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A novel locus for brachydactyly type A1 on chromosome 5p13.3-p13.2

C M Armour, M E McCready, A Baig, A G W Hunter, D E Bulman

The brachydactylies are a group of inherited disorders characterised by shortened or malformed digits that are thought to be the result of abnormal growth of the phalanges and/or metacarpals. First classified by Bell into types A, B, C, D, and E, they were reclassified by Temtamy and McKusick[1] and Fitch.[2] Brachydactyly type A1 (BDA1, MIM 112500) is characterised by shortened or absent middle phalanges. Often the second and fifth digits, as well as the first proximal phalanx, are the most severely affected. In addition, all of the small tubular bones tend to be reduced in size and the metacarpals may be shortened, particularly the fifth metacarpal. Radial/ulnar clinodactyly, as well as malformed or absent epiphyses, have also been reported.[1][2] Complex syndromes have been described in which BDA1 is one of a number of manifestations.[3]

Recently, genetic linkage for a BDA1 locus has been reported to map to 2q35-q36 in two unrelated Chinese families.[4] Subsequent sequence analysis identified mutations in the Indian Hedgehog gene (*Ihh*) in affected subjects.[5] There have been no other reports of linkage for BDA1, although identification of a balanced translocation between 5q11.2 and 17q23 in a girl with Klippel-Feil anomaly and BDA1 suggests that there may be a BDA1 locus on either chromosome 5 or 17. Mastrobattista *et al*[6] examined a number of candidate genes, including *HoxD*, *Msx1*, *Msx2*, *FGF1*, and *FGF2*, in two families with BDA1, but did not find evidence of linkage. Genes involved in two of the other types of brachydactyly have been described. Mutations in *CDMP1*, a member of the TGF-β superfamily, have been found in a variant of autosomal dominant brachydactyly type C in which the middle phalanges of the second, third, and fifth fingers are shortened.[7] Mutations in this gene also cause Hunter-Thompson and Grebe acromesomelic dysplasias, two autosomal recessive conditions.[8] In addition, dominant mutations in *ROR2*, an orphan receptor tyrosine kinase, have been shown to cause brachydactyly type B.[9][10]

We have recently described a three generation family with mild BDA1,[3] in which 13 affected subjects exhibited shortened middle and distal phalanges, proximal first phalanx, and fifth metacarpal. Consistently, the middle phalanges of affected members were below 2 SD of age matched norms. Most of the proximal first phalanges and fifth metacarpals of affected subjects were similarly 2 SD below the norms. Affected members also tended to be of short stature. The children who were studied had coned and prematurely fused epiphyses. Several members, such as the proband, had clinodactyly of one or more digits. A number of subjects also had a broad distal hallux and/or broad, slightly adducted forefoot. Since our initial report, we have ascertained an additional 15 family members including nine affected subjects. The phenotype of these additional family members is similar to those subjects in the family already described, and no additional clinical findings were associated with BDA1 in this family. We now report linkage of BDA1 in this kindred to a novel locus on chromosome 5.

METHODS

The linkage study comprised 34 members including 20 affected subjects and was conducted after approval by the Children's Hospital of Eastern Ontario Ethics Review Committee.

Peripheral blood samples were taken with informed consent from all participating family members, and a standard protocol was used to isolate DNA. A genome wide scan was initiated using 36 primer sets from the MAPPAIRS™ microsatellite markers (Research Genetics, Huntsville, Alabama), encompassing markers from 16 chromosomes. Particular emphasis was placed on markers from chromosome 5 and 17, based on the report by Fukushima *et al*[11] describing a translocation between 5q11.2 and 17q23 in a girl with Klippel-Feil anomaly and BDA1. Additional markers from Marshfield's sex averaged genetic map were examined. These included three markers from chromosome 17 (D17S1301, D17S1290, and D17S1303) and 14 markers from chromosome 5 (table 1). Each of the loci examined were individually amplified in 10 μl PCR containing 10 mmol/l Tris HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 100-200 ng genomic DNA, 0.2 mmol/l dNTP, 0.12 μmol/l M13 tailed forward primer, 0.12 μmol/l reverse primer, 0.12 μmol/l IRD-700 labelled M13 forward(-29) primer (LI-COR, Lincoln, NE), and 1 Unit *Taq* enzyme. DNA was amplified over 16 cycles at 94°C for 30 seconds, 66-50°C for 30 seconds (−1°C per cycle), and 72°C for 30 seconds, followed by 23 cycles at 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. PCR products were size separated on 6% acrylamide gels, using the LI-COR DNA sequencer Model 4000 (LI-COR, Lincoln, NE) and analysed using RFLPscan software (version 3.0). Haplotypes were subsequently generated using Cyrillic software (version 2.1).

Two point lod scores were calculated using MLINK, ILINK, and LODSCORE from the FASTLINK version[12][13] of the LINK-AGE software package.[14] The LINKMAP program from the same software package was used to calculate location scores for multipoint linkage analyses. All lod and location scores were calculated using an autosomal dominant model, penetrance of 100%, and a disease frequency of 0.000001. Equal recombination frequencies between males and females were assumed.

RESULTS

Initial genotyping and linkage analysis of markers from chromosome 17 failed to yield evidence for linkage in the pedigree (data not shown). Lod scores greater than 3.0, however, were observed for two consecutive markers, D5S2848 and D5S1506, on chromosome 5p. Additional markers around this region were subsequently analysed, providing a maximum lod score of 6.91 at D5S477 (recombination fraction (θ) = 0.00) (table 1). Haplotype analysis provided evidence for an 11 cM critical region that cosegregates with the disease (fig 1). The distal end of this critical region was defined by an inferred recombination event in II.9 between markers

Table 1 Lod score calculations for microsatellite markers on chromosome 5

Marker	Map position	Lod score at θ =							Zmax	θmax
		0.0	0.01	0.05	0.1	0.2	0.3	0.4		
D5S817	22.88 cM	−∞	−2.68	0.43	1.43	1.85	1.53	0.81	1.85	0.19
D5S2846	33.04 cM	−∞	−0.02	1.73	2.19	2.14	1.61	0.81	2.26	0.14
D5S2845	36.25 cM	0.60	0.58	0.52	0.43	0.27	0.13	0.03	0.60	0.00
D5S2848	39.99 cM	0.51	2.42	3.43	3.53	3.05	2.20	1.10	3.54	0.09
D5S819	41.06 cM	1.46	5.34	5.58	5.27	4.26	2.96	1.45	5.60	0.04
D5S1986	44.73 cM	4.19	4.10	3.77	3.35	2.49	1.59	0.69	4.19	0.00
D5S477	45.34 cM	6.91	6.79	6.33	5.73	4.43	3.01	1.45	6.91	0.00
D5S111	47.09 cM	5.51	5.41	5.03	4.53	3.47	2.33	1.08	5.51	0.00
D5S1506	49.54 cM	3.22	3.16	2.90	2.57	1.89	1.18	0.46	3.22	0.00
D5S663	51.99 cM	1.48	5.37	5.60	5.30	4.28	2.98	1.46	5.62	0.04
D5S1457	59.30 cM	−∞	−0.57	1.81	2.47	2.51	1.90	0.95	2.61	0.15
D5S2500	69.23 cM	−∞	−5.59	−2.31	−1.08	−0.17	0.07	0.05	0.09	0.68
D5S1501	85.25 cM	−∞	−4.70	−1.47	−0.32	0.43	0.52	0.34	0.53	0.28
D5S1725	97.82 cM	−∞	−11.36	−5.36	−3.01	−1.04	−0.24	0.04	0.06	0.44
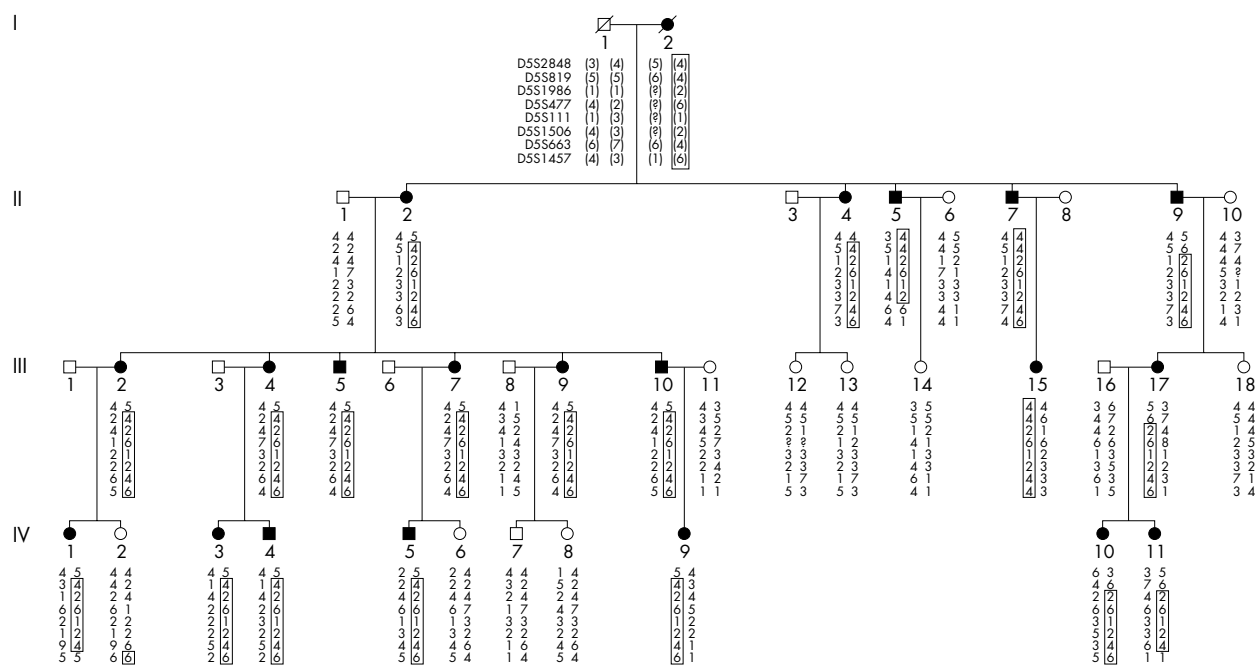
Figure 1 Haplotype analysis of polymorphic markers from chromosome 5p in a family with mild brachydactyly. Filled symbols represent affected subjects. The disease haplotype is highlighted by the open boxes around the haplotypes.

D5S819 and D5S1986. The proximal boundary was further defined by an inferred recombination in II.5 between markers D5S1506 and D5S663. The location of the disease locus within the region between D5S819 and D5S1986 was further supported by multipoint linkage analysis (data not shown). As our initial linkage results were obtained before the report of Yang *et al*,[4] the BDA1 locus at 2q35-36 was not evaluated for exclusion.

There is a discrepancy between the position of our locus and the reported translocation breakpoint found in the girl with BDA1 and Klippel-Feil anomaly.[11] If this girl's features are the result of a disruption in a gene at 5p13.2-13.3, the translocation may also be associated with a more complex, cryptic rearrangement. Similar phenotypic characteristics between the girl reported by Fukushima *et al*[11] and our kindred include brachydactyly type A1 and wide spacing of the first and second toes. Although not reported, the radiographs in Fukushima *et al*[11] also appear to show shortened fifth metacarpals, a feature also found in our kindred.

DISCUSSION

In this paper, we report a family with a relatively mild form of BDA1. Affected subjects were characterised by short middle phalanges of digits 2 to 5, a hallmark feature of BDA1, but lacked traits common in more severe forms, such as the absence of middle phalanges and terminal symphalangism.[3] Other common findings in this family included shortened distal phalanges, a short fifth metacarpal, malformed epiphyses, and clinodactyly. Previous reports of families with brachydactyly have shown that subjects can inherit the disease as an isolated malformation[15–18] or in association with other disorders. Slavotinek and Donnai[19] described a boy with severe brachydactyly, valgus deformities, developmental delay, nystagmus, and scoliosis. Scoliosis was also described in a family with BDA1 and degenerative arthritis owing to discoid menisci,[20] and in a family with brachydactyly, tall stature, and foot deformities.[21] In the examination of affected members described in this report, there were no features observed other than BDA1.

We have identified a second BDA1 locus at 5p13.2-13.3 in a Canadian kindred, indicating that BDA1 is genetically heterogeneous. A previous report by Yang *et al*[4] identified a BDA1 locus on chromosome 2q35-36 in two Chinese families. The families, while linked to the same region on chromosome 2, did not have a common haplotype within the defined interval. Our kindred had features similar to the families described by Yang *et al*[4] although some differences in phenotype were noted. Many members in their study were completely lacking middle phalanges, a phenotype that was not observed in our family. Similarly, some subjects had shortened third metacarpals while this was not a marked feature in our affected patients. As originally described by Fitch,[2] all hand bones may be proportionally reduced in size. For affected members in our kindred the distal and proximal phalanges as well as the metacarpals were often shortened below 2 SD compared to age related norms. The first proximal and fifth metacarpal were also frequently short.[3] Yang *et al*[4] described a number of features within their family that were apparently beyond Fitch's description, such as severely shortened distal phalanges, shortened third metacarpals, and shortened proximal phalanges of the fifth digit. These differences are not surprising given that different loci have been implicated.

Despite being the first disease described in terms of Mendelian autosomal dominant inheritance, a gene that causes BDA1 had not been identified until recently. During the preparation of this manuscript, Gao *et al*[5] reported mutations of the *Ihh* gene in three Chinese families with BDA1. Having excluded *PAX3* as the cause of the disease, Gao *et al*[5] examined *Ihh* based on its position within the critical region on chromosome 2q35-36, and its involvement in regulating chondrocyte growth and differentiation during endochondrial ossification.[22 23] Three different point mutations were identified, each causing amino acid substitutions in a portion of the protein predicted to interact with the Patched receptor.[5] These mutations probably cause BDA1 through a haploinsufficiency of the wild type Ihh, resulting in decreased chondrocyte proliferation and attenuation of the negative feedback loop that regulates chondrocyte maturation during endochondrial ossification. Although *Ihh* mutations have been identified in people with BDA1, we are not aware of any genes within the chromosome 5 critical region that fit in the Ihh pathway. Despite this, there are a number of candidate genes in the 11 cM region in which we have found linkage. Two prime candidates include cadherin-6 and *Npr3*.

Cadherin-6 is part of a superfamily of proteins that mediate cell-cell interactions, some of which have been previously implicated in osteoclast differentiation.[24–27] Experiments with dominant negative cadherin-6 isoforms suggest that cadherin-6 may be necessary for osteoclast differentiation by mediating heterotypic interactions between stromal cells and pre-osteoclast cells during osteoclast differentiation.[28] This effect has not yet been shown in vivo.

Npr-3, a receptor involved in natriuretic peptide clearance, has been reported to be involved in mouse skeletal development. Homozygote *Npr3* mutated mice have increased body lengths and longer digits because of aberrant proliferation in vertebral growth plates and delayed forepaw ossification.[29] The delay was most marked in the hind and fore paws and was most noticeable at the extremities of the metacarpals and phalanges.

Our kindred show premature fusion of growth plates as well as malformed epiphyses resulting in clinodactyly. Fitch described BDA1 as "proportionate dwarfing of limb bones with the middle phalanges most affected as these are the last to ossify".[2] These patterns are very nearly the complete opposite to those seen by Jaubert *et al*.[29] While *Npr3* is implicated in an overgrowth syndrome, it is conceivable that a different mutation resulting in up regulation could give rise to the BDA1 phenotype seen in our kindred.

In summary, we have shown that BDA1 is linked to an 11 cM critical region on chromosome 5p13.2-13.3 in a four gen-

eration family affected with the disease. As previous studies have shown linkage of BDA1 to chromosome 2q35-36,[4] linkage to a different locus in this study suggests that BDA1 is genetically heterogeneous. Further analysis of the 11 cM region is necessary to identify the gene involved.

ACKNOWLEDGEMENTS

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A Val227Ala polymorphism in the peroxisome proliferator activated receptor α (*PPARα*) gene is associated with variations in serum lipid levels

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Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors that belong to the nuclear receptor superfamily.[1 2] PPARs regulate gene transcription by heterodimerising with retinoic X receptors and binding to DNA sequences, termed PPAR response elements (PPRE), in the promoters of target genes.[2 3]

Three different PPAR genes (α, δ/β, and γ), each displaying distinct tissue and developmental expression patterns, have been identified.[4 5] *PPARα*, the first member of the PPAR family to be identified, was cloned as an orphan receptor activated by agents that induce peroxisome proliferation.[6] It is expressed primarily in tissues with high levels of fatty acid oxidation, such as those in liver, kidney, heart, and muscle.[4 6] Most target genes of PPARα encode enzymes involved in oxidation of cellular fatty acids, lipid transporters, and apolipoproteins.[1 7] Furthermore, PPARα is known to mediate the actions of fibrates, which are hypolipidaemic drugs that decrease plasma triglycerides and increase high density lipoprotein (HDL) cholesterol concentrations.[8 9] Numerous in vivo and in vitro studies have suggested that PPARα is a key metabolic regulator involved in lipid and glucose homeostasis.[1 10–12] Recently, a Leu162Val polymorphism was identified in the *PPARα* gene, and this polymorphism was shown to influence plasma lipid concentrations, especially in type II diabetes patients.[13 14] In the present study, we have screened for polymorphisms in the *PPARα* gene to determine whether genomic variations in this gene influence serum lipid levels, glucose levels, and body mass index (BMI) in healthy subjects.

SUBJECTS AND METHODS

Taking part in this study were 401 unrelated and apparently healthy subjects (207 men and 194 women) who visited medical clinics in Tokyo for routine medical check ups. They were all Japanese. They ranged in age from 29 to 75 years with a mean age (SD) of 47.7 (SD 9.1) years. The age range of the subjects is broad because we selected them without consideration of their age. The clinical characteristics and serum lipid and fasting glucose levels of the subjects are shown in table 1.

People with medical histories that included liver or renal abnormalities or diabetes mellitus and people taking medication known to affect lipid metabolism were excluded from the study. Blood samples were taken after an overnight fast. Informed consent was obtained from all subjects and the study was approved by the Ethics Committee of the University of Tsukuba.

Serum total cholesterol, HDL-C, triglycerides, and glucose levels were measured by standard enzymatic methods as described elsewhere.[15] Serum LDL cholesterol (LDL-C) levels were estimated according to Friedewald's equation.[16] Lipid and glucose levels are presented as mg/dl.

The genomic structure of the *PPARα* gene was deduced through a BLAST search of GenBank (Accession numbers NM_005036 and AL032818). Potential mutations in coding exons and exon-intron boundaries of the *PPARα* gene were screened in 48 randomly selected subjects by single strand conformation polymorphism (SSCP) analysis with the GenePhor System (Amersham-Pharmacia-Biotec). The genotype of the Val227Ala (T→C, nt 892 in NM_005036) polymorphism was assessed by PCR restriction fragment length polymorphism (RFLP). PCR primers were 5′-CCCTCCAAACCCTA GCGATTCGTT-3′ and 5′-CTGGTTCCATGTTGCCAAGAGAAC-3′, and the annealing temperature for amplification was 55°C. The 266 bp PCR product was digested with 2 units of *Sau*96I (New England BioLabs). The Val227 allele was resistant to digestion, whereas the Ala227 allele was digested into 185 bp and 81 bp fragments. The accuracy of this genotyping method was confirmed by sequence analysis of 10 randomly selected samples. The Leu162Val polymorphism of the PPARα gene was analysed with the method described by Vahl *et al*.[14] To avoid genotyping errors, we repeated the analysis twice.

