ORIGINAL ARTICLE

Identification of a 650 kb duplication at the X chromosome breakpoint in a patient with 46,X,t(X;8)(q28;q12) and non-syndromic mental retardation

J J Cox, S T Holden, S Dee, J I Burbridge, F L Raymond

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See end of article for authors' affiliations

Correspondence to:
Dr F L Raymond,
Cambridge Institute for
Medical Research,
University of Cambridge,
Addenbrooke's Hospital,
Cambridge CB2 2XY, UK;
flr24@cam.ac.uk

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A female patient with non-syndromic mental retardation was shown by high resolution GTL banding to have inherited an apparently balanced translocation, 46,X,t(X;8)(q28;q12)mat. Replication studies in the mother and daughter showed a skewed X inactivation pattern in lymphocytes, with the normal X chromosome preferentially inactivated. The mother also had significant intellectual disability. To investigate the possibility that a novel candidate gene for XLMR was disrupted at the X chromosome translocation breakpoint, we mapped the breakpoint using fluorescence in situ hybridisation (FISH). This showed that the four known genes involved in non-syndromic mental retardation in Xq28, *FMR2*, *SLC6A8*, *MECP2*, and *GDI1*, were not involved in the translocation. Intriguingly, we found that the X chromosome breakpoint in the daughter could not be defined by a single breakpoint spanning genomic clone and further analysis showed a 650 kb submicroscopic duplication between DXS7067 and DXS7060 on either side of the X chromosome translocation breakpoint. This duplicated region contains 11 characterised genes, of which nine are expressed in brain. Duplication of one or several of the genes within the 650 kb interval is likely to be responsible for the mental retardation phenotype seen in our patient. Xq28 appears to be an unstable region of the human genome and genomic rearrangements are recognised as major causes of two single gene defects, haemophilia A and incontinentia pigmenti, which map within Xq28. This patient therefore provides further evidence for the instability of this genomic region.

Characterisation of breakpoints in patients with apparently balanced constitutional chromosome rearrangements and phenotypic abnormalities has proved an invaluable strategy for identifying disease causing genes, especially those on the X chromosome.^{1–2} To date, four genes responsible for non-syndromic X linked mental retardation have been identified using this method.^{3–6} Phenotypic abnormalities seen in cases with apparently balanced chromosome rearrangements have usually been explained by the disruption of a gene at the breakpoint causing the loss of gene function. However, other mechanisms of disease causation have also been described where (1) a breakpoint disrupts or alters gene expression via a position effect⁷ or (2) a cryptic deletion or duplication is identified at the translocation breakpoint.^{8–11}

We have investigated a female patient with non-syndromic mental retardation who has inherited an apparently balanced X;autosome translocation, 46,X,t(X;8)(q28;q12)mat. Her mother also had significant intellectual disability from childhood. Four genes within Xq28 have previously been identified that when mutated result in non-syndromic mental retardation.^{12–17} However, there is still further allelic heterogeneity within this region as there are additional XLMR families published that map to Xq28 with a significant lod score but do not have abnormalities in any of these genes.^{18–21} Xq28 is a highly gene rich region of the human X chromosome and we focused on the X chromosome breakpoint as the identification of a disrupted gene here would be a rapid way to identify a further candidate gene for X linked mental retardation within Xq28.

MATERIALS AND METHODS

Clinical summary

The child was born by SVD at 38 weeks' gestation. Birth weight was 2670 g (10th centile), head circumference 33.2 cm

(10th centile), and Apgar scores were 6 and 9 at five and 10 minutes respectively. She had marked plagiocephaly but no other dysmorphic features. Head circumference measurements remained between the 10th and 50th centile while her weight remained below the 3rd centile over the first nine months. She was closely monitored in the early period because of concerns about the social circumstances of the family and possible neglect owing to the intellectual disability of the mother. Developmental assessment at 2 years 1 month found the child to be functioning at a 10–12 month level with global developmental delay. She sat unsupported at 16 months and walked by 2 years 10 months. At 3 years 2 months, assessment of her expressive language and verbal comprehension showed her to be functioning at a 12–17 month old level. She attended a special school for severe learning disability from 5 years. There were continuing concerns about the welfare of the child from the child protection agencies and she and her sibs were removed from the parental home aged 7 years. Her sibs have a normal karyotype and attend mainstream school. Since her adoption, her development has progressed rapidly suggesting that part of her disability was the result of the extremely poor social circumstances. She is due to be transferred to a school for moderate intellectual disability. However, despite her care she remains significantly developmentally delayed.

