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Prenatal identification of a girl with a t(X;4)(p21;q35) translocation: molecular characterisation, paternal origin, and association with muscular dystrophy

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Abstract

There are 23 females known with Duchenne or Becker muscular dystrophy (DMD or BMD) who have X;autosome translocations that disrupt the X chromosome within band p21. A female with a t(X;4)(p21;q35) translocation was identified prenatally at routine amniocentesis. At birth, she was found to have a raised CK level, consistent with a diagnosis of Duchenne muscular dystrophy. Her cells were fused with mouse RAG cells and the translocated chromosomes were separated from one another and from the normal X chromosome by segregation in the resulting somatic cell hybrids. Southern blot analysis of the hybrids indicated that the translocation occurred on the X chromosome between genomic probes GMGX11 and J-66, both of which lie within the DMD gene. Further localisation with a subfragment of the DMD cDNA clone placed the translocation breakpoint in an intron towards the middle of the gene, confirming that the de novo translocation disrupted the DMD gene. RFLP analysis of the patient, her parents, and the hybrid cell lines showed that the translocation originated in the paternal genome. This brings to six out of six the number of DMD gene translocations of paternal origin, a fact that may be an important clue in future studies of the mechanism by which X;autosome translocations arise.

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Duchenne and Becker muscular dystrophies (DMD and BMD) are X linked muscle wasting disorders caused by mutation in a gene located at Xp21.[1] Twenty-three females have been described with DMD and de novo X;autosome translocations with the X chromosome breakpoint at p21 and the autosomal breakpoint variable in location.[2] Expression of the disease in these females is the result of non-random inactivation of the normal X chromosome and the variable severity of the disease is probably dependent on the percentage of active normal X chromosomes in the muscle fibres.[2]

Although all of the translocations break on the X chromosome at p21, detailed cytogenetic examination has mapped the translocation exchange points over a large region of Xp21.[3] The DMD gene has been estimated by pulsed field gel analysis to be as large as 2000 kb[4–7] and has a minimum of 60 exons.[8] Southern blot analysis using DMD genomic probes on mouse-human somatic cell hybrids containing one of the two translocation derived chromosomes from several patients has confirmed that the translocation breakpoints are scattered throughout the gene.[9 10] Further localisation of translocation breakpoints has been possible with the use of the DMD cDNA clones,[8 11 12] and analysis of four translocation patients has shown breakpoints within large introns, two in intron 1 and two in intron 7.[13]

To date, all of the published translocation cases were ascertained as females with muscular dystrophy. We have recently had the opportunity to study a t(X;4) translocation first detected in amniotic fluid from a 31 year old undergoing routine prenatal diagnosis. At birth, the child was confirmed to have the translocation, and had a grossly raised CK level consistent with DMD. Molecular analysis has mapped the translocation exchange point to an intron near the middle of the gene, and has shown that the translocation originated in the paternal genome.

Materials and methods

CHROMOSOME ANALYSIS

Amniotic fluid cells were cultured by standard tech-

niques and harvested by the suspension technique. Lymphoblastoid cells from the patient were transformed with Epstein-Barr virus. Metaphase chromosomes were G banded with trypsin. The replication pattern of the X chromosomes was evaluated by RBG staining.

SOMATIC CELL HYBRIDS

Mouse-human somatic cell hybrids were made by fusing 2×10^6 lymphoblastoid cells from the patient with 2×10^6 mouse RAG cells in the presence of Sendai virus (800 HAU) according to a standard protocol.[14] Cells were plated onto five 100 mm plates and grown for 48 hours in α-MEM containing 10% fetal bovine serum. They were changed to selective medium containing HAT (hypoxanthine, methotrexate, thymidine; Flow Laboratories, Inc) and 3 μmol/l ouabain. After three weeks colonies were picked from selective medium and grown continuously in HAT medium to ensure retention in the hybrids of the derivative(X) chromosome carrying the active human hprt gene. After six weeks, clones that retained the der(X) chromosome were grown for further analysis. Clones that contained both translocated chromosomes, but not the unrearranged X chromosome, were back selected in 6-thioguanine (10^{-5} mol/l) to select for clones that had lost the der(X) chromosome.

