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 Témamty and McKusick and Fitch. 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. Complex syndromes have been described in which BDA1 is one of a number of manifestations.

Recently, genetic linkage for a BDA1 locus has been reported to map to 2q35-q36 in two unrelated Chinese families. Subsequent sequence analysis identified mutations in the Indian Hedgehog gene (Ihh) in affected subjects. 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 examined a number of candidate genes, including HexD, 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. Mutations in this gene also cause Hunter-Thompson and Grebe acromesomelic dysplasias, two autosomal recessive conditions. In addition, dominant mutations in ROR2, an orphan receptor tyrosine kinase, have been shown to cause brachydactyly type B.

We have recently described a three generation family with mild BDA1, 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 halo 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 MAPPAIRSTM microsatellite markers (Research Genetics, Huntsville, Alabama), encompassing markers from 16 chromosomes. Particular emphasis was placed on markers from chromosomes 5 and 17, based on the report by Fukushima et al 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 MgCl2, 100-200 ng genomic DNA, 0.2 mmol/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 of the LINKAGE software package. 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 112500 and 112500. The distal end of this critical region was defined by an inferred recombination event in II.9 between markers.
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., 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 transloca-
tion may also be associated with a more complex, cryptic rear-
rangemem. Similar phenotypic characteristics between the
girl reported by Fukushima et al.4 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.4 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 epi-
physes, and clinodactyly. Previous reports of families with
brachydactyly have shown that subjects can inherit the
disease as an isolated malformation1-16 or in association with
other disorders. Slavotinek and Donnai17 described a boy with
severe brachydactyly, valgus deformities, developmental delay,
nyctagiasmus, and scoliosis. Scoliosis was also described in
a family with BDA1 and degenerative arthritis owing to discoid
menisci,18 and in a family with brachydactyly, tall stature, and
foot deformities.19 In the examination of affected members
described in this report, there were no features observed other
than BDA1.

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**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.
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 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 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, all hand bones may be proportionately 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 related age norms. The first proximal and fifth metacarpal were also frequently short. Yang et al 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 reported mutations of the Ihh gene in three Chinese families with BDA1. Having excluded PAX3 as the cause of the disease, Gao et al examined Ihh based on its position within the critical region on chromosome 2q35-36, and its involvement in regulating chondrocyte growth and differentiation during endochondral ossification. Three different point mutations were identified, each causing amino acid substitutions in a portion of the protein predicted to interact with the Patched receptor. 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 endochondral 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.

Caderhin-6 is part of a superfamily of proteins that mediate cell-cell interactions, some of which have been previously implicated in osteoclast differentiation. 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. 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 mutant mice have increased body lengths and longer digits because of aberrant proliferation in vertebral growth plates and delayed forepaw ossification. 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”. These patterns are very nearly the complete opposite to those seen by Jaubert et al. 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-generation family affected with the disease. As previous studies have shown linkage of BDA1 to chromosome 2q35-36, 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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AUTHORS’ AFFILIATIONS

C M Armour, A G W Hunter, Eastern Ontario Regional Genetics Programme, Children’s Hospital of Eastern Ontario, Ottawa, Ontario, Canada

C M Armour, M E McCready, A Baig, D E Bulman, Ottawa Health Research Institute, and the University of Ottawa Centre for Neuromuscular Disease, Ottawa, Ontario, Canada

M E McCready, A Baig, D E Bulman, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada

D E Bulman, Department of Medicine, Division of Neurology, University of Ottawa, Ottawa, Ontario, Canada

Correspondence to: Dr D E Bulman, Ottawa Health Research Institute, 501 Smyth Road, Room 4G-101, Ottawa, Ontario, Canada K1H 8L6; dbulman@ohri.ca

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

K Yamakawa-Kobayashi, H Ishiguro, T Arinami, R Miyazaki, H Hamaguchi

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors that belong to the nuclear receptor superfamily. 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. Three different PPAR genes (α, δ, and γ), each displaying distinct tissue and developmental expression patterns, have been identified. PPARα, the first member of the PPAR family to be identified, was cloned as an orphan receptor activated by agents that induce peroxisome proliferation. It is expressed primarily in tissues with high levels of fatty acid oxidation, such as those in liver, kidney, heart, and muscle. Most target genes of PPARα encode enzymes involved in oxidation of cellular fatty acids, lipid transporters, and apolipoproteins. 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. Numerous in vivo and in vitro studies have suggested that PPARα is a key metabolic regulator involved in lipid and glucose homeostasis. Recently, a Leu162Val polymorphism was identified in the PPARα gene, and this polymorphism was shown to influence plasma lipid concentrations, especially in type 2 diabetes patients. 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. Serum LDL cholesterol (LDL-C) levels were estimated according to Friedewald's equation. 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-Biotech). 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′-CCCTCCAACACCTA GGGATTCGT3′ and 5′-CTGTTTCAATGTGCAAGAAG-3′, and the annealing temperature for amplification was 55°C. The 266 bp PCR product was digested with 2 units of Sau96I (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. 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 significant.
significant. Statistical analyses were performed with the JMP software package (SAS Institute).

RESULTS AND DISCUSSION

A missense polymorphism, Val227Ala (GTC→GCG), 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 allele, 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. It is assumed that oestrogen signalling plays a role in lipid and glucose homeostasis via regulation of fatty acid utilisation pathways. 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 1 Clinical characteristics and serum lipid and glucose levels of the subjects

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Values shown are mean (SD).

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

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<td>91.1 (57.6)</td>
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</tr>
<tr>
<td>AA+AV</td>
<td>85.8 (50.7)</td>
<td>[39]</td>
<td>114.0 (62.5)</td>
<td>[16]</td>
<td>66.2 (28.2)</td>
<td>[23]</td>
</tr>
<tr>
<td>VV</td>
<td>125.6 (31.8)</td>
<td>0.10</td>
<td>125.5 (32.0)</td>
<td>[19]</td>
<td>258.8 (31.7)</td>
<td>[17]</td>
</tr>
<tr>
<td>AA+AV</td>
<td>112.6 (31.2)</td>
<td>[39]</td>
<td>115.2 (29.2)</td>
<td>[16]</td>
<td>110.7 (33.0)</td>
<td>[23]</td>
</tr>
<tr>
<td>VV</td>
<td>92.4 (7.4)</td>
<td>0.083</td>
<td>92.8 (7.5)</td>
<td>[19]</td>
<td>92.0 (7.3)</td>
<td>[17]</td>
</tr>
<tr>
<td>AA+AV</td>
<td>89.7 (9.6)</td>
<td>[39]</td>
<td>90.4 (9.0)</td>
<td>[16]</td>
<td>89.3 (10.2)</td>
<td>[23]</td>
</tr>
<tr>
<td>VV</td>
<td>22.6 (3.0)</td>
<td>0.18</td>
<td>22.8 (2.5)</td>
<td>[19]</td>
<td>22.4 (3.4)</td>
<td>[17]</td>
</tr>
<tr>
<td>AA+AV</td>
<td>21.8 (2.6)</td>
<td>[39]</td>
<td>21.8 (2.4)</td>
<td>[16]</td>
<td>21.8 (2.9)</td>
<td>[23]</td>
</tr>
</tbody>
</table>

Values shown are mean (SD).

p value for linear terms was calculated by multiple linear regression analysis 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.

*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 ($p=0.023\quad \nu\quad p=0.046$) (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.\textsuperscript{11} \textsuperscript{14} \textsuperscript{20} Vohl\textit{ et al}\textsuperscript{15} found an association between the Leu162Val polymorphism and hyperapobetalipoproteinaemia in non-diabetic subjects. Flavell\textit{ et al}\textsuperscript{16} reported associations between the Leu162Val polymorphism and total cholesterol, HDL-C, and apolipoprotein A1 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.\textsuperscript{17} \textsuperscript{18} \textsuperscript{20} However, we did not find this polymorphism in our Japanese subjects. In contrast, the Val227Ala polymorphism has not been detected in white populations,\textsuperscript{13} \textsuperscript{14} \textsuperscript{20} suggesting that there is variability in the frequencies of PPARR\textalpha\ 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.\textsuperscript{11} \textsuperscript{15} \textsuperscript{20} However, many of the relationships appear to differ between populations with the exception of the apolipoprotein E polymorphism.\textsuperscript{21} \textsuperscript{22} Further epidemiological and genetic studies are needed to understand the relationship between the Val227Ala polymorphism in the PPARR\textalpha\ gene and serum lipid levels. It will also be interesting to examine the relationship between responses to fibrates and the Val227Ala genotype of the PPARR\textalpha\ gene.

ACKNOWLEDGEMENTS

We are grateful to the subjects for participating in this study. This study was supported by a Grant-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture.

Authors' affiliations

K Yamakawa-Kobayashi, H Ishiguro, R Harimoto, H Hamaguchi, Department of Medical Genetics, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

R Miyazaki, Department of Internal Medicine, Kudanza Hospital, Kudan-minami, Tokyo 102-0074, Japan

Correspondence to: Dr K Yamakawa-Kobayashi, Department of Medical Genetics Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan; kkimikoi@md.tsukuba.ac.jp

REFERENCES

ATM mutations in Finnish breast cancer patients


In Finland, mutations in the two major breast cancer susceptibility genes BRCA1 and BRCA2 appear to account for a considerably lower proportion of hereditary breast cancer than in other western European countries. In addition, the recently identified new susceptibility locus in 13q21 is expected to explain, at most, one quarter of the hereditary susceptibility. The recently identified new susceptibility locus in 13q21 is associated with certain rare hereditary syndromes, such as Li-Fraumeni syndrome or ataxia-telangiectasia (ATM). ATM is a recessive neurodegenerative disorder resulting from mutations in the ATM gene. It is characterised by progressive cerebellar ataxia, telangiectasia, 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 leukemic malignancies, but also breast cancer. Also AT heterozygotes have an increased risk of developing breast cancer, yet a clear discrepancy remains between the epidemiological data and the observed low frequency of ATM mutations in breast cancer patients.

Recently, Laake et al. 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). Given the fact that many disease related gene defects are increased risk of developing breast cancer, 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.

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.

METHODS

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. 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 mutation 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.

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 658 amino acids from the carboxy terminus of the ATM protein. The 6903insA mutation had previously been 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.
be 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 56. 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 group or healthy controls. Furthermore, neither Arg45Trp nor Ser49Cys resides in a known functional 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 patients, 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. The remaining three variants (133C>T, 146C>G, and IVS62+8A>C) were classified as polymorphisms. This assumption was based both on their location

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>nt change</th>
<th>Predicted change in protein</th>
<th>Family ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–5</td>
<td>Unknown</td>
<td>Unknown</td>
<td>200 bp deletion</td>
<td>FIAT8</td>
</tr>
<tr>
<td>14</td>
<td>602–603</td>
<td>IVS1+3delAT</td>
<td>Skipping of exon 1.4</td>
<td>FIAT4</td>
</tr>
<tr>
<td>37</td>
<td>1773–1774</td>
<td>IVS33–34delG</td>
<td>Insertion of Val, Ser, and Stop</td>
<td>FIAT3, FIAT5</td>
</tr>
<tr>
<td>48</td>
<td>2260</td>
<td>6790–6791delTA</td>
<td>Truncated protein</td>
<td>FIAT7</td>
</tr>
<tr>
<td>49*</td>
<td>2303</td>
<td>6903insA</td>
<td>Truncated protein</td>
<td>FIAT4, FIAT5</td>
</tr>
<tr>
<td>53*</td>
<td>2524</td>
<td>7522G&gt;C</td>
<td>Ala&gt;Glu, Pro</td>
<td>FIAT6, FIAT7</td>
</tr>
<tr>
<td>62</td>
<td>2904–2905</td>
<td>8710–8715delGAGACA</td>
<td>Deletion of Gly and Thr</td>
<td>FIAT2</td>
</tr>
<tr>
<td>65</td>
<td>3947</td>
<td>9139C&gt;T</td>
<td>Arg&gt;Stop</td>
<td>FIAT1</td>
</tr>
</tbody>
</table>

*Unpublished mutations.

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

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Phenotype*</th>
<th>Exon</th>
<th>nt change</th>
<th>Sporadic breast cancer</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>003</td>
<td>Br(50)†, Br(59), Br(51), Th(47), Br(71), Sto, Pul (55), Csu</td>
<td>53</td>
<td>7522G&gt;C</td>
<td>0/85</td>
<td>0/200</td>
</tr>
<tr>
<td>005</td>
<td>Br(57)+Slo(41), Br(70), Sto, Slo(41), Csu, Th(55)</td>
<td>53</td>
<td>7522G&gt;C</td>
<td>0/85</td>
<td>0/200</td>
</tr>
<tr>
<td>004</td>
<td>Br(50)†, Br(47)+Sar(18)†, Br(40)†, Sto, Br(45)†, Br(37)†, Br(34)†, Sto, Slo, Br(30)†, Br(31)†</td>
<td>49</td>
<td>6903insA</td>
<td>0/85</td>
<td>0/200</td>
</tr>
</tbody>
</table>

Br, breast; Bs, basal cell; Br, 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

<table>
<thead>
<tr>
<th>nt change</th>
<th>aa change</th>
<th>Frequency in the breast cancer patients studied</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>133C&gt;T</td>
<td>Arg45Trp</td>
<td>1/215 (0.5%)</td>
<td>0/85</td>
</tr>
<tr>
<td>146C&gt;G</td>
<td>Ser49CyS</td>
<td>1/215 (0.5%)</td>
<td>0/85</td>
</tr>
<tr>
<td>IVS62+8A&gt;C</td>
<td>None</td>
<td>5/215 (2.3%)</td>
<td>2/85 (2.4%)</td>
</tr>
</tbody>
</table>

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away from essential functional domains, and for 146C>G also on previous studies by Vorechovsky et al.\textsuperscript{22} and Izatt et al.\textsuperscript{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\textsuperscript{91 3} 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 identified in Finnish AT patients (partly unpublished data) and recognized from all known Finnish AT patients (partly unpublished results). Because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations. Therefore, and also because of the anticipation of seeing breast cancer related genetically recurrent mutations, we concentrated on sample sets for missense mutations.
A single base alteration in the CGG repeat region of FMR1: possible effects on gene expression and phenotype

J Tarleton, A Kenneson, A K Taylor, K Crandall, R Fletcher, R Casey, P S Hart, D Hatton, G Fisch, S T Warren

Frailty X syndrome is characterised by mild to severe mental retardation, minor physical anomalies such as large ears, long faces 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.\(^2\) 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.\(^3\) 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 varying degrees of methylation (termed “methylation mosaics”). These authors observed that the presence of >50% FMRP expressing mosaicism and methylation mosaicism. 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 FMR1 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 at a young age.

### Table 1: Clinical assessment for the proband

<table>
<thead>
<tr>
<th>Evaluation tool</th>
<th>Age and scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 y 16 mth</td>
</tr>
<tr>
<td>Stanford-Binet Intelligence Scale (4th ed)</td>
<td></td>
</tr>
<tr>
<td>Verbal reasoning</td>
<td>ND</td>
</tr>
<tr>
<td>Abstract/visual reasoning</td>
<td>ND</td>
</tr>
<tr>
<td>Quantitative reasoning</td>
<td>ND</td>
</tr>
<tr>
<td>Short term memory</td>
<td>ND</td>
</tr>
<tr>
<td>Test composite</td>
<td>ND</td>
</tr>
<tr>
<td>Vineland Adaptive Behavior Scale</td>
<td></td>
</tr>
<tr>
<td>Communication domain</td>
<td>69</td>
</tr>
<tr>
<td>Daily living skills domain</td>
<td>93</td>
</tr>
<tr>
<td>Socialisation domain</td>
<td>76</td>
</tr>
<tr>
<td>Motor skills domain</td>
<td>93</td>
</tr>
<tr>
<td>Adaptive behaviour composite</td>
<td>77</td>
</tr>
</tbody>
</table>

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 EagI 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 *EcoRI* and *EagI* (or other methylation sensitive restriction enzymes) generates a 2.8 kb fragment in males having trinucleotide repeats within the normal range (<54 repeats). Polymerase chain reaction (PCR) analysis specific for the *FMR1* CGG repeat segment 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). 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. 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](https://example.com/image1.png)

**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 *EagI* site is capitalised.
levels for a child of his age. Unlike same aged males with fragile X syndrome who show declines in IQ and adaptive behaviour levels, 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 Eagl was used as the methylation sensitive restriction enzyme in the original study, another methylation sensitive enzyme, NruI, 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 Eagl site 356 bp downstream from the Eagl 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 Eagl in the Southern blot procedure) in both the proband’s mother (fig 1) and older sister (data not shown).
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, \(1\) 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. Quantification 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\(^{19}\) and using an anti-FMRP antibody.\(^{10-17}\) The FMRP level was normalised to that of a control protein, eIF-4e, which was determined using a similar method with purified eIF-4e\(^2\) 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 worldwide using the double restriction enzyme digestion protocol with EcoRI and EagI, no other subjects have been reported with a G to C transversion creating a new EagI 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 EagI site (CGGCGG), and several possible new sites in surrounding DNA for other commonly used methylation sensitive restriction enzymes, BstIII (GCGGCC), NruI (TCGCGA), or SacII (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 the list 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 \textit{et al}. 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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Authors’ affiliations

J Tarleton, K Crandall, R Casey, Fullerton Genetics Center, Mission St Joseph’s Health System, 267 McDowell Street, Asheville, NC 28803, USA

A Kennerson, Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, USA

A K Taylor, Kimball Genetics Inc, Denver, CO, USA

R Fletcher, North Carolina Department of Health and Human Services, Asheville, NC, USA

P S Hart, Department of Pediatrics, Wake Forest University, Winston-Salem, NC, USA

D Hatton, Frank Porter Graham Child Development Center, University of North Carolina, Chapel Hill, NC, USA

G Fisch, General Clinical Research Center, Yale University. New Haven, CT, USA

S T Warren, Howard Hughes Medical Institute and Departments of Genetics, Biochemistry, and Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

Correspondence to: Dr J Tarleton, Fullerton Genetics Center, Mission St Joseph’s Health System, 267 McDowell Street, Asheville, NC 28803, USA; jack.tarleton@msj.org

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Mosaicism for FMR1 and FMR2 deletion: a new case

S Fengler, S Fuchs, R König, J Arnemann

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. 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. 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. However, the X inactivation pattern can vary between different cell types and during development and thus, usually, causes a milder phenotype in female patients. 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. This report adds a further case with mosaicism for a FMR1 and FMR2 deletion to the few described cases with a large FMR1 deletion.

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, short nose, and long, smooth philtrum. (Photograph reproduced with permission.)

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. 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; CTCGAGGGCCGCCGG; FRAXA-A; GCTGGAGGCT- GAAGAGAAGATTG) and FMR2 genes (FMR2-S: GTCAACACACACACACCGA; FMR2-A: CACGTGGAGGTTAACCAGG) were applied to genomic DNA from leucocytes and from fibroblasts, clear bands were obtained which suggested the presence 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 of 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 - S: GTCACACACA- GGCTACCGGC; FMR2-A: CACGTGGAGGTTAACCAGG) 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 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 (LDDB; 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, while small mosaic deletions are usually associated with pre-mutations. 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, or inherited deletions, while small mosaic deletions are usually associated with pre-mutations. The basic events giving rise to these deletions are not yet understood.

**REFERENCES**

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

M G Butler, D Bittel, Z Talebizadeh

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. PWS syndrome is considered to be the most common genetic cause of marked obesity in humans. Two breakpoint clusters have been reported centromeric to locus 2NF127 with the most proximal breakpoint (BP1) accounting for 44% of cytogenetic deletions while 56% of deletions occur at the second proximal breakpoint (BP2). The second breakpoint (BP2) lies between loci D15S541/S542 and D15S543 and breakpoint BP1 is proximal to D15S541/S542. 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. 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). 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.

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. Paternal duplications have no apparent impact on the phenotype although a patient reported by Mohandas et al 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 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 paternally duplicated 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, D15S511 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, downturned 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. 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, D15S563, 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 siblings. No other person tested in the family showed the deletion or duplication (table 1).

DISCUSSION

This 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 had an
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 recognizable 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.

Authors’ affiliations
M G Butler, D Bitzel, Z Talebizadeh, Section of Medical Genetics and Molecular Medicine, Children’s Mercy Hospitals and Clinic, University of Missouri-Kansas City School of Medicine, Kansas City, Missouri, USA
Correspondence to: Dr M G Butler, William R Brown/Missouri Chair of Medical Genetics and Professor of Pediatrics, 2401 Gillham Road, Kansas City, MO 64108, USA; mgbutler@cmh.edu

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

R G F Gray, P A Davies, A Marshall, S K Heath

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) and Leigh's syndrome. Conventionally, it is detected by polymerase chain reaction (PCR) amplification of the region containing the mutant sequence followed by digestion with restriction enzymes HapII or HpaII (recognition sequence c↓ccgg) which recognise both sequence changes. 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 HaeIII 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. Patients without the polymorphism have three HaeIII restriction sites in the amplified region resulting in four fragments (190 bp, 174 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.
An aetiological study of 25 mentally retarded adults with autism

C D M van Karnebeek, I van Gelderen, G J Nijhof, N G Abeing, P Vreken, E J Redeker, A M van Eeghen, J M N Hoovers, R C M Hennekam

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. The prevalence in the general population was estimated at 5.5/10 000 but more recent investigations report higher rates. Males are affected more often than females, with a predominance of 3 to 1. Mental retardation is present in about 75 to 85% of patients with autism, and almost half of all autistic patients are functionally mute.

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. 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. However, the aetiology often remains obscure and earlier studies reported that causal medical conditions were detectable in a relatively small percentage of autistic patients. There is increasing evidence that genetic factors may well play a major role in the remaining idiopathic cases. In support of this hypothesis is the recent identification of deletions in the short arm of the X chromosome, duplications of the Prader-Willi/Angelman critical region on chromosome 15q, and linkage to 7q31 and 2q as well as the high monozygotic twin concordance rates, and high recurrence risk among sibs of patients with idiopathic autism.

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, 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 and subsequently classified based on DSM-IV criteria. 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.
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 technique, 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 methods: (1) FISH analysis using the D15S510 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. 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. 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.

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.
### Table 1  Patient characteristics

| Patient No | 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 | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M |
| **Age** | 20 | 22 | 27 | 29 | 31 | 32 | 33 | 34 | 35 | 34 | 35 | 36 | 36 | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 38 | 43 | 45 |   |
| **Degree of MR** | P | M | S | S | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M | M |
| **Family history** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Paternal age** | 48 | 28 | 33 | 23 | 47 | 53 | 44 | 25 | 30 | 41 | 31 | 35 | 31 | 36 | 31 | 31 | 36 | 31 | 31 | 36 | 31 | 31 | 36 | 31 |
| **Maternal age** | 29 | 30 | 27 | 24 | 40 | 39 | 43 | 26 | 27 | 34 | 29 | 29 | 34 | 34 | 34 | 34 | 34 | 34 | 34 | 34 | 34 | 34 | 34 | 34 |
| **Consanguinity** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Psychiatric disorder** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Clinical history** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Teratogens** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **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 (cm)** | 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** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Minor anomalies** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **Malformations** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| **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=paternal; m=maternal.
¶¶Malformations: UH=umbilical hernia; US=ureteric stenosis.
**Pigmentation defects: ++=one or two café au lait spots; +++=three or more café au lait spots.

**Additional investigations**

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

Table 2

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Dosometric 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 MECPP2 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. 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. Cerebral 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.

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
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. However, autism should be considered a symptom, caused by a variety of

<table>
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<tr>
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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.\(^1\)

**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%,\(^{2-5,7-12}\) \(^{19}\) \(^{20}\) \(^{26}\) \(^{28}\) 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.\(^1\)

**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.\(^13\) However, this approach is especially important in linkage studies, but less so for finding organic causes, as in the present study.\(^1\)

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.\(^7\) \(^9\) \(^13\) \(^15\) 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\(^41\) and alcohol.\(^16\) The exact teratogenic mechanism of alcohol is unknown, although a clue may be the disturbance in the migration of neuronal and glia cells,\(^41\) supported among other things by the cerebellar anomalies present in both animal models of FAS\(^38\) and subjects with autism.\(^41\) 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.\(^41\) The latter characteristic may well explain the autistic behaviour reported in patients with kernicterus,\(^41\) 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,\(^1\) 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.\(^1\)\(^-\)\(^4\) 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).\(^1\)

Many syndromes are known to have a distinct behavioural phenotype, indicating the potential for the causative genes to influence human cognitive development.\(^41\) Autism is considered to be such a behavioural phenotype associated with several well described syndromes, such as Williams syndrome and Moebius syndrome.\(^41\) 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\(^11\) 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.\(^41\) Most frequently documented are (de novo) structural and numerical abnormalities of sex chromosomes and anomalies of chromosome 15.\(^15\) In our cohort, two (8%) subjects were identified with numerical chromosome anomalies, a rate similar to the 5% reported by Bailey et al.\(^17\) in 1996 and 6.3% by Konstantareas et al in 1999.\(^19\) Three patients similar to our case with 45,X/46,XY mosaic and autistic behaviour have been described.\(^1\)\(^1\) 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.\(^21\) Although supernumerary marker chromosomes have been reported in subjects with autism,\(^2\)\(^5\)\(^\)\(^6\) to our knowledge no other cases with a marker derived from chromosome 13 were reported to have autism.\(^2\)\(^5\)\(^6\) Interestingly, a chromosome 13q region was the most significant result in one collaborative linkage study (CLSA) genome scan.\(^28\) Other linkage studies also showed increased sharing of this locus, although weaker.\(^1\) It seems likely that the marker 13 chromosome has affected the phenotype, including the presence of autism.

| 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 |
| Densityomtry 15q 11.13 region | 6 | 0 |
| Real time PCR 15q 11.13 region | 0 | 0 |
| Fragile X screening | 1 | 0 |
| MEC2P mutation screening | 1 | 0 |
| Metabolic screening | 2 | 1 |
| EEG | 8 | 2 |
| Neuroimaging | 2 | 2 |
| Ophthalmological investigations | 4 | 1 |
| ENT investigations | 6 | 0 |
Submicroscopic deletions

Patients with the velocardiofacial (VCF) syndrome have a distinct behavioural phenotype and often suffer psychiatric disorders, including autism. 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. Submicroscopic rearrangements involving (sub-)telomeric regions are emerging as an important cause of mental retardation. 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. The region may harbour a potential susceptibility gene or genes for autism. In a large study performed by Schroet et al., a cohort of 100 consecutive patients with autism was screened and abnormalities were identified in four (4%); two with supernumerary bisatelleted 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. 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. 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”.

Metabolic screening

Phenylketonuria, hypersucinylpurinemia, changes of aromatic acids and monoamines, lactate acidosis, and cholesterol anomalies 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. The so-called enzyme/brain-barrier has also been suggested to be involved. 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. It has been hypothesised that mitochondrial dysfunction may act through excessive nitric oxide production.

Finally, abnormalities in the glycosylation process cause the CDG syndrome phenotypes. As these are still in the process of delineation, 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. In autistic subjects with a single EEG, abnormalities especially in diagnostic cancer research. A recent exemplary study was performed by Aarskog et al., who used Real time PCR to detect cryptic PMP22 duplications and deletions in patients with Charcot-Marie-Tooth type 1A. 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.

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, but later on autism was proven to occur more frequently in a fra(X) population than in the mentally retarded population in general. 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.

MECP2 gene screening

The recent study by Lam et al. 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. 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.
are present in about 40% versus 65% with multiple EEGs.\textsuperscript{56} 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.\textsuperscript{57} It has been difficult to integrate these findings in an explanatory model.\textsuperscript{58} Of the present 10 patients in whom neuroimaging was performed, findings concurring with the diagnosis of kericniterus 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.\textsuperscript{59} 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 multiply affected families in order to identify autism susceptible loci,\textsuperscript{60} 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.\textsuperscript{61}

**ACKNOWLEDGEMENTS**

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**Authors’ affiliations**

C D M van Karnebeek, E J Redeker, A M van Eeghen, J M M Hoovers, R C M Hennekam, Department of Clinical Genetics, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

C D M van Karnebeek, R C M Hennekam, Department of Paediatrics, Emma Children’s Hospital, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

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

D Concolino, M A Iembo, E Rossi, S Giglio, G Coppola, E Miraglia del Giudice, P Strisciuglio

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 or partial generalised clonic seizures at an early age. We describe the proband, a male, as the second child of unrelated parents, born at 40 weeks of gestation after an uneventful pregnancy. Appgar 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), and head circumference was 35 cm (50-75th centile). The clinical examination was normal except for the presence of hypotelorism. The proband showed hypotelorism without other clinical or dysmorphic features. No other relatives of the proband had hypotelorism or other clinical features.

CASE REPORT
The proband, a male, was the second child of unrelated parents, born at 40 weeks of gestation after an uneventful pregnancy. Appgar 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), and head circumference was 35 cm (50-75th centile). The clinical examination was normal except for the presence of hypotelorism. The proband showed hypotelorism without other clinical or dysmorphic features. No other relatives of the proband had hypotelorism or other clinical features.

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

www.jmedgenet.com
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.

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) (D5S1954p,D5S2076p).

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, 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. In the last few years, two loci for BFNC have been mapped to chromosomes 20q13.3 and 8q24 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, suggesting that KCNQ2 was considered to be a major gene locus for BFNC.

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. 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 chromosomes 20q13.3 and 8q24 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.

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
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.

Authors’ affiliations
D Concolino, M A Iembo, P Strisciuglio, Department of Paediatrics, University of Catanzaro “Magna Graecia”, Italy
E Rossi, S Giglio, Department of General Biology and Medical Genetics, University of Pavia, Italy
G Coppola, E Miraglia del Giudice, Department of Paediatrics, 2nd University of Naples, Italy

Correspondence to: Dr P Strisciuglio, Department of Paediatrics, Faculty of Medicine, University of Catanzaro, C/o Ospedale “A Pugliese”, Viale Pio X, 88100 Catanzaro, Italy; Strisciuglio-unicz@libero.it

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

V P Sybert

Turner syndrome, gonadal dysgenesis with sex chromosome abnormalities, occurs in approximately 1/3000 liveborn 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%) of subjects with Turner syndrome are mosaic for a triple X (47,XXX) cell line. The triple X syndrome in the nonmosaic state is associated with a decrement in intelligence from that expected based on partial and sib accomplishment, normal stature, and normal fertility.

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). 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. 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

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Clinic X/XXX</th>
<th>Clinic X/XX/XXX</th>
<th>Published reports X/XXX</th>
<th>Published reports X/XX/XXX</th>
<th>Clinic 45,X</th>
<th>Clinic X/XX</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>11</td>
<td>6</td>
<td>18</td>
<td>62</td>
<td>227</td>
<td>69</td>
</tr>
<tr>
<td>Age range (y)</td>
<td>5–54</td>
<td>1.5–60</td>
<td>0.55</td>
<td>1–67</td>
<td>0–80</td>
<td>0–74</td>
</tr>
<tr>
<td>Mean age (y)</td>
<td>20.7</td>
<td>21.4</td>
<td>15.4</td>
<td>29.4</td>
<td>18.9</td>
<td>17.4</td>
</tr>
<tr>
<td>Reason for diagnosis (No)</td>
<td>11</td>
<td>6</td>
<td>11</td>
<td>56</td>
<td>224</td>
<td>65</td>
</tr>
<tr>
<td>Oedema</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>89 [40%]</td>
<td>4 [6%]</td>
</tr>
<tr>
<td>Short stature</td>
<td>8 [73%]</td>
<td>1 [17%]</td>
<td>4 [33%]</td>
<td>8 [14%]</td>
<td>66 [29%]</td>
<td>16 [24%]</td>
</tr>
<tr>
<td>Amenorrhoea</td>
<td>2 [18%]</td>
<td>1 [17%]</td>
<td>0</td>
<td>16 [29%]</td>
<td>44 [20%]</td>
<td>12 [18%]</td>
</tr>
<tr>
<td>Prenatal</td>
<td>1 [9%]</td>
<td>2 [33%]</td>
<td>0</td>
<td>2 [4%]</td>
<td>11 [5%]</td>
<td>24 [37%]</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>2* [33%]</td>
<td>7† [66%]</td>
<td>30‡ [53%]</td>
<td>14§ [8%]</td>
<td>9 [14%]</td>
</tr>
</tbody>
</table>

*Recurrent pregnancy loss (1), behavioural problems (1).
†Mental retardation (3), secondary amenorrhoea (3), hypoplastic left heart (1).
‡Secondary amenorrhoea or fetal wastage (13), hypoplastic left heart (1), abnormal offspring (6), cancer (5), ambiguous genitalia (1), newborn screening (1), Hirschsprung disease (1).
§Mental retardation (1), failure to thrive (1), developmental delay (2), 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).
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/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., 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 and those with monosomy X or 45,X/46,XX in our clinic were mentally retarded, 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 and those with monosomy X or 45,X/46,XX in our clinic were mentally retarded,

| Karyotype | Clinic X/XXX | X/XXX/XXX | Published reports X/XXX | X/XXX/XXX | Clinic 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 | 1/123 (0.8%) | 1/3 (33%) | 9/13 (69%) | 20/44 (45%) | 1/123 (0.8%) | 6/32 (19%)
| G2/SA2 | 7/8 (87%) | 27/46 (59%) | 70/95 (74%) | 17/28 (61%)

*Includes only those women who have achieved final adult height.
†Some the result of medically induced ovulation.

G, gravida; P, para; SAB, spontaneous abortion; TAB, therapeutic abortion.

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 X/XXX | X/XXX/XXX | Published reports X/XXX | X/XXX/XXX | Clinic 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 monosomy 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 normal-abnormal cells in all tissues and the proportion in one tissue 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 syndrome 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 counselling 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 antverted 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 insignificant taller mean adult height.3 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.4 A much smaller proportion in each group will maintain menses and even fewer will be fertile. Other physical features common to Turner syndrome, including congenital heart disease, kidney malformations, multiple naevi, etc, show no consistent relationship to karyotype.5

Triple XXX or 47,XXX occurs in 1/1000 of liveborn females.6 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.7–75 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 recognised to be adversely affected. Caution must be used in making blanket predictions, as the total number of patients studied 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.7 In our clinical experience, the recognition of this chromosome constitution prenatally can be particularly distressing for prospective 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 associated with a 47,XXX cell line may be a pivotal factor in decisions regarding termination or maintenance of pregnancy. Thus, it seemed particularly worthwhile to try to establish phenotype-karyotype correlations based on postnatal study for this subset of people.

On review of medical publications and our own clinical population, there are some major differences between those females with a 47,XXX cell line and those with 45,X 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 tuberculous meningitis at the age of 11 months,73 a second had neurofibromatosis,74 and a third had Duchenne muscular dystrophy,75 and a fourth woman had structural brain abnormalities including lissencephaly, cerebral atrophy, and pachygria of the frontal lobe and cerebellar atrophy.76 None of these is associated with 47,XXX specifically. A fifth subject was ascertained through screening of inmates in a mental institution.76 Bias of ascertainment must always be considered a contaminant of published data.

Short stature is not invariably in this subset of women. There are no data to suggest that treatment with growth promoting agents in this subset is more or less effective than 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 normal, 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 mosaicism77 and 45,X/46,XX mosaicism78 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 unimpaired, are only 1½, 5, and 7 years old.

In summary, I recommend that prenatal and postnatal predictive 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, the general with regard to likelihood of mental 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) and should be counselled appropriately.

Authors’ affiliations
V P Sybert, Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA

Correspondence to: Dr V P Sybert, Box 356524, Division of Dermatology, University of Washington School of Medicine, 1959 NE Pacific Street, Seattle, Washington 98195, USA; flk01@u.washington.edu

REFERENCES


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

J A Goodship, J O'Sullivan, P F Chinnery, A K Ryan, N Ziakas, C M Hall, M Clarke

We report a young woman with dilated cardiomyopathy, severe visual dysfunction, and short stature secondary to a previously unrecognized 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 (50-75th centile). There was no clinical indication to have skeletal radiographs taken. She had two pregnancies, leading to the proband and her sister.

The proband was a term delivery, weighing 2700 g (10th centile). At the age of 5 years, her visual acuity was of perceptible 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 had two pregnancies, leading to the proband and her sister.

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 died suddenly and unexpectedly at a school disco, aged 12 years. A retinal detachment was repaired surgically and she retains visual acuity of 6/60 in that eye. Examination under anaesthesia at this time showed
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 trac-tional 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 fainty episodes, 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 histol-oogy was consistent with a diagnosis of dilated cardiomyopa-thy. 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 arrhythmia 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).
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 \text{Glu}^{*}(UUR), tRNA^{ATP}, tRNA^{A}{A}{U}(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 unidentifiable 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 arrhythmias. 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). 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. 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. 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. 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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Authors’ affiliations

J A Goodship, A K Ryan, Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK

J O’Sullivan, Department of Paediatric Cardiology, Freeman Hospital, Newcastle upon Tyne, UK

P F Chinnery, Department of Neurology, Newcastle University, Newcastle upon Tyne, UK

N Ziakas, M Clarke, Department of Ophthalmology, Royal Victoria Infirmary, Newcastle upon Tyne, UK

C M Hall, Department of Radiology, Great Ormond Street Hospital, London, UK

Correspondence to: Dr J Goodship, Institute of Human Genetics, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK; j.a.goodship@ncl.ac.uk

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