Chromosome analysis of cultured blood sample and X chromosome inactivation analysis

GTL banding at the 550 band level was performed by standard techniques.²² The X inactivation status of the patient and her

Abbreviations: XLMR, non-syndromic X linked mental retardation; FISH, fluorescence in situ hybridisation; HR-CGH, high resolution comparative genomic hybridisation

Figure 1 Diagrammatic representation and partial karyotype of the apparently balanced translocation, $t(X;8)(q28;q12)$.

mother was determined cytogenetically by determining early and late replication status of the two X chromosomes. PCR based X inactivation assays were also performed at the *AR*, *MAOA*, and *PGK-1* loci.^{23–25}

DNA isolation from genomic clones

Clones from the RPCI-1, 5, 11, and 13 BAC and PAC libraries were obtained from BACPAC resources. Cosmid U-58E6 is from the chromosome specific library prepared at the Lawrence Livermore Laboratory and was obtained from the UK HGMP Resource Centre. Clones were amplified on plates containing LB and 25 µg/ml kanamycin (PACs and cosmid) or 20 µg/ml chloramphenicol (BACs), and incubated overnight at 37°C. Single colonies were picked and inoculated into 5 ml cultures of LB supplemented with the appropriate antibiotic and grown overnight at 37°C at 215 rpm. DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen).

Fluorescence in situ hybridisation (FISH)

A total of 500 ng of purified PCR products or miniprep DNA was labelled with SpectrumOrange dUTP by nick translation (Vysis). After incubation at 15°C for 15 hours, the probe was ethanol precipitated and resuspended in 6 µl TE. Then 80 ng of probe was hybridised to metaphase chromosomes with 1 µg cot⁻¹ DNA, 15 µl hybrisol VI (Appligene-oncor), and 10 ng of chromosome X and 8 centromere specific probes (Vysis). Slides were analysed using a fluorescence microscope and images recorded using SmartCapture 2 software.

PCR products for FISH analysis from dyskerin, *MPP1*, and factor VIII genes

PCR products for FISH analysis were generated using the Expand Long Template PCR System (Roche). Primers for PCR probe 1 were designed to amplify exons from the dyskerin gene (*DKC1*) and membrane protein palmitoylated 1 gene (*MPP1*) (probe 1 fwd: CTG CCT TGA TGC AGG AGT ATG TC and probe 1 rev: TCT TTC ACT GAG AAG TAT GAC GTT G; 12 868 bp product). Primers for PCR probe 2 amplified the largest exon and flanking intronic sequence from the factor VIII gene (probe 2 fwd: GTT GAG ACT TGC TAT ATG CCC AGT G; probe 2 rev: GAG GCT AAA CAA TGT CGT CAC AGA G; 9971 bp product). A total of 50–150 ng of miniprep DNA from non-overlapping clones (RP1-248B23 for probe 1, RP11-296N8 for probe 2) was used as template in a 25 µl reaction mixture containing 0.3 µmol/l forward and reverse primers, 0.5 mmol/l dNTPs, 2.5 µl buffer 3, and 1.3 U enzyme mixture. The PCR reactions were performed using the PTC-200 DNA Engine (MJ Research) with cycling conditions as follows: initial denaturation at 94°C for two minutes; 10 cycles of 94°C for 10 seconds, 62°C for 30 seconds, 68°C for 10 minutes 30 seconds; 25 cycles

of 94°C for 10 seconds, 62°C for 30 seconds, 68°C for 10 minutes 30 seconds + 20 seconds per cycle; and 68°C for 10 minutes. The PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen) and fluorescently labelled as above.

Checking the identity of the clones by PCR of sequence tagged sites

Published STS primers (Xq3649, DXS7067, GDB:179459, RH69764, DXS7532, WI-13882, and DXS7060) were used to check the identity of the clones. In addition, primers within the *GDII* gene were designed to determine whether cosmid U-58E6 contained *GDII* (*GDII* fwd: GAG TTG ACG GAG CTC TTC CTG TG, *GDII* rev: GCA TCT CCA AAG GCC ACT ACA G; 918 bp product). A total of 50–150 ng of miniprep DNA was used as template in 25 µl reaction mixtures containing 0.2 µmol/l forward and reverse primers, 0.2 mmol/l dNTPs, 1.5 mmol/l MgCl₂, 2.5 µl 10 × buffer, and 0.625 U *Taq* DNA polymerase (Promega). Cycling conditions were 95°C for four minutes; 35 cycles of 95°C for 30 seconds, 55–65°C for 30 seconds, 72°C for 30 seconds-one minute; and 72°C for three minutes.

RESULTS

Karyotype

Chromosome analysis of cultured blood lymphocytes from the patient presenting with developmental delay showed an abnormal karyotype, 46,X,t(X;8)(q28;q12)mat (fig 1). The hypothesis was that the learning disability in this family was associated with the balanced translocation. Two possible mechanisms were proposed for the developmental delay in the family: a random X inactivation pattern resulting in a functional imbalance of chromosome material and/or the disruption of a gene at the X;autosome translocation breakpoint that was responsible for intellectual development.

X chromosome inactivation analysis

Using blood lymphocyte DNA we investigated the X inactivation status of the patient using the (CAG)_n polymorphism at the androgen receptor locus.²³ This was uninformative as the two *AR* allele sizes were indistinguishable by PAGE and DNA sequence analysis confirmed that the patient was homozygous for the *AR* polymorphism. Unfortunately, the patient was also uninformative for two other PCR based X inactivation assays at the *MAOA* and *PGK-1* loci.^{24–25} We therefore determined the X inactivation status of the patient cytogenetically via replication studies. In 50 out of 50 cells analysed, the derivative X chromosome was found to be early replicating and the normal X chromosome to be late (fig 2). Late replication is associated

Figure 2 Replication studies to identify which X chromosome is late replicating (inactive). In 50/50 cells analysed the normal X chromosome was late replicating and the der(X) chromosome was early replicating, thus indicating a skewed X inactivation pattern.

with inactive methylated DNA and so the results indicated a skewed X inactivation pattern with the normal X chromosome preferentially inactivated. Her mother was also shown, by replication studies, to have a skewed X inactivation pattern. The skewed X inactivation pattern would result in a functional chromosome balance of material. We therefore concluded that the mental retardation phenotype seen in this family was likely to be related to abnormalities at the chromosome translocation breakpoints. To this end we determined the location of the X chromosome breakpoint in the translocation.

FISH analysis of the X chromosome breakpoint

Initially we identified a panel of BAC, PAC, and cosmid clones that mapped to Xq28 through public databases and publications.^{26, 27} Using fluorescence in situ hybridisation (FISH) with the genomic clones as probes, we identified the X chromosome translocation breakpoint. A series of clones (RP13-156P1, RP5-865E18, RP5-1087L19, RP11-196H18, RP11-115M6, RP1-248B23, RP11-524G17, RP11-296N8, RP11-402H20, and RP11-430K16) were all found to hybridise to the normal X, der(X), and der(8) chromosomes (fig 3). The boundaries of the translocation region were defined using cosmid clone U-58E6 and BAC RP11-405N23, which defined the centromeric and telomeric boundaries respectively. The identity of the clones was checked by PCR using published sequence tagged sites and agrees with the contig published by Aradhya *et al.*,²⁷ with the exception of RP11-430K16 which does not contain DXS7060 (fig 4).