SOUTHERN BLOTTING

DNA from lymphoblastoid cells, mouse RAG cells, and hybrids was prepared using standard procedures. Restriction digests were done using 5 μg of DNA from human and RAG cells, or 10 to 15 μg of DNA from hybrids. Suppliers' recommended digestion conditions were followed. DNA fragments were separated on agarose gels and transferred to Hybond-N (Amersham). Labelled probes (^{32}P) were prepared by random oligonucleotide primed synthesis.[15] Hybridisation was carried out overnight at 42°C in 50% formamide, 3 × SSC, 0·05 mol/l $NaPO_4$, 1% SDS, 0·5% (w/v) non-fat milk powder, 0·3 mg ml^{-1} sheared herring sperm DNA, 10% dextran sulphate, and 1 to 3×10^6 cpm ml^{-1} of labelled probe. Washing conditions varied depending on the probe used. Autoradiography was at −70°C for one to seven days.

PROBES

Probes used to characterise the hybrids were 754 (*DXS84*),[16] which is centromeric to the DMD gene and recognises a *Pst*I polymorphism of 12 kb and 16 kb, and 99–6 (*DXS41*),[17] which is telomeric to the DMD gene and recognises a *Pst*I polymorphism of 13 kb and 22 kb. Probes used to map the translocation within the DMD gene were GMGX11 (*DXS239*),[18] J–66 (*DXS268*),[19] and DMD cDNA probe cDMD–8 (American Type Culture Collection).[8] Polymorphic probes used in addition to 754 and 99–6 to determine parental origin were C7 (*DXS28*),[20] which is telomeric to the DMD gene and recognises *Eco*RV alleles of 7·5 kb and 8·0 kb, P20 (*DXS267*),[21] which lies within the DMD gene and recognises *Eco*RV and *Msp*I alleles of 7·0 kb/7·5 kb and 3·5 kb/6·8 kb respectively, p87–15 and p87–30 (*DXS164*),[22] which lie within the DMD gene and recognise *Taq*I alleles of 3·3 kb and 3·5 kb, and *Bgl*II alleles of 8·0 kb and 30 kb respectively, and cX5·7 (*DXS148*),[23] which is centromeric to the DMD gene and recognises *Msp*I alleles of 3·5 kb and 7·0 kb.

Results

CLINICAL DESCRIPTION

In 1983, amniotic fluid was obtained from a 31 year old woman. She and her 31 year old husband presented for prenatal testing for personal reasons. Parental chromosomes were normal. The amniotic fluid cell karyotype was that of a female with a translocation 46,X,t(X;4)(p21;q35).[24] A partial karyotype showing the translocation is shown in fig 1. The normal X chromosome was late replicating in all of 100 RBG stained amniotic fluid cells examined, and

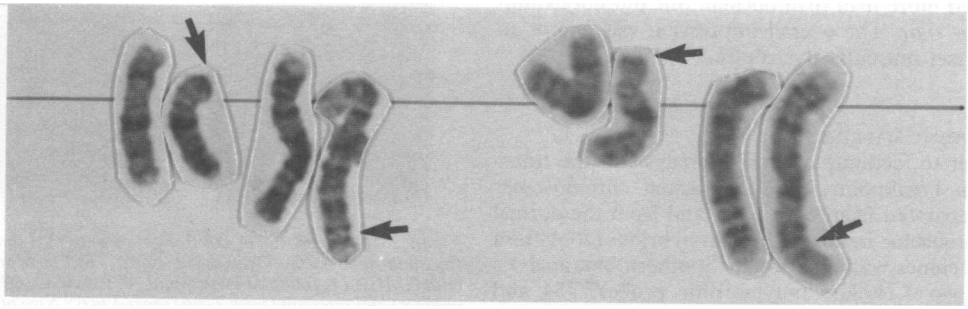

Figure 1 Partial karyotypes of lymphoblastoid cells with the t(X;4). Two GTG banded sets of chromosome 4 and X are shown, with arrows showing the approximate breakpoints on the derivative 4 (der (4) contains the centromere of chromosome 4) and the derivative X (der(X) contains the centromere of the X chromosome) in each set.

only the structurally normal X chromosome exhibited X inactivation associated folding at Xq12–q21.[25] This is evidence favouring preferential inactivation of the normal X chromosome in the cultured amniotic fluid cells. Alphafetoprotein and detailed ultrasound examination were normal.