Differences in lipid levels owing to the genotype of the Val227Ala polymorphism were analysed by multiple linear regression analyses incorporating age and BMI as covariates. Differences in fasting glucose levels and BMI owing to the genotype of the Val227Ala polymorphism were analysed by multiple linear regression analyses incorporating age as a covariate. A p value of <0.05 was considered statistically

Table 1   Clinical characteristics and serum lipid and glucose levels of the subjects

	Total subjects	Men	Women
Numbers of subjects	401	207	194
Age (y)	47.7 (9.1)	49.2 (8.9)	46.2 (9.1)
Total cholesterol (mg/dl)	199.2 (34.1)	195.5 (33.1)	203.2 (34.8)
HDL cholesterol (mg/dl)	53.8 (16.1)	46.5 (13.2)	61.5 (15.3)
Triglycerides (mg/dl)	105.4 (62.1)	121.7 (63.7)	88.1 (55.5)
LDL cholesterol (mg/dl)	124.4 (32.0)	124.7 (31.9)	124.0 (32.2)
Fasting glucose (mg/dl)	92.2 (7.7)	92.6 (7.7)	91.7 (7.7)
BMI (kg/m^2)	22.6 (3.0)	22.8 (2.5)	22.3 (3.4)

Values shown are mean (SD).
BMI=body mass index.

significant. Statistical analyses were performed with the JMP software package (SAS Institute).

RESULTS AND DISCUSSION

A missense polymorphism, Val227Ala (GTC→GCC), was identified in the *PPARα* gene by SSCP analysis followed by sequence analysis. Of 401 subjects, 362 were homozygous for the Val227 allele, 37 were heterozygous for the Val227 and Ala227 alleles, and two were homozygous for the Ala227 allele. The distribution of these genotypes did not deviate from Hardy-Weinberg equilibrium.

Table 2 shows the mean values for serum lipid levels, fasting glucose levels, and BMI according to the *PPARα* genotype for both men and women and total subjects. Because only two subjects were homozygous for the Ala227, we classified the subjects into two groups, carriers of the Ala227 allele and non-carriers.

Sex, age, and BMI affect serum lipid levels; therefore, we examined the relationship between the Val227Ala genotype and lipid levels separately in men and women, and p values were calculated after adjustments for age and BMI by multiple linear regression analysis. It is also known that dietary habits, exercise, smoking, and alcohol intake influence serum lipid levels, but we could not obtain information regarding such factors for our subjects.

Among women, the mean serum total cholesterol and triglyceride levels in carriers of the Ala227 allele were significantly lower than those in non-carriers (p=0.046 and p=0.038,

respectively). The mean LDL-C level in carriers of the Ala227 allele was lower than that in non-carriers; however, the difference was not significant (p=0.066). In men, a similar trend was observed, but the difference was not significant. Mean HDL-C, fasting glucose levels, and BMI did not differ significantly between the genotype groups in either women or men. In the total subjects, the mean serum total cholesterol level in carriers of the Ala227 allele was significantly lower than that in non-carriers (p=0.033). The lipid profiles of the carriers with the Ala227 allele appear favourable compared with those of non-carriers.

The Val227Ala site is located in the region between the DNA binding and ligand binding domains of the *PPARα* gene, which is also thought to contain the dimerisation domain. It is possible that the substitution of Val to Ala at codon 227 causes a functional change in *PPARα* and that the Ala227 isoform has higher activity than the Val227 isoform. However, we have no experimental evidence to support this hypothesis. It will be necessary to examine differences in the transactivation activities in vitro as a result of this amino acid substitution.

It was reported that phenotypes related to lipid and glucose metabolism are strongly influenced by sex in *PPARα* deficient mice.[17 18] It is assumed that oestrogen signalling plays a role in lipid and glucose homeostasis via regulation of fatty acid utilisation pathways.[18 19] In our present study, the findings suggest that *PPARα* gene polymorphisms affect serum lipid levels, especially in women. Menopause has a significant effect on serum oestrogen and lipid levels in women; however, we could not obtain information regarding the menopausal status

Table 2   Effects of the Val227Ala polymorphism of the *PPARα* gene on the serum lipid and glucose levels and BMI

	Genotype	Total subjects	p	Men	p	Women	p	Women (<45 y)	p
Total cholesterol (mg/dl)	VV	200.7 (34.2) [362]	**0.033**	196.7 (33.5) [191]	0.30	205.2 (34.5) [171]	**0.046**	190.7 (29.0) [83]	**0.023**
	AA+AV	185.3 (30.6) [39]		181.6 (25.0) [16]		188.0 (34.3) [23]		170.1 (22.3) [12]	
HDL cholesterol (mg/dl)	VV	53.6 (16.2) [362]	0.58	46.7 (13.2) [191]	0.17	61.2 (15.8) [171]	0.66	64.1 (15.6) [83]	0.96
	AA+AV	55.6 (15.5) [39]		43.5 (12.4) [16]		64.0 (11.3) [23]		65.4 (9.3) [12]	
Triglycerides (mg/dl)*	VV	107.6 (62.9) [362]	0.26	122.3 (63.9) [191]	0.54	91.1 (57.6) [171]	**0.038**	79.3 (60.8) [83]	0.055
	AA+AV	85.8 (50.7) [39]		114.0 (62.5) [16]		66.2 (28.2) [23]		52.8 (16.1) [12]	
LDL cholesterol (mg/dl)	VV	125.6 (31.8) [362]	0.10	125.5 (32.0) [191]	0.55	125.8 (31.7) [171]	0.066	110.8 (26.9) [83]	0.054
	AA+AV	112.6 (31.2) [39]		115.2 (29.2) [16]		110.7 (33.0) [23]		94.2 (18.7) [12]	
Fasting glucose (mg/dl)	VV	92.4 (7.4) [362]	0.083	92.8 (7.5) [191]	0.27	92.0 (7.3) [171]	0.15	91.4 (7.6) [83]	**0.031**
	AA+AV	89.7 (9.6) [39]		90.4 (9.0) [16]		89.3 (10.2) [23]		86.1 (7.1) [12]	
BMI (kg/m^2)	VV	22.6 (3.0) [362]	0.18	22.8 (2.5) [191]	0.13	22.4 (3.4) [171]	0.48	21.6 (3.8) [83]	0.30
	AA+AV	21.8 (2.6) [39]		21.8 (2.4) [16]		21.8 (2.9) [23]		20.6 (1.6) [12]	

Values shown are mean (SD).
p value for lipid levels was calculated by multiple linear regression analyses incorporating age and BMI as covariates.
p value for fasting glucose levels and BMI was calculated by multiple linear regression analysis incorporating age as a covariate.
For multiple linear regression analysis of total subjects, sex was also incorporated as a covariate.
*Statistical tests for triglyceride levels were calculated on log transformed values.
Statistically significant p values are shown in bold.
Numbers of subjects are shown in square brackets. BMI=body mass index.

of our subjects. For convenience, we selected 95 women under 45 years from our subjects, assuming that most women under 45 years are premenopausal. A stronger association between the genotypes of the Val227Ala polymorphism and total cholesterol levels was observed in women under 45 years than in the total population of 194 women (p=0.023 v p=0.046) (table 2). Such data appear to support the above assumption for a relationship between oestrogen signalling and lipid homeostasis.

Recently, a Leu162Val polymorphism was identified in white populations.[13 14 20] Vohl et al[14] found an association between the Leu162Val polymorphism and hyperapobetalipoproteinaemia in non-diabetic subjects. Flavell et al[13] reported associations between the Leu162Val polymorphism and total cholesterol, HDL-C, and apolipoprotein AI levels in type II diabetic subjects. Furthermore, it was reported that the Val162 isoform increases PPRE dependent transcriptional activity compared with the Leu162 isoform in in vitro transient transfection assays.[13 20] However, we did not find this polymorphism in our Japanese subjects. In contrast, the Val227Ala polymorphism has not been detected in white populations,[13 14 20] suggesting that there is variability in the frequencies of *PPAR*α gene polymorphisms between ethnic groups.

Many association studies of polymorphic markers in candidate genes related to lipoprotein metabolism and interpersonal variations in serum total and LDL cholesterol levels have been reported.[21–23] However, many of the relationships appear to differ between populations with the exception of the apolipoprotein E polymorphism.[21–24] Further epidemiological and genetic studies are needed to understand the relationship between the Val227Ala polymorphism in the *PPAR*α gene and serum lipid levels. It will also be interesting to examine the relationship between responses to fibrates and the Val227Ala genotype of the *PPAR*α gene.

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ATM mutations in Finnish breast cancer patients

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In Finland, mutations in the two major breast cancer susceptibility genes *BRCA1*[1] and *BRCA2*[2] appear to account for a considerably lower proportion of hereditary breast cancer than in other western European countries.[3–5] In addition, the recently identified new susceptibility locus in 13q21 is expected to explain, at most, one quarter of the hereditary predisposition to breast cancer.[6] Inherited breast cancer susceptibility may also be the result of mutations in genes associated with certain rare hereditary syndromes, such as Li-Fraumeni syndrome[7] or ataxia-telangiectasia (AT).[8–10]

AT is a recessive neurodegenerative disorder resulting from mutations in the *ATM* gene.[11] It is characterised by progressive cerebellar ataxia, telangiectasias, sensitivity to ionising radiation, and immunodeficiencies. Furthermore, owing to *ATM* involvement in double strand break repair, defects in its protein function cause genetic instability and, as a consequence, an increased risk of cancer. AT patients are especially prone to developing lymphatic and leukaemic malignancies, but also breast cancer. Also AT heterozygotes have an increased risk of developing breast cancer,[8 10] yet a clear discrepancy remains between the epidemiological data and the observed low frequency of *ATM* mutations in breast cancer patients.[12]

Recently, Laake *et al*[13] screened 41 AT families from the Nordic countries for *ATM* mutations. In seven Finnish AT families included in the study, they observed eight distinct mutations, some of which were recurrent (partly unpublished results).[13] Given the fact that many disease related gene defects are clearly enriched in the Finnish population owing to founder effects, genetic drift, and isolation,[14] we anticipated that these "Finnish" *ATM* mutations might also be found in breast cancer patients in Finland, possibly contributing to increased breast cancer susceptibility. Recurrent *ATM* mutations have been reported in several countries and within many different ethnic groups.[9 15–19]

On these grounds, we decided to screen 162 breast cancer families and 85 sporadic breast cancer patients for *ATM* germline mutations previously identified in Finnish AT families. We were interested to determine the frequency of these mutations among these different categories of breast cancer patients, as well as to see whether there were any indications of geographical clustering.

SUBJECTS AND METHODS
Subjects
A total of 215 breast cancer patients from 162 families originating from central and northern Finland were chosen for *ATM* mutation screening. All families, except one in which two members tested positive for a newly discovered *BRCA2* mutation (5797G>T, exon 11) after the *ATM* study had been performed, had previously been excluded for *BRCA1*, *BRCA2*, and *TP53* mutations.[3–5 7] Inclusion criteria for the families with moderate to high genetic susceptibility to breast cancer were one or more of the following: (1) at least three (two in combination with other selection criteria) cases of breast cancer in first or second degree relatives, (2) early disease onset (≤35 years alone, or <45 in combination with other inclusion criteria), (3) bilateral breast cancer, or (4) multiple tumours including breast cancer in the same person. Additionally, 85

sporadic breast cancer cases from the Oulu area were included. All patients provided informed consent for obtaining pedigree data and blood specimens for a study on cancer susceptibility gene mutations. Reference blood samples from 200 healthy, geographically matched controls were used to validate the observations from the two test groups. Approval to perform the study was obtained from the Ethical Board of the Northern Ostrobotnia Health Care District and the Finnish Ministry of Social Affairs and Health.

Methods
DNA extraction from blood lymphocyte specimens was performed using the standard phenol-chloroform method. The screening of eight distinct *ATM* germline mutations (table 1) previously detected in Finnish AT families (partly unpublished results)[13] was mainly done by conformation sensitive gel electrophoresis (CSGE).[5] Simultaneously, the complete exons and exon-intron boundary regions at the sites of the known mutations were evaluated for other possible aberrations. Samples with a band shift were reamplified and purified with the QIAquick PCR purification Kit (Qiagen). Sequencing analysis was performed with the Li-Cor IR[2] 4200-S DNA Analysis system (Li-Cor Inc, Lincoln, USA) and using the SequiTherm EXCEL™ II DNA Sequencing Kit-LC (Epicentre Technologies). Oligonucleotides for exons 14, 37, 62, and 65 were as reported by Shayegi *et al*.[20] For exons 4, 5, 48, 49, and 53, the oligonucleotides were designed by using the Primer3 software. Primer sequences and PCR conditions for CSGE and sequencing are available upon request.

RESULTS
Identification of pathogenic *ATM* variants
All detected germline alterations are summarised in tables 2 and 3. Two of the eight *ATM* mutations previously identified in Finnish AT families were also detected in the breast cancer patients studied. 7522G>C (exon 53, Ala2524Pro) was originally observed in two AT families, FIAT6 and FIAT7, and was now identified in two women with breast cancer, belonging to separate cancer families (table 2, fig 1, families 003 and 005). The woman in family 003 had breast cancer at the age of 50. Her sister had breast cancer at the age of 59, but owing to lack of DNA we were unable to determine her carrier status. However, of her four children, the two sons carried the 7522G>C aberration, both cancer free at ages 49 and 54. Her two daughters, on the other hand, tested negative for the mutation, even though they had breast and thyroid cancer at the ages of 51 and 47, respectively. Interestingly, there were also multiple cases of cancer on the other side of the family. In family 005, an identified mutation carrier was diagnosed with both breast and stomach cancer at 57 and 41, respectively. In her relatives, other observed cancers included a basal cell carcinoma in the proband's uncle, several cases of stomach cancer (in the maternal grandmother, paternal uncle, and cousin), as well as an unknown cancer in the paternal aunt and thyroid cancer in a cousin.

In breast cancer family 004 (table 2, fig 1), an alteration in exon 49 was observed. The insertion of adenine (6903insA) results in a loss of 685 amino acids from the carboxy terminus of the ATM protein. The 6903insA mutation had previously

Table 1 *ATM* germline mutations observed in Finnish AT families (partly unpublished results)[13]

Exon	Codon	nt change	Predicted change in protein	Family ID
4–5	Unknown	Unknown	200 bp deletion	FIAT8
14	602-603	IVS14+3-4delAT	Skipping of exon 14	FIAT4
37	1773-1774	IVS37+9A>G	Insertion of Val, Ser, and Stop	FIAT3, FIAT5
48	2260	6779-6780delTA	Truncated protein	FIAT7
49*	2303	6903insA	Truncated protein	FIAT4, FIAT5
53*	2524	7522G>C	Ala>Pro	FIAT6, FIAT7
62	2904-2905	8710-8715delGAGACA	Deletion of Glu and Thr	FIAT3
65	3947	9139C>T	Arg>Stop	FIAT2

*Unpublished mutations.

Table 2 Pathogenic *ATM* germline changes in families with breast cancer, sporadic breast cancer, and controls

Family ID	Phenotype*	Exon	nt change	Sporadic breast cancer	Controls
003	Br(50)†, Br(59), Br(51), Th(47), Pro(59), Br (71), Sto, Pul (55), Csu	53	7522G>C	0/85	0/200
005	Br(57)+Sto(41)†, Bs(70), Sto, Sto, Sto(41), Csu, Th(55)	53	7522G>C	0/85	0/200
004	Br(50)†, Br(47)+Sar(18)†, Br(40)†, Sto, Bt, Pan(40), Br(54), Sto, Pan, Br(45)‡, Br(37)‡, Bt, Sto, Sto, Br(30), Bt, Pro, Sto	49	6903insA	0/85	0/200

Br, breast; Bs, basal cell; Bt, brain; Csu, cancer site unknown; Pan, pancreas; Pro, prostate; Pul, pulmonary; Sar, sarcoma; Sto, stomach; Th, thyroid.
*The mutation status has not been defined for all subjects.
†Initially studied probands.
‡Patients subsequently tested positive for *BRCA2* mutation 5797G>T.

Table 3 Polymorphisms detected in the *ATM* gene

nt change	aa change	Frequency in the breast cancer patients studied		Controls
		With family history	Without family history	
133C>T	Arg45Trp	1/215 (0.5%)	0/85	0/200
146C>G	Ser49Cys	1/215 (0.5%)	0/85	0/200
IVS62+8A>C	None	5/215 (2.3%)	2/85 (2.4%)	4/200 (2%)

been detected in two AT families, FIAT4 and FIAT5. Interestingly, this alteration was now found in three sisters, who all had breast cancer at ages 40, 47, and 50. Further investigation of family 004 showed ambiguity in the genotype-phenotype association (fig 1), as the probands' cousin who had been diagnosed with breast cancer aged 54 did not carry the mutation. Her sister, however, who was unaffected at the age of 49, was a carrier. Also, two additional breast cancer patients (diagnosed at ages 37 and 45) from another branch of this cancer family tested negative for 6903insA. Interestingly, however, after completion of our *ATM* mutation screening, these two cancer cases tested positive for a newly discovered *BRCA2* mutation (5797G>T, exon 11), which probably explains their hereditary susceptibility to breast cancer. We then tested all the other available family members for this particular *BRCA2* mutation, but no additional carriers were found. At present, it appears unlikely that the *BRCA2* mutation positive branch of family 004 would segregate the 6903insA *ATM* mutation.

Neither 6903insA nor 7522G>C was seen in the sporadic breast cancer cases studied or in the healthy controls, indicating that in addition to being instrumental to AT, these two *ATM* mutations could also be related to hereditary predisposition to breast cancer.

Polymorphic variants

We also found three other germline sequence variants, two in exon 5 (133C>T and 146C>G) and one in the intronic region between exons 62 and 63. All these changes were regarded as polymorphisms (table 3). 133C>T leads to an amino acid substitution Arg45Trp and was observed in a woman with bilateral breast cancer at the age of 45. The second exon 5 variant, 146C>G, results in Ser49Cys and was detected in a woman diagnosed with breast cancer at 60. She had two sisters, both with breast cancer at ages 48 and 58, but neither of them carried the alteration. We did not observe any of these alterations in the sporadic breast cancer group or healthy controls. Furthermore, neither Arg45Trp nor Ser49Cys resides in a known functional *ATM* domain, and are thus less likely to interfere with ATM kinase function. The third sequence alteration, IVS62+8A>C, was detected in 5/215 (2.3%) of the familial breast cancer patients, 2/85 (2.4%) of the sporadic breast cancer cases, and 4/200 (2.0%) of the control samples.

DISCUSSION

We have examined the prevalence of eight different *ATM* germline mutations, originally found in Finnish AT families, in a cohort of 215 breast cancer cases from 162 cancer families, as well as in 85 sporadic breast cancer patients. Altogether, we detected five different *ATM* sequence alterations (tables 2 and 3), two of which (7522G>C and 6903insA) potentially relate to breast cancer susceptibility. The 7522G>C mutation was previously found in two AT families also displaying signs of genetic predisposition to breast cancer (see below). The 6903insA mutation present in two known AT families (see below) may also be associated with susceptibility to cancer, as this mutation leads to a truncated ATM protein with loss of 685 amino acids, including the region containing the PI-3 kinase motif.[21] The remaining three variants (133C>T, 146C>G, and IVS62+8A>C) were classified as polymorphisms. This assumption was based both on their location
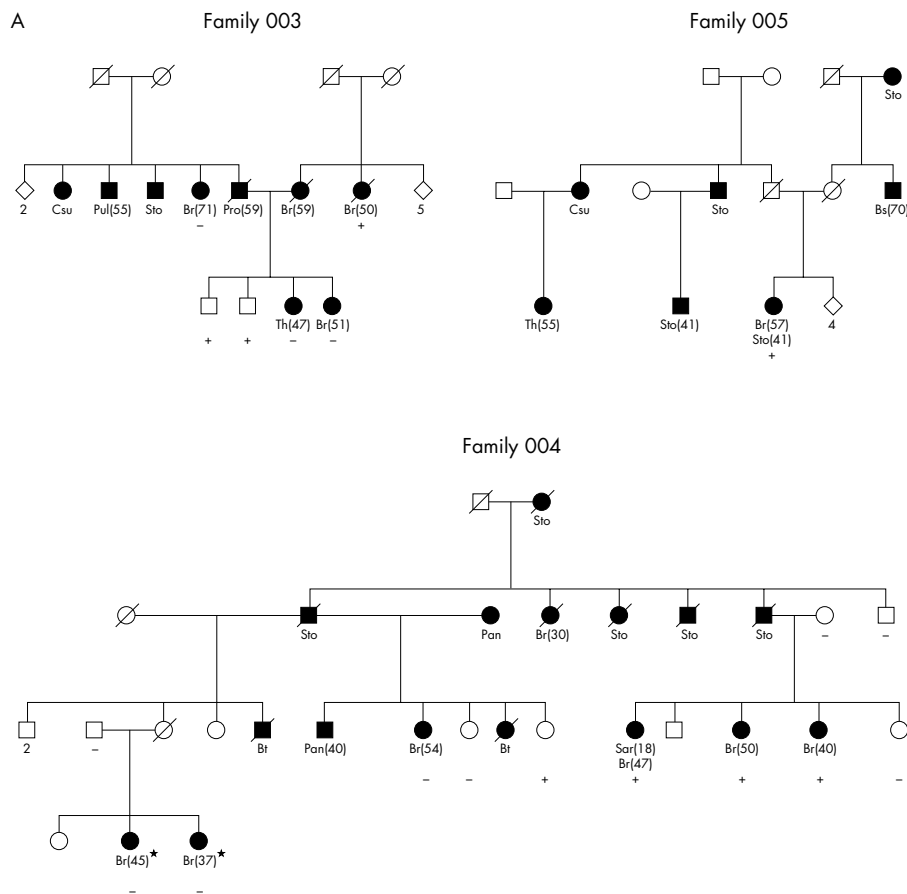
Figure 1 Families with breast cancer exhibiting (A) 7522G>C and (B) 6903insA *ATM* germline mutations. Filled/open symbols indicate cancer/non-cancer status. Age at diagnosis, when known, is shown in brackets after the cancer type (Br, breast; Bs, basal cell; Bt, brain; Csu, cancer site unknown; Pan, pancreas; Pro, prostate; Pul, pulmonary; Sar, sarcoma; Sto, stomach; Th, thyroid). Subjects tested for a specific mutation are marked + if positive and − if negative. In addition, in a subsequent *BRCA2* study of a previously unscreened branch of family 004, two subjects marked with an asterisk tested positive for the 5797G>T mutation. Therefore, other available members of this family were also evaluated but were all found to be negative for this mutation.

away from essential functional domains, and for 146C>G also on previous studies by Vorechovsky *et al*[22] and Izatt *et al*,[23] who did not observe loss of heterozygosity (LOH) of the relevant chromosome region in tumours carrying this alteration. Both groups also identified 146C>G among healthy controls.

Both of the two Finnish AT families (FIAT6 and FIAT7) showing 7522G>C originated from northern Finland. In family FIAT7, the AT patient carried two different mutant *ATM* alleles, 7522G>C and a 2 bp deletion in exon 48. The patient's mother, who carried the 7522G>C mutation, had been diagnosed with childhood leukaemia at the age of 4, followed by bilateral breast cancer at the age of 37. Tumour cells from her left breast showed LOH of the wild type allele, as determined by using the intragenic *ATM* marker D11S2179 (data not shown). Also, the AT patient's grandmother had died from breast cancer at the age of 52. In family FIAT6, the AT patient was a missense 7522G>C homozygote. It is known that at least the proband's maternal grandmother (aged 61) and also one of her sisters had breast cancer. So far, only a few missense homozygotes have been identified[9 13] and it has been suggested that these patients would express a milder AT phenotype. However, no clear differences were observed in the phenotypes of these two AT patients from families FIAT6 and FIAT7. The appearance of 7522G>C in two AT families from northern Finland suggested that this mutation could also be more frequent in breast cancer patients originating from the larger Oulu region. Indeed, the mutation was found in two families with breast cancer (table 2, families 003 and 005).

Besides 7522G>C, *ATM* 6903insA was the other putative breast cancer related alteration seen in this study. The geographical origin of the 6903insA allele, first identified from AT families FIAT4 and FIAT5, appeared to be a rural area slightly west of the city of Tampere. Both AT families showed excessive cases of breast cancer in the branches segregating the 6903insA allele, and at relatively young ages (FIAT4, the proband's paternal grandmother (aged 53); FIAT5, the proband's mother (aged 50) and maternal grandmother's sister (aged 51)). Interestingly, the branch of breast cancer family 004 displaying the 6903insA mutation also came from west of Tampere. Neither 7522G>C nor 6903insA was detected among 200 controls originating from the same geographical region, suggesting that these two mutations could have some effects on the carrier phenotype, even when heterozygous.

In families 003 and 004, where we were able to study additional family members, some discrepancies regarding cancer phenotype and carrier status were seen (fig 1). Even though this observation raises questions about the contribution of these mutations to breast cancer susceptibility, incomplete penetrance and occurrence of sporadic cancer cases must also be taken into account. Furthermore, the dominant inheritance model of cancer predisposition cannot automatically be assumed to apply to these mutations.

The large size of the *ATM* gene sets certain limitations to mutation screening. Most studies have used the protein truncation test, which only detects alterations resulting in a premature stop codon and therefore a shortened protein

- We screened Finnish breast cancer patients for eight different germline mutations previously found in Finnish AT patients to determine their occurrence and possible geographical clustering.
- Two of these alterations (6903insA in exon 49 and 7522G>C in exon 53) were detected in three families with breast cancer, but not in any of the sporadic cases. Thus, our results suggest that ATM mutations contribute to a small proportion of the hereditary breast cancer risk.
- The ancestors of the mutation positive cancer families and the AT families exhibiting the corresponding ATM aberration originated from the same geographical areas.

product. Missense mutations, however, contributing to the other of the two putative phenotypically distinct populations of AT carriers, are not detected with this method.[24][25] Also, if DNA is the only starting template available, as was the case in this study, it is quite impossible to cost effectively screen large sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related geographically recurrent mutations, we concentrated on studying only the ATM germline mutations previously recognised from all known Finnish AT patients (partly unpublished data)[13] and used CSGE as the primary screening method because of its simplicity and high accuracy followed by DNA sequencing, if required.

ATM is one of the central components of the DNA damage response, and it interacts with several key proteins such as p53, Mdm2, BRCA1, Chk2, Nbs1, and Rad17.[26][27] Viewed against this background and considering the existing epidemiological evidence,[10] the fact remains that ATM is a strong candidate contributing to cancer susceptibility. The low frequency of mutations within the protein encoding region in the breast cancer material analysed, however, raises the question of whether epigenetic mechanisms (for example, promoter region hypermethylation) could play an additional role in ATM silencing. These studies are currently under way in our laboratory.

We have analysed both familial and sporadic breast cancer patients for eight ATM germline mutations previously identified from Finnish AT families (partly unpublished results)[13] and found a pathogenic mutation present in 3/162 (1.9%) of them displaying various indications of hereditary breast cancer. These mutations were not observed among sporadic breast cancer patients or healthy controls. Both of the detected mutations were also found in AT families originating from the same geographical region as the breast cancer families displaying the corresponding mutation, implying the possibility of a founder effect concerning the distribution of 7522G>C and 6903insA. However, to follow this preliminary lead, a more extensive screening for ATM mutations among breast cancer families is indicated. In addition, it would be interesting to investigate whether similar trends are seen with mutations in other AT families from different parts of the country.

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A single base alteration in the CGG repeat region of *FMR1*: possible effects on gene expression and phenotype

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Fragile X syndrome is characterised by mild to severe mental retardation, minor physical anomalies such as large ears, long facies with a high forehead, joint hyperextensibility, and macro-orchidism, behaviour such as hyperactivity and social avoidance, and speech and developmental delays.[1] Trinucleotide repeat (CGG) expansion mutations account for nearly all cases of fragile X syndrome with only a few patients reported with deletions or point mutations in the *FMR1* gene.[2–4] Expansion beyond approximately 200 repeats (normal range 5-54 repeats), commonly referred to as a "full mutation", usually results in hypermethylation of the *FMR1* promoter region with a concomitant loss of *FMR1* transcription and the protein product of the gene, FMRP.[5] People with 55-200 repeats have a premutation, which is unstable and prone to expansion when transmitted by females to their offspring. Premutation carriers produce FMRP and typically have few manifestations of the syndrome.

It is the loss or severe reduction in FMRP that results in the fragile X syndrome.[6–8] However, there are numerous reports of subjects with mild phenotypes related to modest FMRP production.[9] Such subjects may have learning disabilities but are not retarded (IQ>70), and the milder phenotype appears related to FMRP production arising from transcription of unmethylated alleles. Typically, such patients are cellular mosaics having both full mutations and premutations (termed "full mutation/premutation mosaics"), or having full mutations with varying degrees of methylation (termed "methylation mosaics"). Using a detection technique comprising immunocytochemical staining and counting of lymphocytes expressing FMRP,[7] Tassone *et al*[8] identified a significant correlation between FMRP expression and full scale IQ in males with full mutation/premutation mosaicism and methylation mosaicism. These authors observed that the presence of >50% FMRP expressing lymphocytes correlated with non-retarded status (IQ>70). These "high functioning" males, however, displayed some learning disabilities and behavioural manifestations of fragile X

syndrome. The findings of improved function in these patients show that the fragile X syndrome phenotype can be modified by the production of substantial amounts of FMRP. The recognition that substantial *FMR1* expression ameliorates the phenotype suggests that there may be a general threshold of FMRP expression beyond which normal intellect results. Attempts to define this possible expression threshold are complicated by difficulties in precise quantification of intracellular FMRP, possible differences in FMRP expression between blood and other tissues, and background genetic and social effects on intellect.

We report here on a subject who has a point mutation in the CGG repeat region of *FMR1* which reduces gene expression by approximately 24% in a lymphoblastoid cell line established from his peripheral blood. This young male has a history of speech and developmental delay and low-normal IQ measures

Table 1 Clinical assessment for the proband

Evaluation tool	Age and scores		
	2 y 10 mth	6 y 10 mth	8 y 11 mth
Stanford-Binet Intelligence Scale (4th ed)			
Verbal reasoning	ND	83	89
Abstract/visual reasoning	ND	89	79
Quantitative reasoning	ND	108	93
Short term memory	ND	58	67
Test composite	ND	81	81
Vineland Adaptive Behavior Scale			
Communication domain	69	101	94
Daily living skills domain	93	95	106
Socialisation domain	76	109	104
Motor skills domain	93	ND	ND
Adaptive behaviour composite	77	102	101

ND=test or domain not done.

(table 1). He was initially categorised as having fragile X syndrome based on his clinical presentation as a 2 year old with delays in speech and also on the results of molecular diagnosis for *FMR1*, which mistakenly indicated a deletion within the gene. Later investigations have shown that the proband does not have a *FMR1* deletion but a single base alteration within the CGG repeat segment creating a new *Eag*I restriction enzyme site and hence leading to an unexpected change in migration on Southern blot analysis (fig 1). To our knowledge, this is the first report of a point mutation within the CGG repeat of *FMR1*. This patient is instructive for two reasons. One, he may represent a "natural experiment" whereby a single base alteration within the CGG repeat sequence affects FMRP production and results in mild phenotypic effects. Two, the single base alteration creates a new restriction enzyme site and illustrates the potential for misinterpretation of molecular diagnostic tests for fragile X syndrome.

CASE REPORT

The proband was first evaluated for developmental and speech delay in late 1993 at 30 months of age. He had a five word vocabulary at this time and often used these words inappropriately. A physical examination indicated height and weight on the 10th centile, head circumference >95th centile, prominent forehead, and speech delay. A follow up examination at 4½ years showed height on the 45th centile, weight on the 25th centile, head circumference >95th centile, long face, prominent forehead, speech delay, but no hyperactivity. Molecular genetic testing for *FMR1* was ordered based on the clinical findings.

Initially, the *FMR1* alteration was identified as a deletion of approximately 400 bp when a DNA fragment of ~2.4 kb was observed on Southern blot analysis (using probe StB12.3)

instead of the expected 2.8 kb fragment (fig 1A). Routinely, Southern blot analysis using *FMR1* specific DNA probes near the CGG repeat and double restriction enzyme digestion with *Eco*RI and *Eag*I (or other methylation sensitive restriction enzymes) generates a 2.8 kb fragment in males having trinucleotide repeats within the normal range (<54 repeats).[10] Polymerase chain reaction (PCR) analysis specific for the *FMR1* CGG repeat segment[11] showed that the proband had 31 repeats (his mother was homozygous for alleles with 31 repeats), suggesting the "deletion" did not involve the *FMR1* region containing the CGG repeat and the nearby flanking sequence. Based on the Southern blot results and clinical presentation of delays typical of fragile X syndrome in young boys, the proband was expected to have fragile X syndrome. Subjects with *FMR1* deletions resulting in the loss of gene expression invariably have fragile X syndrome indistinguishable from those who have the common trinucleotide repeat expansion (full mutation).[12] The PCR result was unusual since it indicated no disruption of the region in *FMR1* exon 1 containing the trinucleotide repeat, a known "hotspot" for many deletion events.[13] After several years of follow up evaluation, however, it is clear that the proband has significantly higher cognitive abilities than most males with fragile X syndrome (table 1).

The proband's development has been frequently evaluated since 1994, and he was enrolled in the Carolina Fragile X Project, a long term study to follow the development of young males with fragile X syndrome. Investigators in the Project frequently noted that he was an outlier when compared to other boys of a similar age with fragile X syndrome. More recent cognitive-behavioural assessments at the ages of 6 years 10 months and 8 years 11 months found borderline to low-normal cognitive skills and average adaptive behaviour

Figure 1   Southern blot and DNA sequence analysis of DNA from the proband. (A) Southern blot analysis. Lane 1 = patient; lane 2 = patient's mother; lanes 3, 5, and 6 = male controls (patients with normal alleles with <54 repeats); lane 4 = female control with both *FMR1* alleles <54 repeats; lane 7 = full mutation control patient with complete methylation. (B) DNA sequence of *FMR1* repeat region from the patient. The trinucleotide repeat segment containing 31 repeats is underlined. The G to C transversion is noted by an asterisk and the newly created *Eag*I site is capitalised.

Figure 2 *FMR1* repeat segment and flanking DNA. (A) Diagram of a portion of the *FMR1* gene showing commonly used restriction enzymes for Southern blot analysis, fragment sizes, the StB12.3 probe, and the location of the repeat segment. The clustered methylation sensitive restriction enzyme sites appear underlined in (B). (The second *Bss*HII site contains overlapping restriction sites.) (B) DNA sequence of *FMR1* promoter (GenBank Accession L29074) and repeat regions illustrating numerous potential single base alterations which may create new *Eag*I (E), *Bss*HII (B), *Sac*II (S), or *Nru*I (N) restriction enzyme sites. Bases followed by * indicate potential new sites (* marks bases at any position within the six base target recognition) and letters over the asterisks indicate which restriction enzyme site could be created by a single base change. Within the CGG repeat segment, single base changes similar to that found in the proband can create only *Eag*I or *Sac*II sites (of the four listed enzymes). However, these potential new sites can be created by point mutation at any consecutive CGGCGG (denoted as SE when either a *Sac*II or *Eag*I site can be created by a single base change in the same position). The arrow indicates the location of the proband's mutation and the FMRP translation start site is denoted.

levels for a child of his age. Unlike same aged males with fragile X syndrome who show declines in IQ and adaptive behaviour levels,[14] the proband's test-retest IQ and adaptive behaviour scores were quite stable (table 1).

As a part of the Project's evaluation, a blood sample was obtained from the proband in 1998 to evaluate his production of FMRP (performed at Kimball Genetics Inc). Fragile X DNA testing was repeated along with FMRP testing, but Southern blot analysis at this clinical testing laboratory showed a normal result with a 2.8 kb fragment and no 2.4 kb fragment, as seen in the previous testing. After consultation between the laboratories involved in testing the proband, it was realised that the respective Southern blot analysis protocols differed

slightly. While *Eag*I was used as the methylation sensitive restriction enzyme in the original study, another methylation sensitive enzyme, *Nru*I, was used in the second study. This realisation led to DNA sequencing of the region around the trinucleotide repeat segment. A single base transversion (G to C) was identified embedded within the CGG repeat segment (fig 1B). This alteration created a new *Eag*I site 356 bp downstream from the *Eag*I site normally detected, and led directly to production of the 2.4 kb "deletion" fragment originally detected in the proband. Thus, his "deletion" is, in fact, not a deletion but a single base alteration. The 2.4 kb fragment has been found (using *Eag*I in the Southern blot procedure) in both the proband's mother (fig 1) and older sister (data not

shown), neither of whom have any characteristics of fragile X syndrome.

The effect of the G to C alteration on FMRP production was assessed in the patient. Using FMRP detection by immunocytochemistry of blood smears,[7] he was found to express FMRP from 85% of lymphocytes inspected, which is within the range found in people without fragile X syndrome. This confirms that he is producing detectable levels of FMRP in a normal percentage of cells. Quantitation of the total amount of FMRP is not possible by immunocytochemistry, however. In order to investigate whether total FMRP is affected by the G to C alteration, FMRP quantitation assays were performed on an Epstein-Barr virus transformed B cell line established from a peripheral blood sample obtained from the patient. The amount of FMRP produced by his cell line and two control cell lines was determined in a slot-blot based assay relative to known amounts of purified FMRP[15] and using an anti-FMRP antibody.[16][17] The FMRP level was normalised to that of a control protein, eIF-4e, which was determined using a similar method with purified eIF-4e[18] and an anti-eIF-4e antibody (Transduction Labs). The molar ratio of FMRP:eIF-4e for the two normal cell lines (29 repeats each) was 2.34 (SE=0.194, n=11). The molar ratio for the patient's cell line was 1.78 (SE=0.146, n=11) representing a reduction of 24%. This reduction is statistically significant by a paired t test (t=5.6455, p=0.0002). Although findings of reduced FMRP in a cell line may not reflect an in vivo reduction, the results were obtained reproducibly on several occasions with the same cell line. After long term culturing of the cell line, the FMRP production eventually returned to levels comparable with controls, presumably as an artefact of culture selection for direct mutation reversion or a suppressor mutation. The phenotypic consequences of a minor reduction in FMRP are unclear without additional studies of males with the G to C transversion. The proband's extended family history does show several maternal great uncles with possible learning deficiencies, but we have been unable to study these people despite repeated requests.

DISCUSSION

It is tempting to speculate regarding the effect on the patient of possible reduced FMRP production. A clear correlation exists between FMRP production and the intellectual/behavioural phenotype in patients with *FMR1* mosaicism. Yet the amount of FMRP production that is enough for rescue or modification of the phenotype has not been definitively established owing to the impossibility of FMRP quantitation in the brain, the limited number of fragile X male patients expressing substantial amounts of FMRP, and the difficulty of isolating the effect of a single gene defect on performance. The uncovering of a point mutation possibly affecting *FMR1* expression may provide an example of a quantitative phenotypic effect by minor FMRP reduction, especially if other patients with identical or similar mutations in the repeat segment confirm the effect of the mutation described here. The patient functions in the low average intellectual range, yet has specific deficiencies in some areas, for example, short term memory, which lower his overall scores. Although we have only speculative thoughts regarding a mechanism for his reduced FMRP production, possible effects on FMRP production may occur related to mRNA stability or translatability, or to the association of the 5'-(CGG)(n)-3' binding protein (also known as CGGBP1 or p20). This latter protein binds non-methylated, but not methylated, CGG repeats and appears to modulate the activity of the *FMR1* promoter.[19] Perhaps subtle effects on FMRP production approximating the 24% found in the patient's cell line lead to minor deficiencies in learning.

Despite nearly a decade of *FMR1* molecular analysis with many laboratories world wide using the double restriction enzyme digestion protocol with *Eco*RI and *Eag*I, no other sub-

jects have been reported with a G to C transversion creating a new *Eag*I site. Although precise numbers of subjects tested for possible *FMR1* repeat expansion using this Southern blot protocol have not been tabulated, the protocol is used extensively and the number of tested subjects is likely to number in the tens of thousands or greater. Inspection of the CGG repeat segment shows many sites where a single base alteration may create a new *Eag*I site (CGGCCG), and several possible new sites in surrounding DNA for other commonly used methylation sensitive restriction enzymes, *Bss*HII (GCGCGC), *Nru*I (TCGCGA), or *Sac*II (CCGCGG) (fig 2). Based on the large number of tested subjects, the repeat segment is not frequently prone to mutations which create a new site. However, as this case has instructed, it is prudent to confirm potential *FMR1* "deletions" by using a second methylation sensitive enzyme from those listed above or by restriction mapping of the deletion endpoints.

Patients with point mutations in the promoter or 5' untranslated region of *FMR1* containing the CGG repeat may be underascertained since the usual molecular diagnostic approaches of assaying for repeat expansion do not identify subjects with small alterations. Mila *et al*[20] recently identified two patients with mental retardation (one reportedly with a phenotype highly suggestive of fragile X syndrome) having alterations in the *FMR1* promoter. If similarly affected patients with alterations in the *FMR1* promoter or repeat segment are identified, consideration should be given to follow up DNA sequence investigations in patients with phenotypic characteristics of fragile X syndrome yet no repeat expansion. In addition, it is possible that some subjects diagnosed with a "deletion" in *FMR1* may actually have a point mutation in a fashion similar to our patient.

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Mosaicism for *FMR1* and *FMR2* deletion: a new case

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Fragile X syndrome is the most common disorder causing mental retardation with an estimated prevalence of between 1 in 4000 to 1 in 6000 males.[1 2] In nearly all cases, the molecular basis of this X linked mental impairment is an amplification in excess of 200 copies of an unstable CGG trinucleotide repeat sequence within the 5' untranslated region of the *FMR1* gene (FRAXA, MIM 309550) at Xq27.3.[3] This causes an inhibition of the transcription leading to a loss of function effect in the male. In the female, the amplified gene is preferentially X inactivated by methylation.[4] However, the X inactivation pattern can vary between different cell types and during development and thus, usually, causes a milder phenotype in female patients.[5] In close distal proximity to the *FMR1* gene, a second gene, *FMR2* (FRAXE, MIM 309548) involved in mental retardation, has been described with amplification of an unstable GCC trinucleotide repeat sequence as the underlying pathological mechanism.[6] This report adds a further case with mosaicism for a *FMR1* and *FMR2* deletion to the few described cases with a large *FMR1* deletion.[7–11]

CASE REPORT

A boy of 3½ years of age was referred to our Clinical Genetics Unit because of severe developmental delay, behavioural difficulties, and delayed speech development. He is the first child of healthy, unrelated Turkish parents. The boy was born two weeks before term with a birth weight of 2690 g, length of 49 cm, and OFC of 32.5 cm. He had feeding and eating problems which still persisted at the age of 3½ years. At 3 months he was treated with physiotherapy for muscular hypotonia.

Clinical investigation at 3½ years showed height (97.5 cm) and weight (14.2 kg) between the 25th-50th centile, a long face with prominent forehead and small and pointed chin, periorbital fullness, big ears, a short nose with anteverted nares, a long, smooth philtrum (fig 1), microdolichocephaly (OFC 48.5 cm, <3%), marked joint hypermobility, broad hands and feet, short toes, and truncal hypotonia. He only speaks a few words. He is hyperactive and his behaviour is characterised by aggression and self-mutilation, but he calms

Figure 1 The proband. Note the long face with prominent forehead, pointed chin, periorbital fullness, big ears, short nose, and long, smooth philtrum. (Photograph reproduced with permission.)

down while listening to music. There is no history yet of epilepsy as has been described in similar cases.[7 12] He suffers from recurrent otitis media with effusion which may have caused subsequent language and articulation deficits.

Cytogenetic analysis of peripheral lymphocytes at a 700 band level showed a normal male karyotype, 46,XY. Routine molecular analysis for trinucleotide amplification of the *FMR1* gene by Southern blot hybridisation of genomic DNA from white blood cells with an *FMR1* probe (GL30, a kind gift from

A Poustka's laboratory) failed to give any result for our patient, leaving the possibility of a deletion. Hybridisation with an *FMR2* probe (Oxe20, a kind gift from Kay Davies's laboratory) confirmed a deletion covering at least *FMR1* and *FMR2*. The same results were obtained testing genomic DNA from the patient's fibroblasts. However, when PCR based assays for the non-variable regions of the *FMR1* (FRAXA-S: CTGCAGGAGGCGGCCCGG; FRAXA-A: GCTAGCAGGGCT-GAAGAGAAGATG) and *FMR2* genes (*FMR2*-S: GTCACACACA-GAGCTACCGGC; *FMR2*-A: CACGTGGAGGGTTTAACCGAG) were applied to genomic DNA from leucocytes and from fibroblasts, clear bands were obtained which suggested the existence of a cellular mosaicism as a likely explanation of the laboratory findings. Given the established sensitivity of our Southern blot hybridisation conditions, we estimated the presence of the second cell line to be less than 10% (based on other data not shown) in the tested tissues. This level, however, does not necessarily reflect the state of mosaicism in the brain. Standard Southern blot hybridisation as well as methylation analysis showed a carrier pattern with amplification of the trinucleotide repeats within the premutation range for his mother, while his father was normal. Testing for a possible amplification of the CGG repeats in the remaining cell line by PCR (*FMR1*-A: GGAACAGCGTTGATCACGTGACGT-GGTTTC; *FMR1*-571R: GGGGCCTGCCCTAGAGCCAAGTAC-CTTGT) did not give a normal sized band in the patient, suggesting that the *FMR1* gene of the remaining cell line most likely originated from the mother's premutated gene. The deletion in the prominent cell line must have been a postzygotic event covering the region between the *FMR1* and *FMR2* genes with an estimated size of at least 900 kb, based on knowledge of the distance between *FMR1* and *FMR2* as reported in the Genetic Location Database (LDB; http://cedar.genetics.soton.ac.uk/pub/chromX/gene.html).

DISCUSSION

A few cases with large deletions in a complete or mosaic pattern have been published, which were preferentially de novo or inherited deletions,[10 11 13–15] while small mosaic deletions are usually associated with premutations.[8] The basic events giving rise to these deletions are not yet understood.

The small head, the severe developmental delay, and the persistent feeding and eating problems are not typical for fragile X syndrome, but might reflect a functional or developmental imbalance caused by the deletion of additional genes in the majority of cells. Similar clinical phenotypes with moderate to severe mental retardation, epilepsy, and deletion of the *FMR1* and *FMR2* genes have been described previously.[7 10] Microcephaly is atypical in fragile X syndrome; however, the patient has a fragile X-like facial appearance. Two other published patients with a large deletion[10] had head circumferences at or below the 3rd centile as well, while a third patient with a deletion of the *FMR1* and *FMR2* genes[7] was macrocephalic. While the clinical findings of our patient suggest that the deletion may be larger, encompassing more genes than just *FMR1* and *FMR2*, the deletion has apparently arisen on the background of an inherited triplet repeat mutation. Usually, in

previous reports, deletions arising in the presence of a triplet repeat expansion have been small while large deletions have not been associated with triplet repeat expansion as in our case.[16] We thus speculate that our patient has mosaicism of cells with an *FMR1* gene functionally inactivated by repeat amplification and cells with a large deletion of *FMR1*, *FMR2*, and possibly neighbouring genes, thus spreading the classical fragile X phenotype.

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Prader-Willi syndrome and a deletion/duplication within the 15q11-q13 region

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Prader-Willi syndrome (PWS) is characterised by infantile hypotonia, feeding difficulties, hypogonadism, small hands and feet, mental deficiency, obesity in early childhood, a particular facial appearance, and a paternally derived 15q11-q13 deletion (approximately 4 million bp in size) in about 70% of subjects, maternal disomy 15 (both 15s from the mother) in 25% of subjects, or an imprinting mutation in 2-5% of subjects.[1–4] PWS syndrome is considered to be the most common genetic cause of marked obesity in humans.[1]

Two breakpoint clusters have been reported centromeric to locus ZNF127 with the most proximal breakpoint (BP1) accounting for 44% of cytogenetic deletions while 56% of deletions occur at the second proximal breakpoint (BP2).[5 6] The second breakpoint (BP2) lies between loci D15S541/S542 and D15S543 and breakpoint BP1 is proximal to D15S541/S542.[5 6] A third breakpoint (BP3) is distally located within the 15q11-q13 region and mapped telomeric to the P locus (involved in hypopigmentation) in nearly all deletion subjects studied.[5–13] The apparent genetic instability in the 15q11-q13 region may be attributed to DNA sequences identified in low copy repeats in the vicinity of the common breakpoints occurring in patients with PWS. These END repeats are derived from large genomic duplications of a novel gene (*HERC2*).[8 12] The END repeats flanking the 15q11-q13 region may be involved with inter- and intrachromosomal misalignment and homologous recombination resulting in the common PWS deletion and facilitated by active transcription of the END repeats in male and female gametogenesis.[12 14]

Duplication, triplication, and tetrasomy of the 15q11-q13 region have been reported with varying degrees of clinical manifestation. Generally, duplications of maternal origin have been associated with developmental delay and autistic behaviour.[15–18] Paternal duplications have no apparent impact on the phenotype although a patient reported by Mohandas *et al*[19] with an interstitial duplication of proximal 15q of paternal origin had non-specific developmental delay and partial agenesis of the corpus callosum. In addition, Ungaro *et al*[6] reported molecular characterisation of four subjects presenting with mild to severe mental retardation and features of PWS or Angelman syndrome along with intrachromosomal triplications of the chromosome region 15q11-q14.

Here, we present a patient with the typical 15q11-q13 deletion seen in PWS who also carried a familial duplication of centromerically located 15q11-q13 loci. The paternally derived deletion of 15q11-q13 occurred at breakpoints BP2 and BP3 and the paternal duplication was centromeric to breakpoint BP2.

CASE REPORT

Our patient was born at 30 weeks' gestation by vertex vaginal delivery. She weighed 1531 g and was 40 cm long. Bleeding problems were reported during the pregnancy but no admission to hospital was required. Decreased fetal activity was noted. Hypotonia and a poor suck reflex with feeding difficulties were observed shortly after birth. Chromosome studies showed the typical chromosome 15q11-q13 deletion confirmed by fluorescence in situ hybridisation using probes

for *SNRPN*, D15S11 and *GABRB3*. In addition, polymerase chain reaction (PCR) methylation testing was diagnostic for PWS. Historically, she had the typical features seen in PWS including a narrow forehead, small upturned nose, down-turned mouth, hypotonia, small hands and feet, obesity in early childhood, learning and behavioural problems, and skin picking. She also had hypopigmentation in relation to other family members, typically seen in those with a 15q11-q13 deletion.[13 20] She has not been diabetic and had normal thyroid function tests in the past although she had a long history of obesity. She has had a history of unusual eating patterns with hyperphagia and food foraging leading to early childhood obesity. The refrigerator and kitchen cabinets are locked in the home environment. She has not had sleep apnoea but does experience drowsiness. At 17 years of age she weighed 98.9 kg (>>97th centile) and was 149.9 cm (3rd centile) tall. She has not been treated with psychotropic medication although behavioural problems including skin picking were present in early childhood. She had developmental delay and mild mental retardation. She had a history of reading and maths difficulties requiring special education and speech therapy. She enjoys swimming but does not like to cycle, walk, or dance. She enjoys working with crafts and jigsaw puzzles.

Microsatellite analysis with PCR using 19 short tandem repeats from the 15q11-q13 region showed a paternally derived deletion of several informative loci (for example, D15S817, D15S63, D15S210, *GABRA5*, D15S822) supporting breakpoints at BP2 and BP3. Interestingly, she had three alleles at D15S541, D15S542, and D15S1035, which are centromeric to breakpoint BP2 within the 15q11-q13 region, indicating a duplication of these loci (table 1). The same duplication was shared by her phenotypically normal father and uncles by PCR analysis but not by the proband's two unaffected female sibs. No other person tested in the family showed the deletion or duplication (table 1).

DISCUSSION

Our report illustrates the clinical and molecular findings of a subject with classical features of PWS with the typical deletion of 15q11-q13 seen in 70% of PWS subjects. In addition, she had a familial duplication of centromerically located 15q11-q13 loci inherited from her father. The clinical presentation is described in the text and the molecular findings summarised in table 1 with microsatellite data shown in fig 1. Our laboratory has analysed DNA microsatellite data from the 15q11-q13 region including D15S541, D15S542, D15S543, and D15S1035 loci in over 50 PWS families including the parents. However, this was the only family we identified with a duplication of loci centromerically located within the 15q11-q13 region. To our knowledge, this PWS subject is the first reported with the typical 15q11-q13 deletion and a duplication of genetic loci inherited from the father centromeric to breakpoint BP2.

Interstitial duplications of 15q11-q13 of maternal origin have been identified in patients with developmental delay and learning/behavioural problems while subjects with paternally derived duplications generally have a normal phenotype as seen in our unaffected father and the proband's two paternal uncles. However, a patient reported by Mohandas *et al*[19] had an

Table 1 PCR and FISH data in linear order from the 15q11-q13 region

	D15S1035	D15S541	D15S542	D15S543	D15S11	D15S817	D15S63	D15S128	MN1	SNRPN	D15S122	D15S210	D15S113	GABRB3	GABRA.5	D15S822	D15S156	D15S219	D15S217	D15S165
Mother	1,4	2,4	2,3	2,2	2,4	2,3	3,3	1,1	1,1	1,1		3,4	1,3	1,3	1,2	2,2	1,3	1,2	1,2	1,3
PWS proband	3,4,5*	2,3,4*	3,4,5*	2,2 or 2,-	4†	2†	3†	1†	1‡	1†	(-)	4†	3†	3 (-)	2†	2†	3,3 or 3,-	2,2 or 2,-	1,1 or 1,-	2,3§
Father	1,3,5	1,2,3	1,4,5	1,2	1,3	1,3	1,2	1,2	1,2	2,2		1,2	2,3	2,3	3,3	1,3	2,3	2,2	1,1	2,3
Sib 1	1,1			1,2																
Sib 2	1,1			1,2																
Paternal uncle 1	1,3,5			1,2																
Paternal uncle 2	2,3,5			1,2																

*Paternal duplication status; †paternal deletion status; ‡deletion inferred by proximal and distal loci status; §biparental (normal) inheritance. (-) = deletion status determined by FISH.

interstitial duplication of proximal 15q containing the Prader-Willi syndrome/Angelman syndrome region (for example, *GABRB3* locus) of paternal origin and an abnormal phenotype. The duplication they reported was larger than in our patient. They reported that their patient's genetic findings were consistent with the origin of the duplication from unequal crossing over between the two chromosome 15 homologues from the father.

Our family lends support with anecdotal and laboratory data that a parent (father) with a genomic duplication within the 15q11-q13 region may predispose to unequal crossing over in meiosis leading to a deletion event within the region and therefore PWS. Additional studies are needed to determine if the apparent duplication of the proximal 15q loci seen in our PWS proband and her father and paternal uncles without recognisable clinical findings is a common polymorphism or impacts on meiosis and crossing over events leading to a deletion. In addition, a study to compare the frequency of this molecular finding in fathers of PWS children compared to fathers in the general population would be of importance to address this observation seen in our PWS family.

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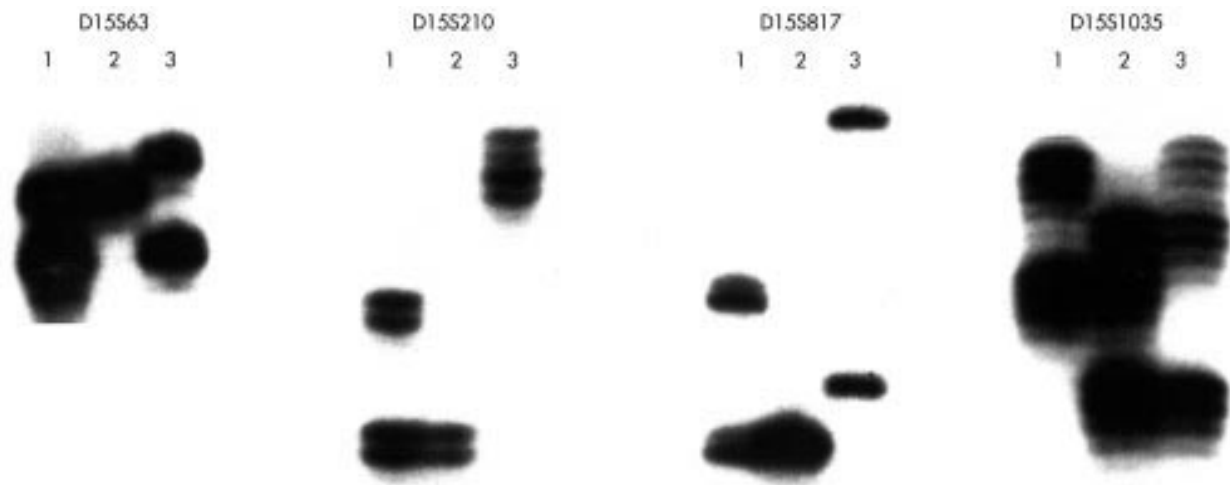
Figure 1 Polymerase chain reaction (PCR) from genomic DNA of several informative loci (D15S63, D15S210, and D15S817) from the 15q11-q13 region showing only one allele for the PWS patient (lane 2) consistent with a paternal deletion. PCR result of the D15S1035 locus from the centromeric end of the 15q11-q13 region shows three alleles, consistent with a paternal duplication. Lanes 1, 2, and 3 represent the mother, PWS subject, and father, respectively.

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The frequency of mtDNA 8994 polymorphism and detection of the NARP 8993 mutation

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The highly polymorphic nature of the mitochondrial genome (mtDNA) has proved valuable to the population geneticist, but can cause serious problems in the identification of disease causing mutations.

A T→C or T→G transition at nt 8993 in human mtDNA is associated with an array of clinical phenotypes including Neuropathy, Ataxia and Retinitis Pigmentosa (NARP)[1] and Leigh's syndrome.[2] Conventionally, it is detected by polymerase chain reaction (PCR) amplification of the region containing the mutant sequence followed by digestion with restriction enzymes *Hap*II or *Hpa*II (recognition sequence c↓cgg) which recognise both sequence changes.[1] The presence of either mutation results in the PCR product being cut into two fragments (343 bp and 206 bp), which can be separated and identified by agarose gel electrophoresis.

A polymorphic G→A transition in the adjacent base (nt 8994) abolishes the recognition site for the relevant restriction enzymes and, hence, in patients who also have the NARP 8993 mutation can result in a false negative diagnosis.

As a consequence, a method has been developed to detect the 8994 polymorphism using the restriction enzyme *Hae*III applied to the same undigested PCR product used for detecting the 8993 mutation, which is in use by some laboratories who test for the 8993 mutation.[3] Patients without the polymorphism have three *Hae*III restriction sites in the amplified region resulting in four fragments (190 bp, 174 bp, 156 bp, 31 bp), while those with the polymorphism have one of these sites abolished giving rise to three fragments (190 bp, 187 bp, 174 bp). Under the gel electrophoresis conditions used (2% agarose with ethidium bromide staining), the 190 bp and 187 bp fragments run as a single band and the 31 bp fragment is usually not visible as it runs off the gel. Thus, in the presence of the 8994 polymorphism two fragments are seen, while in its absence three fragments are detected.

To exclude the NARP 8993 mutation, we routinely test all patients for both the 8994 and 8993 mutations. If the 8994 polymorphism is detected, the PCR product is sequenced to exclude the 8993 mutation.

The 8994 polymorphism has been reported in mtDNA haplogroup W, one of the rarer haplogroups specific to people of European origin.[4] However, the polymorphism may also exist on its own in other ethnic groups. We have analysed muscle and blood samples for the 8993 mutation and the 8994 polymorphism in 547 patients from the West Midlands over a five year period. Analyses were performed using blood cell lysates and DNA extracted from muscle biopsies.

Six patients (1.1%) did not show the normal pattern of fragment sizes on *Hae*III digestion for the 8994 polymorphism. On sequencing, four (0.7%) showed the 8994 polymorphism for which all were homoplasmic. One of these was of European origin, while three were from the Indian subcontinent or of Middle Eastern origin. The remaining two patients showed a different abnormal digestion pattern with *Hae*III. On sequencing this was found to be the result of a G→A change at nucleotide 9025. This change has previously been reported as a polymorphism in the MITOMAP[5] database. While this polymorphism resulted in an atypical restriction pattern, it would not have caused a misdiagnosis for the 8993 mutation.

In our study, the frequency of the 8994 polymorphism observed in the West Midlands population was very low (0.7%). Over a five year period we have diagnosed one family group with the NARP 8993 mutation in 547 samples from adults and children. Hence, the probability of a false negative diagnosis if a test for the 8994 polymorphism was not performed is 0.0013%. Despite the low frequency of the 8994 polymorphism in our population, it will remain necessary for us to use the restriction digestion method to detect this sequence change in order to exclude the 8993 mutation. This illustrates the problem of restriction digestion based methods for the detection of mutations in the highly polymorphic mitochondrial genome. A method using direct sequencing or a primer extension assay would obviate this problem and could be automated for high throughput diagnostic screening.

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An aetiological study of 25 mentally retarded adults with autism

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Autism is a chronic and severe neuropsychiatric disorder with an early onset, characterised by qualitative impairments of social interaction, deviant development of language and other communicative skills, delayed cognitive development, and restricted repetitive and stereotyped interests and behaviours.[1][2] The prevalence in the general population was estimated at 5.5/10 000 but more recent investigations report higher rates.[3] Males are affected more often than females, with a predominance of 3 to 1.[4] Mental retardation is present in about 75 to 85% of patients with autism,[1][5][6] and almost half of all autistic patients are functionally mute.[5]

The causes and mechanisms underlying autism are heterogeneous, varying from genetic causes, that is, chromosome abnormalities and conditions with Mendelian inheritance such as metabolic disturbances, to infectious causes and teratogenic influences.[7-9] The prevailing view is that autism is caused by a pathophysiological process arising from the interaction of an early environmental insult and a genetic predisposition.[10] However, the aetiology often remains obscure and earlier studies reported that causal medical conditions were detectable in a relatively small percentage of autistic patients.[11][12] There is increasing evidence that genetic factors may well play a major role in the remaining idiopathic cases.[13] In support of this hypothesis is the recent identification of deletions in the short arm of the X chromosome,[14] duplications of the Prader-Willi/Angelman critical region on chromosome 15q,[15][16] linkage to 7q31 and 2q,[17][18] as well as the high monozygotic twin concordance rates,[19][20] and high recurrence risk among sibs of patients with idiopathic autism.[21]

Autism poses an extremely heavy burden for affected subjects, their families, and society. Research focusing on biological causes and on guidelines for these studies in each specific person is important, both in diagnostics, management, and genetic counselling. Here we report on the results of a full diagnostic work up of 25 patients with autism, all residing in an institute for the mentally retarded.

METHODS
Study subjects
In 1993, a behavioural study concerning stereotypic movements in autism was initiated in 25 adults with autism residing in an institute for the mentally retarded ("Eemeroord", Baarn, The Netherlands). Inclusion criteria at that time were: confirmed diagnosis of autism using DSM-IV criteria,[1] age >14 years, and residency in either of the two wards chosen for the study for practical reasons. The level of functioning was assessed in each patient using the SRZ scale[22][23] and subsequently classified based on DSM-IV criteria.[1] All subjects participated after written informed consent was obtained from parents or other legal care givers. The study was approved by the Medical Ethical Committee of the Academic Medical Centre in Amsterdam.
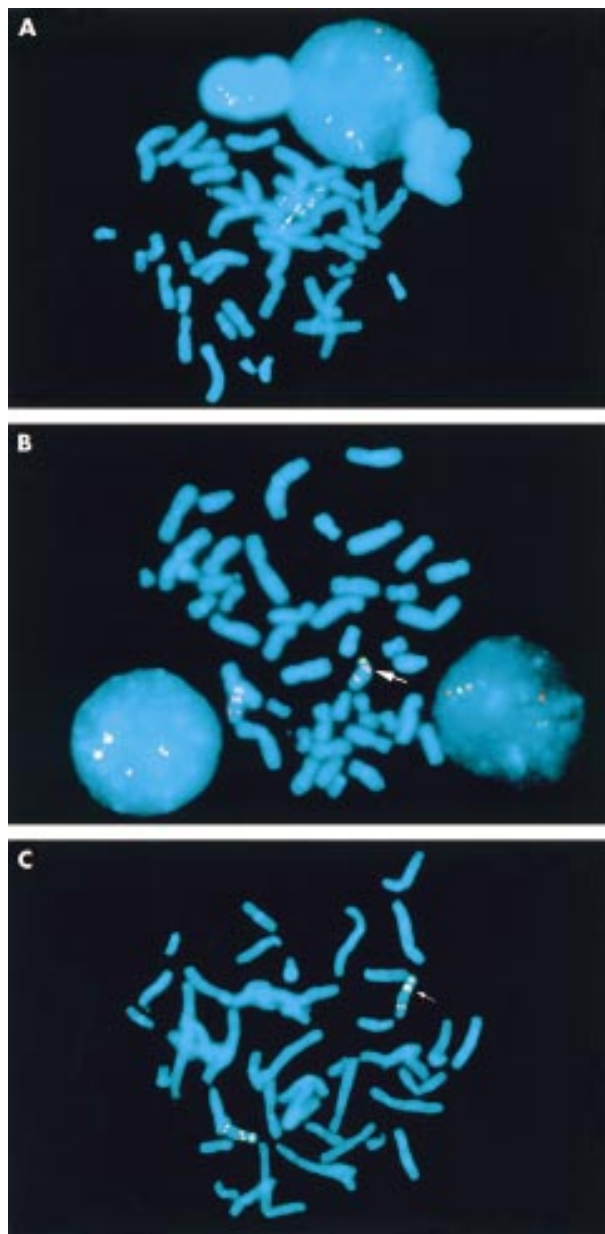
Figure 1 Fluorescence in situ hybridisation with LSI SNRPN/PML/CEP 15 dual colour probe (Vysis, Inc) which hybridises to the PW/AS critical region 15q11-13 (SpectrumOrange SNRPN), and as a control also to 15p11.2 (SpectrumGreen CEP 15p11.2, D15Z1) and to 15q22 (SpectrumOrange PML). (A) Metaphase cell showing a single orange signal (normal) at locus 15q11-13 (arrow). (B) Metaphase cell showing double orange signals at locus 15q11-13 (arrow), indicating a possible interstitial duplication. (C) Metaphase cell showing a large merged orange signal at locus 15q11-13 (arrow), indicating a possible interstitial duplication.

Data collection
Archives were searched to collect data retrospectively on each patient.

Physical examination
All patients were examined by one physician for the mentally retarded and one clinical geneticist.

Additional investigations
Chromosomes
Chromosome preparations from peripheral blood cultures and cytogenetic analyses were performed using standard techniques.

Fluorescence in situ hybridisation (FISH)
FISH analysis of the minimal DiGeorge critical region was performed using the cosmid M51 probe. FISH analysis of the subtelomeric regions of all chromosomes was performed using the Cytocell Ltd Multiprobe[T] technique,[24] and scored by two investigators following the protocol described in Appendix 1 (see www.jmedgenet.com). The FISH probes used to detect the origin and size of the marker chromosome identified in one of the patients are described in Appendix 1.

Screening for 15q11-13 interstitial duplications
The Prader-Willi/Angelman syndrome critical region (15q11-13) was screened for duplications by applying three different methods: (1) FISH analysis using the D15S10 and *SNRPN* probes (Vysis Inc), (2) densitometry using the microsatellite markers described in Appendix 1, (3) quantitative Real time PCR of loci D15S122 and *GABRA5* using probes and primers chosen with the assistance of the Primer Express software program (Applied Biosystems) and ordered from Applied Biosystems as well (for detailed description see Appendix 1) (fig 1).

Other molecular analysis
The presence of an *FMR1* gene expansion was analysed using standard molecular PCR procedures.[25] *MECP2* gene mutation screening is described in detail in Appendix 1.

Neuroimaging and EEG
Neuroimaging (CT and/or MRI scanning) of the brain was performed in all patients with either neurological signs at physical examination or in patients with microcephaly or macrocephaly (that is, an occipitofrontal head circumference below the 2nd centile or above the 98th centile, respectively) (n=10). Electroencephalography was performed on all patients using standard methods.

Metabolic investigations
These included a general urine screen as well as a search for peroxisomal, mitochondrial, glycosylation, and cholesterol metabolism disturbances (for detailed description see Appendix 1).

Ophthalmological and ear, nose, and throat (ENT) investigations
The investigations were performed according to international standards in all patients.

Other investigations
If clinical history, physical examination, or one of the other above mentioned studies produced clues for a specific diagnosis, further investigations in search of this diagnosis were initiated.

RESULTS
Patient characteristics (table 1)
Detailed information on single patients is provided in Appendix 2 (see www.jmedgenet.com). All patients are of Dutch extraction except for one Indonesian male and one Nigerian female. Age at physical examination varied from 22 to 45 years (mean 33.6 years). Intellectual abilities were limited in all 25 patients, the severity of mental retardation being mild (IQ 50/55-70) in two, moderate (IQ 35/40-50/55) in 11, severe (IQ 20/25-35/40) in 11, and profound (IQ <20/25) in the remaining two patients.[1] Six patients had mothers whose age at conception was 36 years or older. Mental retardation was present in seven relatives; psychiatric disorders occurred in five relatives.

Supplementary information in Appendices 1 and 2 can be found on the Journal website at www.jmedgenet.com

Table 1 Patient characteristics

Patient characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
General																									
Gender	F	M	F	M	F	M	F	M	M	M	M	M	M	M	M	M	M	M	F	M	M	M	M	M	M
Age	20	22	27	29	29	31	31	32	33	33	34	34	34	35	35	35	36	36	37	37	37	37	38	43	45
Degree of MR*	P	Mo	S	S	S	Mo	Mo	Mo	Mo	Mo	Mo	S	S	Mo	S	S	Mi	Mo	Mi	Mo	S	P	Mo	S	S
Family history†																									
Paternal age	48	28	33	23	47	53	44	25	30	41	31	35	27	30	38	31	36	31	29	47	39	39	27	33	44
Maternal age	29	30	27	24	40	39	43	26	27	34	29	29	22	32	38	22	33	22	23	32	34	34	28	40	39
Consanguinity‡	–	–	–	–	–	–	–	–	–	+7th	–	–	–	–	–	+3rd	–	–	–	–	–	–	–	–	–
Mental retardation§	–	–	–	+p	+c	–	–	+c	–	+s	+s	–	–	–	+u/a	+s	–	–	–	–	–	–	–	–	+c
Psychiatric disorder¶	–	–	–	+p	–	–	–	+u	–	–	–	–	–	–	–	+u	–	–	–	–	+gf	–	–	–	–
Other**	–	–	–	–	–	RM	–	–	–	–	–	–	–	–	–	SB	–	–	–	–	–	–	–	–	–
Clinical history																									
Teratogens††	–	–	–	ac	–	–	–	–	–	–	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–
Illnesses‡‡	–	Kink	–	SA	–	–	–	–	–	–	Hb	–	–	–	–	–	BP, Hi	–	–	Ha	–	–	–	–	–
Epilepsy	–	–	+	+	–	–	–	–	–	–	+	–	+	–	+	–	+	+	–	–	+	+	+	–	–
Phenotype																									
Height (m)	1.65	1.82	1.53	1.60	1.53	1.78	1.65	1.82	1.85	1.80	1.80	1.77	1.62	1.78	1.75	1.60	1.68	1.80	1.64	1.82	1.80	1.66	1.76	1.71	1.52
Head circumference (centile)	60	>98	<2	10	25	<2	70	10	50	>98	10	50	10	>98	10	<3	40	40	25	>98	>98	50	50	10	10
Dysmorphic features		98																							
Minor anomalies §§	+	–	+++	+++	–	++	+	+	–	+	+	++	+	+++	+	++	–	+	–	–	–	+++	–	++	+++
Malformations¶¶	UH	–	–	–	–	–	–	–	–	–	US	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Pigmentation defects***	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	++	–	–	–

*Mi=mild; Mo=moderate; S=severe; P=profound.
†Only data pertaining to first and second degree relatives.
‡Degree of consanguinity.
§¶c=cousins; gf=grandfather; p=parents; s=sibs; u/a=uncle/aunt.
**RM=recurrent miscarriages; SB=sib with spina bifida.
††c=present, type unknown; a=alcohol; ac=anticonvulsants.
‡‡Kink=kink in development; BP=blood pressure increase; Hb=hyperbilirubinaemia; Ha=haemangioma; HI=hernia inguinalis; SA=severe acne.
§§Minor anomalies: +=one or two minor features; ++=three or four minor features; +++=multiple minor features, strongly suggestive of a syndromal genesis.
¶¶Malformations: UH=umbilical hernia; US=ureteric stenosis.
***Pigmentation defects: +=one or two café au lait spots; ++=three or more café au lait spots.

In two patients, teratogenic influences were present during pregnancy: excessive maternal alcohol abuse in one and anticonvulsant use in the other. The patient born to the mother with alcohol abuse had multiple features fitting fetal alcohol syndrome; in the other patient no stigmata of fetal anticonvulsant syndrome were present. In a third patient, phenotypic features were strongly suggestive of a teratogenic influence. However, the mother denied use of medication or abuse of alcohol or other drugs during pregnancy, although she had been known to have periods of alcohol abuse before.

On physical examination, microcephaly or macrocephaly was diagnosed in eight patients, and neurological examination showed abnormalities (signs of a confirmed HNP L5-S1, drowsiness, tremor, and cogwheel rigidity) in three patients, while neurological side effects of medication were present in two patients. Minor congenital anomalies were present in 17 patients, varying from only one to multiple dysmorphic features. In eight of them the features were suggestive of the presence of a multiple congenital anomalies syndrome. More expressed congenital anomalies (urethral stenosis, umbilical hernia) were found twice. Skin depigmentation disturbances including café au lait spots and erythema were found in three patients; none had symptoms of neurofibromatosis, tuberous sclerosis, or other neurocutaneous disorders.

Additional investigations
The results of all additional investigations performed are listed in table 2. In this section only more specific details are provided.

Cytogenetics
The supernumerary marker chromosome detected in one patient was positive for FISH analysis with the centromere 13/21 probe (pZ21A), as well as for the whole chromosome 13 paint. Further studies showed the marker to be dicentric, without proximally located long arm material (13q11-12). In another patient, a mosaic pattern of the sex chromosomes was found, indicating his karyotype to be 45,X (75%)/46,XY (25%). In a third patient, subtelomeric FISH analysis yielded an Xpter deletion using cosmid probe CY29. However, this finding was not reproduced by the BAC probe 98C4 nor by molecular analysis. We concluded that the subtelomeric Xp abnormality was a polymorphism.

Screening for 15q11-13 interstitial duplications
The scoring results of *SNRPN* and D15S10 probes in interphase and metaphase showed considerable intra- as well as interobserver variability (data not shown). Thus results were difficult to interpret. The number of scored interstitial duplications, applying the definitions described in the Methods section, for the *SNRPN* probe varied between 0 and seven (28%) patients and for the D15S10 probe between one (4.2%) and 11 (45.8%) patients. There was no patient in whom a duplication was scored in both interphase and metaphase by both technicians.

Densitometric studies did not show a true duplication in the patients in our cohort, but

Table 2 Results of additional investigations and diagnosis

Investigation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Chromosomes																									
Karyotype†	46,XX	46,XY	46,XX	46,XY,+m13	46,XX	46,XX	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XX	46,XY	46,XY	46,XY	46,XY	46,XY	45,X/46,XY
Subtelomeric regions	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Dup 15q11-13																									
Densitometry‡	mdup	N	N	mdup	N	N	N	N	N	N	N	N	mdup	N	N	mdup	N	N	N	mdup	N	N	N	mdup	N
Real time PCR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
DiGeorge (22q11)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Fragile X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MECP2 gene§	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Metabolic screening¶	N	N	N	N	N	N	Pm	N	N	N	N	PKU	N	DCM	N	N	N	N	N	N	N	N	N	N	N
Neuroimaging††	N	N	N	N	N	N	N	N	N	N	CA	N	N	N	N	N	N	N	N	N	N	CEA	N	N	N
Ophthalmological investigation‡‡	N	N	N	RV/KC	N	N	N	N	N	N	N	N	N	N	N	RV/KC	N	N	N	N	N	OA/RV	N	N	RV
ENT investigation§§	N	N	N	PHL	N	N	N	N	N	N	PHL	N	N	CHL	N	N	PHL	N	N	N	N	N	PHL	N	PHL
Diagnosis¶¶		**Orstavik	**Teratogen	*m13						**Orstavik	*Kernicterus	*PKU		*FAS								**Private s			*mos 45,X/46,XY

N=normal.
†m13=marker chromosome 13.
‡mdup=mosaic pattern of duplication.
§Pm=polymorphism.
¶DCM=disturbed cholesterol metabolism; PKU=phenylketonuria.
††CA=cerebellar atrophy; CEA=cerebral atrophy.
‡‡KC=keratoconus; OA=optic atrophy; RV=reduced vision.
§§CHL=conductive hearing loss (as a result of radical eradication); PHL=perceptive hearing loss.
¶¶*=unequivocal diagnosis; **=probable diagnosis.
Orstavik=Orstavik syndrome; PKU=phenylketonuria; Private s=private syndrome; Teratogen=teratogenic influence; mos 45,X/46,XY=mosaicism for XY/XO.

dosimetric abnormalities were found in six patients, indicating a mosaic pattern of duplication in the region of marker D15S122 (n=4) and in the GABRA5 region (n=2) (fig 2). These results did not correspond to those of FISH analysis. Real time quantitative PCR analysis of the D15S122 region in the former four patients did not show any abnormalities, nor did analysis of the GABRA5 region in the latter two. As a positive control, a patient known in our Department with a cytogenetically visible duplication of the region 15q11-13 was used, in whom the duplication was confirmed with Real time quantitative PCR. Thus, by defining the latter technique as the gold standard, no person in our cohort had an interstitial 15q duplication.

Molecular analysis
In one female, analysis of MECP2 showed a C to T transition at position 1125 (the C-terminal region) causing a substitution of serine for proline at amino acid position 376. In the subsequent screening of 200 X chromosomes of normal controls, a similar mutation was detected in one control, indicating that it was most probably a polymorphism.

Metabolic investigations
In one patient, a previously established diagnosis of phenylketonuria (PKU) was reconfirmed by detection of raised plasma concentration of phenylalanine. In another patient, in whom the clinical diagnosis of fetal alcohol syndrome (FAS) was established, an abnormality of the distal cholesterol biosynthesis was detected: plasma concentrations of 5;7-dehydrocholesterol as well as 5;8-dehydrocholesterol were raised and plasma bile acid concentrations were low. Molecular analysis of the complete gene gave normal results, however. It seems likely that the detected cholesterol abnormalities can be attributed to the patient's intake of haloperidol, as has been reported before.[26] No abnormalities were detected in the cholesterol biosynthesis of five other patients in our cohort also using haloperidol.

Neuroimaging
In 10 patients neuroimaging studies were performed. Cerebellar atrophy was found in one and in another possibly diffuse cerebral atrophy was present; in all others neuroimaging showed no abnormality.

Diagnosis
The individual case histories of the following nine patients are described in detail in Appendix 2. In five (20%) patients the following unequivocal aetiological diagnoses were established: a prenatal factor in one (FAS, case 1), a perinatal factor in one (kernicterus, case 2), a metabolic disturbance in one (PKU, case 3), and a chromosome abnormality in two (marker chromosome 13, case 4) (mosaic karyotype, 45,X (75%)/46,XY (25%), case 5). In four (16%) patients a diagnosis was probable but without firm proof: a private syndrome in one patient (case 6), a teratogenic factor in one (case 7), and Orstavik syndrome in two patients (cases 8 and 9). Features of the latter two are compared to published cases in table 3.[27]

Diagnostic yield of individual investigations
The diagnostic yield of each investigation differed widely (table 4). A detailed clinical history identified abnormalities in 13 patients and in five of them this investigation contributed significantly to the diagnosis. Family history showed abnormalities in 12 patients, which contributed significantly in one patient. Physical examination showed abnormalities in 20 patients and in seven of them these findings contributed to establishing a diagnosis. Karyotyping led to diagnosis in two patients, while the results of FISH screening for subtelomeric rearrangements as well as for 22q11 deletions were normal. Screening for duplications in the PW/AS region through densitometry indicated possible mosaic duplications in six
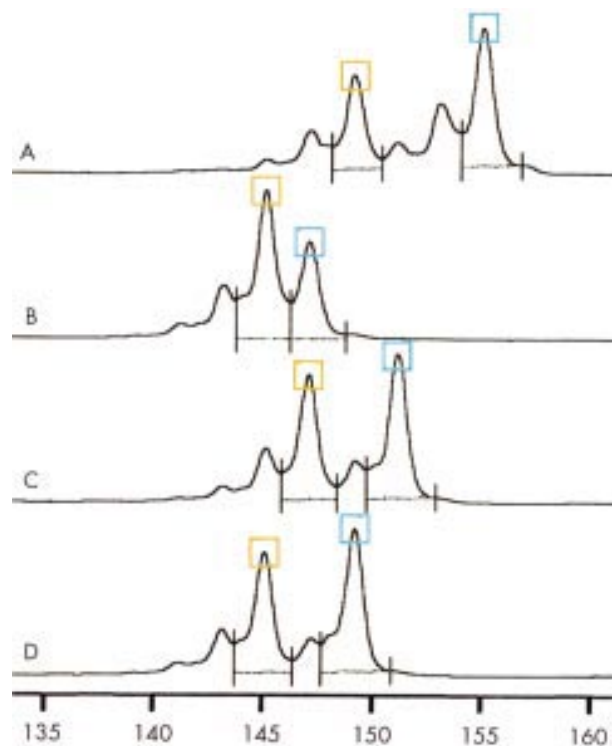
Figure 2 Electropherograms for marker D15S122 in four patients. (A) Positive control with visible duplication of the 15q11-13 region. The amplitude ratio of the 154.8 mobility unit (mu) peak (blue) v the 148.9 mu peak (orange) is 55.5:37.7 (=1.5). (B) Normal patient without a duplication. The amplitude of the 146.8 mu peak (blue) v the 144.9 mu peak (orange) is 42.4:64.6 (=0.7). (C) Patient with a possible mosaic duplication of this locus. The blue (150.9) v orange (146.8) peak ratio is 61.4:53.1 (=1.2), which is similar to, but smaller than, gold standard (A). (D) Another patient with a possible mosaic duplication of this locus. The blue (149) v orange (144.9) mu peak ratio is 43.6:36.7 (=1.2), which is also similar to but smaller than (A).

patients, but these could not be confirmed by Real time PCR. Molecular analysis for *FMR1* gene expansions did not contribute to the diagnosis, nor did *MECP2* gene mutation screening. Finally, EEG abnormalities were detected in eight patients, contributing to a diagnosis in two. Based on neuroimaging findings a diagnosis could be established in two subjects.

Remaining patients

Of the remaining 16 (64%) patients without any diagnosis, one had a major congenital malformation and eight had minor anomalies on physical examination. Of the latter group, no person had four or more anomalies, which would have been suggestive of a MR/MCA syndrome.

DISCUSSION

Since Kanner's description of autism as a developmental disorder characterised by "extreme autistic loneliness" and "an obsessional desire for the maintenance of sameness", autism has often been referred to as a diagnosis on its own.[28] However, autism should be considered a symptom, caused by a variety of

Table 3 Orstavik syndrome: comparison of our patients to published reports[27]

	Orstavik 1	Orstavik 2	Case 5	Case 13
Gender	F	F	M	M
Age	14	<14	24	33
Family history:				
Mental retardation	–	–	–	+
Seizures	–	–	–	–
Pregnancy	Term	Term	Term	Term
Weight at birth (g)	3600	2660	3480	N
OFC at birth (centile)	36 cm (75)	34 cm (25)	?	?
Developmental delay	+	+	+	+
Autism	+	+	+	+
Seizures	+	+	+	+
Coeliac disease	–	+	–	–
Dysmorphic features				
Macrocephaly	+	+	+	+
High, broad forehead	+	+	–	+
Deep set eyes	+	+	+	+
Short philtrum	+	+	+	+
Laboratory investigations				
EEG abnormalities	+	+	+	+
Neuroradiological abnormalities	N	N	?	?
Muscle biopsy	N	N	?	?
Spinal fluid (protein content and electrophoresis)	N	N	?	?
Urinary metabolic screening	N	N	N	N
Glutaryl CoA dehydrogenase	N	N	N	N
Karyotype	46,XX	46,XX	46,XY	46,XY
Molecular studies for fra(X) syndrome	N	N	N	N

Table 4 Yield of investigations performed in all 25 patients

Investigation	Abnormalities identified (No of patients)	Contributing to a diagnosis (No of patients)
Patient clinical history	13	5
Family history	12	1
Physical examination	20	7
Karyotype	2	2
FISH subtelomeric regions	0	0
FISH DiGeorge region	0	0
Densitometry 15q11-13 region	6	0
Real time PCR 15q11-13 region	0	0
Fragile X screening	0	0
MECP2 mutation screening	1	0
Metabolic screening	2	1
EEG	8	2
Neuroimaging	2	2
Opthalmological investigations	4	1
ENT investigations	6	0

underlying disorders. Most investigators at present assume that the underlying pathophysiological process causing autism arises from the interaction between a genetic predisposition and an early environmental insult.[10]

Comparison to other studies

In the present study, a diagnosis was made in nine (36%) patients, in five unequivocally and in four probably. These results fall in the middle range when compared to other similar studies: an organic aetiologic factor for autism was found to range from 10-60%.[2 5 7–9 12 19 20 29–32] The variation in the results of these studies may be explained by a difference in diagnostic criteria used to establish the diagnosis of autism, in patient selection, and in diagnostic work up.[11]

Present study: advantages and limitations

In the present study we analysed a group of patients assembled previously for another study (with a different scope), all residing in the same institute for the mentally retarded, fulfilling all the DSM-IV criteria for autism,[1] and with a standardised work up in all patients that was (almost) complete for present standards. The work up is limited only in the absence of screening of family members for components of the broad autism phenotype, as suggested by Piven et al.[33] However, this approach is especially important in linkage studies, but less so for finding organic causes, as in the present study.[33]

The fact that all patients were mentally retarded adults may have created a bias. However, as 75-85% of patients with autism are mentally retarded and as the distribution of the different degrees of delay within the present study group is similar to the distribution within persons with autism in general, the introduced bias is limited.[7 8 29 32] For subjects with autism whose characteristics differ from those of our cohort, such as children and subjects without mental retardation, the results and conclusions of the present study are only partly applicable. Therefore the yield expected for a diagnostic work up should be adjusted to the age and developmental level of the autistic patient.

Value of each diagnostic investigation
Clinical history and physical examination

Clinical history and physical examination have proven to be the most rewarding parts of the diagnostic work up. A patient's history may provide clues to prenatal causes, for example, fetal teratogen exposure such as thalidomide[34] and alcohol.[35] The exact teratogenic mechanism of alcohol is unknown, although a clue may be the disturbance in the migration of neuronal and

glia cells,[36] supported among other things by the cerebellar anomalies present in both animal models of FAS[37] and subjects with autism.[38] Perinatal history may provide clues for factors such as neonatal jaundice. Before the advent of phototherapy and exchange blood transfusions, neonatal jaundice often caused kernicterus, characterised by sensorineural hearing loss, mental retardation, and evidence on MRI of damaged basal ganglia, especially the globus pallidus.[39] The latter characteristic may well explain the autistic behaviour reported in patients with kernicterus,[40] as there is increasing evidence that the volume and function of basal ganglia are different in autistic subjects compared to controls.[41]

Detailed physical examination, including anthropometric, neurological, and dysmorphology examinations, may provide clues to many possible aetiologies. In general, the incidence of minor physical anomalies in patients with autism is increased when compared to controls,[42] indicating that in the former group structural development was disrupted during early embryogenesis as a result of underlying disorder. Abnormal cephalic measures occur among autistic persons in a significantly higher proportion than in the general population, and have been suggested to be the single most consistent physical characteristic of autistic subjects.[12 43] In our cohort, the frequency of macrocephaly (20%) and microcephaly (12%) is similar to the frequencies among the general autistic population (20% and 15%, respectively).[44]

Many syndromes are known to have a distinct behavioural phenotype, indicating the potential for the causative genes to influence human cognitive development.[45] Autism is considered to be such a behavioural phenotype associated with several well described syndromes, such as Williams syndrome and Moebius syndrome.[46] In our cohort, a syndromic genesis was suspected for two subjects whose phenotype strongly resembled the autosomal recessive syndrome described by Orstavik et al,[27] and which is characterised by epilepsy, mental retardation, facial dysmorphism, and macrocephaly (table 3). This combination of features may have been described before in 1995 by Andermann[47] in four patients. As neuroimaging data are lacking in both our patients, an unequivocal diagnosis of Orstavik syndrome is currently not possible.

Karyotyping

A broad spectrum of chromosome anomalies in autism has been reported, involving almost all chromosomes and many types of rearrangements.[48] Most frequently documented are (de novo) structural and numerical abnormalities of sex chromosomes and anomalies of chromosome 15.[13] In our cohort, two (8%) subjects were identified with numerical chromosome anomalies, a rate similar to the 5% reported by Bailey et al[49] in 1996 and 6.3% by Konstantareas et al in 1999.[50] Three patients similar to our case with 45,X/46,XY mosaic and autistic behaviour have been described.[2 51] The present patient with mosaic Turner karyotype showed bilateral shortening of the fourth metatarsal bones, a common feature in Turner syndrome. This is additional evidence that the chromosome anomaly exerts its effect on the phenotype. Furthermore, the maternal origin of the remaining X in the present patient is in agreement with the hypothesis of a parent of origin effect in the X chromosome influencing social cognition.[52] Although supernumerary marker chromosomes have been reported in subjects with autism,[50 53] to our knowledge no other cases with a marker derived from chromosome 13 were reported to have autism.[54] Interestingly, a chromosome 13q region was the most significant result in one collaborative linkage study (CLSA) genome scan.[55] Other linkage studies also showed increased sharing of this locus, although weaker.[56] It seems likely that the marker 13 chromosome has affected the phenotype, including the presence of autism.

Submicroscopic deletions

Patients with the velocardiofacial (VCF) syndrome have a distinct behavioural phenotype and often suffer psychiatric disorders, including autism.[57] FISH analysis in all the present patients was performed despite the absence of the dysmorphic features of VCF syndrome, and no deletion was identified. This concurs with a recent paper, in which no 22q11 deletions were found in autistic patients without a phenotype suggesting VCF syndrome.[58] Submicroscopic rearrangements involving (sub-)telomeric regions are emerging as an important cause of mental retardation.[59] In our cohort, no subtelomeric rearrangements were found. Owing to the limited number of patients in our cohort, however, no firm conclusions can be drawn. To ascertain the presence and frequency of these cryptic rearrangements among autistic subjects, further similar, and if possible larger, studies are required.

15q11-13 abnormalities

Over the past decade, numerous reports have mentioned abnormalities of chromosome 15 associated with autism, with or without mental retardation. Frequently, they concerned supernumerary isodicentric chromosomes 15, less frequently interstitial duplications, and rarely triplications of the 15q11-13 region, almost all maternally derived.[60–69] The region may harbour a potential susceptibility gene or genes for autism [13 70 71] In a large study performed by Schroer et al,[15] a cohort of 100 consecutive patients with autism was screened and abnormalities were identified in four (4%): two with supernumerary bisatellited marker chromosomes 15, one with a 15q11-13 deletion, and one with an interstitial duplication of region 15q11-13. In most published cases, an interstitial duplication (or triplication) was already visible on G banded chromosomes. However, there is as yet no generally accepted reconfirmation method, nor a method to screen for submicroscopic duplications in this region. FISH analysis, sometimes followed by PCR microsatellite analysis, is the most widely applied method.[15 16 62 67] There is only a single report of subtle 15q11-13 interstitial duplications, which according to the authors might have been missed in routine chromosome or FISH analysis: microsatellite densitometry analysis was used to detect this abnormality in two sibs with autism, which was also present in their unaffected mother.[72] In our cohort, we found no patients with cytogenetically visible abnormalities of chromosome 15. We first performed FISH analysis, but this technique is not suitable for detecting cryptic 15q11-13 duplications because the company making the probes (Vysis, Inc) states in their "Quality Assurance Certificate" that "Occasionally, the LSI probes may appear as three or four signals, depending upon condensation of the DNA and the relative distances between chromatids. All probe signals may also appear diffuse or split". No validated method has been published to quantify and qualify results of duplication screening of region 15q11-13 by FISH, and various subjective terms have been used, such as "a large merged signal", "a double signal", or "a split signal"[16 73]; interobserver and intraobserver variability studies are lacking. Consequently, we have performed densitometric studies. This technique yielded possible interstitial duplications (in mosaic form) in several patients; however, these did not coincide with those found by FISH. Furthermore, we doubted its validity, as the amplitude ratio of the electropherogram peaks in our six patients was less than the ratio in the positive control (fig 2). Therefore, quantitative Real time PCR was performed, but no abnormalities were found, indicating that no person in our cohort had such a duplication. FISH analysis and densitometry would have led to an overestimated frequency of interstitial 15q duplications in our cohort. Hence, this seems to be a less frequent cause of autism than suggested by previous clinical studies.[15 72]

Real time PCR has proven to be a sensitive, specific, and reproducible method for diagnosing changes in gene dosage, especially in diagnostic cancer research.[74] A recent exemplary study was performed by Aarskog et al,[75] who used Real time PCR to detect cryptic PMP22 duplications and deletions in patients with Charcot-Marie-Tooth type 1A.[75] For our study, its technical validity was proven by confirmation of a duplication in a cytogenetically positive control.

Whether duplications in the PWAS region are really pathophysiologically significant in autism remains to be elucidated. Recently, duplications of the *GABRA5* gene were detected in unaffected subjects, suggesting that (some) 15q11-13 duplications may be benign polymorphisms.[76]

Fragile X syndrome

No patients were identified with fra(X). Indeed, the relationship between these two conditions remains uncertain. At first, males and females with fra(X) were reported to display autistic behaviour frequently,[77] but later on autism was proven to occur no more frequently in a fra(X) population than in the mentally retarded population in general.[78] Also, no linkage with the *FMR1* gene region was proven in a study of families multiplex for autism but without cytological evidence of fragile X expression.[79]

MECP2 gene screening

The recent study by Lam et al[80] reporting a *MECP2* gene mutation in one of the 21 screened (non-RTT) subjects with infantile autism and mental retardation motivated us to perform a similar screening in our cohort. No true mutations were found, as the single point mutation we found is probably a (hitherto unreported) polymorphism.

DNA repair disorder

One of the present patients (case report 6) showed many features compatible with a DNA repair disturbance, although firm proof at the cellular level is still lacking. To our knowledge, only one earlier report mentioned autism in a patient with a DNA repair disturbance.[81] It remains uncertain how this relationship, if any, might be explained. Well known repair disturbances such as Bloom, Cockayne, Rothmund-Thomson, and Werner syndromes do not include autism.[82]

Metabolic screening

Phenylketonuria,[83] hypersuccinylpurinaemia,[84] changes of aromatic acids and monoamines,[85] lactate acidosis,[86] and cholesterol anomalies[87] are metabolic disturbances reported in patients with autism. In our cohort, extensive screening of multiple metabolic pathways resulted in the (re)confirmation of metabolic disturbances in two patients: phenylketonuria (PKU) in one and an abnormality in the distal cholesterol biosynthesis in another patient. The mechanism through which PKU causes autism remains uncertain, but has been suggested to involve dopaminergic pathways.[88] The so-called enzyme/brain-barrier has also been suggested to be involved.[89] In our cohort, no proof of a mitochondrial dysfunction was present. A disturbance in brain energy metabolism owing to mitochondrial dysfunction has been proposed as a cause of autism, supported by reports of lactate acidosis and urinary excretion of Krebs cycle metabolites in autistic patients.[86 90] It has been hypothesised that mitochondrial dysfunction may act through excessive nitric oxide production.[91]

Finally, abnormalities in the glycosylation process cause the CDG syndrome phenotypes.[92] As these are still in the process of delineation,[93] we investigated all patients, all with negative results.

EEG

The recognition of a high incidence of EEG abnormalities and of seizure disorders involving all areas of the cerebral cortex was among the earliest evidence of an organic basis of autism.[94] In autistic subjects with a single EEG, abnormalities

are present in about 40% versus 65% with multiple EEGs.[95] In our cohort, single EEGs identified abnormalities suggestive of epilepsy in about one-third of patients (n=8). However, epilepsy should not be considered a separate aetiological entity but a symptom.

Neuroanatomical imaging

Neuroanatomical abnormalities are frequently found in autistic subjects, affecting the cerebral cortex, thalamus, brain stem, and most often the cerebellum.[96] It has been difficult to integrate these findings in an explanatory model.[97] Of the present 10 patients in whom neuroimaging was performed, findings concurring with the diagnosis of kernicterus were identified in one patient, and in the patient with a possible DNA repair disorder signs of cerebral atrophy were present. Otherwise all neuroradiological studies gave normal results. Thus, in our cohort, neuroimaging provided few clues for previously unsuspected diagnoses.

ENT and ophthalmological investigation

Investigation of both vision and hearing is important in any person with developmental delay for adequate support and care and to detect specific causes for the mental handicap.[98] Although in our cohort the ENT and ophthalmological investigations did not provide new aetiological insights, a considerable number of subjects were identified with insufficient sensory function, with therapeutic consequences.

CONCLUSIONS

In autism, a specific aetiological diagnosis is of considerable value, both to establish prognosis and to provide adequate care, as well as for genetic counselling and for unravelling its pathogenetic mechanisms. The present study shows that an extensive, structured work up yields a diagnosis in at least 20%, and possibly up to 36% of adult autistic subjects with mental retardation. As these underlying medical conditions encompass teratogenic, metabolic, and syndromic influences, a prerequisite for such a yield is a multidisciplinary approach, of which clinical history taking and physical examination form the basis. In future similar studies, diagnostic data should be gathered on a larger number of patients, allowing more firm conclusions. Linkage studies, used to screen multiplex families in order to identify autism susceptible loci,[56 99] will profit from an initial diagnostic work up of all subjects before inclusion, as all other causes of autism will be ruled out. The diagnostic yield of linkage studies will undoubtedly increase if the cohorts comprise only those remaining cases with truly idiopathic autism.[18]

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- Twenty-five consecutive mentally retarded adults (20 males) with autism (DSM-IV criteria) were investigated for somatic causes of their developmental disorder.
- Each patient underwent complete physical examination, ophthalmological and ENT investigations, EEG studies, karyotyping, FISH analysis of 22q11 region and all sub-telomeric regions, molecular analysis for *FMR1* gene expansions, *MECP2* gene mutations, and duplications of the 15q11-13 region, metabolic investigations including a general urine screen, search for peroxisomal, mitochondrial, glycosylation, and cholesterol metabolism disturbances, and neuroradiological studies (if neurological symptoms were present).
- In five patients an unequivocal diagnosis was established: fetal alcohol syndrome, kernicterus, phenylketonuria, disturbed cholesterol metabolism, marker chromosome 13, and mosaicism for XY/XO. A probable diagnosis was made in four other patients: Orstavik syndrome in two, a private syndrome in one, and a teratogenic cause in one.
- A complete work up of autistic mentally retarded adults yields a diagnosis responsible for autism in at least 20% and possibly up to 36%. If such studies are performed in cohorts of familial cases used for linkage analysis, such studies may well be more successful.

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Familial pericentric inversion of chromosome 5 in a family with benign neonatal convulsions

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We describe a family in whom a pericentric inversion of chromosome 5 segregates with benign familial neonatal convulsions (BFNC). BFNC is an autosomal dominant form of epilepsy characterised by spontaneous partial or generalised clonic convulsions beginning within the first months of life. Seizures usually disappear by the age of 6 months; intercritical electroencephalogram and subsequent psychomotor development are normal. BFNC loci have been mapped to human chromosomes 20q13.3 (*BFNC1*) and 8q24 (*BFNC2*), based on linkage analysis.[1 2] Recently, two potassium channel genes (*KCNQ2* and *KCNQ3*), located in these two regions, were shown to be mutated in BFNC1 and BFNC2, respectively.[3 4]

We report a family with BFNC and a pericentric inversion of chromosome 5 cosegregating with BFNC. Fluorescence in situ hybridisation (FISH) experiments were performed to define the breakpoints at YAC level. The linkage of BFNC to *KCNQ2* and mutations in the *KCNQ3* gene were excluded. This report raises the possibility of a new locus for BFNC on chromosome 5.

CASE REPORT

The proband, a male, was the second child of unrelated parents, born at 40 weeks of gestation after an uneventful pregnancy. Apgar score was 10 at one and five minutes. The parents were both 32 years old at his birth. Birth weight was 3400 g (50-75th centile), length was 51 cm (50-75th centile), head circumference was 35 cm (50-75th centile), and the clinical examination was normal except for the presence of hypotelorism. On the third day of life he had five episodes of generalised clonic convulsions treated with phenobarbital (5 mg/kg/day). Two additional episodes of seizures occurred during the first months of life and therefore long term therapy was continued. Routine laboratory investigations including plasma ammonia levels and acid base status were normal. Plasma and urinary amino acids were normal. Brain CT scan, electroencephalogram, echocardiography, and abdominal echography were all normal. At 8 months of age he was developmentally normal and continued to take phenobarbital.

The mother and maternal grandmother appeared to be intellectually normal but reported a clear history of convulsions in infancy and afebrile seizures later in childhood. Both showed hypotelorism without other clinical or dysmorphic signs. No other relatives of the proband had hypotelorism or a history of idiopathic neonatal convulsions.

Cytogenetic and molecular analysis

Chromosome analysis was performed on QFQ and GTG banded metaphases from synchronised peripheral lymphocyte cultures using standard procedures. The proband's
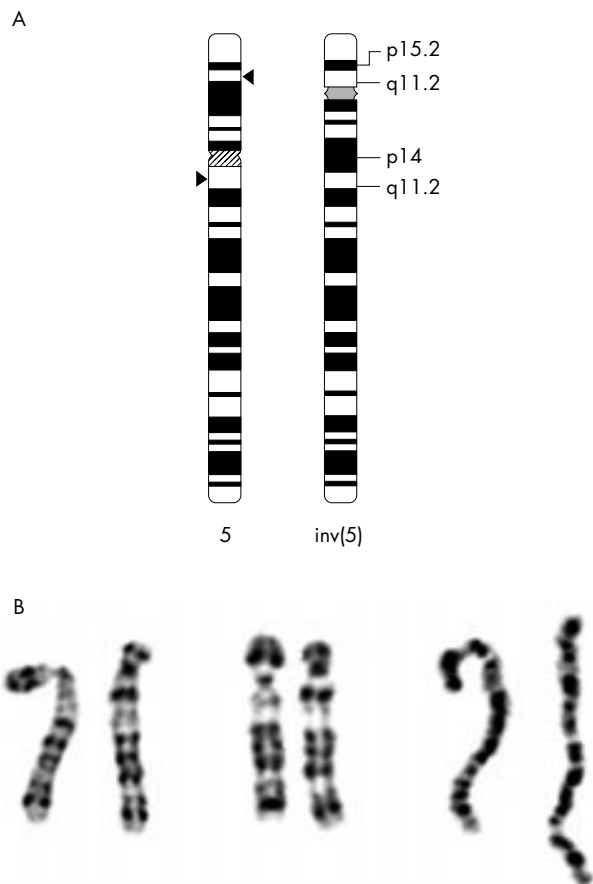
Figure 1 (A) Ideograms of the normal and inverted chromosome. (B) GTG banded partial karyotype of the patient. The normal chromosome 5 is on left and the inverted one on the right.

Figure 2 Family pedigree.

karyotype was interpreted as 46,XY,inv(5)(p15q11) (fig 1) and the same inversion was present in the proband's mother and maternal grandmother but not in other relatives (fig 2).

Inversion breakpoints were defined at the YAC level by fluorescence in situ hybridisation (FISH) to chromosome preparations from the proband. YACs containing chromosome 5 specific sequences from several locations on the p and q arms were selected according to the Genome Database (www.genome. wi.mit.edu/cgi-bin/conting/phys_map). YAC DNA was labelled with biotin using nick translation. The labelled probes were visualised with FITC-avidin (Vector) and the chromosomes were counterstained with DAPI (Sigma). Hybridisations were analysed with a Zeiss Axioplan epifluorescence microscope and images were captured with the Power Gene FISH System (PSI).

Breakpoints were found at YACs 956a11 (5p15.1, D5S1954-D5S416 at 28 cM from the short arm telomere) and 854b12 (5q11.2, D5S2076-D5S664 at 63 cM) (fig 3). Therefore the full karyotype of the proband was 46,XY,inv(5)(p15q11).ish inv (5)(p15.1q11.2)(D5S1954sp,D52076sp).

Segregation of the polymorphic markers D8S1385 and D8S558 closely linked to *KCNQ3* and of the Thr752Asn polymorphism located in exon 16 of the *KCNQ2* gene was studied in the family members. This study excluded linkage of BFNC to *KCNQ2* while linkage to *KCNQ3* was not excluded. Therefore, the *KCNQ3* coding region, including the exon-intron boundaries, was analysed in the patient and his mother. Polymerase chain reactions (PCR) were performed using primers and PCR conditions kindly provided by Shinichi Hisose, Department of Paediatrics, Fukouka University, Japan. PCR products were directly sequenced using ABI PRISM 310 sequencer and the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA). No mutations were found.
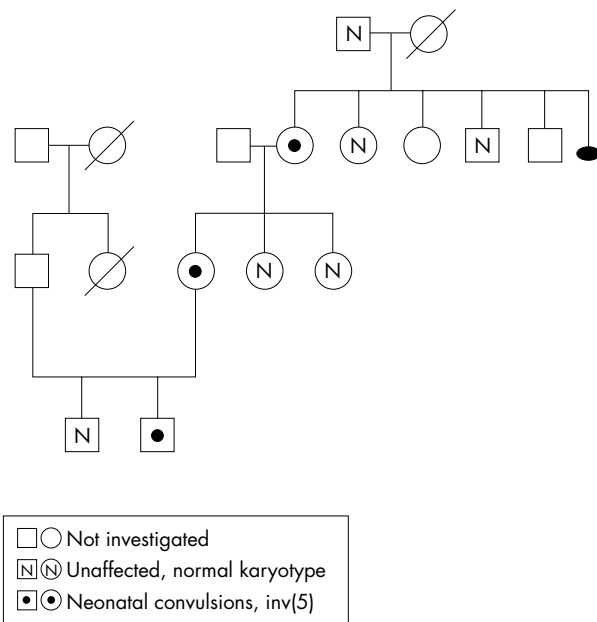
DISCUSSION

This report is another example of a rare case of an inherited pericentric inversion of chromosome 5 with the presence of clinical findings. Other pericentric inversions of the same region of chromosome 5 have been described,[5][6] but not with the same breakpoints in both arms. In our family, FISH analysis localised the 5p breakpoint at YAC clone 956a11 located in proximal 5p15.1 and the 5q breakpoint at YAC 854b12 located in 5q11.2. The breakpoint in the short arm of chromosome 5 in this report is not in the cri du chat syndrome critical region, mapped in 5p15.3 for high pitched cry and in 5p15.2 for the remaining features.[7] Therefore, this is the first example of a family with a pericentric inversion of chromosome 5 and BFNC.

In BFNC, seizures begin in the neonatal period and generally have a favourable outcome with spontaneous remission in the first year of life. The clinical heterogeneity of this disorder has been suggested by differences among pedigrees in the risk of developing epilepsy in later life.[8] In the last few years, two loci for BFNC have been mapped to chromosomes 20q13.3 and 8q24[1][2] showing that this disorder is also genetically heterogeneous. The majority of families are linked to chromosome 20q with mutations in the potassium channel gene *KCNQ2*. Only two families have been detected with mutations in the highly homologous gene *KCNQ3* at 8q24,[9][10] so *KCNQ2* was considered to be a major gene locus for BFNC.[3][4][9] Mutations in both genes are heterozygous, according to the autosomal dominant inheritance pattern of BFNC. A new benign idiopathic epilepsy in early life, with a favourable outcome and autosomal dominant inheritance, has been reported.[11] In this family, the seizures began between 4 and 7 months of life and for this reason the condition was called benign infantile familial convulsions (BFIC). A locus for BFIC has been mapped to chromosome 19q12-13,[12] suggesting that BFNC and BFIC are not allelic diseases with variable expressivity but are different entities. However, in the seven families with BFIC recently investigated, no evidence of linkage with chromosome 19 markers has been found.[13]

In conclusion, there are several lines of evidence for clinical and genetic heterogeneity of this condition and at least three loci responsible for autosomal dominant benign epilepsy of early life. Our family shows a strong correlation between the chromosome 5 inversion and BFNC. In fact, the disease was present only in the three inv(5) subjects but not in the two
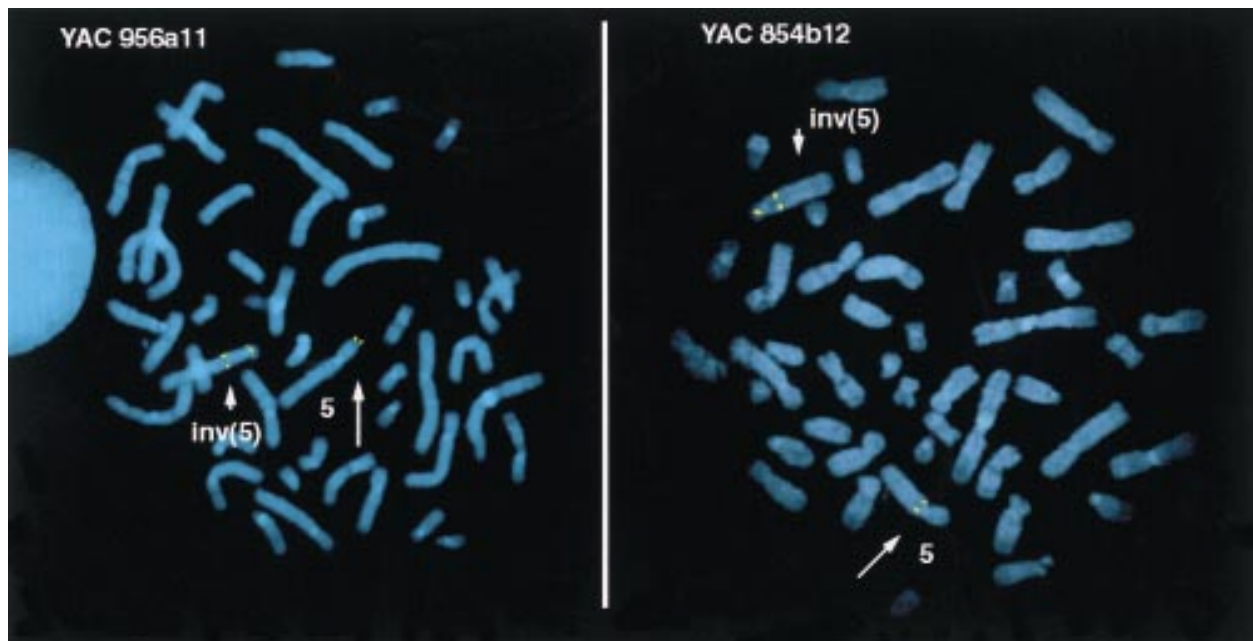
Figure 3 FISH analysis with the YAC 956a11 (left) and YAC 854b12 (right) crossing the inv(5) breakpoints on the short and long arm, respectively.

aunts of the proband or in his brother. We hypothesised that breakage or transcription silencing of a gene at one of the inversion breakpoints is responsible for the disease. Moreover, the exclusion of linkage of BFNC to *KCNQ2* and *KCNQ3* in this family supports this hypothesis.

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Phenotypic effects of mosaicism for a 47,XXX cell line in Turner syndrome

V P Sybert

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Turner syndrome, gonadal dysgenesis with sex chromosome abnormalities, occurs in approximately 1/3000 live-born females. Of females diagnosed with the condition, half are monosomic for the X chromosome. Among the rest, a multiplicity of chromosomal aberrations has been described. The more frequent are the presence of an isochromosome of the long arm of the X (i(Xq)) and ring X and mosaicism for two or more normal or abnormal cell lines (for example, 45,X/46,XX; 45,X/46,X,i(Xq); 45,X/46,XY). A small proportion (3-4%)[1 2] of subjects with Turner syndrome are mosaic for a triple X (47,XXX) cell line. The triple X syndrome in the non-mosaic state is associated with a decrement in intelligence from that expected based on parental and sib accomplishment, normal stature, and normal fertility.[3–8]

Is the prognosis for females with Turner syndrome mosaic for a triple X cell line substantially different from that for females with 45,X? Does the presence of a third, normal 46,XX cell line in some of these females predictably affect phenotype? Is the risk for mental retardation or the likelihood of preserved fertility or normal stature greater in this category of people than in those with 45,X or 45,X/46,XX alone?

I have reviewed our experience with 17 patients with Turner syndrome mosaic for a triple X cell line (11 with 45,X/47,XXX and six with 45,X/46,XX/47,XXX), and that of an additional 80 published case reports (18 with 45,X/47,XXX and 62 with 45,X/46,XX/47,XXX).[1 9–64] These data are compared with those for the 227 girls and women with 45,X Turner syndrome and the 69 with 45,X/46,XX in our clinic database.

METHODS

Information gathered from medical records, direct examination, and self-report has been collected in a computer database as part of an ongoing, long term study of the natural history of Turner syndrome begun in 1977.[2 65 66] Subjects were ascertained through self-referral, advertisement, review of hospital records, and referral for diagnosis or management from paediatricians, obstetricians, perinatologists, endocrinologists, internists, and family practitioners.

All subjects have had karyotype confirmation of their clinical diagnosis. A minority has had two or more tissues analysed. In most of these, the two cell lines were amniotic fluid cells and confirmatory postnatal peripheral lymphocyte karyotyping.

I performed a search in PubMed using: Turner syndrome, sex chromosome, 47,XXX, triple X, and triple X females as search terms. I used the bibliographies from the citations generated to find case reports published before the inception of the electronic databases, as well as for any publications overlooked by the search strategy used. Reports in English, Italian, French, and Spanish were reviewed. I discarded any case reports without karyotype confirmation. Complete information was not available for all subjects. I analysed the results using the appropriate denominator for each feature.

RESULTS

Table 1 lists the ages and reasons for the diagnosis of Turner syndrome, comparing the subjects from our clinic population and those in the published reports to our clinic population with 45,X and 45,X/46,XX chromosome constitutions.

The women in the X/XX/XXX group described in the published reports were older than the subjects in the other groups. This is most likely because a significant proportion, one quarter, of these women were diagnosed in adult life, during evaluation of recurrent pregnancy loss. Among our clinic populations, prenatal diagnosis was a more common avenue of diagnosis than for the published cases. This reflects the nature of our clinic, which is a referral centre for such cases. We are less likely to be involved in the evaluation of recurrent pregnancy loss or fetal wastage and thus less likely to ascertain adult patients with Turner syndrome through this route.

Oedema is the primary reason for the diagnosis of Turner syndrome in infancy. Its absence as a clinical feature in the

Table 1 Comparison of subjects mosaic for a 47,XXX cell line with 45,X and 45,X/46,X Turner syndrome: data from clinic population and medical publications

Karyotype	Clinic		Published reports		Clinic	
	X/XXX	X/XX/XXX	X/XXX	X/XX/XXX	45,X	X/XX
No	11	6	18	62	227	69
Age range (y)	5–54	1.5–60	0–55	1–67	0–80	0–74
Mean age (y)	20.7	21.4	15.4	29.4	18.9	17.4
Reason for diagnosis (No)	11	6	11	56	224	65
Oedema	0	0	0	0	89 (40%)	4 (6%)
Short stature	8 (73%)	1 (17%)	4 (33%)	8 (14%)	66 (29%)	16 (24%)
Amenorrhoea	2 (18%)	1 (17%)	0	16 (29%)	44 (20%)	12 (18%)
Prenatal	1 (9%)	2 (33%)	0	2 (4%)	11 (5%)	24 (37%)
Other	0	2* (33%)	7† (66%)	30‡ (53%)	14§ (8%)	9¶ (14%)

*Recurrent pregnancy loss (1), behavioural problems (1).
†Mental retardation (3), secondary amenorrhoea (3), hypoplastic left heart (1).
‡Secondary amenorrhoea or fetal wastage (15), hypoplastic left heart (1), abnormal offspring (6), cancer (5), ambiguous genitalia (1), newborn screening (1), Hirschsprung disease (1).
§Mental retardation (1), failure to thrive ± developmental delay (3), unusual facies (4), congenital heart disease (3), multiple malformations (1), GI bleeding (1), premature ageing (1).
¶Mental retardation (1), failure to thrive (1), unusual facies (4), hirsutism (1), pregnancy loss (2).

Table 3 Height and ovarian function in subjects mosaic for a 47,XXX cell line compared with 45,X and 45,X/46,XX Turner syndrome

Karyotype	Clinic		Published reports		Clinic	
	X/XXX	X/XX/XXX	X/XXX	X/XX/XXX	45,X*	X/XX*
Height <3%	5/11 (45%)	2/6 (33%)	7/8 (87%)	27/46 (59%)	70/95 (74%)	17/28 (61%)
Spontaneous menses	4/7 (57%)	2/3 (67%)	11/13 (84%)	30/44 (68%)	13/123 (11%)	11/32 (34%)
Fertility	1/7 (14%)	1/3 (33%)	9/13 (69%)†	20/44 (45%)†	1/123 (0.8%)	6/32 (19%)
	G1P1	G2 SAB2	G10P9 SAB2	G76P34 SAB43 TAB1	G2P2	G1P1
						G2P2
						G2P2
						G2P1 TAB1
						G8P1 SAB7
						G4P1 SAB3

G, gravida; P, para; SAB, spontaneous abortion; TAB, therapeutic abortion.
*Includes only those women who have achieved final adult height.
†Some the result of medically induced ovulation.

females with a triple XXX cell line constitutes a major difference between these subjects and those with 45,X or 45,X/46,XX. Although oedema in the newborn period was not recognised, in utero oedema most likely had been present, based on the presence of nuchal webbing in a similar proportion of the subjects with mosaicism for a 47,XXX cell line and those with monosomy X alone.

Approximately a third of patients with Turner syndrome are diagnosed during evaluation for short stature. Among the published X/XX/XXX cases, short stature accounted for only 14% of diagnoses, despite the fact that almost two-thirds of the women were below the 3rd centile in height.

Tables 2 and 3 provide data regarding those features of Turner syndrome hypothesised most likely to be different in subjects with mosaicism for XXX: intellectual function, height, and fertility.

I did not try to establish the presence or absence of specific learning disabilities known to be associated with Turner syndrome, such as difficulties with spatial reasoning, processing, etc,[67–72] as there was inadequate information available for most of the published cases. Only frank mental retardation, as defined by a tested IQ below 70, inability to function independently in adult life, or as stated as such in the case reports, was considered. None of the females in our clinic population with a 47,XXX cell line are mentally retarded, compared with a prevalence of 8-9% in our "control" groups. However, the number of patients with mosaicism for 47,XXX is small. The proportion of published cases with mental retardation and 45,X/47,XXX (42.8%) is much higher than for the other groups. However, one was ascertained as part of a screening evaluation of residents in an institution for the mentally retarded,[45] one had a brother and a half sister with similar delays, both of whom had been extensively evaluated without a diagnosis,[44] and a third woman was karyotyped at the age of 25 during an evaluation for seizures, mental retardation, and structural brain malformations.[61] One subject was

described as having normal mentality but was slightly slow[47] and two others were evaluated for pregnancy loss; normal intellectual function was implied but not clearly stated.[41 53] I did not include these three women in the analysis.

Short stature was seen in the majority of published cases. Some of our 45,X/47,XXX patients have received treatment with androgens or human growth hormone. Two of the three treated were above the 3rd centile in height. Three of eight who had not been treated were above the 3rd centile. None of our 45,X/46,XX/47,XXX patients had been treated with these agents; four of six were above the 3rd centile.

Spontaneous menarche is much more likely in subjects with mosaicism for an XXX cell line, with or without the presence of a normal 46,XX component (70%), than in women with 45,X without mosaicism (11%). Women with mosaicism for a 47,XXX cell line were also more likely to have spontaneous menarche than those with 45,X/46,XX (70% compared with 34%). A significant number of these women did not maintain normal menstrual cycles and secondary amenorrhoea or premature menopause were common occurrences.

Pregnancy is clearly more probable for women with the presence of either a 46,XX or a 47,XXX cell line or both, compared with those with monosomy X alone. Successful pregnancy outcome was least likely in the X/XX/XXX group, with less than 50% of pregnancies resulting in livebirths. Again, this may reflect how the subjects were ascertained, rather than a true a priori risk.

There was inadequate information provided for the published case reports to assess reliably the risk for other features known to be associated with Turner syndrome, including cardiac and renal malformations, thyroid disease, otological complications, etc. There were no obvious differences in the presence of these features between those with a 47,XXX cell line and those with monosomy X or 45,X/46,XX in our clinic population.

Establishing phenotype-karyotype correlations in Turner syndrome has been problematical. Many have been proposed

Table 2 Intellectual function in subjects mosaic for a 47,XXX cell line compared with 45,X and 45,X/46,XX Turner syndrome: comparison of clinic population and data from the medical publications

Karyotype	Clinic		Published reports		Clinic	
	X/XXX	X/XX/XXX	X/XXX	X/XX/XXX	45,X	X/XX
No	11	6	7	31	218	59
Normal intelligence	10 (91%)	5 (83%)	3 (42.8%)	23 (74%)	187 (86%)	52 (88%)
Mental retardation	0	0	3 (42.8%)*†	4 (13%)	19 (9%)	5 (8%)
Psychiatric diagnosis	1 (9%)	1 (17%)	2 (28.5%)*	4 (13%)	12 (5%)	2 (3%)

*One with psychiatric disease and mental retardation.
†One with half sister and brother with similar unexplained developmental delays.

and few confirmed. Overt chromosomal mosaicism for mono-somy X and a second or third cell line occurs in almost 50% of patients with Turner syndrome and it is difficult to assess the relative contribution of each cell line to each organ system. All 45,X/46,XX subjects do not share the same proportion of nor-mal:abnormal cells in all tissues and the proportion in one tis-sue does not necessarily predict that in others. Further, there may be selection in tissue culture for one cell line over another, so that the results in the laboratory do not represent the true in vivo situation. Moreover, occult mosaicism may mask differences conferred by the presence of cell lines with other X chromosome or Y chromosome abnormalities in ostensibly monosomic 45,X subjects. Most patients with Turner syn-drome have been diagnosed based on karyotyping of only one tissue, usually peripheral blood. Thus, mosaicism for a chromosomally normal or differently aneuploid cell line may have remained undetected in the majority of cases.

Despite these limitations, the demand for prognostic coun-selling in Turner syndrome has increased, especially for the more uncommon karyotypes and in mosaicism, and even more so when the sex chromosome aneuploidy is detected prenatally.

DISCUSSION

In general, females with Turner syndrome, gonadal dysgenesis with sex chromosome aneuploidy, have oedema at birth or show physical evidence of in utero lymphoedema, are short, with adult height below the 3rd centile, and have streak gonads. These three features are the most consistently seen hallmarks of the condition. One-third of females with Turner syndrome are diagnosed at birth because of the presence of lymphoedema; two-thirds of those with a 45,X karyotype are so diagnosed.[2] Many of the infants without postnatal oedema show stigmata of prenatal oedema such as webbing of the neck and anteverted ears. Short stature is an almost invariable feature of the condition. Almost no predictions regarding final adult height can be made based on karyotype. Girls with mosaicism for a 46,XX cell line show a statistically insignifi-cant taller mean adult height.[73] Those with a deletion of the long arm of the X (Xq−) are much more likely to have normal stature and to be diagnosed for primary amenorrhoea. Gonadal dysgenesis is a feature in almost all girls with Turner syndrome. Spontaneous menses occurs in approximately 10% of the entire population of girls with Turner syndrome. Almost 25% of girls who are mosaic for a normal 46,XX cell line will have spontaneous menarche.[2] A much smaller proportion in each group will maintain menses and even fewer will be fer-tile. Other physical features common to Turner syndrome, including congenital heart disease, kidney malformations, multiple naevi, etc, show no consistent relationship to karyotype.[2]

Triple XXX or 47,XXX occurs in 1/1000 of liveborn females.[74] Most of the information regarding these patients comes from several long term follow up studies of subjects ascertained through newborn screening programmes or from case reports.[3–8 75–80] Although there are relatively few studies, in general, it is believed that subjects with 47,XXX are more likely to have verbal processing deficits, a decrement in IQ predicted by sib achievement, and global delays. Height is usually normal and menses and fertility have not been recog-nised to be adversely affected. Caution must be used in mak-ing blanket predictions, as the total number of patients stud-ied has been small, and most girls with 47,XXX are likely to go undetected or unreported.

In the subset of subjects with mosaicism for a monosomy X cell line who have been diagnosed with Turner syndrome, approximately 3-4% are mosaic for a 47,XXX cell line.[2] In our clinical experience, the recognition of this chromosome constitution prenatally can be particularly distressing for pro-spective parents and physicians. The most common reason for

detection of 45,X/46,XX/47,XXX or 45,X/47,XXX prenatally is screening for Down syndrome. Prospective parents are usually concerned about the possibility of mental retardation and an increased likelihood of significant intellectual handicap asso-ciated with a 47,XXX cell line may be a pivotal factor in deci-sions regarding termination or maintenance of pregnancy. Thus, it seemed particularly worthwhile to try to establish phenotype-karyotype correlations based on postnatal survey for this subset of people.

On review of medical publications and our own clinic popu-lation, there are some major differences between those females with a 47,XXX cell line and those with 45,X only or 45,X/46,XX. The former are unlikely to present with perinatal oedema and more likely to be diagnosed in childhood for short stature, or later for primary or secondary amenorrhoea or during evaluation for pregnancy loss.

Despite the association of mental retardation with 47,XXX alone, there appears to be no significant difference in the risk for retardation in 45,X/47,XXX and 45,X/46,XX/47,XXX compared with monosomy X. None of our 16 patients is retarded. Although a much higher proportion (7/39, 18%) of published cases with 45,X/47,XXX or 45,X/46,XX/47,XXX had mental retardation, one had tubercular meningitis at the age of 11 months,[30] a second had neurofibromatosis,[12] another had Duchenne muscular dystrophy,[38] and a fourth woman had structural brain abnormalities including lissencephaly, cer-ebral atrophy, and pachygyria of the frontal lobe and cerebel-lar atrophy.[61] None of these is associated with 47,XXX specifi-cally. A fifth subject was ascertained through screening of inmates in a mental institution.[45] Bias of ascertainment must always be considered a contaminant of published data.

Short stature is not invariable in this subset of women. There are no data to suggest that treatment with growth pro-moting agents in this subset is more or less effective than for other karyotypes associated with Turner syndrome. I believe it is reasonable and fair to counsel parents that approximately 60% of girls with 45,X/46,XX/47,XXX or 45,X/47,XXX will have short stature.

Gonadal dysgenesis and infertility are the primary features of Turner syndrome that are a major concern for parents and, in my opinion and experience, play a significant role in the weighing of consideration for termination or continuance of a pregnancy when the prenatal diagnosis of an X chromosome abnormality is made. It is clear that the presence of an XXX cell line carries with it a greater likelihood of residual ovarian function. However, it must be understood that while the development of secondary sexual characteristics may be nor-mal, fertility may be impaired in the majority of these women.

Prenatal diagnosis of mosaicism for a sex chromosome abnormality always carries with it uncertainty of prognosis based on the limitations of the testing itself. The detection of mosaicism in chorionic villus cells or amniotic fluid may reflect placental rather than true embryonic/fetal mosaicism and the proportion of chromosomally abnormal cells in vitro may not represent the true proportion in the embryo/fetus, nor the specific tissue distribution of the mosaicism. It has been shown for both 45,X/46,XY mosaicism[81 82] and 45,X/46,XX mosaicism[83] that there is a much better prognosis and greater likelihood of normality when the diagnosis is made prenatally rather than postnatally. At this time, we do not have a sufficient number of patients with mosaicism for 47,XXX that have been ascertained prenatally to come to any useful conclusions. Our three prenatally diagnosed patients, while growing within normal parameters and intellectually unim-paired, are only 1½, 5, and 7 years old.

In summary, I recommend that prenatal and postnatal pre-dictive counselling for subjects with 45,X/46,XX/47,XXX and 45,X/47,XXX Turner syndrome be essentially the same as for Turner syndrome in general with regard to likelihood of men-tal retardation, short stature, and structural abnormalities. For any particular infant, it is impossible to predict specific

outcome. It is fair to suggest that residual ovarian function is possible and to caution that premature ovarian failure is common. Those women who do become pregnant, with or without medication, are at higher risk for chromosomally abnormal offspring (7/44 livebirths, two with trisomy 21, the rest with sex chromosome aneuploidy[13 20 27 32 42]) and should be counselled appropriately.

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Dilated cardiomyopathy, sudden cardiac death, hypoplastic discs, and retinal detachment: a new autosomal dominant syndrome

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We report a young woman with dilated cardiomyopathy, severe visual dysfunction, and short stature secondary to a previously unrecognised skeletal dysplasia. Her mother and sister died before we met the proband; both had had a sudden cardiac death and both also had impaired vision and we include the information gleaned from their hospital records.

CASE REPORTS

The proband's mother had attended a school for the partially sighted and was of normal intelligence. Her visual acuities as an adult were 6/60 in her right eye and 3/60 in her left eye. Unfortunately, there is no information regarding the appearance of her optic discs or retinae. She was of average build, with an adult height of 163 cm (50th centile). There was no clinical indication for skeletal radiographs to be taken. She had two pregnancies, leading to the proband and her sister who are described below. A heart murmur noted during childhood had persisted into adult life and an ECG showed frequent ectopic beats. She died suddenly and unexpectedly aged 30, while sitting in a motor vehicle. There was no history of prodromal symptoms or previous episodes of syncope or presyncope. Necropsy showed slight hypertrophy of both ventricles with a congested myocardium. The cardiac histology was consistent with a diagnosis of dilated cardiomyopathy. The proband's father has not been examined by us but has no history of visual, cardiac, or skeletal problems.

The proband's sister's visual acuities were 4/60 in the right eye and 6/36 in her left eye at 6 years of age. She had nystagmus and a high myopic refractive error. Examination under anaesthesia showed hypoplastic discs with an oblique/inverse appearance and myopic peripapillary atrophy. Her macula and retinal vessels were normal and her electroretinogram (ERG) was within normal limits. She attended a school for the blind from the age of 7 years and was intellectually normal. At 12 years, her height was 148 cm (25th centile) and weight was 47 kg (50-75th centile). She had no clinical indication to have skeletal radiographs taken. A heart murmur had been noted during childhood, but not investigated. She collapsed and died suddenly and unexpectedly at a school disco, aged 12 years. There was no history of previous symptoms or episodes of syncope or presyncope. Necropsy showed some thickening of the left ventricular free wall with extreme thinning and fibrosis of the interventricular septum and some thickening of the septum in the subaortic area.

The proband was a term delivery, weighing 2700 g (10th centile). At the age of 5 years, her visual acuity was of perception of light in her right eye and 6/36 in her left eye. She had hypoplastic optic discs, a right divergent squint, nystagmus, and was mildly myopic. Her ERG was very reduced. She attended a school for the blind and her situation remained stable until the age of 15 years when she complained of deterioration of vision in her left eye. A retinal detachment was repaired surgically and she retains visual acuity of 6/60 in that eye. Examination under anaesthesia at this time showed
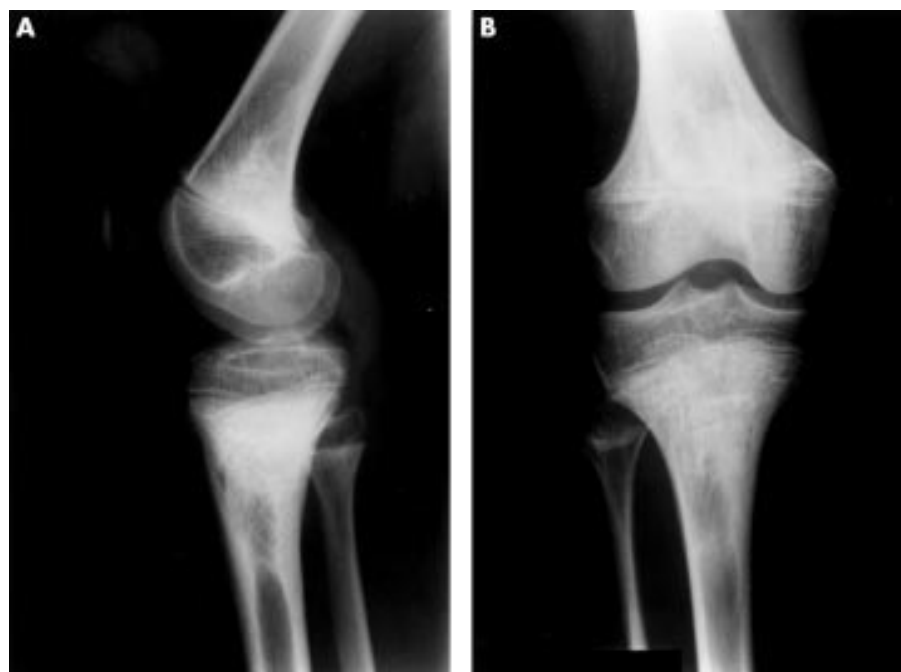
Figure 1 The proband.

tilted/colobomatous optic discs with full thickness retinal folds, vitreous veils, temporal peripheral retinal scarring, but normal vasculature in both eyes. She also had a shallow tractional retinal detachment and a dragged disc. Her ERG showed low amplitude responses. At this time, a diagnosis of familial exudative vitreoretinopathy was made.

During childhood, she had six fractures of her long bones and she has a mild idiopathic non-progressive scoliosis. Her height and weight were <3rd centile throughout childhood, and she attained an adult height of 152 cm (3rd centile) and weight of 47 kg (10th centile). There was no joint hypermobility or skin laxity. She is of normal intelligence. She has a short philtrum and thin upper lip (fig 1).

She had a number of faints when aged 12 years. An ECG at that time was normal apart from a prominent "u" wave in the precordial leads. The symptoms resolved and no further investigations were instituted. She was well up until her second pregnancy, when she had a number of episodes of dizziness associated with palpitations and two episodes associated with loss of consciousness which were not investigated. A few months following delivery, she had an episode of collapse followed by palpitations. Clinical examination showed a murmur of mitral regurgitation which was clinically mild. There was no evidence of heart failure. Her resting ECG at this point showed sinus rhythm with inferolateral repolarisation abnormalities. Exercise test showed a number of short episodes of ventricular tachycardia and a 24 hour tape showed numerous ventricular ectopic beats with one eight beat run of ventricular tachycardia. Her echocardiogram showed moderate global left ventricular dilatation and impaired function, without hypertrophy. MRI scan showed areas of thinning of the free wall of the right ventricle and a fine line of subendocardial infiltrate in the lateral wall of the left ventricle. Cardiac biopsy of the interventricular septum was carried out and the histology was consistent with a diagnosis of dilated cardiomyopathy. She was started on angiotensin converting enzyme inhibitors but was intolerant of beta blockers. Amiodarone exacerbated the arrhythmia and she was unable to tolerate Mexiletine. She continued to have non-sustained ventricular tachycardia following exercise. An automatic defibrillator was implanted and she was also started on Nicorandil. The defibrillator discharged three months following implantation suggesting that a life threatening arrythmia had occurred.

Chromosome analysis showed an apparently normal 46,XX female karyotype. Analysis of cultured fibroblasts indicated a normal collagen profile. A skeletal survey showed abnormally short long bones, with flared metaphyses and patchy areas of sclerosis and lysis within the metaphyses and metadiaphyses (fig 2). These findings did not fit into a recognised pattern. A full blood count, electrolytes, calcium, phosphate, liver function tests, and amino urinary acids were normal. Alkaline phosphatase was slightly raised at 133 U/l (reference range 41-117 U/l).

Figure 2 (A) Lateral and (B) AP views of the proband's knee radiographs, showing abnormal modelling, flared metaphyses, and areas of both lysis and sclerosis within the metaphyses and metadiaphyses.

A mitochondrial aetiology was considered and a quadriceps muscle biopsy taken. Histological and histochemical examination of the muscle biopsy showed minor abnormalities (type 2B fibre atrophy). There was no histological or histochemical evidence of mitochondrial disease: there were no ragged red fibres and no cytochrome c oxidase negative fibres seen. Respiratory chain studies were undertaken on mitochondria isolated from a fresh muscle biopsy. The activity of respiratory chain complexes I, II, and IV fell comfortably within the age and sex matched control range. No mtDNA rearrangements were identified by Southern blot analysis of skeletal muscle DNA. Three mitochondrial genes in which mutations have been associated with cardiomyopathy (tRNA^{leu}(UUR), tRNA^{Ille}, tRNA^{leu}(CUN)) were sequenced and only well established polymorphisms were identified.

DISCUSSION

The common findings in this family are dilated cardiomyopathy and visual impairment. It seems likely that the cause of death in both the mother and older daughter was an arrhythmia, secondary to their cardiomyopathy, as the proband had ventricular dysrhythmia necessitating defibrillator implantation. This striking combination of features has not been described previously.

The proband has an unidentified skeletal dysplasia with short stature. Unfortunately, no information is available on whether the mother or older sister had these features as neither had any radiological examination. The changes are not those of a recognised dysplasia. Retinal and skeletal abnormalities are often associated with collagen disorders, but there was a normal collagen profile in cultured fibroblasts from the proband. Dilated cardiomyopathy consists of ventricular enlargement, abnormal systolic and diastolic left ventricular function, symptoms of congestive heart failure, and premature death resulting predominantly from heart failure and cardiac arrythmias.[1] The aetiologies are varied, and although frequently idiopathic, a familial basis has been identified in 20-30%. Genes implicated to date in dilated cardiomyopathy are of two types: those which encode structural proteins such as dystrophin and muscle LIM protein, and those encoding transcription factors which control the expression of cardiac myocyte genes, such as the cyclic AMP response element binding protein (CREB).[1] While the vast majority of familial forms of dilated cardiomyopathy appear to be autosomal dominant, autosomal recessive, X linked recessive, and mitochondrial inheritance have all been described.[2] This family pedigree is not consistent with autosomal recessive or X linked recessive inheritance and, in the light of the investigations of mitochondrial function, mitochondrial inheritance is unlikely.

Although a diagnosis of familial exudative vitreoretinopathy had been made in the proband, this seems unlikely as there was no evidence of retinovascular abnormalities in either the proband or her sister at a time when both had poor acuity. A diagnosis of Wagner's vitreoretinal dystrophy, with features of myopic refractive error, and inverse, pale optic discs and retinal detachment was considered. However, the ERG in this condition shows subnormal b waves, which were not a feature in these patients, and the temporal retinal scarring with dragged optic discs seen in the family are not features of Wagner's vitreoretinal dystrophy.[3] Some of the Wagner-like syndromes (Kniest dysplasia, diastrophic variant and spondyloepiphyseal dysplasia congenita) have short stature as one of their characteristics, but the normal collagen profile and atypical x rays exclude these diagnoses.[4-6] The appearance of retinopathy of prematurity is similar to that in the proband, but she was not born prematurely and did not receive oxygen supplementation neonatally. Thus, the visual problems are not typical of any recognised condition.

In conclusion, this family represents a distinct entity consisting of dilated cardiomyopathy, arrhythmia predisposing to sudden death, visual impairment, and possibly skeletal dysplasia. The most likely inheritance pattern in this family is autosomal dominant.

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