Evidence for an X chromosome duplication between DXS7067 and DXS7060

To identify the degree of overlap between the series of BACs and PACs that all appeared to map on either side of the translocation breakpoint, we identified the length of each clone insert and compared the end sequences of the clones, where available in GenBank, using the local alignment program BLAST. This showed that the BAC/PAC contig between DXS7067 and DXS7060 that gave signals on the X, der(X), and der(8) chromosomes spans approximately 650 kb of genomic sequence (fig 4). Two models were proposed to explain why clones from the 650 kb region all hybridised to the X, der(X), and der(8) chromosomes: (1) there was a common sequence (either from a segmental duplication or low copy repeat)

Figure 3 FISH analysis with genomic clones from each end of the duplicated region: (A) RP13-156P1, (B) RP11-430K16. These two clones do not contain overlapping sequence, yet both hybridise to the normal X, derivative X, and derivative 8 chromosomes (red signals) owing to the duplication of sequence at the X chromosome translocation breakpoint. Centromeres labelled in green.

found within the genomic clones of the contig which hybridised to sequence at each side of the translocation breakpoint, or (2) there was a duplication of unique genomic sequence at the translocation breakpoint in the patient.

Analysis of the clone sequences indicated that there was no common sequence that could explain why non-overlapping clones hybridised to the X, der(X), and der(8) chromosomes by FISH. Firstly, although a 35.5 kb duplication is known to exist within clones RP5-865E18 and RP5-1087L19, which includes *NEMO* and its pseudogene,²⁸ this duplication could

Figure 4 Clone contig of distal Xq28. STS markers and the genes *GD11*, *DKC1*, and *FVIII* are indicated at the top. Arrows denote the known *NEMO/LAGE2* 35.5 kb duplicated region. FISH experiments showed cosmid U-58E6 and BAC RP11-405N23 to map proximal and distal to the translocation breakpoint respectively. Clones indicated by a dark line showed FISH signals on the X, der(X), and der(8) chromosomes.

not explain why clones RP1-248B23, RP11-524G17, RP11-296N8, RP11-402H20, and RP11-430K16 appeared to be breakpoint spanning as these clones do not overlap RP5-865E18 and RP5-1087L19 (fig 4). Secondly, we analysed the sequence of one of the clones from the 650 kb region (RP1-248B23) to see if this clone contained sequence that would enable it to cross hybridise to either side of the translocation breakpoint. Using BLAST we compared the complete non-repeat masked finished sequence of RP1-248B23 in 5 kb sections to the non-redundant (nr) and unfinished high throughput genomic sequences (htgs) databases, which showed that from our panel only the known overlapping clones, RP11-196H18, RP11-115M6, and RP11-524G17, showed significant alignment to RP1-248B23.

The possibility remained that the translocation breakpoint was within RP1-248B23 and that adjacent non-overlapping clones contained common sequence. To test this hypothesis we compared the non-repeat masked sequences of RP11-296N8, RP11-402H20, and RP11-524G17 (RP11-430K16 sequence was unavailable) to clones RP5-865E18 and RP5-1087L19 using a pairwise BLAST. Results showed a 618 bp sequence with 99% identity within each of the clones. In our experience this small section of sequence is unlikely to result in such intense breakpoint spanning signals by FISH. The most likely explanation for the FISH results was therefore a genomic sequence duplication at the translocation breakpoint with the breakpoint lying within RP11-430K16.

The GenBank sequence entries for clones RP11-296N8, RP11-402H20, and RP11-524G17 do not contain their entire inserts and so further sequence unknown to us may exist within these clones that is common to RP5-865E18 and RP5-1087L19. So, to confirm the existence of a duplication at the translocation breakpoint, primer pairs were designed to amplify unique sequences from two separate non-overlapping clones. One PCR product was a 12.9 kb fragment amplified from RP1-248B23 and contained exons from the dyskerin gene (*DKC1*) and the membrane protein palmitoylated 1 gene (*MPP1*). The second PCR product was a 10 kb fragment amplified from RP11-296N8 and contained the largest exon of the factor VIII gene and flanking intronic sequence. The PCR products were fluorescently labelled with SpectrumOrange dUTP. FISH on metaphase spreads showed that both of these probes hybridised to the normal X, der(X), and der(8), thus confirming the presence of a duplication of unique sequence on either side of the translocation breakpoint in this patient.

Exclusion of disruption or duplication of known XLMR genes in Xq28

The gene *GD11*, which is known to cause X linked mental retardation when mutated, lies in this region of Xq28. We tested whether this gene is either duplicated or disrupted by the translocation. FISH with cosmid U-58E6, which is telomeric to *GD11*, showed a signal on only the normal and

Table 1 Known genes within the duplicated region at the X chromosome translocation breakpoint

Gene	UniGene Accession No	Brain ESTs in UniGene Database	Hippocampal ESTs in UniGene Database
<i>G6PD</i>	Hs.80206	+	–
<i>NEMO</i>	Hs.43505	+	+
<i>CTAG1</i>	Hs.167379	–	–
<i>CTAG2</i>	Hs.87225	–	–
<i>GAB3</i>	Hs.102630	+	–
<i>DKC1</i>	Hs.4747	+	+
<i>MPP1</i>	Hs.1861	+	+
<i>F8</i>	Hs.79345	+	+
<i>HCBP6</i>	Hs.356050	+	–
<i>MTCP1</i>	Hs.3548	+	–
<i>C6.1A</i>	Hs.301927	+	+

derivative X chromosomes. We therefore conclude that *GD11* is not disrupted or duplicated by the complex rearrangement in this patient. *FMRI1*, *FMRI2*, *SLC6A8*, and *MECP2* map a significant distance from the rearrangement and are therefore unlikely to be involved.

Candidate genes for XLMR within the duplicated region

Owing to the presence of the 650 kb duplication within which the translocation occurs, any disrupted genes at either end of the duplication or at the translocation breakpoint will be compensated for by the presence of that gene as an intact copy on the other rearranged chromosome.

Eleven characterised genes exist within the duplicated region (table 1). Analysis of the UniGene database shows that there are brain ESTs corresponding to nine of these genes. Furthermore, for five of the genes in the duplicated region (*NEMO*, *DKC1*, *MPP1*, *F8*, and *C6.1A*) there are ESTs showing expression in the hippocampus, the region of the brain considered important in the processes of learning and memory.^{29, 30}

DISCUSSION

Using fluorescence in situ hybridisation (FISH) with genomic clones and PCR products as probes, we have identified a small ~650 kb duplication at the X chromosome breakpoint in a female patient with an apparently balanced chromosome translocation t(X;8)(q28;q12) and non-syndromic mental retardation. To our knowledge, this is the first reported duplication to accompany an apparently balanced chromosomal rearrangement in Xq28.

Characterisation of breakpoint sequences in patients with apparently balanced chromosome translocations has in some

instances shown deletions or duplications involving megabases of sequence.⁸⁻¹⁰ Indeed, it is known that approximately 6-10% of apparently balanced de novo translocations are pathogenic^{31,32} and a large proportion of these are likely to be unbalanced but cannot be distinguished from true balanced translocations by conventional cytogenetic techniques.³³ Genomic deletions at a breakpoint appear to be more common than duplications. This is likely to be the result of under-reporting of duplications because of the technical difficulties of detection. Loss of genomic material is easier to see cytogenetically both by G banding and by FISH than gain of material. Detecting duplications by FISH requires sufficient material to be duplicated such that two fluorescent signals can be resolved by fluorescence microscopy (that is >3 Mb apart on metaphase spreads). In our patient the presence of a translocation breakpoint within the duplicated region facilitated the detection of the duplication as FISH using probes across the duplicated region resolved onto separate chromosomes, the derivative X and derivative 8. This enabled us to estimate the length of the duplication to be approximately 650 kb, which would have been beyond the resolution of FISH and would have been undetected if the duplication had remained on the X chromosome alone. This raises the possibility that the duplication in this region occurs more frequently in the population but as a result of technical limitations remains undetected. High resolution comparative genomic hybridisation (HR-CGH) is emerging as an important technique for identifying genome imbalances in patients who by G banding have normal karyotypes or apparently balanced translocations.³⁴ HR-CGH has been used successfully in identifying deletions as small as 3 Mb, and further improvements in sensitivity will make this technique an important tool for identifying disease causing submicroscopic deletions and duplications.

The non-syndromic mental retardation phenotype observed in the patient is likely to be related to the duplication of one or several genes within the 650 kb duplicated region at the X chromosome translocation breakpoint. Eleven characterised genes are located within the duplicated interval, of which nine are expressed in the brain. Five of these genes (*NEMO*, *DKC1*, *MPPI1*, *F8*, and *C6.1A*) are expressed in the hippocampus, a region of the brain important in learning and memory. It is of interest that *NEMO* and *DKC1* are genes mutated in syndromic forms of X linked mental retardation (incontinentia pigmenti and dyskeratosis congenita respectively) and perhaps it is specifically the duplication of these genes that is responsible for the patient's non-syndromic mental retardation phenotype. There are published examples where a whole gene duplication alone or a point mutation in the single copy of the gene are associated with specific diseases, as in Charcot-Marie-Tooth disease and Pelizaeus-Merzbacher disease (PMD).³⁵ It is possible that the patient's abnormal phenotype is the result of the disruption of a gene at the autosomal translocation breakpoint and we have yet to exclude this possibility. Similarly, an alternative explanation for the patient's phenotype could be that there is a random X inactivation pattern in brain tissue resulting in a functional gene imbalance. However, it is likely that the major contributor to the phenotype in this patient is abnormal gene dosage within the 650 kb duplicated sequence at the X chromosome translocation breakpoint.

The clinical manifestation of gene dosage imbalance in Xq28 is highlighted by cases of XY_q syndrome.³⁶ Males affected with this syndrome have a 46,XY_q karyotype and show the presence of a small portion (5-10 Mb) of distal Xq on the long arm of the Y derivative. The phenotypic consequences of this partial X disomy are severe mental retardation, generalised hypotonia, and microcephaly. Similarly, a male with BPNH/MR syndrome (bilateral periventricular nodular heterotopia, cerebellar hypoplasia, severe mental retardation, epilepsy, and syndactyly) was reported with a 2.25-3.25 Mb inverted duplication of distal Xq28.³⁷ Our patient shows disomy for a subset of the genes involved in XY_q syndrome

and BPNH/MR syndrome and hence helps to narrow down the search for possible phenotypically critical, dosage sensitive candidate genes for mental retardation.

In our family, both the mother and daughter were phenotypically similar although the daughter appears more affected than the mother because of the additional effect of poor social circumstances. Both the mother and the daughter carry the same X;8 translocation cytogenetically. Unfortunately, we were not able to obtain further samples from the mother to determine whether she also carried the 650 kb duplication. It is probable that she does, otherwise two rather than one rare independent rearrangement events need to be hypothesised to explain the karyotype in the daughter.

The distal region of Xq28 has been described as being unstable and large internal rearrangements are reported.^{27,28} Approximately 70-80% of mutations in incontinentia pigmenti are the result of a deletion of exons 4-10 of the *NEMO* gene. This recurrent deletion occurs between two identical 878 bp MER67B repeats which are located in intron 3 of *NEMO* and ~4 kb telomeric to the gene.³⁸ Similarly, a genomic rearrangement is the most frequent cause of severe haemophilia A and approximately 45% of all affected males have a ~500 kb inversion which disrupts *FVIII*.^{39,40} This inversion occurs by homologous recombination between a 9.5 kb *int22h* repeat located in intron 22 and either of the two *int22h* repeats oriented in the opposite direction approximately 500 kb distal to the gene.⁴¹ The duplication at the X chromosome translocation breakpoint in this patient provides further evidence for the instability of the Xq28 region of the X chromosome. The mechanism of formation of the duplication in this case is unknown. In our BAC/PAC sequence analysis, we identified a small 618 bp inverted repeat sequence within the genomic clones at either end of the duplicated region. We hypothesised that these were directly involved in the rearrangement mechanism but Southern blot analysis showed that this did not appear to be the case. Refinement of the X chromosome and autosomal breakpoints and sequence analysis of these regions is required to provide further insights into the rearrangement mechanism.

In summary, analysis of this patient with mental retardation has shown a 650 kb duplicated region of Xq28 in association with a reciprocal translocation. It is likely that the duplication is the cause of disease in this patient and raises the question of whether submicroscopic duplications are a cause of disease in more people. Currently, such small duplications remain undetected owing to limitations in routinely available technologies. At least 11 genes are duplicated within the 650 kb region, and for five of these there is evidence for expression in the hippocampus. Dosage and mutation analysis of all candidate genes within the duplicated region may show a further gene important in non-syndromic mental retardation.

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Authors' affiliations

J J Cox, S T Holden, S Dee, J I Burbridge, F L Raymond, Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, UK

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