At the time this case was observed, six other females were known to have an X;autosome translocation with an X chromosome breakpoint at p21, and all were ascertained because they had Duchenne or Becker muscular dystrophy. The parents were advised that the fetus was at some increased risk for having malformations and mental retardation because she had a de novo translocation, and for having muscular dystrophy because of the Xp21 breakpoint. The absolute risk for muscular dystrophy was uncertain because it was unknown how many phenotypically normal females have a similar translocation. The parents continued the pregnancy. A serum CK assay from the patient at 24 hours of age was 21 450 IU/l. The subsequent assays have ranged from 1260 to 4200 IU/l, grossly raised over the normal range.

At 3 months of age the infant had no dysmorphic features, normal developmental milestones, and a normal neurological examination. Since the age of 3 months she has been examined on two occasions. Although the parents have been hesitant to have muscle biopsies taken, we have been able to obtain blood specimens from her and her parents for lymphoblastoid transformation and molecular analysis.

At 2½ years the patient was talking in short sentences, and was alert and active. The only concern was that she tended to pull herself up from a sitting position on the floor, and although she would walk down stairs, preferred to crawl up stairs. At 4½ years, her height was 99 cm, weight 18·1 kg, and head circumference 51·5 cm. She had normal intellectual development. There was no evidence of lumbar lordosis and her gait was normal. In climbing stairs she showed weakness and preferred to use the handrail. She exhibited weak facial and shoulder girdle musculature and her gastrocnemius muscles appeared more firm than normal. She did not exhibit a Gower sign. The overall impression was one of an early onset muscular dystrophy.

BREAKPOINT MAPPING

In order to facilitate molecular analysis of the translocation breakpoint, the translocated chromosomes were separated from each other and from the normal X chromosome in mouse-human hybrids. DNA from hybrid clones was analysed by Southern blot analysis using two X linked polymorphic probes, 754 and 99–6, which flank the DMD locus. The patient was heterozygous for both probes. Clones which contained only the der(X) chromosome, and not the der(4) or the unrearranged X chromosome, had only the 16 kb *Pst*I fragment detected by probe 754. Probe 99–6 did not detect any bands in these hybrid clones. Clones with both the der(X) and der(4) chromosomes, but not the unrearranged X chromosome, had the 16 kb fragment of probe 754 and the 22 kb fragment of probe 99–6. Following back selection of these clones in 6-thioguanine to select for the loss of the der(X) chromosome, hybrids were identified which carried only the der(4) chromosome and had the 22 kb fragment of probe 99–6, but not the 754 hybridising fragment. Thus, the t(X;4) translocation breakpoint mapped between probes 754 and 99–6. Detailed chromosome analysis was not done to determine which other human chromosomes were present in the hybrids.

The breakpoint was mapped in the DMD gene by analysis with DMD genomic probes (fig 2). DNA from the der(X) hybrid contained hybridising fragments with GMGX11 (lane 2), while DNA from the der(4) hybrid did not (lane 3). When the same blot was probed with J–66, DNA from the der(4) hybrid contained hybridising fragments (lane 6), while DNA from the der(X) hybrid did not (lane 5). This indicated that the breakpoint was within the DMD gene distal to GMGX11 but proximal to J–66. Hybrid cell DNA was then probed with a cDNA fragment (cDMD–8) from this region of the gene. Fig 3 shows *Hind*III digests probed with cDMD–8, indicating seven exon containing bands in the control lanes (lanes 1 and 5). The mouse specific bands found with this probe are shown in lane 4. The der(X) hybrid in

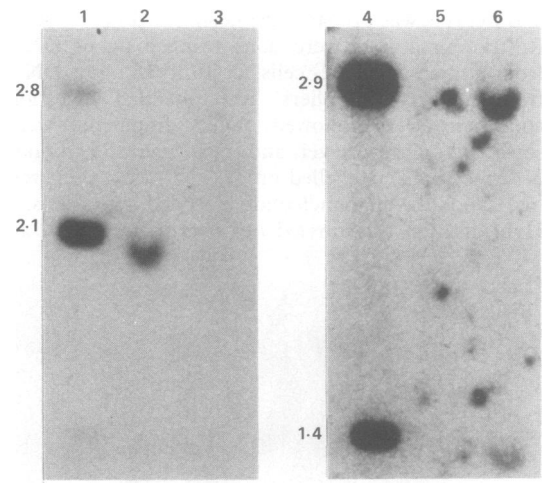

Figure 2 Southern blot of DNA digested with PstI and fractionated on a 0·8% agarose gel. Lanes 1 to 3 were probed with GMGX11, followed by stripping (0·1 × SSC, 80°C, 10 minutes) and reprobing with J–66 (lanes 4 to 6). Size of bands in kb are labelled on the left of each blot. Lanes: (1) and (4) female control, (2) and (5) der(X)t(X;4) hybrid, (3) and (6) der(4)t(X;4) hybrid.

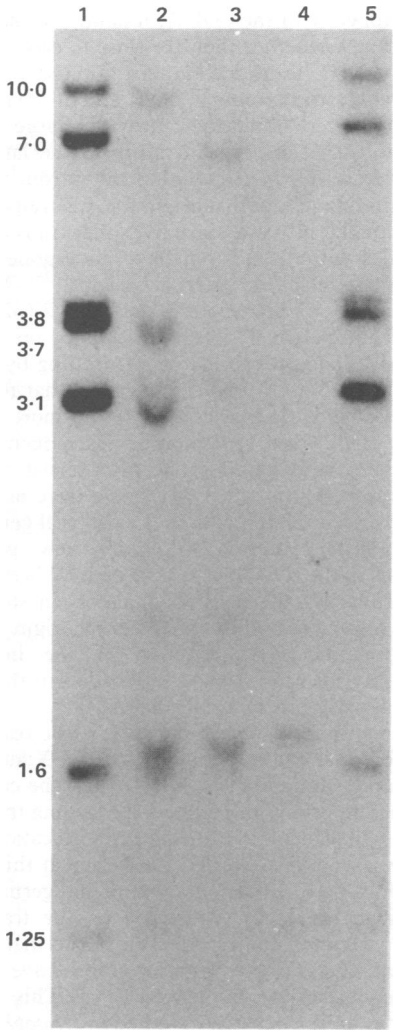

Figure 3 Southern blot of DNA digested with HindIII and fractionated on a 0·9% agarose gel. The blot was probed with a 0·9 kb EcoRI fragment of the DMD cDNA (cDMD–8). This fragment detects approximately exons 47 to 52 out of 74 exons in total. Size of exon containing bands in kb are labelled on the left of the blot. Lanes: (1) t(X;4) patient, (2) der(X)t(X;4) hybrid, (3) der(4)t(X;4) hybrid, (4) mouse (RAG), (5) control female.

lane 2 shows the presence of six human specific bands. The der(4) hybrid in lane 3 shows the presence of one human specific band of 7·0 kb. When the adjacent cDNA clone 3′ to cDMD–8 was used as a probe, all exon bands were located in the der(4) hybrid lane (data not shown). The order of exon containing *Hind*III fragments (size in kb) in this region of the gene is 5′–10, 1·25/3·8, 1·6, 3·7, 3·1, 7·0–3′,[18] leading to the conclusion that the translocation breakpoints are within the intron between the 3·1 kb and the 7·0 kb exon containing fragments (equivalent to about nucleotide 7675 in the 13 900 nucleotide DMD cDNA).

ORIGIN OF TRANSLOCATION

Southern blot analysis of the hybrids carrying the translocated chromosomes made possible the determination of the phase of the polymorphic markers for which the patient was heterozygous, and thus the parental origin of the translocation. A number of markers, both flanking and within the DMD gene, were used. Fig 4, lanes 1 to 6, shows an *Eco*RV polymorphism with the probe C7, which detects heterozygous bands in the translocation patient (lane

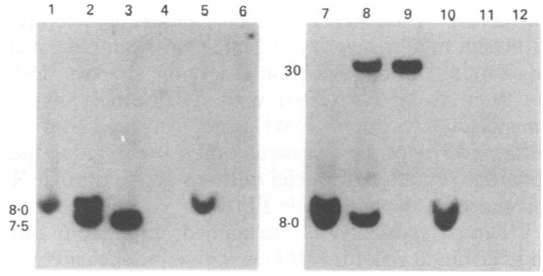

Figure 4 Lanes 1 to 6: Southern blot of DNA digested with EcoRV and fractionated on a 0·6% agarose gel. The blot was probed with C7. Lanes 7 to 12: Southern blot of DNA digested with BglII and fractionated on a 0·6% agarose gel. The blot was probed with p87–30. Size of bands in kb are labelled on the left of each blot. Lanes: (1) and (7) father of patient, (2) and (8) t(X;4) patient, (3) and (9) mother of patient, (4) and (10) der(X)t(X;4) hybrid, (5) and (11) der(4)t(X;4) hybrid, (6) and (12) mouse (A9).

Parental inheritance of the translocation.

Probe	Size of polymorphic bands (kb)				
	Father	Mother	Patient	der(X)	der(4)
99–6	22	13/22	13/22	—	22
C7	8·0	7·5/7·5	7·5/8·0	—	8·0
P20 *Eco*RV	7·5	7·0/7·0	7·0/7·5	7·5	—
P20 *Msp*I	3·5	6·8/6·8	3·5/6·8	3·5	—
p87–30	8·0	30/30	8·0/30	8·0	—
p87–15	3·5	3·3/3·3	3·3/3·5	3·5	—
754	16	12/16	12/16	16	—
cX5.7	3·5	7·0/7·0	3·5/7·0	3·5	—

2). The 8·0 kb band in the father (lane 1) is the same as the band in the der(4) hybrid (lane 5). Lanes 7 to 12 show a *BglII* polymorphism with p87–30, which detects heterozygous bands in the translocation patient (lane 8). The father has the 8 kb band (lane 7), which is the same as the band in the der(X) hybrid (lane 10). The table shows a summary of the results with all probes tested. In every case the band in the hybrid carrying one of the two translocation derived chromosomes is the same band as in the father, indicating paternal origin of the translocation.

Discussion

This is the first report of a female with an X linked muscular dystrophy whose translocation was ascertained prenatally through routine amniocentesis. Although at the age of 4 she has not yet shown signs of acute disease, her CK values and her neurological examination are consistent with the early stages of the disease. Her t(X;4)(p21;q35) translocation was confirmed at birth and the breakpoint has been mapped by a combination of somatic cell hybrid and molecular analyses to an intron near the middle of the DMD gene.

The CK data and the translocation breakpoint mapping predict that the patient is developing the Duchenne or Becker form of muscular dystrophy. Although milder severity of DMD has been observed in a few of the translocation cases, the severity does not seem to be correlated with the location of the translocation within the DMD gene.[9] Any modification in the severity of the disease could be attributed to the potential presence of nuclei with an active normal X chromosome in the muscle fibres.[2]

When our patient was ascertained, a moderate but undetermined risk for DMD was given to the parents. Should a similar translocation be ascertained today, it would still not be possible to assign an accurate risk, since the proportion of normal females with translocations involving Xp21 is still unknown. However, with the current availability of DMD probes, and the powerful technique of in situ hybridisation, it should be possible to localise probes from opposite ends of the gene on the derivative chromosomes. Should such probes localise on alternate translocation derived chromosomes, it would then be reasonable to conclude that the gene was disrupted by the translocation and assign a high risk figure for DMD.

In a study on the position of nine translocations within the DMD gene using genomic probes, there was no apparent clustering of breakpoints, although there were more in the distal half (3′ end) of the gene than the proximal half (5′ end). Two of these cases have been mapped to the same general region as the t(X;4) translocation described here, between GMGX11 and J–66.[9] Since the distance between the two probes is 250 to 400 kb,[5] and the other two cases have not yet been mapped with the cDNA, it is impossible at this time to know whether they are close to each other or to the t(X;4) translocation. In a study of four translocation breakpoints at the 5′ end of the gene, all breakpoints were found to occur within large introns, two in intron 1 and two in intron 7, the latter two being at least 40 kb apart within the intron.[13]

The finding of paternal origin for this translocation is of particular interest, since five other translocations in females with DMD that have undergone similar analysis are also of paternal origin[26–28] (Bodrug, unpublished observations on t(X;2)(p21;q37); