R133C, P152R,

T58M, and R306C, were also detected in 16 cases with RTT (44.4%) (fig 2, table 2). Mutations with a nucleotide deletion resulting in a frameshift with a premature stop codon were detected in five patients with RTT. The clinical courses and symptoms in RTT patients with mutations of *MECP2* did not differ from those in four RTT patients who had no mutations detected in this study. Recently, Amir *et al*⁵ and Wan *et al*⁶ reported 10 mutations, five missense and five nonsense, in *MECP2* in patients with RTT. Among the five missense mutations, four mutations (R106W, R133C, F155S, and T158M) were in a highly conservative methyl binding domain (MBD) of *MECP2* and the R106W, R133C, and T158M mutations were also detected in patients in our study. In addition, three new missense mutations, L124F, S134C, and P152R, were detected in the MBD region of *MECP2* in Japanese patients with RTT. Methyl CpG binding protein 2 (MeCP2), an abundant chromosomal protein with a high affinity for methyl CpG pairs, is a transcriptional repressor⁵ and is essential for embryonic development in mouse.⁶ Since MBD, which consists of 85 amino acids, is essential for chromosomal localisation of the protein as determined by a deletion study of *MECP2*,⁵ these amino acid changes may decrease its binding affinity for chromatin.³

As a result of an extensive mutation search of CpG hotspots based on the *MECP2* coding sequence, five R to X mutations, R168X, R255X, R270X, R294X, and R452X, were predicted by Wan *et al*.³ All mutations resulted from a C to T substitution in an Arg codon (CGA). Two nonsense mutations, R168X and R255X, were identified in a previous report.^{2,3} Five RTT patients in our study had the nonsense mutation R168X, resulting in a loss of the transcriptional repression domain (TRD) of *MECP2*. Furthermore, two similar nonsense mutations, R270X and R294X, were detected in three and four patients, respectively, with RTT (table 2). Twelve (33.3%) of 36 patients with known DNA mutations of *MECP2* had one of these nonsense mutations in our series and had a truncated C-terminal half of MeCP2. The C-terminal half of the protein is needed for the efficient repression of transcription *in vitro*.⁷ These data suggest that a C to T substitution in an Arg codon (CGA) is the most common mutation in *MECP2* in patients with RTT.

Seven mutations, R133C, P152R, T158M, S306C, R168X, R270X, and R294X, in exon 3 of *MCEP2* were detected in 28 cases (77.8%) in our study. Six mutations resulted from a C to T transition and only one C to G

Table 1 PCR primer sets for amplifying exons of *MECP2*

Exon	Primer sets	PCR product (bp)
Exon 1	F 5' ACAGAGGCCAAACCAGGAC 3'	416
	R 5' TAGAGGTGACAAGGCTTGTG 3'	
Exon 2	F 5' CTCCATGAGGGATCCTTGTG 3'	463
	R 5' AGACTGGCATGTTCTCTGTG 3'	
Exon 3-1	F 5' ACATTGCTATGGAGAGTTCTC 3'	427
	R 5' GTTTGATCACCATGACCTGG 3'	
Exon 3-2	F 5' GAAGCTCCTTGTCAAGATGCC 3'	477
	R 5' TTTGGGGACTCTGATGGTG 3'	
Exon 3-3	F 5' ACTGAAGACCTGTAAGAGCC 3'	632
	R 5' AATGCTCCAACCTACTCCCAC 3'	

F: forward primer, R: reverse primer.

DNA sequence cited from Gen Bank accession number AJ132917.

Table 2 Mutations of *MECP2* identified in patients with RTT

Mutation type	DNA change	Predicted protein change	No of patients
Missense	C391T	R106W	1
	G447C	L124F	1
	C472T	R133C	3
	C476G	S134C	1
	C530G	P152R	3
	C548T	T158M	5
	C991T	R306C	5
Nonsense	C577T	R168X	5
	C883T	R270X	3
	C956T	R294X	4
	450(C)	1 bp deletion	1
Frameshift and stop	770(G)	1 bp deletion	1
	771(C)	1 bp deletion	1
	829-832(GGCA)	4 bp deletion	1
	882(C)	1 bp deletion	1

DNA sequence cited from Gen Bank accession number AJ132917.

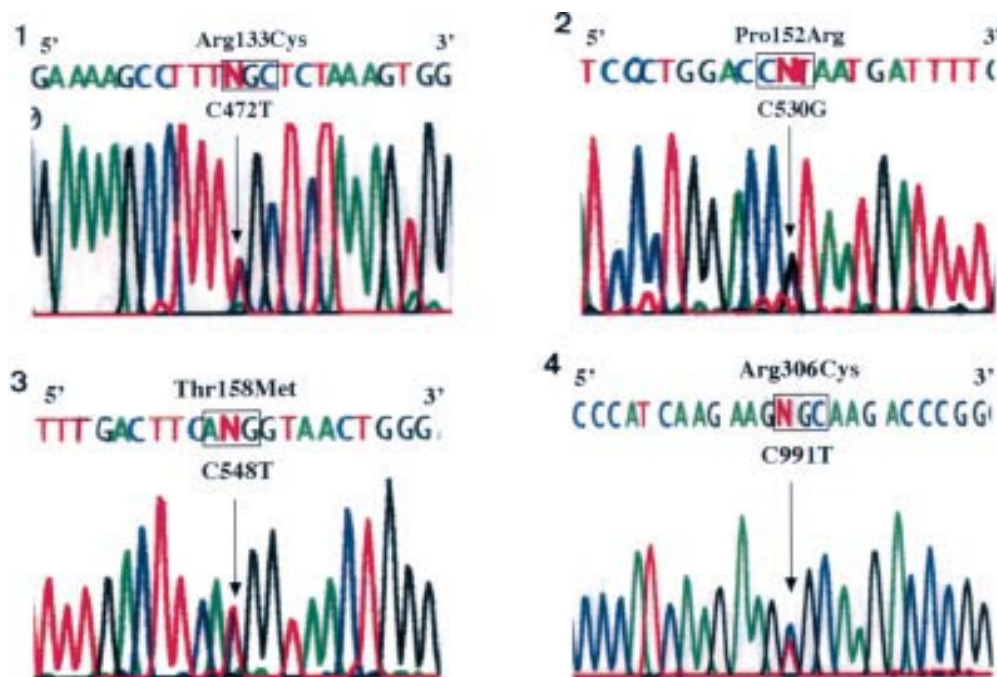


Figure 1 Nucleotide sequence of four common missense mutations of *MECP2* in patients with RTT. (1) R133C mutation, (2) P152R mutation, (3) T158M mutation, (4) S306C mutation.

transversion was observed in P152R. Thus, mutations in patients with RTT are restricted to some portions of *MECP2*.

The clinical symptoms and their causes were slightly different in patients with different DNA mutations. Patients with the P152R mutation were severely handicapped; none of them can walk now. Patients with the R306C mutation had a mild form and all of them can walk and one patient can speak several significant words. In our study, the patients with R306C and R133C have the preserved speech variant. Further study of these links are necessary to confirm the genotype-phenotype correlation of RTT.

Amir *et al*² reported that five (23.8%) of 21 patients with RTT had DNA mutations in *MECP2* in their preliminary study. Wan *et al*³ detected DNA mutations of *MECP2* in half of the patients with RTT. Furthermore, Zoghbi⁸ later found DNA mutations of *MECP2* in approximately 70% of patients with RTT. We found 15 different DNA mutations of *MECP2* in 36 (90%) of 40 patients with RTT, which is a much higher percentage than in the previous reports.^{2,3,8} We have determined the whole DNA sequence in encoding and splicing regions of *MECP2* in patients who did not show heteroduplex DNA bands by the DGGE method. Thus, the rate of mutations of *MECP2* in our study may increase compared with that in other studies.^{2,3,8} However, no mutations in the coding and splicing portions of *MECP2* were detected in four of the patients with RTT screened so far. We have not analysed the 3' untranslated region (3'-UTR) of *MECP2*. The long 3'-UTR of *MECP2* is differently expressed in brain and other tissues, suggesting that both the primary sequence and the three dimensional structure of the 3'-UTR have essential roles in the post-transcriptional regulation of *MECP2* expression.⁶ Therefore, DNA mutations in the 3'-UTR of *MECP2* may be responsible for the symptoms in patients with RTT. However, there is another possibility, that RTT is genetically heterogeneous and caused by other gene(s).

In conclusion, we have confirmed that mutations in *MECP2* are responsible for RTT. Fifteen different mutations in *MECP2* have been detected in 90% of patients with RTT and seven common mutations were

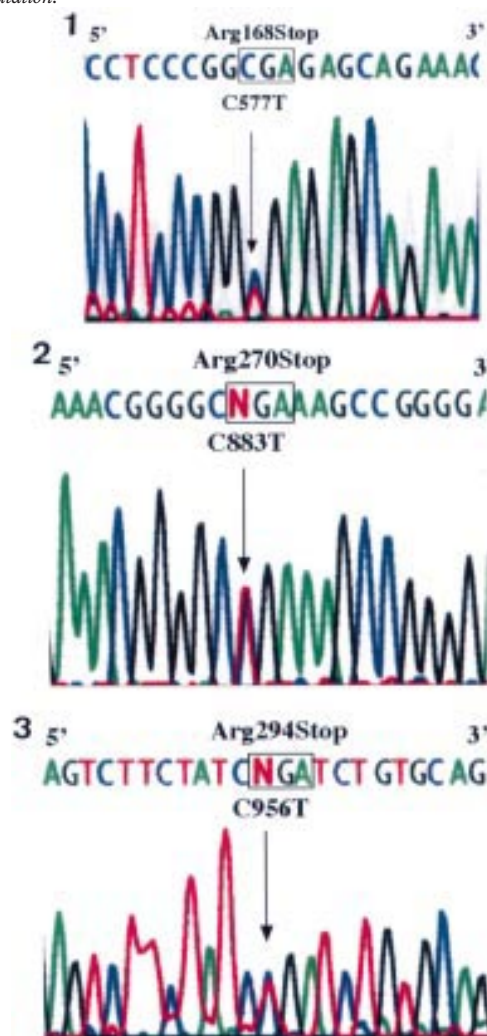


Figure 2 Nucleotide sequence of three common nonsense mutations of *MECP2* in patients with RTT. (1) R168X mutation, (2) R270X mutation, (3) R294X mutation.

defined. Early diagnosis of RTT is now possible in patients with neurodevelopmental problems using DNA analysis of *MECP2*. Particular clinical symptoms were associated with DNA mutations in some patients with RTT and it may therefore be more heterogeneous than reported previously.

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Mutations in the *MECP2* gene in a cohort of girls with Rett syndrome

EDITOR—Rett syndrome is a severe, progressive, neurodevelopmental disorder which almost exclusively affects females. At first the affected girls appear to develop normally but after a year to 18 months they begin to deteriorate. Not only do they fail to progress but they lose skills already learnt until finally they have severe developmental delay with dementia and autistic behaviour, an apraxic gait, breathing dysfunction, and stereotyped hand movements, such as excessive hand wringing. Lost skills are not regained.¹ The disease, which affects ~1 in 10 000 girls, accounts for about 10% of profound handicap in females. More than 95% of cases are sporadic leading to the assumption that the syndrome must be the result of an X linked dominant gene with male lethality. Thomas² has also suggested that the lack of males with the syndrome could be accounted for by the increased rate of de novo germline mutations in males. This would imply that affected females arise as a consequence of de novo mutations in their fathers. Marked skewing of X inactivation has not been detected in sporadic cases of the syndrome either in affected girls or in their mothers,³ but in one familial case the mother of three affected girls was found to have

>95% skewing of inactivation in favour of the normal chromosome remaining preferentially active.⁴ The few available familial cases allowed the gene to be mapped to Xq28⁵ and in 1999 Amir *et al*⁶ reported that mutations in the *MECP2* gene, located in Xq28, were associated with Rett syndrome in 5/21 of de novo cases. Amir *et al*⁶ and Wan *et al*⁷ reported a total of 10 mutations in the *MECP2* gene of which five were missense and five were nonsense mutations. Four out of the five missense mutations were located in the highly conserved methyl binding domain (MBD), the fifth being in the equally highly conserved transcription repression domain (TRD) of the gene. The nonsense mutations, causing truncation of the protein product, were located both within and between these two functional domains. Wan *et al*⁸ also found that certain of the truncating mutations were hot spots, the R168X mutation in particular being detected seven times. Because of this, despite the identification of equal numbers of individual missense and nonsense mutations, out of a total of 18 mutations detected, 12 were found to lead to a truncated product. Several other incidences of multiple recurrence were also detected, indicating the presence of further hot spots which all involved C→T transitions at CpG dinucleotides.

We report a mutation analysis of the *MECP2* gene undertaken on a further 40 patients with Rett syndrome.

Table 1 Detection of mutations and variants in the *MeCP2* gene in Rett syndrome patients

Mutation type	Nationality	Domain	Nucleotide change	Amino acid change	Occurrence	Previous detection
Missense	European	MBD	376C→G	P101R	1	No
	European	MBD	390C→T	R106W	1	Amir <i>et al</i> ⁶
	European	MBD	547C→T	T158M	2	Wan <i>et al</i> ⁸
	European	TRD	990C→T	R306C	1	Amir <i>et al</i> ⁶
Protein truncation	European (2)		576C→T	R168X	3	Wan <i>et al</i> ⁸
	Asian (1)					
	European	TRD	837C→T	R255X	3	Amir <i>et al</i> ⁶
	European					Wan <i>et al</i> ⁸
Variants	European (1)	TRD	882C→T	R270X	2	No
	European Asian (1)	TRD	954C→T	R294X	2	No
	European		1263G→A	E397K	1	Wan <i>et al</i> ⁸
	European		971C→T	T299T	1	
	European		22939ΔA	Intron	1	
European		23668G→C	Intron	1		

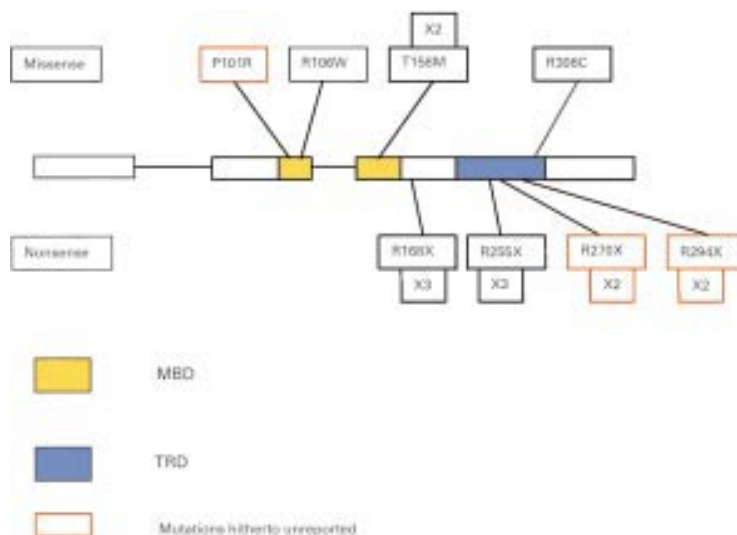


Figure 1 Mutations detected in the *MECP2* gene in Rett syndrome.

All of the girls entering the study had a clinical diagnosis of Rett syndrome. There were 27 singleton girls of European extraction, three Asian girls with consanguineous parents, and 10 girls from a further five British families.

DNA was extracted by standard methods from blood lymphocytes obtained from the girls diagnosed with Rett syndrome and from their families. The DNA was subjected to SSCP analysis using the primer sets published by Amir

*et al.*⁷ The eight sets of primers covered all 3 exons of the *MECP2* gene but neither the 5' untranslated region nor the very large 3' untranslated region were investigated.

All band shifts detected on SSCP gels were sequenced by standard methods using an ABI 377 automatic sequencer. Both forward and reverse primers were used for sequencing PCR products with fluorescent dye terminators. The results were then compared to the *MECP2* reference sequences

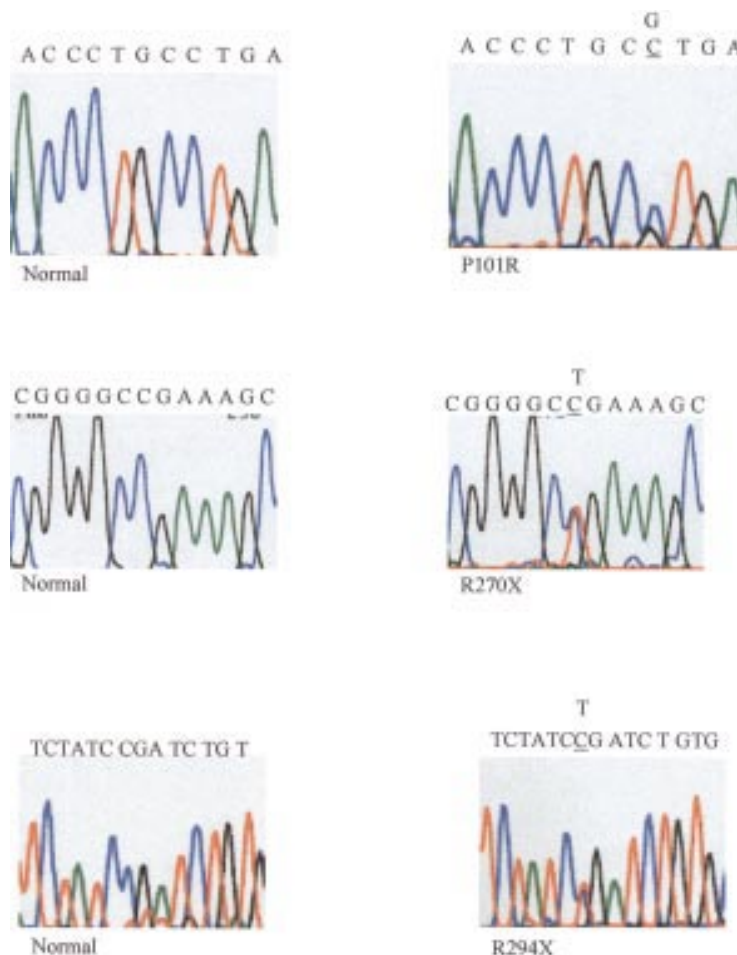


Figure 2 Sequences of the new missense and premature termination of translation mutations and normal controls (only forward sequences are shown).

published in the Gene Bank, X99686 for the transcribed and AF030876 for the complete gene sequence.

A total of 15 probands were found to have a mutation in the *MECP2* gene. Two of these girls also showed a variant and a further two girls had variants alone (table 1). Altogether, eight different disease causing mutations were found (fig 1), three were detected twice and a further two were found on three different occasions. Our results confirmed the presence of hot spots for mutation as suggested by Wan *et al.*,⁸ as the R168X mutation which they found on seven occasions was detected a further three times in our cohort and the R255X which they found to reoccur once was also detected a further three times. Of the eight mutations detected, five had been previously reported by Amir *et al.*⁷ or Wan *et al.*,⁸ but three have not been reported previously (fig 2). Interestingly two of these were detected twice in our cohort of affected girls, R270X in two European girls and R294X in another European and in an Asian girl. R270X is located in exon 3 and changes a CGA arginine codon to TGA stop codon, and R294X is also located in exon 3 and again changes a CGA arginine codon to a TGA stop codon. The third hitherto unreported mutation in this series, P101R, substitutes a CGT arginine codon for a CCT proline codon. This mutation is located in the MBD in a position conserved between human, mouse, chicken, and *Xenopus*. It is one of the very few mutations reported so far which substitutes a G and not a T moiety for a C and does not involve a CpG dinucleotide.

One of the four variants detected (E397K) had also been reported by Wan *et al.*,⁸ whereas the others were either silent or found to be located within introns.

Mutations that altered specific restriction enzyme cleavage sites could be confirmed by performing restriction digests. Other family members were also studied by this method if possible. The R106W and T158M mutations could be confirmed using the *Nla*III site which is generated, the R168X mutation creates an *Hph*I site, the R270X mutation creates an *Hae*III site, and the R306C mutation creates an *Hha*I site. No unaffected family members were found to carry a disease causing mutation in the *MECP2* gene.

In the 27 affected ethnically European girls, eight different mutations and two variants were detected. However, in the five families studied, out of 10 affected girls, only one had a mutation in the *MECP2* gene (T158M); her affected cousin did not have this mutation. Linkage analysis with microsatellite markers had previously shown that the two affected girls shared alleles at Xq28 inherited from their respective mothers who are sisters. It is possible that the second cousin has a different mutation in the *MECP2* gene as yet undetected or that she has a different form of neurological impairment. Amir *et al.*⁷ had previously reported that a pair of half sisters both had the R106W mutation. It was suggested that their mother, who did not have the mutation, was a gonadal mosaic.

If only the singleton girls are considered, then 12/27 (44%) of girls with ethnically European parents and 2/3 girls with consanguineous Asian parents had mutations. Thus, mutations have been detected in different racial groups and in some cases they have been shared, including one of the truncating mutations previously unreported.

The pattern of mutations in the *MECP2* gene that are associated with Rett syndrome indicates that they fall into two types, nonsense mutations located in the TRD and missense mutations found almost exclusively in the MBD. The truncating mutations are believed to prevent the association of the 5-methylcytosine/MeCP2 complex with sin3A and histone deacetylase, preventing the deacetylation of histones and, as a consequence, failing to repress transcription.⁸ The missense mutations in the MBD are likely to interfere with the binding of MeCP2 to 5-methylcytosine itself. This would suggest that individual mutations should have different effects upon the phenotype, since MeCP2 is ubiquitously expressed and is believed to be involved in the silencing of many different genes. Mutations in the *MECP2* gene have already been associated with different phenotypes,⁸ but many individual mutations in different areas of the MBD still lead to the specific phenotype of Rett syndrome and it remains to be determined just which genes are affected and to what degree.

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Monosomy and trisomy 1q44-qter in two sisters originating from a half cryptic 1q;15p translocation

EDITOR—Cryptic translocations usually involve the telomeric regions of chromosomes and are not easy to detect by means of conventional cytogenetics.^{1,2} The published cryptic

translocations have been identified through a phenotype corresponding to a chromosome deletion syndrome or as a result of the observation of an anomalous chromosome, in which case they are known as half cryptic translocations because only one of the chromosomes involved can be seen cytogenetically. The presence of satellites at the p or q end of non-acrocentric chromosomes seems to be quite a common phenomenon^{3,4} and represents a type of half cryptic translocation that signals a rearrangement between the satellited chromosome and one of the acrocentrics.

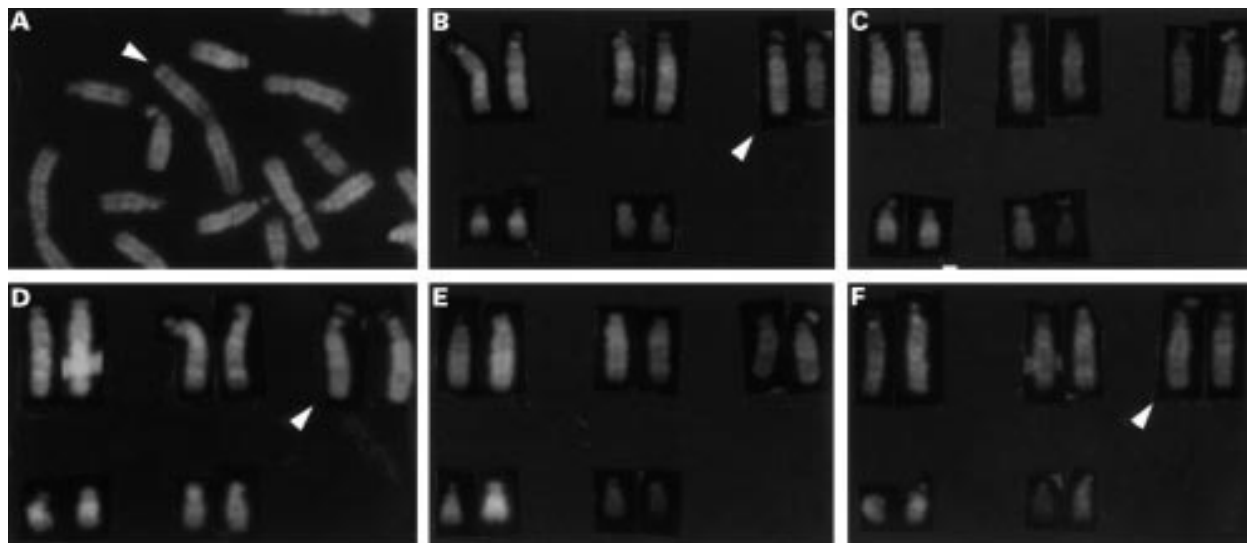


Figure 1 (A) Partial QFQ banded metaphase of the proband. The arrowhead indicates the chromosome 1qs. (B-F) Acrocentric chromosomes of the mother (B), the father (C), the first normal child (D), daughter 1 (E), and daughter 2 (F). The arrowheads indicate the chromosomes 15 carrying the 1q44-qter region instead of the chromosome satellite.

We describe here a family with a half cryptic translocation detected through a dysmorphic child. The reproductive history of the couple and their family was unremarkable.

The proband was the second child of the couple (daughter 1); the first was a phenotypically normal male and the third was a female with a mildly abnormal phenotype (daughter 2). The third month of gestation was characterised by a threatened miscarriage and the seventh by threatened premature labour. Spontaneous delivery occurred at 41 weeks' of gestation. Apgar scores were 9 and 10 at one and five minutes, respectively. Birth weight was 2770 g (>3rd centile), length 48 cm (3rd centile), and head circumference 30 cm (<3rd centile). A CT scan at birth showed microcephaly without any brain anomalies; the results of cerebral and renal echography were also normal. During the early neonatal period the child had feeding problems and her growth was retarded despite artificial feeding. She experienced her first febrile seizure at the age of 11 months, which was followed by a further five episodes with hyperpyrexia over the next five years. Antiepileptic therapy with phenobarbital was unsuccessful and at 6 years of age she was started on carbamazepine. She began sitting with support at the age of 2 years, but never started walking or talking. At the age of 3 years, the proband developed acute lymphoblastic leukaemia and underwent chemotherapy until remission. Echography of the heart and abdomen and laboratory examinations were normal. At 3 and 5 years, CT scans detected slight dilatation of the lateral ventricles at the level of the temporal horns and a moderate enlargement of the cortical sulci. On two subsequent occasions, EEG showed slow background activity during waking EEG recordings. When examined at the age of 5 years, her weight was 17.5 kg (10th centile), length 99 cm (<3rd centile), head circumference 42 cm (<3rd centile), and the inner and outer canthal distances were 3 cm and 8.5 cm respectively (both 75th centile). The lengths of her hand, third finger, and foot were 11 cm (<3rd centile), 4.5 cm (<3rd centile), and 14 cm (<3rd centile), respectively. Physical examination showed marked microcephaly, brachycephaly, and severe psychomotor retardation. Her facial dysmorphism included an oval-round face, a narrow forehead with a prominent metopic suture, synophrys, epicanthic folds, upward slanting palpebral fissures, hypertelorism, a short and broad nose with a flat nasal bridge, a thin, Cupid's bow shaped upper lip,

normal ears with hypoplastic helices, a normal palate and short frenulum, a U shaped tongue, and no tongue protrusion. Her external genitalia were normal with mild clitoral hypertrophy. Her severe growth and mental retardation were confirmed at the age of 10 years.

Daughter 2 was delivered after 39 weeks of gestation. The pregnancy was uneventful and birth weight was 3550 g (75th centile). The results of a CT scan were normal, but cardiac echocardiography showed a ventricular septal defect that closed spontaneously. Physical examination carried out when the child was 5 years old showed that her weight was 20 kg (75th centile), height 108.5 cm (50th centile), and head circumference 55.5 cm (>97th centile). Facial dysmorphism included macrocephaly, a prominent forehead, and hypertelorism. Psychomotor and growth development was normal. The parents refused to allow publication of their children's photographs.

Cytogenetics studies were performed using PHA stimulated lymphocytes; QFQ, high resolution GTG, and RBA banded chromosomes showed a satellited chromosome 1 (1qs) in the proband (fig 1A), which was negative on CBG banding and DA-DAPI and AgNOR staining. Family investigations showed the same satellited chromosome 1 in her mother and brother, whereas her father's and sister's karyotypes were apparently normal. A satellited chromosome 15 present in the mother and inherited by the son (but not by the affected child) was thought to be a candidate partner of the translocation with chromosome 1q (fig 1B-F), and so the proband was considered monosomic for the 1qter region. The finding of the same chromosome 15 with absence of 1qs in daughter 2 suggested the presence of partial trisomy 1q. High resolution banding made it possible to define the translocation breakpoints as 1q44 and 15p12. FISH analysis was performed on lymphocytes of the mother and her two daughters using a β satellite probe (ONCOR) (fig 2). Hybridisation signals were found on 1q in the mother and syndromic child (fig 2A, B), whereas daughter 2 had the maternal translocated chromosome 15 but no signals on either chromosome 1 (fig 2C). WCP1 FISH (CAMBIO) and dual FISH analysis using 1q44-qter cosmid and 15 classical satellite probes (ONCOR) confirmed these results (fig 2D, E).

Molecular analysis of the family was carried out by means of both non-radioactive and radioactive methods, using the polymorphic microsatellite markers D1S2785, D1S2842, D1S2836, and D1S2682 (fig 3). D1S2785 and

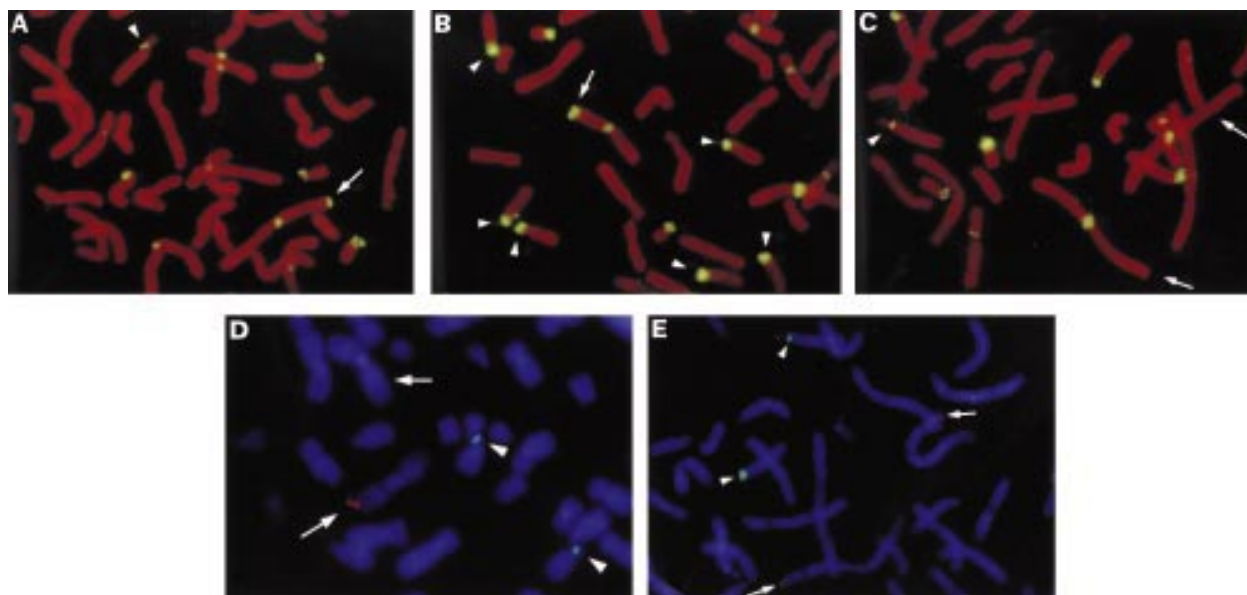


Figure 2 (A-C) FISH using β acrocentric probe. (A) The mother's chromosome 1qs is indicated by the arrow, while the arrowhead indicates the chromosome 15 with only the proximal β region hybridised. (B) Metaphase of the affected child in which all chromosomes of the D group (arrowheads) and the 1qs (arrow) show hybridisation signals. (C) Metaphase of daughter 2 with the arrowed two chromosomes 1. The maternal chromosome 15 is indicated by the arrowhead. (D-E) Dual FISH using 1q44-qter (red signal) and 15 classical satellite (green signal) probes. (D) The proband's partial metaphase showing one normal chromosome 1 (red signal), the 1qs without hybridisation signal, and two normal chromosomes 15 (green signals), corresponding to the partial 1q monosomy. (E) Daughter 2's partial metaphase showing two normal chromosomes 1 (indicated by the arrows), one chromosome 15 with double red-green signals corresponding to the partial trisomy 1q and one normal 15 chromosome (indicated by arrowheads).

D1S2842 were not involved in the rearrangement because both daughters were heterozygous for them, but the two informative distal markers (D1S2836 and D1S2682) showed that the affected child had only one allele of paternal origin, whereas daughter 2 had one paternal and one more intense maternal alleles.

In brief, the mother and her first child (a son) showed the balanced translocation, whereas the karyotypes of the two daughters originated from adjacent I segregation; the first had 1q44-qter monosomy and 15p12-pter trisomy and the second had 1q44-qter trisomy and 15p12-pter monosomy. Since the gain or loss of the acrocentric short arm has no clinical significance, these sisters can be considered as having pure monosomy and pure trisomy 1q44-qter. To the best of our knowledge, there is only one published report describing monosomy involving the 1q44 distal region⁶; the other published deletions all involve larger regions ranging from 1q42 or 1q43 to 1qter.⁷⁻⁹ The 1q deletion syndrome is clinically characterised by growth and psychomotor retardation, seizures, microcephaly, brachycephaly, and typical face, hand, and foot anomalies.⁷⁻⁹ As the clinical features observed in our patient are the same as those described in previously reported cases, the 1q44-qter loss alone seems to be responsible for the clinical pictures of patients with distal chromosome 1q monosomy.

As far as we know, daughter 2 is the first described case of pure 1q44-qter trisomy. Previous reports have described trisomy involving the 1q42-qter region in association with monosomy of the other chromosomes.¹⁰⁻¹² Only three patients have presented pure 1q42-qter trisomy^{13,14} (table 1), all of whom showed growth and mental retardation, macrocephaly, a prominent forehead, and micro/retrognathia. Our patient had macrocephaly and a prominent forehead, but it is worth underlining her normal mental development at 5 years of age. This may be because of the different size of the 1q region involved and, if this is so, macrocephaly would be the only manifestation of the trisomy itself. Alternatively, the short arm of chromosome 15 may influence the expression of segment 1q44 as a result of a positional effect, but the normal phenotype

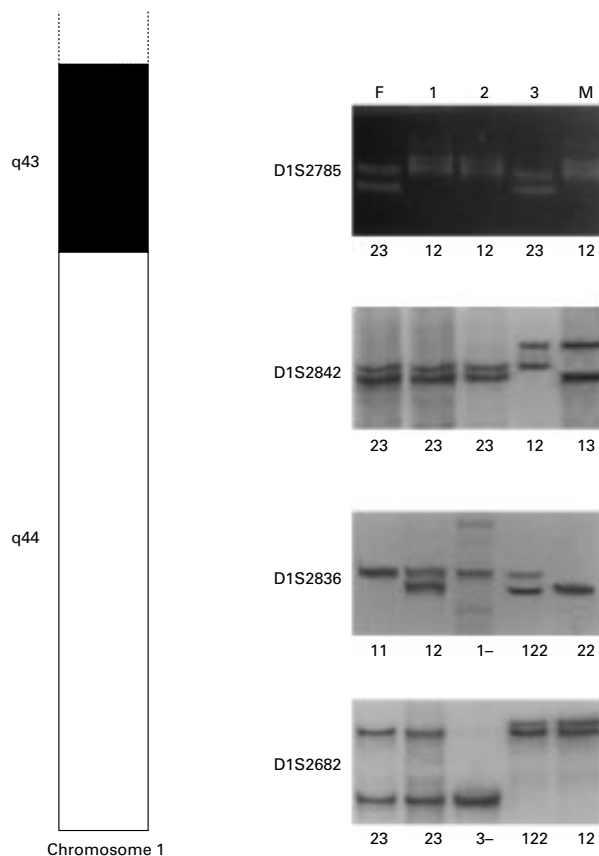


Figure 3 Microsatellite analysis of the family. F: father; 1: normal son, carrier of the balanced translocation; 2: syndromic daughter 1, monosomy of chromosome 1 was evident for the microsatellite D1S2836 and D1S2682; 3: daughter 2, trisomy of chromosome 1 was deduced from the different allele intensity (D1S2836 and D1S2682); M: mother, carrier of the balanced translocation.

allowed us to rule this out in the case of the balanced car-

Table 1 Clinical findings of the trisomic patient compared with published case reports

Clinical findings	Chia et al ¹³	Verschuuren-Bemelmans et al ⁴	Verschuuren-Bemelmans et al ⁴	Present case
Sex	M	F	M	F
Age	5 weeks	22 years	20 years	5 years
1q partial duplication	1q42-qter	1q42-qter	1q42-qter	1q-44qter
Parental origin	t(1;22)(q42;p12)mat	t(1;15)(q42;p11)pat	t(1;15)(q42;p11)pat	t(1;15)(q44;p12)mat
Growth retardation	Prenatal	+	+	-
Mental retardation	/	+	+	-
Macrocephaly	+	+	+	+
Large fontanelle	+	+	-	-
Widely spaced sutures	+	+	-	-
Prominent forehead	+	+	+	+
Downward slanting palpebral fissures	-	+	+	-
Hypertelorism	/	/	/	+
Flat nasal bridge	+	-	-	-
High arched palate	/	+	-	-
Micrognathia/retrognathia	+	+	+	-
Abnormal ears	+	-	-	-
Cerebral anomaly	/	+	-	-
Cardiac defect	+	Systolic murmur	-	VSD spontaneously closed

+ present, - absent, / not reported.

VSD: ventricular septal defect.

riers of t(1;15), because it would have produced monosomy 1q44-qter caused by the lack of gene expression and consequently an abnormal phenotype. However, the absence of other published cases with pure trisomy 1q44-qter does not allow us to draw any definite conclusions. A possible mechanism generating 1q/acrocentric chromosome translocation could be an interchange between the 5S rRNA genes mapped to bands 1q42-q43 and the 18S-28S rRNA genes localised on the p arms of acrocentric chromosomes¹⁵; such a mechanism has been described in Robertsonian translocations.¹⁶ We would finally like to stress the importance of identifying both of the chromosomes involved in the translocation. The clinical picture of the affected child was suggestive of 1q deletion syndrome but, without the cytogenetic and FISH analysis of the translocation, it would have been impossible to identify the trisomic child.

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A constitutional homozygous mutation in the *RB1* gene in a patient with unilateral retinoblastoma

EDITOR—Retinoblastoma, a childhood tumour of the eye, is caused by inactivation in the developing human retina of both alleles of the tumour suppressor gene *RB1*. The product of the human *RB1* gene (p110^{RB}) is a nuclear phosphoprotein composed of 928 amino acids, which regulates the progression through the G₁ phase of the cell cycle by inter-

acting with transcription factors required for the expression of genes involved in cellular proliferation and differentiation.

In the non-hereditary form of the disease (~60% of tumours), both mutations arise in retinal cells. Because in non-hereditary RB patients both *RB1* mutations must occur in the same retinal cell, they are usually unilateral and unifocal. However, based on retrospective surveys of the offspring of patients with unilateral isolated retinoblastoma, Vogel¹ estimated that in 10-12% of these patients the tumour is caused by germline cell mutations.

In the hereditary form of the disease (~40% of RB patients), the initial mutation in one allele of the *RB1* gene is present in germline cells and leads to a predisposition to

retinoblastoma. Since mutations in the second allele can occur independently in several retinal cells carrying this predisposing mutation, all patients with bilateral or unilateral multifocal RB are classified as having hereditary retinoblastoma. Most hereditary cases must result from "de novo" germline mutations, because only 10-15% of the hereditary cases have a previous family history of the disease. In familial cases, the predisposition to RB is transmitted as an autosomal dominant trait with 90% penetrance. Thus, in some familial cases, unaffected or only unilaterally affected subjects can be identified who can transmit the mutant gene.¹ The "two hit" hypothesis predicts the existence of these cases that form part of a Poisson distribution in which, by chance, the second random mutation does not occur.² However, the distribution of cases of incomplete penetrance is not entirely random, and families have been reported in which the majority of the carriers have either unilateral tumours, regressed tumours, or no evidence of malignant disease.³⁻⁶ Some hypotheses have been set forth to explain this phenomenon: the existence of "delayed mutations,"⁷ the "host resistance" model,^{8,9} and the existence of lethal alleles at the cellular level.^{10,11} Cloning and characterisation^{12,13} of the *RB1* gene made it possible to determine the nature of the mutations, and it has been shown that the mutations found in families with low penetrance retinoblastoma, rather than being stop codons or mutations which are presumed to abolish p110^{RB} protein activity completely, as described in severe cases of retinoblastoma, are either missense mutations,⁵ in frame deletions,¹⁴ or mutations affecting the promoter region of the gene,^{6,15} which generate a partially defective protein (low penetrance mutations).^{16,17} In addition to the low penetrance mutations, other causes of incomplete penetrance and reduced expressivity have been described in retinoblastoma: the existence of mosaicism¹⁸ and the aggregation of sporadic genetic events in the same family ("pseudo low penetrance").^{14,19,20}

We present here a family with three carriers of a mutation in the donor splice site of intron 5 (G→A). This change causes a protein lacking only exon 5 because it does not disturb the frameshift. Only one of the carriers is affected by unilateral retinoblastoma. Furthermore, the affected child has mosaicism comprising homozygosity and heterozygosity for the mutation. The characteristics of the mutation, its location, and the existence of unaffected carriers in the family allow us to hypothesise that this is a low penetrance mutation.

Table 1 Intragenic and extragenic polymorphic markers used for the haplotype analysis

Polymorphic marker (probe)	Type	Method of analysis	Localisation
D13S787	Microsatellite	PCR	13q11-q12.1
D13S894	Microsatellite	PCR	13q12.3-q14.2
D13S325	Microsatellite	PCR	13q14.1-q14.2
<i>Bam</i> HI (p123M1.8)	RFLP	Southern	RB1 (intron 1)
Rbi2	Microsatellite	PCR	RB1 (intron 2)
Rbi4	Microsatellite	PCR	RB1 (intron 4)
<i>Xba</i> I	RFLP	PCR	RB1 (intron 17)
<i>Rsa</i> I (p68RS2.0)	VNTR	Southern	RB1 (intron 17)
RB1.20	Microsatellite	PCR	RB1 (intron 20)
RB1.26	RFLP*	PCR	RB1 (intron 26)
D13S788	Microsatellite	PCR	13q14.1-q14.3
D13S800	Microsatellite	PCR	13q21.2-q22
D13S317	Microsatellite	PCR	13q22
D13S793	Microsatellite	PCR	13q31-q32
D13S779	Microsatellite	PCR	13q32
D13S796	Microsatellite	PCR	13q32-q34
D13S173	Microsatellite	PCR	13q32-q34
D13S285	Microsatellite	PCR	13q34

*We observed that the polymorphic nucleotide change described by Yandell and Dryja forms a new *Dra*I site.
GDB: Genome Data Base (<http://www.hgmp.ac.uk/gdb/gdbtop.html>).

The patient in this study was admitted to hospital at 11 months of age as he had leucocoria in his right eye. His mother noticed it first when he was 2-3 months old and he underwent ophthalmological study eight months later. An analysis of the fundus of the right eye showed that the optic nerve could not be seen because detachment of the retina had taken over three quarters of the vitreous cavity. Ophthalmoscopic examination showed a typical exophytic retinoblastoma. The eye globe was 1.8 cm in diameter (maximum) and inside was observed a retinoblastoma which did not infiltrate the choroid, optic nerve, or ciliary body. The child is now 32 months old and the healthy eye has been checked every six months without any change. Ophthalmological study of the patient's father showed no tumour and the fundus of both eyes was normal.

Samples of peripheral blood and tumour tissue were obtained from the child when he was 12 months old. He was diagnosed at 11 months as having a unilateral sporadic retinoblastoma and his affected eye was enucleated. Blood samples were also obtained from his unaffected parents and grandparents. Genomic DNA from leucocytes and fresh tumour tissue was isolated by using standard phenol/chloroform procedures. Total RNA from these tissues was obtained by means of the RNeasy kit (Quiagen) following the recommendations of the manufacturers.

In order to carry out the haplotype analysis, genomic DNA from white cells and tumour tissue was subjected to Southern blot analysis or PCR amplification or both to genotype RFLP and microsatellite markers within and outside the *RB1* gene (table 1). All the intragenic polymorphic markers were detected as indicated in the references. The exception was the Rbi2 and Rbi4 markers, which were amplified by a two step PCR (10 cycles at 94°C for one minute and 65/55°C for one minute with a decrease of 0.5°C/cycle, followed by 20 cycles at 94°C for one minute and 60/50°C for one minute with a final extension at 65°C for five minutes). The extragenic markers were amplified for 35 cycles at 94°C for 40 seconds and 55°C for 30 seconds and a final extension at 72°C for two minutes.

Densitometric analysis was performed using the *Bam*HI polymorphism located in intron 1 of the *RB1* gene. This polymorphic marker is detected by the p123M1.8 probe, which identifies both the 5' end of the *RB1* gene and a band corresponding to a fragment of the 28S rDNA gene.²¹ We used this constant band as an internal control. Quantification of the genetic doses of the *Bam*HI RFLP alleles was performed using computer software (Intelligent Quantifier™, Bio Image®) that measured the intensities of bands from digitised images (Gelstation, TDI).

In order to perform screening for small mutations and sequencing, the promoter region and 27 exons nearest the intronic regions of the *RB1* gene were PCR amplified using the primers described by Hogg *et al.*²² and Shimizu *et al.*²³ PCR products were digested with the appropriate restriction enzymes to produce DNA fragments of 250 bp or less. Mutation screening was performed by SSCP analysis and samples showing altered electrophoretic behaviour were subjected to direct sequencing.

RNA from peripheral blood and tumour tissue was reverse transcribed using the RT-PCR kit (Stratagene) and following the recommendations of the manufacturers. The resulting first strand cDNA was PCR amplified using primers which were designed to amplify six overlapping fragments of the coding sequence from the *RB1* gene.²⁴ The distribution of exons in these fragments was: fragment 1, exons 2-6; fragment 2, exons 6-10; fragment 3, exons 10-16; fragment 4, exons 16-19; fragment 5, exons 19-23; and fragment 6, exons 23-27. Amplification conditions consisted of 35 cycles at 94°C for one minute, 55°C for one minute, and 72°C for one minute, and a final extension at

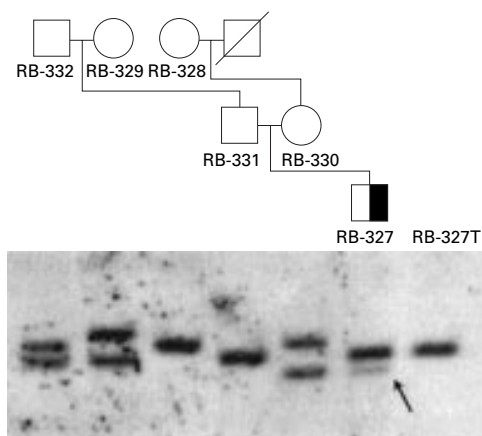


Figure 1 (A) Pedigree of the RB-55 family. The identification number appears below the symbol for each subject. (B) Southern blot analysis of the VNTR marker in intron 17 of the *RB1* gene (detected with probe p68RS2.0) showing that the affected child has a less intense maternal allele (arrow).

72°C for 10 minutes. Individual PCR products were subjected to fragment length analysis, SSCP analysis, and direct sequencing. For the SSCP, digestion of the PCR products 1 to 6 was carried out before analysis with enzymes *RsaI*, *ApaII*, *DraI*, *DdeI*, *NdeI*, and *ApoI*, respectively.

Molecular analysis of the *RB1* gene in the family studied showed a decrease in the dose of the maternal alleles in intragenic polymorphic markers at a constitutional level in the RB-327 patient (fig 1). The densitometric analysis of the *BamHI* marker (fig 2A) showed that, while the internal control band of a normal heterozygote and that of the affected child had similar intensities, the peak corresponding to allele 1 in the affected child (paternally derived allele) had a higher intensity than the heterozygous control in DNA isolated from leucocytes. This increase in the paternal allele was associated with a decrease in the dose of the maternally derived allele (allele 2) with regard to the heterozygous control sample (fig 2B). Analysis of the patient's tumour DNA showed an undetectable maternal allele (LOH), while the paternal allele was similar to that found in a homozygous control (fig 2C). The same loss of the maternal allele was observed for other polymorphic markers on chromosome 13 flanking the *RB1* gene, except for the most centromeric one (D13S787), in which both leucocytes and tumour DNA showed the maternal and paternal alleles at a similar level (fig 3).

On the other hand, gel electrophoresis of the RT-PCR product showed a band of normal size and a smaller band when we used the primers to amplify fragment 1 of cDNA from the child and his father's leucocytes (fig 4). The same fragment obtained from tumour mRNA showed only the smaller band. In the maternal sample, a normal length band was detected. Sequence analysis of that anomalous band showed the loss of exon 5 in fragment 1 (fig 4), but this does not alter the reading frame. SSCP analysis of leucocyte DNA detected an abnormal electrophoretic pattern in the exon 5 PCR product in the proband and his unaffected father and grandmother, as well as in tumour DNA. Direct sequencing of this fragment showed a nucleotide change (G→A) at the splice donor site in intron 5 (position 44707 of the sequence shown in GenBank under Accession No L11910) (fig 5). Because the tumour cells and some constitutional cells of the affected child are homozygous for the paternal allele, they carry the mutation homozygously.

To date, many types of oncogenic mutations have been described as causing retinoblastoma (a summary can be

seen in <http://home.kamp.net/home/dr.lohmann/index.htm>). These mutations are located along the whole *RB1* gene, and the majority of them cause highly penetrant retinoblastoma. We describe a family in which three members are carriers of a point mutation affecting the correct splicing of exon 5 in the mRNA. The fact that the loss of exon 5 does not alter the frameshift and two of the carriers of the mutation are unaffected lends support to the idea that this is not an oncogenic mutation. However, there are also arguments against this assumption. First of all, the screening by SSCP analysis of all other exons and the promoter region of the *RB1* gene did not show any other mutation in this family, although in all tumours studied by other authors, at least one of the mutations in the *RB1* gene has been found. Secondly, some studies of the N-terminal region of the protein encoded by the *RB1* gene have shown that deletions in this region disturb its capacity to polymerise itself in order to form compartments to arrest other molecules.²⁵⁻²⁷ Furthermore, some reports have shown binding of this region of p110^{RB} with other nuclear proteins like laminin A and C and p84,²⁸⁻³⁰ representing a potential site of p110^{RB} interaction in the nuclear matrix. The nuclear matrix has been implicated in most metabolic activities occurring in the nucleus, including replication, transcription, and RNA splicing and transport.³¹⁻³³ p110^{RB} may facilitate the binding of growth promoting factors at subnuclear regions actively involved in RNA metabolism.³⁰ Finally, the third reason we think that the mutation is not a polymorphic variation is that comparison of the amino acid sequences encoded by exon 5 in some species has shown a high degree of conservation, especially in higher vertebrates, and this indicates the importance of this exon.

Although we have only analysed a few subjects in three generations, some of our findings point to a low penetrance mutation: (1) the fact that only one of the three carriers of the mutation is affected by unilateral retinoblastoma; (2) another mutation (deletion of exon 4) probably with similar consequences in the p110^{RB} protein has been described in a family with low penetrance retinoblastoma,¹⁴ where the protein p110^{RB44} was defective for E2F binding but able to activate transcription and promote differentiation³⁴; and (3) the fact that the mutation was found homozygously at a constitutional level. If it were a highly penetrant mutation, it would probably be lethal. Studies done on mice showed that embryos lacking any functional p110^{RB} protein die in utero by day 14.5 of gestation. p110^{RB} is also required in the terminal differentiation of some tissues.³⁵⁻³⁸

The results obtained in blood and tumour from the patient with the different markers used (the most centromeric marker showed the maternal and paternal alleles at a similar level, while the rest of the markers showed a loss of the maternal allele) and densitometric analysis (the increase in the paternal allele dose is associated with a decrease in the dose of the maternally derived allele) suggest that the affected child was a mosaic composed of at least two constitutional cell lines, one of them with a paternal and a maternal allele, and the other with a duplication of the paternal allele.

The mosaicism detected in blood from the patient could have arisen as a consequence of a mitotic recombination event during the earlier stages of embryonic development between markers D13S787 and D13S325 (proximal to the *RB1* gene) (fig 3). The fact that the tumour cells are carriers of a LOH with a double dose of the paternal allele for the same markers in which a reduction of the maternal allele has been shown in blood cells indicates that the tumour arose from a cell with the mutation in double dose. This is remarkable given that, to our knowledge, this is the first time that a low penetrant mutation has been described in

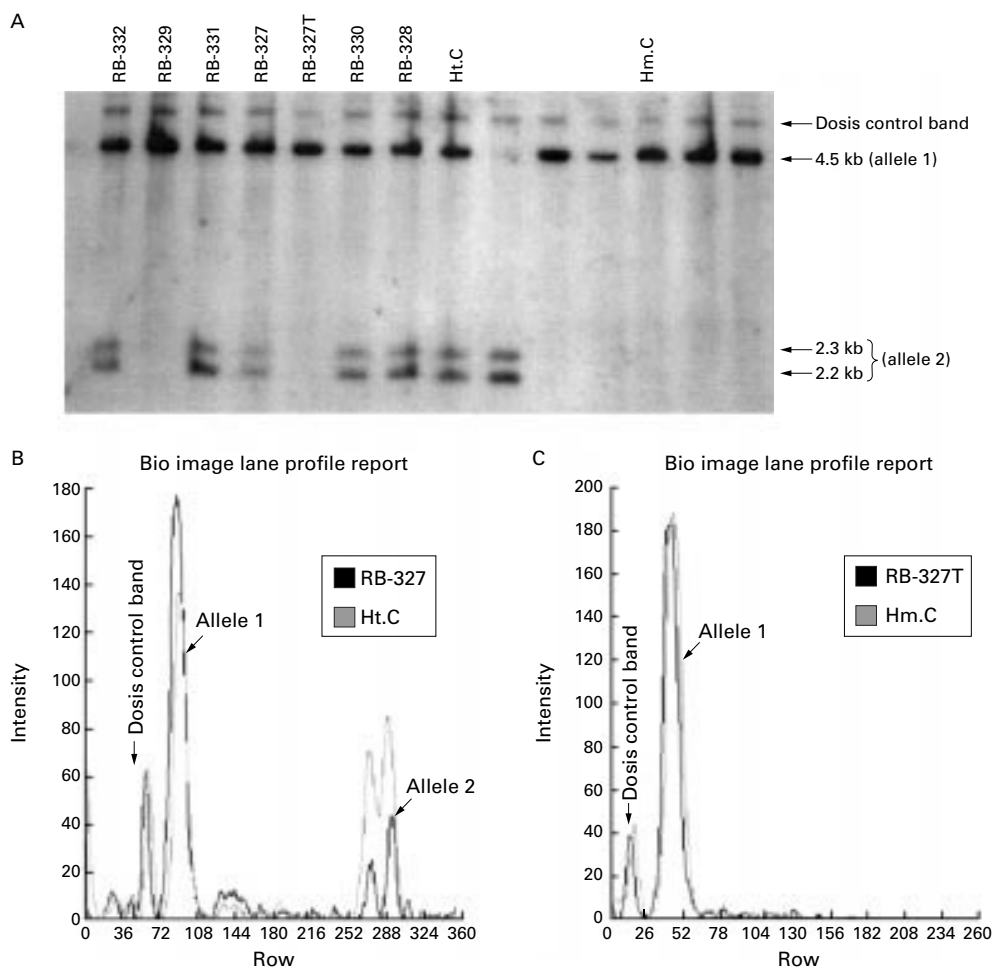


Figure 2 (A) Results of the RFLP marker *Bam*HI (intron 1 of the *RB1* gene) analysis in DNA from peripheral blood of the family and the retinoblastoma tumour of the child (RB-327T). This analysis was performed by means of the intragenic p123M1.8 probe. Individual alleles are either a fragment of 4.5 kb or a pair of fragments of 2.3 and 2.2 kb. A constant band belonging to the rRNA 28S gene was also detected. This band is used as a reference control of the amount of DNA in each row. A heterozygous control (Ht.C) and a homozygous control (Hm.C) have been included. (B) Comparison of the intensities of the control band and alleles of the *Bam*HI marker in the child's blood (black line) and the heterozygous control (grey line). (C) Comparison of the intensities of the control band and alleles of the *Bam*HI marker in the tumour (black line) and the homozygous control (grey line)

homozygosity, both at a tumour and constitutional level. Sakai *et al*⁸ hypothesised that a cell homozygous for a low penetrance mutation would not evolve into a tumour because a minimum threshold of p110^{RB} protein activity is

retained,^{5 16} but in fact only a few tumours in low penetrance retinoblastoma patients have been analysed so far.^{14 39}

There are two possible explanations for the development of the tumour in our patient, in spite of his double dose of

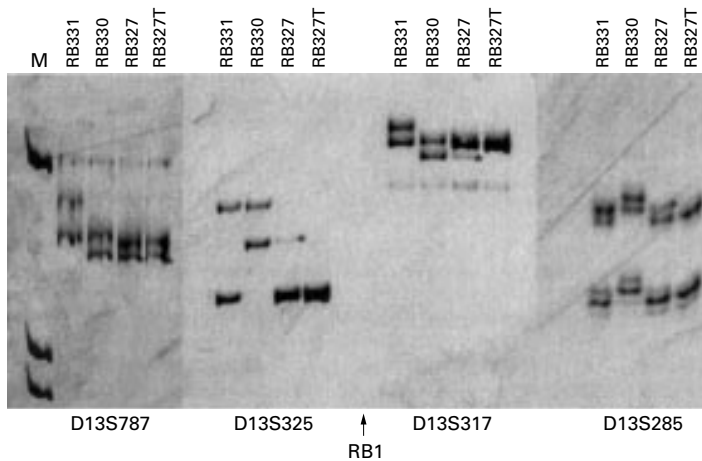


Figure 3 Informative extragenic markers studied in this family. Asterisks indicate the maternal allele in the patient. M: 1 kb ladder. The markers are arranged according to their location in 13q and the position of the *RB1* gene is indicated.

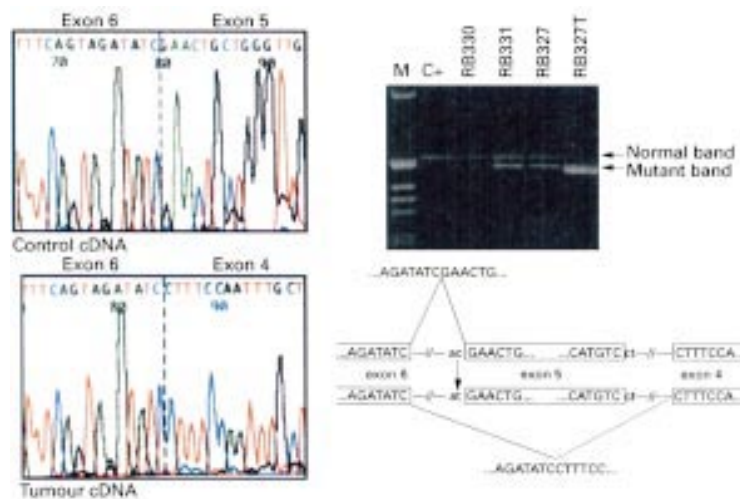


Figure 4 (Left) Results of the sequence analysis of fragment 1 amplified from cDNA obtained from a normal control (top) and from the tumour sample (bottom). The vertical broken line indicates the limits of the exons. (Top right) Agarose gel electrophoresis of fragment 1 (which spans exons 2-6 of the *RB1* gene) amplified from the cDNA obtained from the lymphocytes of the affected child (RB-327), his parents (RB-330 and RB-331), and a normal control (C+), and from the tumour tissue (RB-327T). Lanes RB-327 and RB-331 show a band of normal size and a smaller anomalous band. In the row belonging to the tumour sample only the anomalous band was observed. (Bottom right) Diagram showing the normal spliced sequence of cDNA and that corresponding to the removal of exon 5 observed in the cDNA from some members of the family and the tumour.

a low penetrance mutation. One is the role of p110^{RB} in an unknown mechanism in retinal tissue not screened in the expression studies of mutations with low penetrance. The other explanation, more probable in our opinion, is that a mutation in a gene other than the *RB1* gene or an undiscovered third mutation in the *RB1* gene is necessary for tumour development in this family (three hit model).

To date, only one case of paternal disomy 13 has been described.⁴⁰ A normal phenotype was observed in that case and an unlikely imprinting effect was postulated for this type of disomy.⁴¹ Similarly, the recent clinical assessment of the patient reported here did not show any manifestation other than the retinoblastoma. The serotonin receptor gene (*HTR2*), closely linked to the *RB1* gene, has been reported.^{42,43} and studies with retinoblastoma tumours have shown only expression of the maternal allele of this gene.⁴⁴ Serotonin (5-hydroxytryptamine (5-HT)) is a neurotransmitter that mediates a diverse array of physiological responses by interacting with multiple serotonin receptor

subtypes. Two of them, 5HT2 and 5H1c, modulate similar intranuclear signalling pathways but exhibit different patterns of expression in the brain. The affected child in the family reported here is a mosaic carrier of paternal disomy of almost the whole of q13, so he probably lacks *HTR2* gene expression in a large proportion of his cells. Nevertheless, the patient is too young to assess the putative neurological consequences of the pattern of expression of the *HTR2* gene.

All the unusual findings described in our family, that is, mosaicism and variable expressivity and transmission, have important implications for the DNA based estimates of the prognosis of the disease and accurate genetic counselling.

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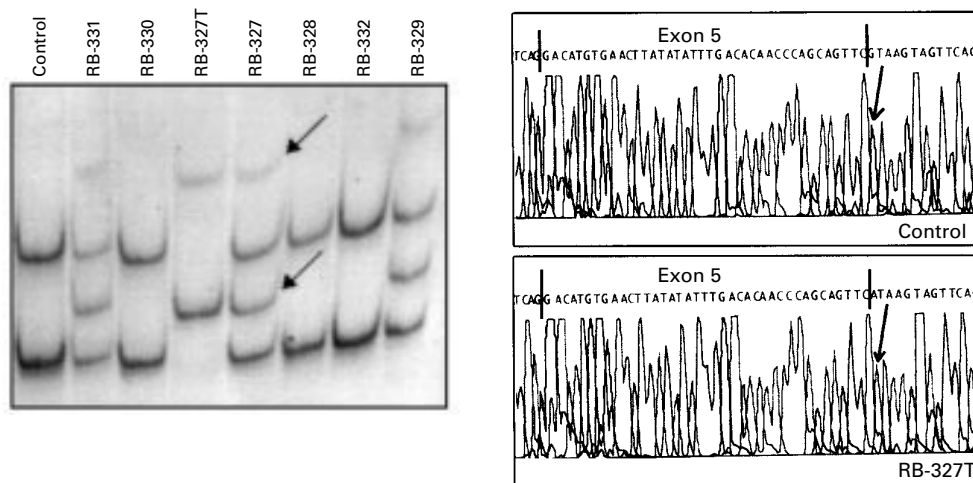


Figure 5 (Left) SSCP analysis of exon 5 in genomic DNA. Arrows indicate the anomalous electrophoretic bands in the samples RB-329, RB-331, RB-327, and RB-327T. (Right) Sequence analysis of exon 5 in tumour tissue. Arrows indicate the position of the change (G→A) detected in the donor splice site in intron 5. Vertical lines in the sequence indicate the limits of exon 5.

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The gene for branchio-oculo-facial syndrome does not colocalise to the *EYA1-4* genes

EDITOR—The branchio-oculo-facial syndrome (BOFS) is characterised by a branchial cleft sinus or linear skin lesion behind the ear, lacrimal duct obstruction, colobomata of the iris/retina, hypertrophy of the lateral pillars of the philtrum ("pseudocleft"), an asymmetrical nose with a broad tip, and auricular and lip pits. Premature greying of the hair is also observed.¹ Inheritance is autosomal dominant (OMIM 113620).² Several anomalies common

to both BOF and BOR (branchio-oto-renal) syndromes have been reported.³ McCool and Weaver⁴ reported three cases with BOF and unilateral renal agenesis. This anomaly is not frequent in BOFS but is characteristic for patients with BOR, and hence a contiguous gene syndrome or the presence of different mutations within a single gene have been suggested.⁴ Recently, the BOR gene was identified by positional cloning on chromosome 8q13.3 and mapped between markers D8S1060 and D8S1807.⁵ The gene was named "eyes absent-like 1" (*EYA1*), the human homologue of the *Drosophila eyes absent* gene. It has been postulated that the *EYA1* gene and two other *EYA* related genes (*EYA2* on chromosome 20q13.1 and *EYA3* on 1p36) play a role in development.⁵

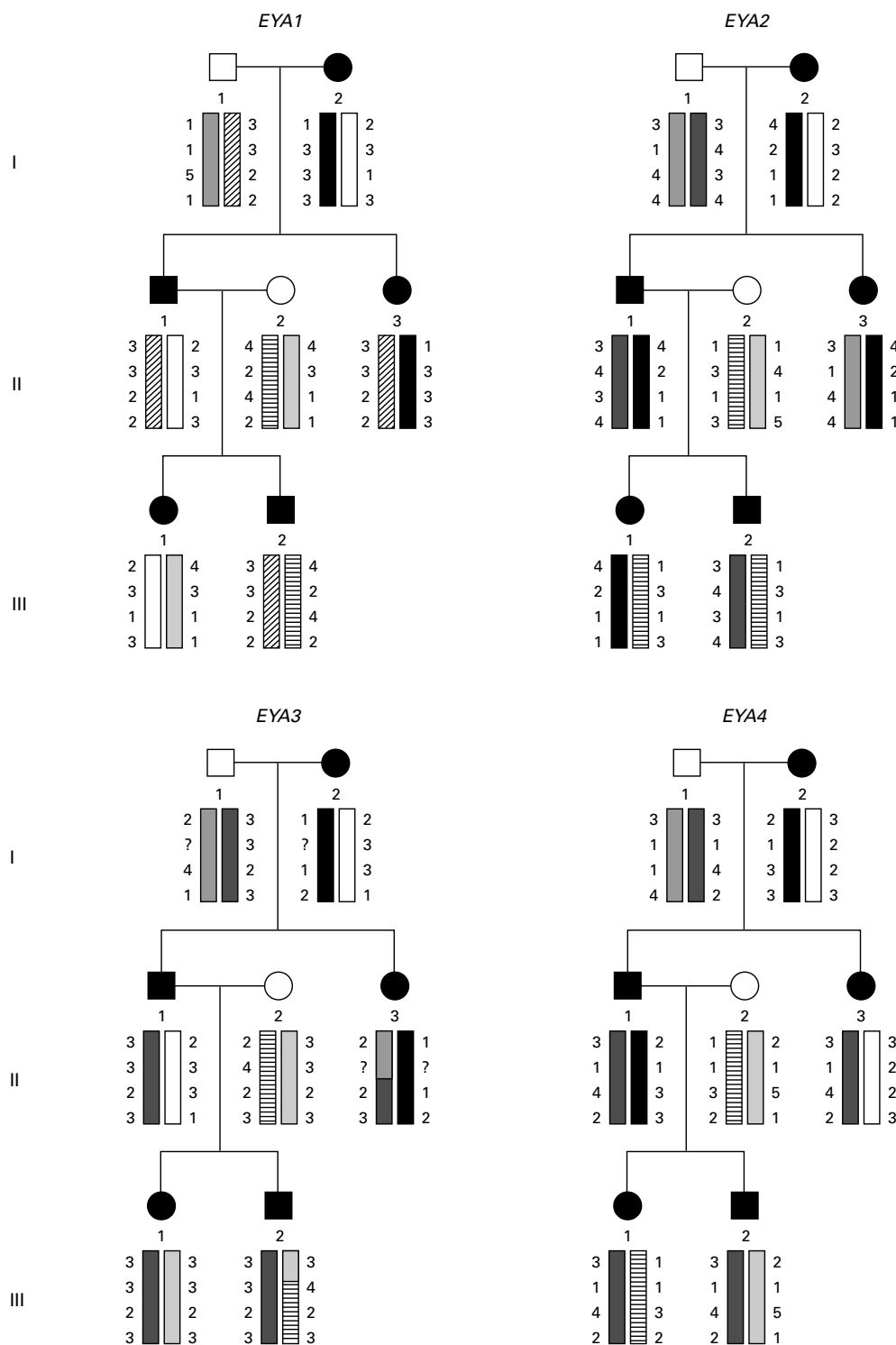


Figure 1 Pedigrees of the family affected with BOF syndrome showing the haplotypes around the EYA1, 2, 3, and 4 genes. Filled symbols represent patients, empty symbols correspond to unaffected subjects. Cosegregation of the disease locus of the BOF syndrome with EYA1 can be excluded because II.1 and II.3 inherited different chromosomes from their mother (I.2). Cosegregation of the disease with the EYA2 gene can be excluded comparing III.1 and III.2. Cosegregation of the disease with the EYA3 gene can be excluded because III.1 and III.2 have inherited the “unaffected” haplotype from their father (II.1). A shared haplotype at the EYA4 locus cannot be found in this pedigree, excluding EYA4 as a BOFS candidate gene too. Markers used (top to bottom): EYA1: D8S543 - (EYA1) - D8S530 - D8S279 - D8S286 7 cM, EYA2: D20S899 - D20S119 - (EYA2) - D20S836 - D20S17 8 cM, EYA3: D1S2893 - D1S214 - (EYA3) - D1S244 - D1S228 21 cM, EYA4: D6S1656 - D6S413 - D6S270 - (EYA4) - D6S292 4 cM.

Based on the largest published family with BOFS⁵ and in order to find a candidate gene for BOFS, we studied four flanking markers in the BOR chromosome region (EYA1 gene)⁷ as well as six markers flanking the EYA2 gene,⁸ four

markers at the EYA3 gene,⁹ and four markers close to the EYA4 gene, which has recently been mapped to 6q23.¹⁰

The family studied here was described in detail by Lin *et al*⁶ (patients 10-14) and includes five affected and two

unaffected members. Autosomal inheritance is shown by a father to son transmission. The affected subjects show intrafamilial variability but their symptoms are all compatible with the clinical diagnosis of BOFS.

Genomic DNA was prepared from peripheral blood lymphocytes from all family members using standard procedures. Four microsatellite markers (D8S543, D8S530, D8S279, and D8S286, covering 7 cM) flanking the *EYA1* gene on chromosome 8q, six markers flanking the *EYA2* gene on chromosome 20q (D20S899, D20S721, D20S911, D20S119, D20S836, and D20S17, covering 8 cM), four microsatellite markers around the *EYA3* gene on chromosome 1p (D1S2893, D1S214, D1S244, and D1S228, covering 21 cM), and four microsatellite markers around *EYA4* on 6q23 (D6S1656, D6S413, D6S270, and D6S292, covering 4 cM) were analysed. PCR amplification was performed on 50 ng of DNA using fluorescently labelled primers (the markers were chosen from GenBank and the primer sequences were taken from <http://www.genome.wi.mit.edu>). The PCR products were run on 6% denaturing polyacrylamide gels in a fluorescence sequencer and analysed with the AlleleLinks™ program (Amersham Pharmacia Biotech). The results were exported to Cyrillic 2.1™ for pedigree drawing. The segregation of the haplotypes was determined using SimWalk,¹¹ the resulting haplotypes re-entered into Cyrillic, and evaluated for cosegregation with BOFS.

The haplotypes at the four loci were determined by SimWalk¹¹ with high probability. The pedigrees with the haplotypes at the different *EYA*n loci are shown in fig 1. The haplotypes of the *EYA1* locus obviously segregate independently of the disease. The children (III.1 and III.2) of II.1 inherited different chromosomes but are both affected. Cosegregation of the BOF syndrome with the *EYA2* gene could also be excluded because different haplotypes were passed to the affected subjects in generations II and III (markers D20S721 and D20S911 were not informative and were excluded from fig 1). A similar situation occurs for the haplotypes around the *EYA3* locus with different haplotypes segregating from the affected mother (I.2) to the affected children (II.1 and II.3). Another apparent example of exclusion of cosegregation of the disorder with a gene locus is shown for *EYA4*, where each haplotype of the affected mother (I.2) was passed to the affected children (II.1 and II.3), whereas only one of the haplotypes around the *EYA4* locus of the unaffected father (I.1) could be found constantly in all affected children. The latter finding is just by chance and the shared haplotype for the paternal (I.1) chromosome 6 in all affected children does not contribute to the phenotype.

From these segregation analyses in the largest family with BOF syndrome reported to date, we conclude that BOF and BOR syndrome may not be allelic. We did not find cosegregation of the disease with the markers from the critical region of the BOR syndrome (*EYA1*) or with the related genes *EYA2*, *EYA3*, or *EYA4*.

The BOF and BOR syndromes were originally postulated to represent one contiguous gene syndrome because of overlapping clinical features.³⁻⁴ However, the variable expression of the BOR syndrome especially with respect to renal anomalies resulted in the delineation of a BO (branchio-oto) syndrome. Recently, it was shown that BOR and BO are allelic defects of *EYA1*.¹² Our patients do not have renal abnormalities typical of BOR syndrome, but share branchial and otological alterations found in BO and BOF syndromes. Intrafamilial variability in BOFS is more indicative of an allelic disorder¹² than of a contiguous gene syndrome. This intrafamilial variability also illustrates the difficulty in delineating distinct syndromes based only on isolated cases.

EYA1 is a member of the *EYA* gene family which at present comprises four genes.¹⁰ It has been postulated that all the *EYA* gene family members may cause developmental defects when mutated.⁵⁻¹⁰ The three first genes (*EYA1-3*) are expressed in the ninth week of human development.⁵ Hence, *EYA2* and *EYA3* could be other candidate genes for BOFS. *EYA4*, a new gene of the *EYA* family, was recently identified.¹⁰ This gene has been localised to chromosome band 6q23. Unlike the other three *EYA* genes it is not expressed in the developing eye, but in the early developing mouse embryo it is expressed in the otic vesicle, the branchial arch region, and in the craniofacial mesenchyme above the nasal process and between the eyes. This expression profile caused us to study a possible cosegregation of *EYA4* with BOF syndrome, which is characterised by branchial and otological alterations. Obviously, *EYA4* is not a candidate gene for BOF syndrome.

From our data, we can conclude that the known genes of the *EYA* family are not involved in the BOFS and BOFS is not allelic to BOR syndrome. Hitherto unknown genes from this gene family cannot be excluded as candidate genes. Final exclusion of the *EYA* genes as candidates, however, can be done only when our data are confirmed by studies on other families. We are aware that genetic heterogeneity may lead to the exclusion of *EYA* genes in our family, while in other families *EYA* may be involved in BOFS. However, the rarity of such cases makes it difficult to present an undisputed candidate gene. A genome wide search for other genes using a panel of polymorphic markers will help in the search for the candidate gene for BOF syndrome.

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Importance of clinical evaluation and molecular testing in the branchio-oto-renal (BOR) syndrome and overlapping phenotypes

EDITOR—The branchio-oto-renal (BOR) syndrome was first reported in the last century by Heusinger, but not clinically defined until 1976 by Melnick *et al.*¹ The major clinical features associated with the BOR syndrome are hearing loss, branchial defects, ear pits, and renal anomalies.¹ Hearing loss can be conductive, sensorineural, or mixed, ranging from mild to profound deafness.² Renal abnormalities are also variable, including renal aplasia, hypoplasia, and dysplasia, as well as anomalies of the collecting system.³ Branchial defects including fistulas or cysts and ear pits are often observed, and minor features such as external ear abnormalities, ear tags, and lacrimal duct aplasia are sometimes present. The BOR syndrome is inherited in an autosomal dominant manner and penetrance is high, although expressivity can be extremely variable.³⁻⁵

The BOR syndrome gene was localised to 8q following the description of a person with an inherited rearrangement, *dir ins(8)(q24.11;q13.3;q21.13)* presenting with features of both the branchio-oto (BO) syndrome and trichorhinophalangeal syndrome.⁶ Linkage analysis confirmed that the BOR syndrome mapped to 8q13.3.^{7,8} Further fine mapping defined the BOR region to be a 450-650 kb interval.⁹⁻¹² In 1997, the gene was cloned by sequencing P1/PAC clones from a contig spanning the region, yielding sequences homologous to the *Drosophila* developmental gene *eyes absent (eya)*. Point mutations and deletions were subsequently detected in the human homologue, *EYA1*, in families with the BOR syndrome.^{13,14}

EYA1 consists of 16 coding exons and encodes a 559 amino acid protein. There are two additional *EYA1* isoforms and all show significant homology to the *Drosophila eya* gene, as well as to the murine homologue, constituting a novel gene family.^{14,15} The *Drosophila eya* gene is essential in the formation of the fly compound eye

and the pathway has been shown to be conserved between flies and vertebrates.¹⁶⁻¹⁸ Preliminary results indicate that *EYA1* is highly expressed in human fetal kidney and brain, as well as in adult heart and skeletal muscle. The murine homologue *Eya1* was found to be strongly expressed in the mouse ear and skeletal muscle, but not shown in mouse kidney or branchial tissues.¹⁵ The product of *EYA1* contains a highly conserved region called the *eyes absent* homologous region (*eyaHR*). This is a 271 amino acid carboxy-terminal region encoded within exons 9-16 and is the site of most mutations identified to date.^{11,12,14,15}

In this study, mutation detection of the *EYA1* gene was performed on a collection of cases with a probable diagnosis of the BOR syndrome and clinically overlapping disorders. We aimed to assess whether cases with overlapping phenotypes are allelic to the BOR syndrome at a molecular level.

The cases for the study were ascertained from several sources, with 16 cases (familial and sporadic) recruited for clinical and mapping studies before gene identification.^{4,13} The remaining 16 cases were identified subsequently through the clinical genetics and nephrourology departments at Great Ormond Street Hospital, London, UK.

Thirty two cases were studied in total, of which 18 (11 familial, seven sporadic) exhibited at least three of the following major features: hearing loss, branchial defects, ear pits, and renal anomalies. These cases were classified as having classical BOR syndrome. Twelve cases (mostly sporadic) exhibited one or two major features, often associated with a minor feature (most commonly external ear anomalies). These were designated as having an atypical form of the disease. Two cases of otofaciocervical (OFC) syndrome were also included. This is an autosomal dominant condition in which there is hearing loss, ear pits, cervical fistulae, hypoplasia of the cervical musculature (sloping shoulders), facial abnormalities, short stature, and mild developmental delay. There is considerable phenotypic overlap between the OFC syndrome and the BOR syndrome.¹⁹ A summary of the clinical features of the cases are given in tables 1 and 2.

Exons 1-3 and 5-16 were amplified from genomic DNA of affected subjects using published intronic primers.¹⁴ Exon 4 was amplified using primers EYA1-4F-gtgatgtggttgaatcggt and EYA1-4R1-

Table 1 Mutations identified in *EYA1* in this study

BOR case	Type	Exon	Nucleotide change	Effect	Phenotype
<i>Nonsense</i>					
Subject 1	Sporadic	8	790 C→T	R265X	D, E, R, C
Family 7	Familial	8	790 C→T	R265X	D, B, E
Subject 2	Sporadic	8	732 C→A	Y244X	D, B, E, L
<i>Missense</i>					
Family 8	Familial	16	1680 A→C	X559Y	D, B, E, R, C, F
Subject 11	Sporadic	16	1649 T→C	L549P	D, B, E, R, F
<i>Frameshifts</i>					
Family 3*	Familial	13	1372 T→AGAGAC	1bp del/6bp insertion	D, B, E
Subject 6	Sporadic	5	387 ins T	1bp insertion	D, B, R
<i>Splice site</i>					
Family 4	Familial	9	868-1 G→A	Loss of acceptor site	D, B, E, R
Family 10	Familial	10	1041+1 G→T	Loss of donor site	D, B, E, R
Subject 9*	Sporadic	14	1498+2 T→G	Loss of donor site	D, B, E, R, C
Subject 5	Sporadic	11	1042-13 23bp inv	Loss of acceptor site	D, E, R, C, H

D = deafness, B = branchial defects, E = ear pits, R = renal anomalies, C = external ear abnormalities, F = facial asymmetry, L = lacrimal duct obstruction, H = sloping shoulders.

Nomenclature as previously described.¹⁴

*Denotes previously published mutations.¹³

Table 2 Clinical details of cases with no mutation identified

BOR case	Type	Phenotype
<i>Classical BOR</i>		
Family 15	Familial	D, B, E, R
Family 21	Familial	D, B, E, R
Family 23	Familial	D, B, E, R
Family 25	Familial	D, B, E, R
Family 26	Familial	D, B, E, C, L
Family 27	Familial	D, B, E, R
<i>Atypical BOR</i>		
Subject 13	Sporadic	B
Subject 14	Sporadic	D, E, R, K
Subject 16	Sporadic	D, R
Subject 17	Sporadic	D, R, C, T, S
Subject 18	Sporadic	D, R
Subject 19	Sporadic	B, E, P, M
Subject 20	Sporadic	E, C, V, H
Family 22	Familial	D, E, C
Subject 29	Sporadic	D, R, C
Subject 30	Sporadic	D, R, C
Subject 31	Sporadic	D, R, C, S, U, A, I
Subject 32	Sporadic	D, R, C
<i>OFC</i>		
Subject 24	Sporadic	D, E, R, C, S, H, V, I
Subject 28	Sporadic	D, B, E, G, Y, H, V

D = deafness, B = branchial defects, E = ear pits, R = renal anomalies, C = external ear abnormalities, F = facial asymmetry, L = lacrimal duct obstruction, K = cataracts, T = skin tags, S = short neck, P = cleft palate, M = micrognathia, V = developmental delay, H = sloping shoulders, U = absent uterus, A = heart abnormalities, I = short stature, G = gustatory lacrimation, Y = dilatation of collecting system.

agaaggtgacaacacgttctaatt. All PCRs were carried out under standard conditions. In familial cases, one affected member was initially analysed and then confirmed in other family members where possible.

The PCR products from exons 1'-15 were denatured and run at 15 W overnight at 4°C on 1 × MDE gels (FMC Bio-Products) using 0.5 × TBE, with and without 10% glycerol. The gels were then silver stained as previously described.²⁰ Owing to its large size, exon 16 was digested with *DdeI* and run as above. Samples with SSCP mobility shifts were then sequenced using an ABI 377 DNA sequencer, using the rhodamine terminator cycle sequencing kit (PE Applied Biosystems). Subjects without SSCP shifts were then sequenced for all exons listed above. Exons 11/12 and 13/14 were sequenced together as the introns between them are 100 bp. Mutant alleles in family 3 and subject 5 were sequenced by excising small fragments of the resolved bands from the SSCP gel, adding straight to a PCR premix, and amplifying.

Of the thirty two cases analysed, twenty nine underwent SSCP analysis, yielding 10 mobility shifts that were not present in 50 normal controls. The remaining three cases who did not undergo SSCP analysis were sequenced for the entire gene. All SSCP shifts were then directly sequenced and subjects without mobility shifts were sequenced for the entire coding region of the gene. An eleventh mutation was identified by sequencing in family 10, which was not detected by SSCP. The mutations are listed in table 1. All mutations were confirmed using either a naturally occurring restriction enzyme site or by designing an artificially created restriction site (ACRS). This involved the use of a mismatched primer to introduce a restriction enzyme site in the presence of the mutation. The primers and enzymes used are shown in table 3.

Subject 1 and family 7, who are apparently unrelated, carried the same mutation, 790C→T, in exon 8. This

results in the creation of a stop codon, which is predicted to cause premature truncation of the protein. The parents of subject 1 were shown not to carry the mutation and non-paternity was excluded. Only one other relative was available for testing in family 7. Family 7 consisted of six affected subjects with deafness, cervical fistulas, and ear pits, but DNA was only available from one relative who was clinically unaffected and who was shown not to carry the mutation. A summary of the sequence and enzyme data is shown in fig 1.

Another nonsense mutation in exon 8, 732C→A, resulted in the substitution of a tyrosine residue for a termination codon. The parents were clinically unaffected, but unavailable for testing.

A missense mutation in exon 16, 1680A→C, in family 8 is predicted to destroy the final stop codon of the gene. The change creates a tyrosine residue, resulting in the addition of five extra amino acids at the end of the protein.

Another missense mutation in exon 16 was identified in subject 11. The change, 1649 T→C, results in a leucine to proline amino acid substitution at position 549 of the gene. No other mutations were found in subject 11 when the remaining coding exons were sequenced, and the change was not present in 85 normal controls tested. Her parents have yet to be tested.

Family 3 was previously reported as having a complex 5 bp substitution/insertion (1372T→AGAGC) in exon 13.¹³ Resequencing of the mutant allele has shown the insertion to be 6 bp (AGAGAC). This combined with the loss of a thymine nucleotide at the same position resulted in an overall gain of 5 bp. This change is predicted to result in a frameshift leading to premature truncation of the protein. All three affected family members were heterozygous for the mutation, while those unaffected were not. Subject 6 was also heterozygous for a frameshift mutation which was the result of an insertion of a T at position 387 in exon 5.

Family 4, family 10, and subject 9 were heterozygous for mutations which are predicted to cause aberrant splicing in exons 9, 14, and 10 respectively. All affect conserved bases and would be predicted to be disease causing. Computer analysis involving a neural network program was used to analyse DNA sequences containing the mutated splice sites and surrounding sequence (50-100 bp). This predicted that the changes would result in the destruction of the consensus splice site and create no other donor/acceptor sites.²¹

A fourth splice site mutation was detected in subject 5, a 23 bp inversion starting at position 1042-13, across the intron/exon boundary of exon 11. This is predicted completely to disrupt the consensus acceptor site of exon 11 and was not present in the unaffected parents; non-paternity was excluded. This is shown in fig 2.

There have been seven exonic *EYA1* polymorphisms published to date. Sequencing of our cohort has confirmed the existence of published polymorphisms 510A→C, 1179C→T, 1233T→C, and 1656T→C.¹⁴ We have identified a new polymorphism in exon 7, 714A→G. This sequence variant occurred in 4/32 of the branchial arch syndrome cases and in 3/20 normal controls.

Comprehensive mutation detection of our cohort has identified 11 mutations from 18 cases with classical BOR syndrome. These 11 cases exhibited at least three of the

Table 3 Primer sequences and enzyme information for ACRS tests

BOR case	ACRS primer	2nd primer	Enzyme
Subject 1/family 7	ccattaaagattcagattctgatcgataacgt	cactgctgtttacgtagcagg	<i>AcII</i>
Family 4	aagacacattgattctgtctctctttta	tgaataacagcttctcagcc	<i>DraI</i>
Family 8	gcaccatgcttggactggagtagccgga	gtggcagacacataacgctg	<i>KpnI</i>
Family 10	ggcagacacacattttttatgacttagac	accaacaaactcgtctcac	<i>AflII</i>

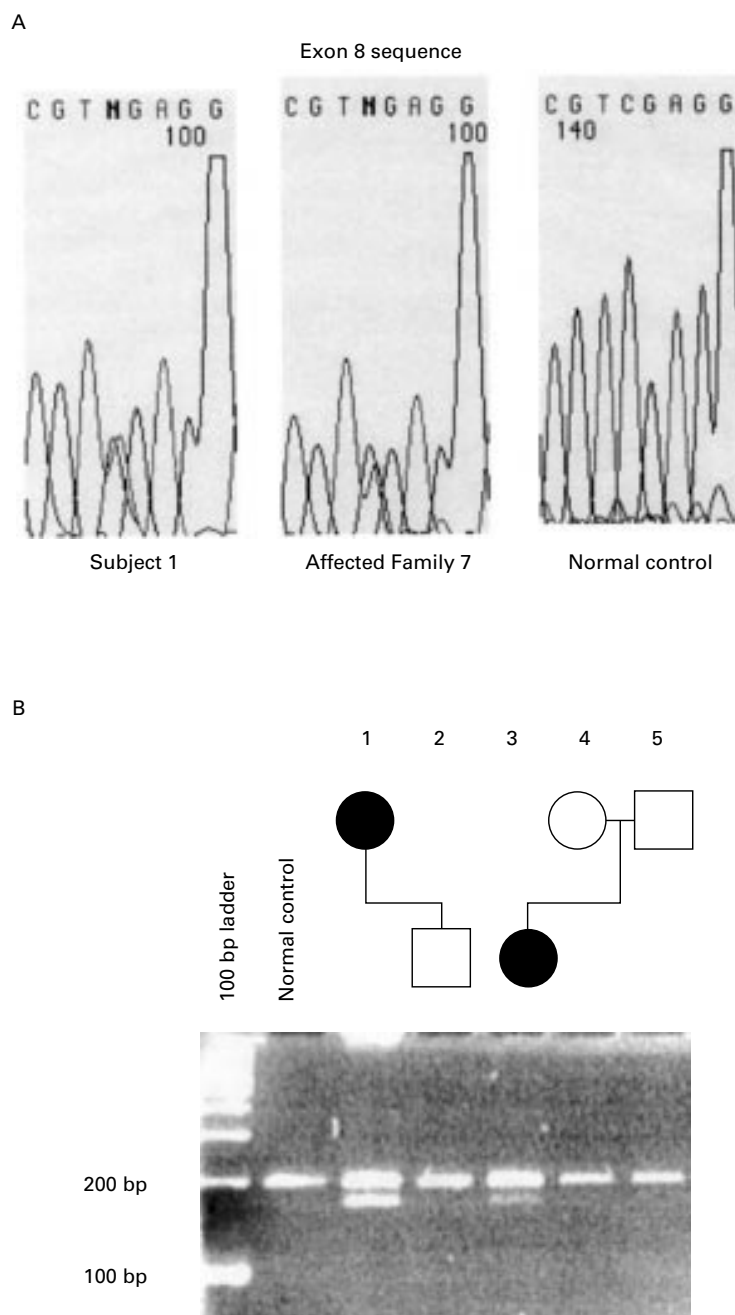


Figure 1 (A) Sequence data showing 790C→T mutation in subject 1 and family 7. (B) Restriction enzyme test confirming the mutation in subject 1 and family 7. The mutation results in a gain of an *AclI* site. Lane 1 is the affected mother in family 7, lane 2 is her unaffected son, lane 3 is subject 1, and lanes 4 and 5 are her unaffected parents.

following major clinical features: deafness, branchial defects, ear pits, and renal anomalies, and gives a detection rate of 61% which is higher than in previous studies.^{13 14 22 23} There were no apparent clinical differences in phenotypic features between the 11 cases with mutations in *EYA1* and the seven classical cases without. It is possible that these seven cases may contain major rearrangements of the gene or mutations in the 3' untranslated or promoter region.

No mutations were detected in any subjects with atypical BOR syndrome or the OFC syndrome. Atypical BOR syndrome cases exhibited only one or two major features of the disease often associated with minor features, most commonly external ear abnormalities. Careful clinical evaluation is therefore essential before determining whether it is appropriate to embark upon mutation screening of the *EYA1* gene.

However, while sporadic cases of BOR syndrome may not fulfil these strict clinical diagnostic criteria, if similar affected members within large families have between them at least three major features, mutation screening would be deemed appropriate. It is therefore possible that such strict clinical diagnostic criteria would result in failure to screen sporadic cases presenting with one or two major features. However, we found no mutations in 13 such sporadic cases studied.

When the results of our mutation detection are combined with published data, the vast majority of mutations occur in exons within the *eyaHR*, that is, exons 9-16.^{13 14 22 23} This region shows 69% identity with the *Drosophila eya* protein and appears essential for normal function of the gene product,¹³ as almost all mutations are clustered in exons within or adjacent to it. Exons 8, 13, 14,

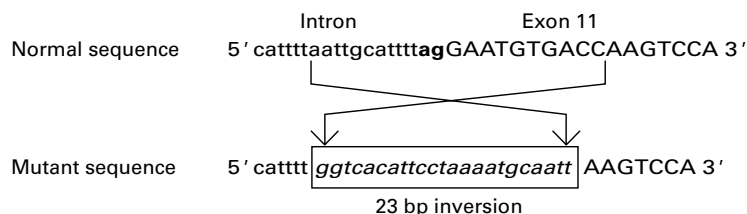


Figure 2 Inversion in subject 5 across the splice acceptor site of exon 11. Intronic sequence is shown in lower case, exonic sequence is shown in upper case.

and 15 contain the largest number of mutations and with one exception all are private.^{13 14 22 23} The missense mutation identified in subject 11 results in an amino acid substitution of leucine to proline in exon 16. The leucine residue is conserved both in *C elegans* and *Drosophila* and falls within the *eyaHR*. Sequencing showed no other changes in this person and the change was not present in 85 normal controls, making it highly likely that it is the disease causing mutation.

A previously reported mutation in a BO syndrome family (no renal anomalies) in exon 4 is the only change reported outside exons 8-16 of *EYA1*.²⁴ Here, we have identified a frameshift in exon 5 in a classical BOR syndrome patient with a single unilateral kidney. Therefore mutations located outside exons encoding the *eyaHR* and immediately adjacent exons do not appear to result in different renal manifestations. No mutations have yet been identified in exons 1'-3 and in exons 6 and 7.

The value of molecular testing shown here is that it can confirm a diagnosis of the BOR syndrome and provide genetic recurrence risk information to families or people. However, variable expressivity is a feature of BOR syndrome and it is not possible to predict the severity of the phenotypic features, even when the mutation is identified in the family.^{3 5} This is highlighted by the occurrence of the same mutation in the following two unrelated families in our study. In family 7, there are no reported renal defects, but a history of deafness, cervical fistulae, and ear pits. However, the sporadic case subject 1 presented with chronic renal failure requiring a kidney transplant at 6 years of age and was noted to have deafness, ear pits, and cupped ears. The identification of the mutation in subject 1 has proven her to be a sporadic case of the condition and greatly reduces the recurrence risk to her parents. Although the possibility of gonadal mosaicism in BOR syndrome cannot be excluded, it has not been reported.

Even with strict clinical criteria, diagnostic uncertainty can still remain as deafness, ear pits, and renal anomalies occur at a relatively high frequency as isolated features in the general population.^{25 26} In our series, two cases of such uncertainty have been resolved by molecular testing. In subject 9, the donor splice site mutation has been previously reported and was initially thought to be familial, owing to a maternal family history of ear pits.¹³ We have proved the mutation to be *de novo*, as it is not present in his parents or sib. In family 8, there is a three generation history of deafness, branchial sinuses and cysts, ear pits, renal abnormalities, facial asymmetry, and abnormal ears. The son of the proband presented with renal abnormalities, but has been shown not to carry the mutation seen in his affected mother. He is therefore very unlikely to be affected with the BOR syndrome and has an unrelated isolated renal anomaly. Molecular testing has allowed confirmation of diagnosis of the BOR syndrome allowing accurate recurrence risks in some of the families in our study.

The absence of renal involvement in some cases with *EYA1* mutations and the difference in renal abnormalities in the two cases with the same mutation (family 7 and subject 1) highlights the variable renal manifestations in the

BOR syndrome. Two cases of the BO syndrome with *EYA1* mutations have been reported,²⁴ but BO syndrome families not mapping to the *EYA1* locus have also been reported.²⁷ Although it is likely that the BOR and BO syndromes are allelic mutations of the *EYA1* gene, other unknown genes can cause branchial arch syndromes. Our study has examined cases with similar phenotypes and is supportive of this hypothesis as no mutations were identified in cases with atypical BOR syndrome (including cases of the OFC syndrome, first and second branchial arch syndrome, and cases of deafness associated with renal defects). Such cases are most unlikely to have *EYA1* mutations and so until mutation detection strategies (including deletion screening and analysis of non-coding regions) yield higher detection rates, screening is probably best limited to cases of classical BOR syndrome. Further research into *EYA1* and its role in branchial arch, ear, and kidney formation is essential in order for us to understand the factors which influence phenotype and variable expressivity of the BOR syndrome.

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Equal expression of type X collagen mRNA from mutant and wild type *COL10A1* alleles in growth plate cartilage from a patient with metaphyseal chondrodysplasia type Schmid

EDITOR—Type X collagen is a short chain collagen consisting of three $\alpha 1(X)$ chains encoded by the *COL10A1* gene. The $\alpha 1(X)$ chains are composed of three structurally distinct domains, an amino-terminal globular domain (NC2), a triple helical region, and a carboxyl-terminal globular domain (NC1).¹ Type X collagen is predominantly synthesised by the hypertrophic chondrocytes of the vertebrate growth plate but its precise function during development remains unclear.² To date, 27 naturally occurring mutations within specific regions of *COL10A1* have been reported to cause the autosomal dominant human disorder metaphyseal chondrodysplasia type Schmid (MCDS), which is characterised by short stature, a waddling gait, and coxa vara.² Of these 27 *COL10A1* mutations, two occur within a single codon and cause single amino acid substitutions at the putative signal sequence cleavage site within NC2,³ 12 mutations cause amino acid substitutions that map to two distinct regions of the predicted structure of the NC1 domain,⁴ and the remaining mutations introduce stop codons or frameshifts plus premature stop codons that affect, at most, 40% of the carboxyl-terminal region of the NC1 domain. No mutations causing MCDS have yet been found altering the collagenous region of type X collagen, and in two unrelated families with MCDS we have not been able to find mutations in the entire coding region of *COL10A1* (unpublished data). The probability of all 27 MCDS mutations clustering within the NC1 and NC2 encoding portions of the gene by chance alone is approximately 1 in 7.6×10^8 and for mutations predicted to truncate the $\alpha 1(X)$ chains is approximately 1 in 10^6 . This restricted distribution of the *COL10A1* mutations causing MCDS strongly suggests that

these mutations alter specific function(s) of the encoded $\alpha 1(X)$ chains.

The molecular mechanism(s) by which mutations in *COL10A1* cause MCDS remain under debate.⁵ In vitro association of MCDS mutant and normal $\alpha 1(X)$ chains has been reported, suggesting that dominant interference may be the underlying molecular mechanism.^{4,6} These in vitro observations have yet to be proven in vivo primarily because of the difficulty of obtaining sufficient growth plate tissue from patients with MCDS for studies of type X collagen biosynthesis. In contrast to the in vitro data, in the only previously reported investigation of the biosynthesis of type X collagen in growth plate cartilage from a patient with MCDS, it has been shown that mRNA representing the mutant allele (which contained a single base pair substitution that introduced a premature termination codon in the NC1 encoding domain) was not present in the growth plate cartilage biopsy.⁷ This finding was explained in that mRNA encoding premature termination codons has been shown to be rapidly degraded by the proof reading machinery of the cell in a number of inherited diseases.⁸ This in vivo data implied that haploinsufficiency is the underlying mutation mechanism causing the MCDS phenotype in this patient and raised the question as to whether other mutations in the *COL10A1* NC1 encoding domain may alter mRNA stability and thereby explain the clustering of the mutations in that domain.

To investigate the mechanism of MCDS pathology fully, there is a clear necessity for direct analysis of the hypertrophic chondrocytes and growth plate cartilage in other cases of MCDS. Although samples of growth plate cartilage from MCDS patients are extremely rare, we were fortunate to acquire such tissue from an affected subject who was heterozygous for a single base pair mutation, T1894C, predicted to cause a single amino acid substitution (S600P) in the NC1 domain of type X collagen.⁵ The patient had a phenotype entirely consistent with MCDS. Length at birth was normal (50 cm) and in the first year of life, the tentative diagnosis was hip dysplasia. In the second year, progressive coxa vara became apparent and at the age of 2½ years the definite diagnosis of MCDS was made. Clinical symptoms included short limbed short stature (80 cm), bowed legs, and waddling gait. Radiological findings consisted of coxa vara and metaphyseal changes including

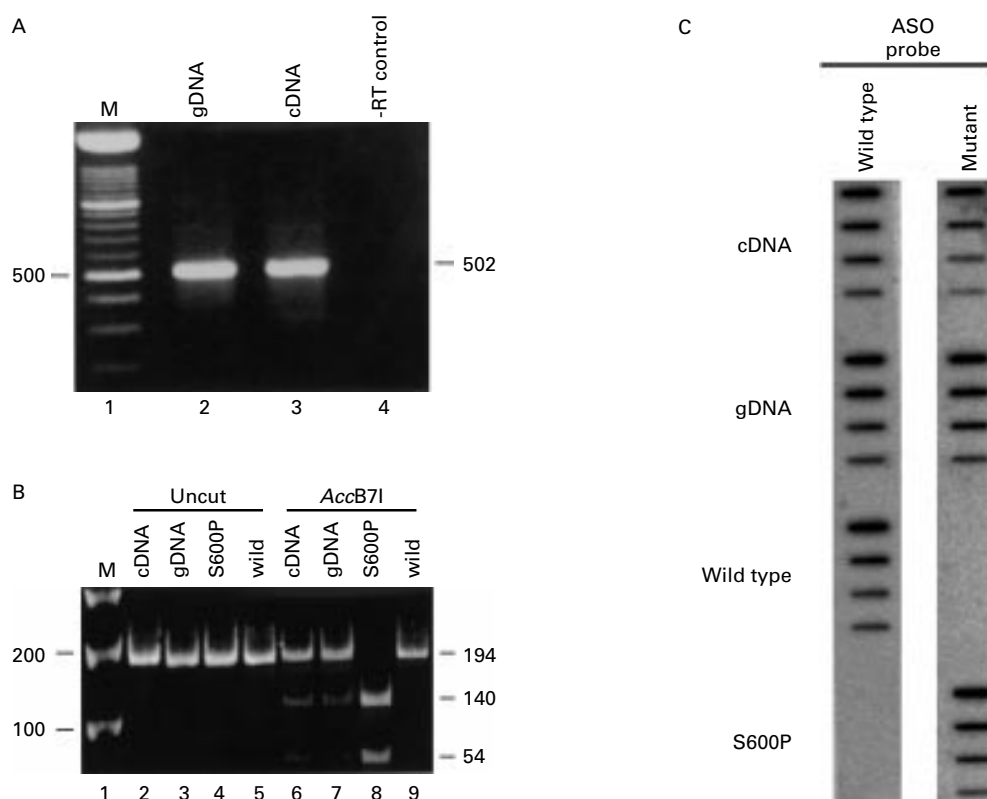


Figure 1 (A) Ethidium bromide stained agarose gel electrophoresis of PCR products of the COL10A1 NC1 encoding domain generated from genomic DNA, from cDNA prepared by reverse transcription of RNA isolated from the MCDS growth plate tissue, and from RNA processed as for the generation of cDNA, but with no reverse transcriptase in the reaction buffer (the cDNA control). Lane 1, 100 bp markers; lane 2, genomic DNA template; lane 3, cDNA template; lane 4, cDNA control. The position of the 502 bp PCR product representing the NC1 encoding domain is indicated. (B) Ethidium bromide stained PAGE analysis of PCR-amplified NC1 encoding DNA modified to incorporate a restriction endonuclease site in fragments harbouring the T1894C mutation. Lane 1, 100 bp markers; lanes 2-5, undigested PCR products amplified from cDNA, genomic DNA (gDNA), and cloned wild type and mutant (T1894C) alleles; lanes 6-9, the corresponding PCR products digested with AccB7I. Bands representing uncut fragments (194 bp) and digestion products (140 bp and 54 bp) are indicated. (C) ASO analysis of slot blots containing PCR amplified NC1 encoding cDNA, genomic DNA (gDNA), and the cloned wild type and mutant alleles. Oligonucleotides complementary to wild type and mutant (T1894C) alleles were hybridised to duplicate filters. For each sample, four slots were loaded containing 0.5 μ g (top), 0.25 μ g, 0.125 μ g, and 0.0625 μ g of DNA.

flaring, signs of sclerosis, irregularities, and growth plate widening, which were more severe at the hips than at the knees. Osteotomy was performed to correct the position of the legs and during this operation iliac crest needle biopsies were carried out to obtain material from the growth plate in this area. Informed consent for this procedure was obtained from the parents.

We used the growth plate biopsy to determine whether in this instance mRNA from both the normal and mutant alleles was available for translation. For this purpose, approximately 50 mg of the cartilage was finely ground under liquid nitrogen and total RNA and genomic DNA was extracted using a standard protocol (Trizol, Gibco BRL).⁹ The purified RNA was treated with RNase free DNase (Promega) and reverse transcribed in two separate reactions using either oligo-dT or random hexamers (Superscript II reverse transcription kit, Gibco BRL). Two further identical reactions were carried out without the addition of reverse transcriptase to control for the contamination of the RNA by genomic DNA. cDNA generated from both the oligo-dT and random primed reactions were pooled. NC1 encoding genomic DNA and cDNA was amplified using oligonucleotides: sense, CCAGCTCATATGGCAACTAAGGGCCTC (nucleotides 1429-1455) and antisense, GGGGTGTACTC-CATTGGAGCCAC (nucleotides 2082-2052). Cycling conditions were 95°C for two minutes, 60°C for two minutes, 72°C for two minutes for 40 cycles. When cDNA was

used as a template, a correctly sized 502 bp fragment was amplified (fig 1A, lane 3). This fragment was not detected in control PCR reactions confirming that there was no detectable genomic contamination of the RNA (fig 1A, lane 4). The COL10A1 NC1 encoding region was also amplified from genomic DNA (fig 1A, lane 2). Direct sequencing of the PCR products generated from both genomic DNA and from cDNA detected the wild type and mutant alleles (data not shown) and the PCR fragments representing both alleles were cloned into the T/A vector, pCR 2.1 (InVitrogen).

In order to introduce a restriction endonuclease site for AccB7I (CCAN₅TGG) into the mutant NC1 encoding DNA, single overlap extension PCR was used as previously described.¹⁰ For this purpose, the 502 bp fragments derived from genomic DNA, cDNA, and the cloned normal and mutant alleles were used as templates in PCR reactions with the mutagenic oligonucleotides (nucleotides 1897-2007, sense, 5' TACCATGGGCATGTGAAAAGGG 3' and antisense, 5' CCCTTTTCACATGCCCATGGTA 3') and the flanking oligonucleotides (sense nucleotides, 1849-1870, 5' AGGACTGGAATCTTTACTTGT 3' and antisense nucleotides, 2027-2048, 5' CTCATTTTCTGTGATCGATGAT 3'), generating a 194 bp fragment. The predicted size of fragments containing the T1894C substitution following cleavage with AccB7I were 140 bp and 54 bp and this was confirmed following digestion of the engineered PCR products generated from the cloned mutant

(fig 1B, lane 8) and wild type (fig 1B, lane 9) alleles. Digestion of the engineered PCR products generated from the MCDS cDNA and genomic DNA confirmed the presence of both alleles (fig 1B, lanes 6 and 7, respectively).

To quantify accurately the levels of wild type and mutant encoding type X collagen mRNA in the MCDS tissue, ASO hybridisation analyses were carried out as described previously.¹¹ For this purpose, PCR generated NC1 encoding DNA generated from genomic DNA, cDNA, and the cloned wild type and mutant alleles was alkali denatured and slot blotted. Duplicate filters were hybridised to ³²P labelled mutant specific (ATACTATTTTCCATAC-CACGT) and wild type specific (ATACTATTTTTCAT-ACCACGT) oligonucleotides (nucleotides 1974-1995) and the relative levels of wild type and mutant specific were digitally imaged (fig 1C) and quantified using the phosphoimaging system (Fuji-Bas). No cross hybridisation of the mutant and wild type oligonucleotides was detected when hybridised against the cloned wild type and mutant alleles, respectively. Quantitative analysis of the hybridisation of the mutant and wild type oligonucleotides to the MCDS growth plate cDNA showed that the mutant and wild type alleles were represented in a 1:1 ratio.

In this study, we have therefore shown that mRNA transcribed from both the wild type and mutant *COL10A1* alleles is available for translation in growth plate cartilage taken from a patient with MCDS. The translation of this mRNA would lead to the synthesis of $\alpha 1(X)$ chains, 50% of which would contain a single amino acid substitution, S600P, in the type X collagen NC1 domain. It has been shown in in vitro studies that NC1 domains containing MCDS mutations are able to trimerise with wild type chains⁴ and lead to the folding of the collagen triple helix.⁶ These data, together with the clustering of mutations in *COL10A1*, can only be rationalised if, in most cases of MCDS, dominant interference of normal type X collagen by MCDS mutant chains is occurring. Thus, the report⁷ that in one patient with MCDS, mRNA representing the mutant allele (which contained a premature termination codon in the NC1 encoding domain) was not present in a growth plate cartilage biopsy remains a conundrum. If, from the analysis of tissue from further patients with MCDS, mRNA instability caused by nonsense mutations is proven, then it must follow that such nonsense mutations only cause mRNA instability when within a restricted region of NC1, as similar mutations causing MCDS have not been found in other regions of *COL10A1*. An explanation would then be needed as to why mutations causing mRNA instability have the same

restricted distribution as those mutations that do not have that effect. These issues could be resolved if mutations in other regions of *COL10A1* causing MCDS are identified. More likely, however, is that conclusive data will come from the detailed examination of the biosynthesis of type X collagen in growth plate tissue either from patients with MCDS or from transgenic mice that harbour MCDS mutations.

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The relationship between neonatal immunoreactive trypsinogen, $\Delta F508$, and IVS8-5T

EDITOR—Neonatal screening for cystic fibrosis (CF) involves measurement of neonatal blood spot immunoreactive trypsinogen (IRT),¹ followed by gene mutation analysis in those with a raised (>99th centile) immunoreactive trypsinogen (IRT).² Screening with this IRT/DNA protocol has been shown, from a number of centres, to detect a greater number of $\Delta F508$ heterozygotes than expected from the known carrier frequency of $\Delta F508$.¹⁻³ The reason for this is unknown but may be explained if $\Delta F508$, on its

own, affects pancreatic function. If this is true, then the frequency of $\Delta F508$ should increase with increasing values of neonatal trypsinogen.

It has also been shown that the intron 8 polythymidine tract sequence 5T (IVS8-5T) is more frequent in neonates with hypertrypsinemia.^{4,5} The intron 8 polythymidine sequence regulates the splicing of exon 9 in transcription of the cystic fibrosis transmembrane conductance regulator protein (CFTR).⁶ IVS8-5T is associated with the least efficient splicing of CFTR, but whether the frequency of IVS8-5T increases with increasing level of IRT, in the absence of another mutation, is unknown.

The relationship between neonatal immunoreactive trypsinogen, $\Delta F508$, and IVS8-5T has not been defined. To answer this question we studied the frequency of $\Delta F508$ and IVS8-5T from neonatal blood spots systematically in

Table 1 Relationship between neonatal IRT, $\Delta F508$, and IVS8-5T

IRT ($\mu\text{g/l}$)	Total No per stratum	No of study subjects	$\Delta F508$ (OR)	IVS8-5T (OR)
0-5	7 942	104	2 (1.0)*	6 (1.0)*
6-10	26 714	95	2 (1.0)	4 (0.7)
11-15	16 543	104	2 (1.0)	10 (1.8)
16-20	6 785	103	4 (2.0)	2 (0.3)
21-25	2 826	98	7 (3.5)	11 (2.2)
26-30	869	101	5 (2.5)	10 (1.9)
Total	61 679	605	22	43

IRT: immunoreactive trypsinogen, OR: odds ratio.

*Baseline category (odds ratio of 1 assigned to this stratum).

IRT strata below the 99th centile cut off normally used for DNA testing.

Newborn screening for cystic fibrosis using day 4 blood spots on filter paper cards is routine in Victoria, Australia. The measurement of immunoreactive trypsinogen (IRT) by fluoroimmunoassay is the primary screen, with the IRT values being normally distributed, and the top 1% being selected for $\Delta F508$ mutation analysis.⁷ We divided newborn screening cards into six strata, based on IRT value below the 99th centile, and 105 cards were selected at random from each strata. We chose an IRT value of 30 $\mu\text{g/l}$ as the upper limit, since the 99th centile is almost always above this figure. Cards from the most recent complete year (1996) were selected to ensure adequate freshness of the blood spot. The study was conducted anonymously and 3 mm punched blood spots labelled to allow correlation between the $\Delta F508$ mutation analysis and the intron 8 polythymidine sequence. DNA was eluted from the blood spot and $\Delta F508$ mutation analysis performed by polymerase chain reaction (PCR) using specific primers and the products detected by gel electrophoresis.^{8,9} The polythymidine sequence was determined by nested PCR and the products sized by gel electrophoresis.^{10,11} The study was approved by the ethics committee of the Royal Children's Hospital, Melbourne.

The strength of the association between IRT level and genotype frequency was assessed by chi-squared test of trend.¹² In order to determine whether the total number of samples with either $\Delta F508$ or IVS8-5T was comparable to the known frequency of these mutations in the population, we calculated a stratum weighted prevalence estimate, using weights based on the total of infants in each IRT stratum for the year of the study.

The results of the relationship between IRT, $\Delta F508$, and the intron 8 polythymidine tract are presented in table 1. In some cases, DNA did not amplify and only subjects for whom both $\Delta F508$ and IVS8-5T results were available are presented.

There were no $\Delta F508$ homozygous infants detected below the 99th centile, the cut off that is currently used for newborn screening. There were 22 $\Delta F508$ heterozygotes detected, representing 3.6% of the study sample. The frequency of $\Delta F508$ increased with increasing level of IRT (χ^2 for trend=4.3, $p=0.04$). The stratum weighted prevalence estimate of the frequency of $\Delta F508$ in the population was 2.5% (95% CI 0.9-4%) which is not different from the expected number, had we selected 605 blood spots cards at random, based on the carriage frequency for $\Delta F508$ of 1/33 in the Victorian population.

Only one $\Delta F508$ heterozygous infant also had the IVS8-5T allele (genotype 5T/7T), and this infant was in the 26-30 IRT cohort. Of the remaining $\Delta F508$ infants, 18 had the 7T/9T genotype, two 7T/7T, and one 9T/9T. Heteroduplex band analysis indicated that one of the subjects with a 7T/7T background was a $\Delta I506/7$ heterozygote (fig 1) while the other 7T/7T subject and the 5T/7T subject were both $\Delta F508$ heterozygotes. Because $\Delta F508$ has always been reported to be in cis with 9T, we performed

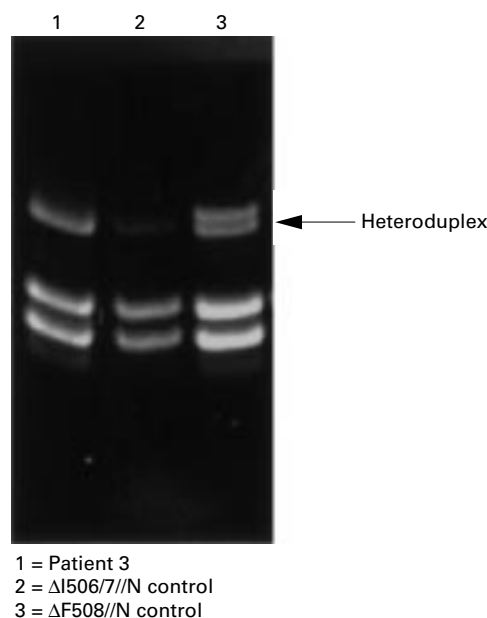


Figure 1 Polyacrylamide electrophoresis gel showing the $\Delta I506/7$ heteroduplex pattern of the subject with a 7T/7T background (lane 1).

gene sequencing on the two $\Delta F508$ subjects with a non-9T background which confirmed the exon 10 mutation as $\Delta F508$ (fig 2).

There were no homozygous 5T/5T infants detected, but 40 had the 5T/7T genotype and three the 5T/9T genotype. The most frequent intron 8 polythymidine genotype was 7T/7T ($n=447$), with 7T/9T ($n=107$) the second most common, while only eight infants had the 9T/9T background. The total number of infants with IVS8-5T detected was 7.1% of the group, and the stratum weighted prevalence estimate of the frequency of IVS8-5T in the study was 6% (95% CI 3.6-8.4%). There was no clear increase in the frequency of IVS8-5T with increasing IRT (χ^2 for trend=2.4, $p=0.12$).

We have shown that the frequency of $\Delta F508$ increases with increasing levels of neonatal IRT and that this is independent of the IVS8-5T allele. We detected the expected number of $\Delta F508$ alleles, but most were found in the higher IRT strata, suggesting that the distribution of $\Delta F508$ heterozygotes is skewed to the higher levels of IRT. It is clear that a single, severe *CFTR* mutation such as $\Delta F508$ can affect neonatal pancreatic function, and explains why the detection of $\Delta F508$ heterozygotes is increased in an IRT/DNA screening protocol.

The effect of $\Delta F508$ on neonatal IRT is interesting in the light of recent reports of an increased frequency of *CFTR* mutations in patients with chronic idiopathic pancreatitis.^{13,14} This suggests that single *CFTR* mutations may be associated with clinical disease, although it is likely that there may be some role for additional environmental exposure such as tobacco smoke or alcohol. We did not detect $\Delta F508$ homozygotes from our group of blood spots taken from IRT values below the cut off normally used for newborn screening. This is reassuring, although we did not sample enough blood spots to be absolutely sure no homozygotes had been missed. We detected the expected number of $\Delta F508$ heterozygotes and it is unlikely that any have a second severe mutation (for example, G551D, G542X, or R553X) as this group of compound heterozygotes have a severe CF phenotype and almost invariably have an IRT above the 99th centile. We studied the group below the 99th centile threshold and it is likely that if a second mutation were present it would be a milder

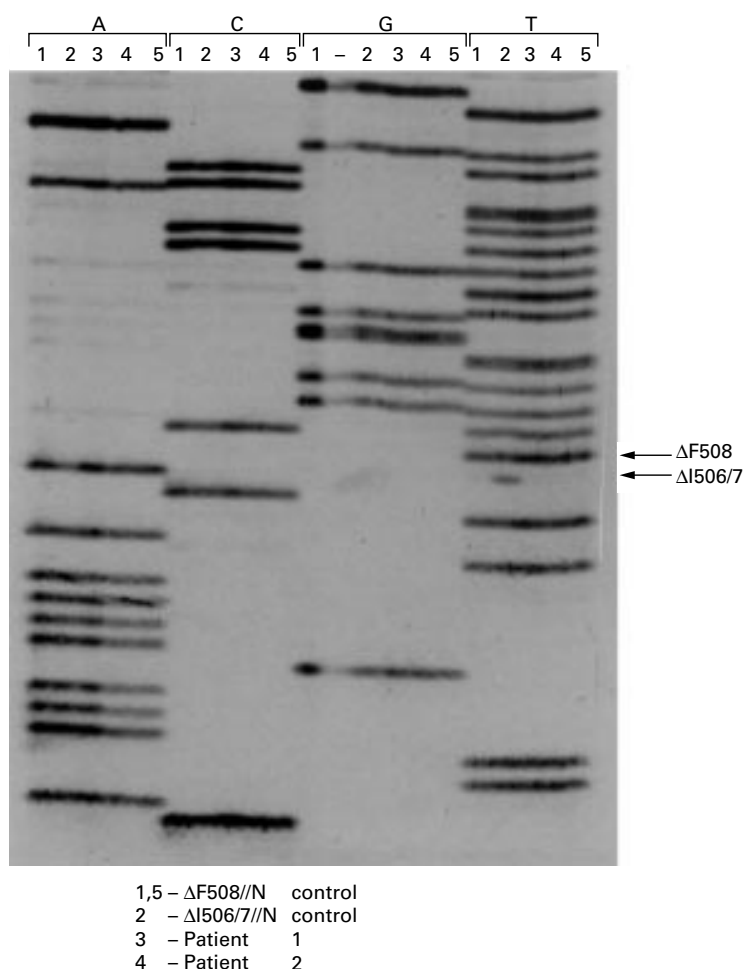


Figure 2 Sequencing gel confirming the exon 10 mutation as $\Delta F508$ for two subjects with a 5T/7T (lane 3) and 7T/7T (lane 4) background.

mutation. The milder mutations are rare in the Victorian population and given the large number of possibilities further mutation analysis is impractical. In the highest two IRT cohorts the frequency of $\Delta F508$ was 6% which is twice the expected frequency from the Victorian population overall, but not high enough to warrant lowering the IRT threshold to test for other mutations or arrange a sweat test. Thus, the 99th centile threshold used in the current screening protocol seems justified.

The results of our study suggest that there is not a clear association between the level of neonatal IRT and the IVS8-5T allele below the 99th centile IRT cut off. The number of IVS8-5T alleles detected was consistent with the 5% reported from other centres,^{11 15 16} and there was only a weak suggestion of higher numbers at the higher IRT levels. This is in contrast to other studies which have suggested the frequency of IVS8-5T is increased over the 99th centile IRT threshold and, by inference, that IVS8-5T can influence neonatal IRT on its own. If this were true, we would expect to have detected a trend, with an increasing frequency of IVS8-5T with increasing IRT, as we have shown with $\Delta F508$.

In our study, we examined the relationship between IRT and IVS8-5T systematically, while in the other studies, a random and small number of subjects below the 99th centile IRT threshold was chosen and compared to subjects with IRT above the 99th centile IRT threshold. The IVS8-5T allele with either a 7T or 9T on the other allele may reduce the production of exon 9 containing CFTR to 30-40%,⁶ a level of CFTR activity which has not been

thought to cause disease.^{11 17} There are reports of an increased frequency of IVS8-5T in patients with chronic, idiopathic pancreatitis, but we did not find an effect on neonatal IRT.¹⁸ With regard to other cystic fibrosis related diseases, IVS8-5T has only been implicated when in association with exonic mutations.^{10 19}

The finding of two $\Delta F508$ heterozygotes from 22 (12%) on a non-9T background challenges current dogma that $\Delta F508$ is always in cis with 9T. The original studies of IVS8 showed that in homozygous $\Delta F508$ subjects only 9T was present on each allele and that in $\Delta F508$ heterozygous subjects at least one IVS8 allele was 9T. Whether the 9T was in cis or trans with $\Delta F508$ in the heterozygous $\Delta F508$ subjects was not determined but was assumed to be in cis, extrapolating from the $\Delta F508$ homozygote data. Our previous experience has suggested that the $\Delta F508$ mutation on a non-9T background may in fact be $\Delta I507$ which has a similar electrophoretic appearance in the polyacrylamide gels used for newborn screening and is known to be associated with a 7T/5T or 7T/7T background. Gene sequencing clearly identified these subjects as having $\Delta F508$, which has not previously been reported on a non-9T background. This makes it unreliable to phase chromosomes using the IVS8 alleles as has been reported.²⁰ It is possible that the infant with the $\Delta F508$ (or $\Delta I507$) 5T/7T genotype, if male, could have congenital absence of the vas deferens (CAVD), although the penetrance of the IVS8-5T allele is variable,¹⁸ and no accurate predictions could be made.

We have shown that $\Delta F508$ alone can affect neonatal immunoreactive trypsinogen, and that this is the explana-

tion for the preponderance of $\Delta F508$ heterozygotes detected by the IRT/DNA newborn screening protocol. The IVS8-5T allele does not appear to influence neonatal pancreatic function but further investigation of its role in CF related disorders is required.

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Epidemiology of neurofibromatosis type 1 (NF1) in northern Finland

EDITOR—Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, is an autosomal dominant neurocutaneous disease characterised by café au lait spots and neurofibromas. The gene responsible for the disorder is located in the chromosome region 17q11.2. The prevalence of NF1 has been estimated to be about 1/3500 in the USA and the United Kingdom,¹⁻³ and its birth incidence has been reported to vary from 1/2558⁴ to 1/4292,⁵ the mutation rate being 3.1×10^{-5} to 6.5×10^{-5} . The first population based study of NF1 was performed in Sweden by Samuelsson⁶ in 1981, who found 74 adult NF1 patients in the Gothenburg region, implying a prevalence of 1/4600. In 1989, Huson *et al*⁷ discovered 135 NF1 patients in 69 families in south east Wales, 83 of whom were index cases and 52 affected relatives, with a prevalence of 1/4150. The highest estimated prevalence for NF1, 1/2190, has been reported in Dunedin, New Zealand, by Fuller *et al*,⁷ who also showed that the prevalence peaked in the age group 20-29 years. A fourth report from north east Italy by Clementi *et al*⁸ quoted a prevalence of 1/6711 and a very high mutation rate of 6.5×10^{-5} gametes per generation.

The purpose of the present work was to determine the prevalence and genetic characteristics of NF1 in northern Finland, including a survey of first degree relatives of patients and linkage data, to assist in the diagnosis of

affected subjects. Clinical data on the patients will be reported separately.

The study was carried out between October 1989 and December 1996 in the region of Oulu University Hospital (OUH) in northern Finland with a total population of 733 037 (31 December 1996).⁸ The basic material consisted of families attending the Department of Clinical Genetics at the OUH for genetic counselling from 1982 onwards. Additional patients with a diagnosis of neurofibromatosis (International Classification of Diseases (ICD), 9th revision), diagnosis codes 2377A or 2251A, or neurofibromatosis (von Recklinghausen) (ICD, 8th revision) diagnosis code 74340, were traced from the records of the University Hospital and the four central hospitals in the area, two in Lapland, one in Kainuu, and one in central Ostrobothnia. In addition, patients were traced by contacting paediatricians, neuropaediatricians, dermatologists, neurologists, ophthalmologists, oncologists, pathologists, audiologists, otologists, surgeons, neurosurgeons, paediatric surgeons, and internists in the area. The OUH records concerning patients treated for neurofibroma, optic glioma, multiple meningiomas, or vestibular schwannomas were reviewed, and all patients with plexiform neurofibroma or congenital pseudoarthrosis were traced in order to examine them for NF. The histological specimens of surgical and necropsy specimens examined at the Department of Pathology, Oulu University Hospital, were reviewed and clinical and necropsy records scrutinised.⁹ Also, the two private pathological laboratories in the area were contacted and searched their records for any surgical

Table 1 Neurofibromatosis type 1: age at diagnosis in different patient groups in 197 cases in northern Finland

Patient groups	No	Age at diagnosis (SD) (y)	Range	p value	Compared group
All cases	197	20 (16)	3 mth–60 y		
Born 1960 to 1995	116	10 (9)	3 mth–36 y	p<0.001	Born before 1960 (n=81)
Males	95	15 (14)	3 mth–54 y	p<0.001	Females (n=102)
Females	102	25 (17)	6 mth–60 y		
Children of an affected parent	81	15 (15)	3 mth–57 y	p=0.113	Sporadic cases (n=77)
Sporadic cases	77	19 (16)	6 mth–60 y		
Sporadic cases born in the 1960s	11	16 (9)	3–33 y	p<0.001	Sporadic cases born before 1960 (n=30)
Sporadic cases born in the 1970s	10	9 (6)	3–22 y	p<0.001	Sporadic cases born before 1970 (n=41)
Sporadic cases born in the 1980s	19	6 (4)	4 mth–13 y	p=0.002	Sporadic cases born in 1960s and 1970s (n=21)

NF findings. Collectively, these sources provided information on 181 families with either confirmed or suspected NF in one or more members. These patients were then contacted through their own physicians to ascertain their willingness to participate in the study. Seven families with one NF1 patient refused to be included in the study, but if the patient had NF1 according to the hospital records they were nevertheless included in the series. The others were assessed clinically at the Department of Clinical Genetics of OUH, and most of them were also examined by a neuro-ophthalmologist.¹⁰

Whenever possible, a family study was undertaken and the first degree relatives living in the area were examined clinically even when it was not possible to confirm the diagnosis of NF in the index case. When the hospital records indicated a positive family history but the index patient had died, relatives in the area were contacted with the help of the patient's doctor.

The NIH criteria for NF1 (National Institute of Health, Consensus Development Conference held in Bethesda, Maryland, July 1987) were used for inclusion.¹¹ Two of the following criteria were needed for NF1: six or more café au lait macules; two or more neurofibromas of any type or one plexiform neurofibroma; multiple freckles in the axillary area or the inguinal regions; optic glioma; two or more Lisch nodules (iris hamartomas); a distinct osseous lesion such as sphenoid dysplasia, or thinning of the bone cortex with or without pseudoarthrosis; or a first degree relative (parent, sib, or offspring) who met the above criteria for NF1.^{11–13}

We were able to do linkage studies in 20 familial cases. DNA was prepared from peripheral blood samples from the patients and their first degree relatives by standard procedures and a linkage analysis was performed using tightly linked flanking DNA markers and intragenic micro-satellite markers. The following polymorphic markers were used in the present study: p11.3C4.2/*MspI*,¹⁴ pHHH202/*RsaI*,¹⁵ EVI-20,¹⁶ AluNF1,¹⁷ IVS27AC28.4,¹⁸ IVS38GT53.0,¹⁹ and pEW206/*MspI*.¹⁴ EVI-20, ALuNF1, IVS27AC28.4, and IVS38GT53.0 are intragenic markers. Intragenic markers were also used to detect possible deletions and to study the parental origin of mutation.

To study the geographical distribution of the disease in the study area, birth places of the patients were used.

The prevalence figures were calculated from the number of affected subjects in the population at a particular time in relation to the total population. The point prevalence on 31 December 1996 was calculated based on the total number of affected persons in the population of 733 037 in the area (Central Statistical Office of Finland, 1997) and the period prevalence from the corresponding figures for a time period 1960 to 1995. For the incidence figures, the number of subjects born with NF was related to the number of live births in the area during 1960 to 1995.⁸ Thus, the youngest patients were 1 year old and the oldest 36 years old on the point prevalence day.

NF1 patients were considered to represent probable new mutations if the clinically examined parents did not show any signs of NF1 when studied by us or by another specialist experienced in NF1. The mutation rate was obtained by calculating the ratio of new mutation cases in a given period to the total number of live births.

Fitness was estimated by the method of Tanaka,²⁰ in which relative fitness was calculated as a fraction comparing the frequency of NF1 among parents of index cases with the frequency of NF1 among offspring of index cases.

The parental age of those cases assumed to represent new mutations was compared with the parental age of the fathers in the general population of Finland and the parental age of the mothers in the study area.⁸ The significance of the differences was evaluated by Student's *t* test. The birth order effect in families representing new mutations was assessed by the method of Haldane and Smith,²¹ in which the sum of the birth orders of all the affected sibs in each family is compared with the theoretical value, calculated on the assumption that there is no birth order effect.

Values are expressed as means (SD). The independent samples *t* test was used to compare differences in mean ages at diagnosis between sexes, age groups, sporadic and familial cases, and sporadic cases in different decades. Two sided *p* values were calculated at a significance level of 0.05.

Segregation analysis was performed by comparing the number of affected offspring of an affected parent having a healthy spouse with the expected number using the χ^2 test.²²

A total of 197 NF1 patients in 119 families were identified. For confidentiality reasons, pedigrees are not shown but are available on request. A total of 77 cases were sporadic and 117 familial. In addition, three patients had a mother with segmental NF (NF5). The diagnosis of NF1 was made by a dermatologist (29%), clinical geneticist (26%), paediatrician or neuropaediatrician (22%), paediatric surgeon or surgeon (13%), general practitioner (4%), and a neurologist (4%). Clinical examination performed by the first author of 239 relatives of 112/119 index cases with at least a 25% a priori risk showed 41 NF1 cases in addition to 37 earlier verified relative cases and excluded the disease in 198 people.

The age distribution of the patients ranged from 3 months to 73 years (mean 29 years). The mean age at the time of diagnosis was 20 years (SD 16), with a range of 3 months to 60 years. This figure was significantly lower in males than in females and in the younger generations. It was also four years lower in children of affected parents compared to sporadic cases. Sporadic cases were diagnosed an average of 10 years earlier in the 1980s (mean age 6 years (SD 4)) than in the 1960s (mean age 16 years (SD 9) (table 1).

By the prevalence day (31 December 1996), 29 of the 197 known NF1 patients had died and three had moved

out of the area. Based on the remaining 165 patients, the prevalence of NF1 in northern Finland was 1/4436 (23/100 000). The period prevalence calculations gave a peak prevalence of 1/2983 (34/100 000) for the age group 10-19 years (fig 1).

There were 116 new NF1 cases and 423 075 live births in the area during the period 1960-1995,⁹ giving an incidence of 1/3647 (27/100 000). By decades, the corresponding incidences were 1/4545 for the 1960s and 1970s and 1/2941 for the 1980s. The incidence for the six year period 1990-1995 was 1/2703. It can be estimated that three new NF1 children will be born in the area annually.

Of the 197 patients, 119 were familial cases. There were four four generation, 10 three generation, and 25 two generation families with NF1. Three patients had a mother with segmental NF (NF5). With 95 male patients and 102 females, the sex ratio was 0.93. The geographical distribution of the patients roughly corresponds to that of the general population in the area. Ninety six of the 197 cases of NF1 identified (49%) represented possible new mutations of the disease gene. Of these, in 39 cases both parents were personally examined by the authors of the present study, in 13 cases only one of the parents was seen, and in 44 cases the family history was obtained from the patient and from hospital records.

The mean maternal age at the birth of a child with NF1 resulting from a probable new mutation was 30 years (SD 6) (range 21-43 years), the corresponding mean overall maternal age in the area during the same time period being 27.5 years ($p=0.006$). The mean paternal ages were 33 years (SD 6) (range 19-50 years) for NF1 children and 30.0 years for fathers in Finland in general ($p=0.008$), respectively. The mean birth order was 2.9 (SD 1.7) calculated from the 33 cases classified as new mutations and the size of the sibship being at least two. The difference between the observed and theoretical sum of the birth orders of affected subjects divided by the standard error of the theoretical mean value was 3.3, showing that the later born sibs are more likely to be affected ($p=0.002$).

Of 20 families studied for genetic linkage, one was shown to have a deletion of the NF1 gene encompassing the loci from EVI-20 to INT-38. In addition, seven other familial and 39 sporadic cases in informative families were screened for deletions with intragenic linked markers. One deletion for the INT-27 locus was found in a sporadic case. Thus,

deletions were found in 2/66 families (3%). The sporadic deletion occurred in the maternally derived chromosome 17.

Among those familial cases in which the parental origin of the new mutation in the first affected subject could be evaluated with linked markers, six of the seven cases studied had the mutation in the paternally derived chromosome 17.

Of the 197 cases, 48 females and 18 males had children. Out of a total of 178 offspring of 66 of the parents with NF1, 78 (44%) were affected, whereas 89 (50%) would have been expected on the assumption of autosomal dominant inheritance. The ratio, 0.44 (78/178), did not differ significantly from the expected ($\chi^2=2.7$). The 48 NF1 mothers had a total of 147 pregnancies, of which 11 ended in a miscarriage, 60 in the birth of a NF1 child, and 76 unaffected children. The corresponding figures for the 18 NF1 fathers were 42 pregnancies, yielding 18 NF children and 24 unaffected ones, but no reported miscarriages.

The relative fitness of the subjects with NF1 was analysed for 68 index cases where both parents were seen by us and for 46 adult index cases where all the children were seen. The relative fitness was 0.48, 0.24 for males and 0.72 for females.

The members of the 20 families with an affected parent and at least one affected child were analysed with linked microsatellite markers in order to search for possible non-penetrance. Out of 87 informative meioses, 54 were associated with the established at risk haplotype of the family. Fifty two of these subjects were affected and two were unaffected. Of the unaffected at risk haplotype carriers, one 15 year old girl had no clinical signs of NF1. In addition, there was a pair of 8 and 11 year old sisters who had inherited different haplotypes from their affected father and again neither of them showed signs of NF. All those 33 persons who had inherited the "non-risk" haplotype were healthy. No recombinants were observed for NF1, nor could any linkage disequilibrium be shown with the linked polymorphic markers used here.

This population based study of NF in northern Finland identified 197 NF1 patients in 119 families. The diagnoses were based both on clinical and imaging findings. In 20 familial cases also, DNA studies with linked DNA markers were carried out.

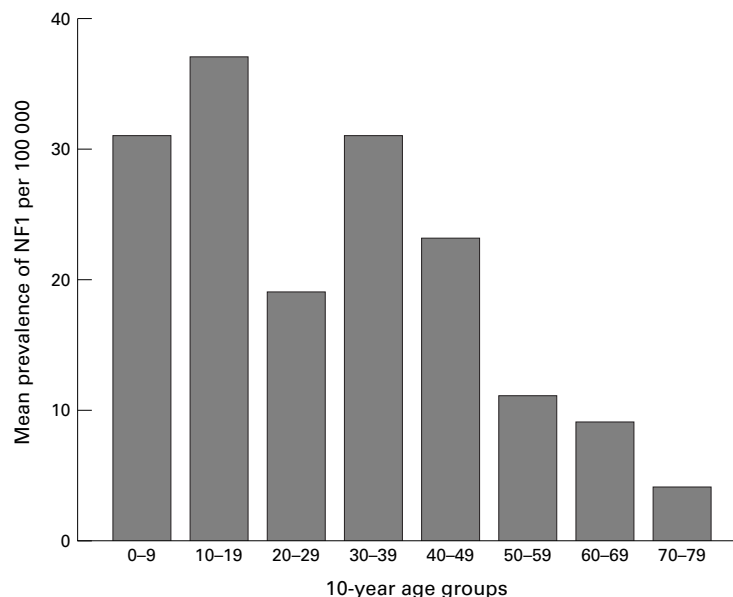


Figure 1 Age related prevalences of NF1 in 10 year age groups.

Table 2 Prevalence, birth incidence, and mutation rate in neurofibromatosis type 1 in different populations

Country	Affected (No)	Families (No)	Size of population	Prevalence	Birth incidence	Mutation rate (direct method) ($\times 10^{-5}$)	Ascertainment	Reference
Denmark	212	84	~3 000 000	~1/14150	NA	NA	Surveys of hospital records and mental institutions	23
USA	223	107	743 000	1/2500–3300	1/2500–3300	14–26	Surveys of hospital records and mental institutions	24
Russia	124	116	94 000	1/7800	NA	4.4–4.9	Population study of 16 year old youths pre-military exam, and surveys of hospital records	25
Sweden	74	63	337 979	1/4600	NA	4.3	Population based	6
United Kingdom	135	69	668 100	1/4150–4950	1/2558	3.07–5.01	Population based	4
New Zealand	52	20	113 700	1/2190	NA	NA	Population based	7
Italy	202	129	2 375 304	1/6711	1/4292	6.5	Population based	5
Canada	242	82	1 500 000	1/6198	NA	NA	Surveys of clinical genetics centres	26
Israel	390	NA	374 440	1/960	NA	NA	Population study of 17 year old youths pre-military exam	27
Present study	197	119	733 037	1/2983–4436	1/2932–3647	3.65–5.09	Population based	Current data

NA = not analysed.

We believe that these 197 patients ascertained represent the great majority of the NF1 patients in northern Finland. In addition to patient diagnosis lists of the hospitals of the region, cases were searched for by asking the paediatricians, neuropaediatricians, dermatologists, neurologists, ophthalmologists, oncologists, pathologists, audiologists, otologists, surgeons, neurosurgeons, paediatric surgeons, and internists in the study area about their NF patients. Mildly affected patients diagnosed and treated only in local health centres, however, may have been missed in our search. In addition, undiagnosed cases certainly exist in the study area. This is reflected by the fact that we diagnosed 41 new cases among the relatives of our index cases. Thus, the figures calculated in this study represent minimal incidence and prevalence of NF in northern Finland.

The observed overall prevalence (1/4436 or 23×10^{-5}) of NF1 in northern Finland and the incidence (1/3647 or 27×10^{-5}) are comparable to findings in other populations studied to date (table 2).^{4–7 23–27} There was no evidence of very large families or of geographical clustering of NF1, neither was there any sign of possible linkage disequilibrium in the DNA studies.

The overall prevalence and incidence figures obtained for NF1 have in most cases proved to be minimum estimates, and this is also apparent in the present study. The fact that the age at diagnosis of NF1 was lower in the younger age groups and the age dependent prevalence/incidence figures were significantly higher in younger age groups, despite the slow accumulation of diagnostic signs in very young children, may reflect a better awareness of the importance of diagnosing NF in suspected cases, improved knowledge of the diagnostic features, and a greater willingness to refer suspected cases for detailed examination. A careful study of the first degree relatives uncovered 41 undiagnosed cases, especially in the relatives of an affected child. The higher prevalence figures among the young may partly be explained by the increased mortality suggested to be associated with NF1.^{28–31} The results suggest that the incidence figures are most reliable for patients under 20 years of age, and for prevalence figures the age related period prevalences are better. The observed sex ratio, 0.93, did not differ significantly from what was expected, as has also been reported in the other studies.^{4–6}

Our linkage studies showed that linked markers/haplotypes show the same result as careful clinical examination in familial cases of NF1, although contradictory results were obtained in two families where linkage data showed the NF1 risk haplotype in a healthy child of an

affected parent. One explanation may be that the children were affected but were still at a presymptomatic stage. This would be exceptional, as all our affected cases (reported separately) had developed café au lait macules by the age of 5 years (96% of all patients), and similar observations have been made in earlier reports.^{1 29} Another explanation would be that even though the affected parents in both families fulfilled the NIH diagnostic criteria for NF1 (in the first family CFS and freckles, and in the second family neurofibromas and Lisch nodules), they both have another type of NF which is not linked to the *NF1* gene. A third explanation would be mosaicism in a parent with NF1 in whom some of the germ cells do not carry the *NF1* mutation. A fourth explanation would be non-penetrance of the *NF1* mutation in the children in question, which has previously been reported in only three cases, a 50 year old woman who had an affected brother, son, and grandson,³² a 45 year old man with an affected mother and daughter,³² and a subject with an affected father and two affected daughters.³³ Although non-paternity is not probable, one should exclude it with other markers. The finding of possible non-penetrance in the two families in this series will be finally answered only after the families' *NF1* mutation has been found.

New mutations accounted for a maximum of 49% of our patients as estimated, a figure that is in agreement with those published earlier.^{4–6}

Genetic fitness of NF1 (0.48) had decreased to about half of the expected, the effect being more marked in males (0.24) than in females (0.72). Similar reductions have been reported by Crowe *et al*,²⁴ Huson *et al*,⁴ and Samuelsson,⁶ who attribute them partly to biological factors and partly to non-biological factors, such as selection against affected subjects marrying.

The mutation rate for the *NF1* gene, $4.37 \pm 0.72 \times 10^{-5}$, is comparable to the published rates (table 2), and confirms the very high mutation rate of this gene. The observation of a birth order effect in new mutation cases (2.9 ± 1.7), showing that later born sibs are more likely to be affected, suggests that parental age has an effect on the mutation rate. The mean paternal age in the cases with a new mutation was significantly higher than in the general population, as also observed by Sergeev,²⁵ Riccardi *et al*,³⁴ Bunin *et al*,³⁵ and Takano *et al*,³⁶ while Borberg,²³ Samuelsson,⁶ Huson *et al*,⁴ Clementi *et al*,⁵ Rodenhiser *et al*,²⁶ and Jadayel *et al*²⁷ did not report any significant effect of paternal age. The observation of significantly increased maternal age is exceptional and has been reported previously by Riccardi *et al*.³⁴ The present study population is, however, too small for a definitive answer to the parental age effect.

More than 246 mutations involved in *NF1* have been reported by the NF1 Genetic Analysis Consortium up to November 1997, 45% of them deletions.³⁸ Our intragenic linkage studies pointed to two cases with a deletion, 3% of those investigated. The small sizes of the families and the low number of families containing several generations, the non-clustering of the cases, and the absence of disequilibrium in linkage studies rule out any founder effect for NF1 in northern Finland. Observations in other population based NF studies are similar and confirm the findings of small family size and few generations.^{4,6} In the familial cases examined by linkage study here, six out of seven of the first affected subjects in the family had inherited the mutation from the father, a phenomenon which has been shown in 34 out of the 37 published cases (92%) including our data.^{37,39,40}

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Genetic registers in clinical practice: a survey of UK clinical geneticists

EDITOR—Genetic registers have now been in use in the United Kingdom for nearly 30 years,¹ although they are not widespread in Europe.² They are an integral part of most UK medical genetics services³ and yet their functions vary from centre to centre. Many registers were originally developed for research purposes, often in connection with one specific inherited disease,⁴ while others, designed for service use, may cater for many genetic disorders. The WHO report of 1969 suggested that a list or register of

pedigree data should be maintained by each genetic centre,⁵ although the purpose of the list was not specified. In its 1972 report,⁶ the WHO recommended setting up of family orientated genetic registries as part of a system to provide counselling and diagnostic services, treatment, and long term follow up for patients with genetic disorders. In 1978, the definition of genetic register functions was clarified by Emery *et al*,¹ who suggested five main roles, which are not mutually exclusive. These were the clinical or therapeutic role (follow up and recall), the reference list, to monitor outcomes of service provision, to act as a research tool, and to assist in the prevention of genetic disease through complete ascertainment and family follow up. Since that time, the use of genetic registers for family

follow up at predetermined times has been advocated to inform younger family members of their genetic risks when they reach maturity⁴ or to carry out interval screening for complications of genetic disorders, such as in Marfan syndrome⁷ or the familial cancers.⁸ Discussion with colleagues suggested that a diversity of practice in the use of genetic registers in different UK genetic centres has arisen, perhaps because of differing funding priorities. This could raise quality issues in clinical genetics, as members of a single family attending different genetic centres may experience a different service from each centre's genetic register. Expectations raised in one centre may not be translated into service at another. To clarify the use of genetic registers in the UK, and to inform the debate as to whether there should or could be an agreed quality standard for genetic registers, we carried out two questionnaire based surveys. The first was designed to ascertain the nature of registers then in use, and the second to ascertain the views of the UK clinical genetics community about what genetic register services should be provided. In these, we considered issues corresponding to the first, second, and fifth roles of a genetic register of Emery *et al*,¹ but did not consider a register's potential role in monitoring service outcomes, nor its use as a valuable research tool.

The first questionnaire was addressed to each UK genetic centre in 1995 and asked for details of registers in clinical use (purely research registers were excluded), staff employed to support them, and precautions taken to maintain data security. Twenty out of 22 questionnaires were returned (91%). Responses to a second, anonymous questionnaire were sought from all members of the UK Clinical Genetics Society of consultant level or equivalent seniority in 1997. Fifty eight replies were received from a possible total of 77 (75%). Two questions asked for a description of a genetic register and its function, while a further 20 questions took the form of statements about genetic registers. These aimed to elicit opinion on the following issues: (1) what is the purpose of a genetic register, (2) how do patients get onto a genetic register, (3) what data should be stored, (4) should there be separate registers for each genetic disease, (5) what form of consent is required for recording details on a register and, (6) who is responsible for the function of the genetic register. Respondents were asked to grade their opinions of the statements on a five point scale, corresponding to "strongly agree", "agree", "no opinion", "disagree", and "strongly disagree". For issues where there was consensus, the responses "strongly agree" and "agree", and "strongly disagree" and "disagree" were added together to simplify presentation of results. Space was available on the questionnaire for additional comments about some of the statements. A computerised family based register was in general clinical use in 18/20 centres. On average, these registers contained data on 17 700 individual patients (range 3000-48 000) in 6050 families (range 5000-16 000). Sixteen centres maintained disease specific registers (DSR) (table 1). Clinical patient data were integrated with clinical laboratory data in 10 centres. Nine centres employed staff primarily to maintain their registers. In four, the staff were medical (average 27 hours per week), in six nursing (average 34.5 hours per week), and in five secretarial (average of 21.5 hours per week). The majority of this dedicated staff time (80.5%) was within three centres. In four centres, the genetic registers were on stand alone computers and the remainder were on a local area network (LAN). One department's register was part of a general hospital network. None was internet accessible. Twelve used the main database computer for purposes other than running the register. Fourteen felt that access to the computers was physically secure. Thirteen used some form of password

Table 1 Disease specific registers in the UK

Disease	No of centres
Huntington's disease	14
Familial cancers	12
Muscular dystrophies	11
Fragile X syndrome	4
Marfan syndrome	3
Neurofibromatosis	2
Adult polycystic kidney disease	2
Chromosome translocations	1
Other	2

protection at machine start up, all used a password at application start up, but only seven changed either password regularly. Only one centre used any form of data encryption. All departments had regular data back up systems although there was considerable variation in the frequency that back ups were carried out.

For the overwhelming majority of clinical geneticists responding to our questionnaire, the primary purpose of a genetic register was to facilitate patient management (Emery's "clinical and therapeutic role"), although one out of 52 thought a register should be regarded only as a research tool. The role of a register as a reference list of diagnostic information for relatives was supported by 46/58 (79%), but there was also strong support for the active role of registers in family follow up. A total of 49/58 (84%) thought registers should be used to recall affected patients for interval clinical screening, and 48/56 (86%) supported recall of patients to update them on new developments. A total of 50/56 (89%) supported the recall of children at risk when they reach the age of maturity (16 years in the UK) to offer genetic counselling.

Although there was no consensus in response to specific questions about whether registers should actively attempt complete ascertainment or rely only on referrals to the genetic service (fig 1), responses to other questions suggested that, in practice, most registers rely on referrals.

Fifty two out of 58 respondents (90%) thought that registers should not be restricted to information about affected patients but should also include information about at risk relatives (53/58 or 91%). Most (51/58 or 88%) believed that registers should record laboratory diagnostic information about affected subjects (for example, mutation results, karyotypes), and similar information about carriers of autosomal or X linked recessive disorders and chromosome rearrangements (48/58 or 83%). There was strong support for recording of identifying information about children at risk of developing genetic disorders (52/57 or 91%), but slightly less support for recording children at risk of being a carrier of a recessive disorder or balanced chromosome rearrangement (39/55 or 71%).

Opinions differed concerning disease specific registers, consent, and continuing care of register families (fig 2). There was no consensus as to whether registers should

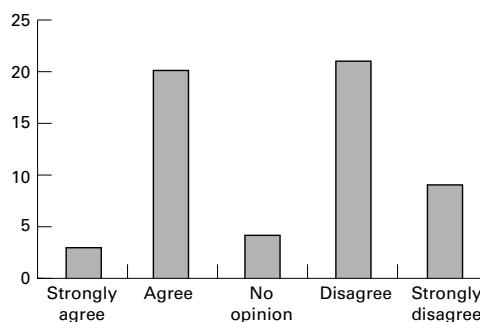


Figure 1 Responses to the statement, "A genetic register should aim for complete ascertainment of genetic disease within the catchment area of the genetic centre".

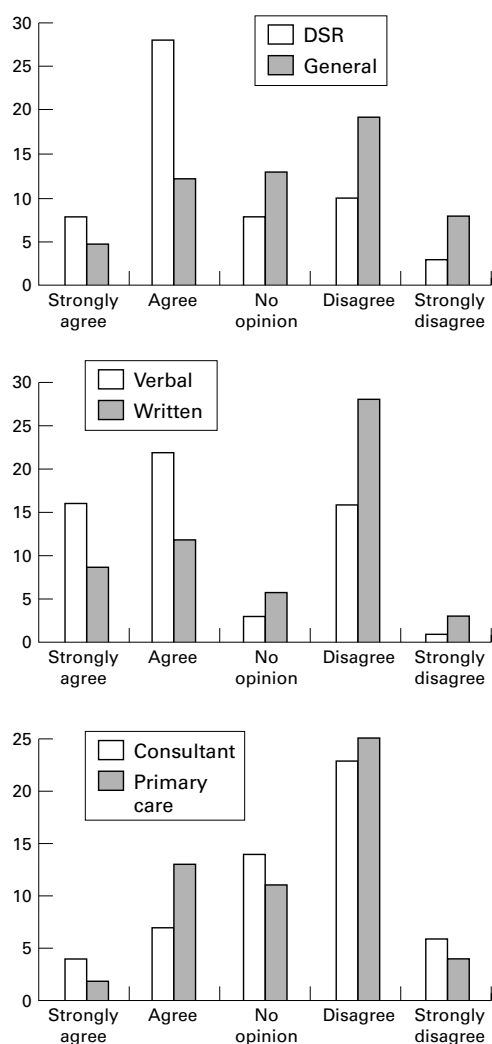


Figure 2 Issues with no consensus. (Top) Should a genetic centre maintain disease specific registers or a more general genetic register? (Middle) Verbal or written consent should be obtained before recording a patient's details on a genetic register. (Bottom) Continuing care of genetic register families should be the responsibility of the clinical genetics consultant or the primary care physician.

record information about one disease only (disease specific registers or DSRs) or whether they should be generic for all genetic disorders referred to the genetics service. There were differences of opinion on the issue of obtaining informed consent. A majority (34/52 or 65%) support seeking verbal consent, but a significant number (16/52 or 31%) oppose this, and the distribution of responses for written consent is more evenly balanced. Having indicated a desire for genetic registers which are involved actively in the management of families with genetic disease, it is interesting to note the dissension from the view that either clinical geneticists or primary care physicians should be mainly responsible for continuing care of such families.

It is clear from the original survey in 1995 that there were at that time considerable differences in resources allocated to operating genetic registers in different centres in the UK, and it seems likely that this would result in different levels of service to patients. The 1997 survey suggests that, in many respects, there is a consensus about genetic register functions, and it seems unlikely that the issues over which there is no agreement (consent, DSRs or generic registers, and responsibility for continuing care) would result in such disparity of resource allocation. The interventionist nature of the genetic register function sup-

ported by the UK clinical geneticists raises concerns about the issues for which there is no consensus (such as consent to be included), and in relation to how such functions can reasonably be supported within the resources available to most centres.

From the responses to the second questionnaire in which there was general agreement, it would be quite possible to draw up a specification for a genetic register service that is seen as desirable by the UK clinical genetics community. It is clear that although a genetic register should function as a reference list of family clinical and laboratory information (Emery's second role), it is also thought desirable for it to have a wider function in the organisation of interval review and follow up of family members (Emery's first role). This is to facilitate timely clinical screening and support of those affected by or at risk of genetic disease, to update family members when new information about their family disease becomes available, and to recall children at risk when they reach maturity. This also contributes to Emery's fifth role (prevention of genetic disease). Thus, a genetic register should comprise a list of people affected by, or at risk of genetic disease, linked as families, and linked to a diagnostic index. The register should include facilities to remind clinical genetics staff to consider further contact with the family under a variety of predetermined circumstances, which could include clinical screening protocols, the occurrence of medical advances, or the attainment of a particular age by a family member.

It is not agreed that registers should attempt complete ascertainment (the first part of Emery's fifth role) and it is clear that most registers do not actively pursue this goal in practice. Perhaps this reflects concern about non-directiveness in the application of genetic services, but it may also reflect the way in which genetic services have outgrown their resources in recent years. The issue of whether registers should be disease specific or general is probably relatively unimportant, as it should be possible to devise software which can handle different follow up protocols or review prompts within the same database system, effectively providing disease specific registers within the framework of a general register. The general register approach should reduce the resource implications of genetic registers, as the maintenance of several DSRs can lead to duplication, and reduced efficiency in responding to enquiries, if it is not immediately apparent which DSR might include a particular family's details. The issue of greatest importance is probably that of informed consent, and this is particularly so in the light of the expressed desire to include details of family members (including children) at risk on the genetic register. The problem of responsibility for continuing care follows on from this.

The Nuffield Council Report on genetic screening⁸ considered that (living) subjects must give informed consent before their information is stored on a genetic register. We believe that from a legal viewpoint this consent need not be written, although the fact of verbal consent should be recorded for the protection of patient and doctor alike. The need for informed consent has three specific implications. Firstly, to be clinically useful as a reference source of family information, a genetic register should record details of all affected subjects known to the genetic centre. If a person refuses consent to be on a register, or if access to a person to obtain consent is not possible for reasons such as confidentiality, this may create problems in recording information necessary to define follow up arrangements made through a genetic register for his or her relatives. However, it would be unreasonable to expect to obtain consent to record the details of every person mentioned in a pedigree chart which forms part of a conventional paper based genetic record and, by analogy, family history information

which is recorded to inform another person's genetic register record may not require specific consent. Secondly, where a DSR is in use clinically but is derived from a research based register, further consent may be required for this altered use of the recorded data. Thirdly, where a child's details are recorded on the register, a parent or guardian may give informed consent. However, it would seem logical that when a child reaches the age of 16, he or she must give his or her own informed consent for his or her details to remain on the register, unless these details are merely part of the family history information recorded about another person. Therefore, the recording of information about younger family members at risk on the genetic register to facilitate recall when they reach maturity may create a legal obligation to contact these people at the age of 16. It is of course only necessary to take "reasonable steps" to contact the child when he or she reaches maturity, but it would seem important that the information given to parents as part of the consenting process should include details of any intention to make contact when the child reaches 16 years of age. It should be clearly stated that this will be facilitated if the family ensures that the genetic centre is made aware of any change of address.

Informed consent also implies transmission of knowledge about why recall is recommended for a particular disease. Reasons might include the risk of developing the disease in the future, the risk of developing complications (such as cancer in familial cancers, or aortic dilatation in Marfan syndrome), the possibility of preventative screening (for example, mammography, echocardiography), the possibility of predictive genetic testing, or the possibility of transmitting a genetic disorder to offspring. Future genetic and medical interventions might allow the avoidance of some adverse outcomes. The possibilities in all of these areas will vary between disorders, and therefore the case for recall and review may be different for different disorders. Other medical specialties may have active follow up clinics for some disorders in one area of the country, but not in another. It is therefore likely that there will be some variation in the clinical need for this aspect of genetic register function for any particular disorder throughout the country. In order to avoid the problem where some members of a family attend a genetic centre with a register with a review policy, while others from the same family attend a centre whose register does not have such a policy and therefore have false expectations of the service available, it

is essential that the information given to the family about the genetic register for the purposes of informed consent should state clearly the intended consequences of recording information on the genetic register, whether follow up is advised, and whether the genetic centre intends to offer active follow up. This could take the form of a supplementary letter to a general information leaflet.

If a genetic register is set up with a review policy for a particular disease, this implies a responsibility for continuing care of a family over time, with the proviso of taking "reasonable steps" to maintain contact as discussed above. Despite the fact that 83-88% of UK clinical geneticists believe that recall of families for updating information or screening is an important function of the genetic register, only 10/35 (29%) believe that this follow up is the responsibility of the clinical geneticist. Interestingly, only a slightly higher proportion believe that it is the responsibility of the general practitioner (15/39 or 38%). North American genetic service providers (physician geneticists, PhD geneticists, and genetic counsellors) expressed a similar opinion in a recent survey, with only 46% agreeing that a "duty to recontact" should be the standard of care.¹⁰ Causing patient anxiety, the burden on staff time, and the fear of litigation were cited as possible burdens of a recontact policy. North American geneticists also considered that primary care physicians could share the responsibility, but might not be very effective. Passing responsibility for recontact to the patient was the most popular option. Unfortunately, our UK questionnaire did not ask as an open question, who should take primary responsibility for maintaining contact. Like our North American colleagues, we believe that some responsibility must devolve on the family. If this is the case, then the issue of proper informed consent becomes even more critical to the satisfactory operation of a genetic register.

The majority of clinical geneticists in the UK regard the provision of recall and review services through a genetic register as an important part of the function of a clinical genetics centre. Provision of services varies throughout the country, partly for historical reasons, and probably partly because of different priorities in the allocation of scarce resources in different regions. Different members of the same family may attend different genetic centres and make false assumptions about clinical genetics services available unless proper information is given about genetic register functions at each centre. This is particularly important

Table 2 Suggested guidelines for the basic operation of a genetic register

1 The genetic register as a reference list

- (a) The genetic register should contain a reference list of people known to a regional genetics service, linked as families, and linked to a diagnostic index.
- (b) The genetic register should include relevant laboratory information about the families or people recorded (eg mutation or linkage results, karyotypes, biochemical findings). Care must be taken to ensure the accuracy of data recorded.

2 Which family members should be recorded on the genetic register

- (a) Adults and children affected by disorders with a genetic aetiology.
- (b) Adults at risk of developing a genetic disorder or its complications.
- (c) Children at risk of developing a genetic disorder or its complications.
- (d) Adults who are at risk of transmitting a genetic disorder to their children (eg a carrier of an autosomal or X linked recessive disorder or of a balanced chromosome rearrangement).
- (e) Children who are at risk of transmitting a genetic disorder to their children (eg a carrier of an autosomal or X linked recessive disorder or of a balanced chromosome rearrangement).

3 Review and recall function of the genetic register

- (a) To prompt recall for review of adults on the genetic register at predetermined intervals, for clinical screening of those at risk of complications of genetic disorders, or to update families on recent medical or scientific developments. The interval set for recall will vary between diseases depending on clinical circumstance and between genetic centres depending on other local service provision.
- (b) To prompt recall for review of children on the genetic register at predetermined intervals, as for adults, or when the child reaches maturity, to offer genetic counselling and further follow up.

4 Informed consent and the genetic register

- (a) Adults should give informed verbal consent for their details to be recorded on the register. The fact of this consent should be recorded.
- (b) Parents or guardians may give informed consent on behalf of children. Children should be given the opportunity to give or withhold their own consent when they reach maturity.
- (c) The purpose of the register should be explained clearly. It should be made plain whether regular follow up through the genetic register is intended, the frequency of the follow up, and the reason for follow up. It should be made clear how much of the responsibility for facilitating this follow up rests with the family, for example, by informing the genetic centre of changes of address, or by recontacting at defined intervals.
- (d) The use of a printed information sheet is suggested as a reasonable means of fulfilling this part of the process of consent, within the limited resources available to most centres. A tear off consent form could be included as part of this information sheet. A supplementary letter describing aspects of the register specific to the patient and the family disorder may be useful.

where there is an intention to offer genetic counselling to children at risk when they reach maturity. The use of a printed information sheet or letter to facilitate informed consent, including details of services offered for genetic disorders, the follow up intentions of the genetic centre, and the need for family members to keep the genetic register informed of change of address would help to resolve this issue. Based on the findings of our two questionnaires, and consideration of their implications, it is possible to draw up guidance about the minimum genetic register function considered important by UK geneticists and its consequences (table 2). Our questionnaires were addressed only to senior physician geneticists in the UK, but genetic registers are increasingly operated and maintained by genetic nurses or associates, and it would be most important to seek their views on the conclusions and implications of this survey. Further discussions involving physicians, genetic nurses and associates, and the families themselves might help to clarify those issues without consensus. As with all clinical services, genetic register functions should be kept under review as service intentions and practices may change in the light of future clinical and scientific developments.

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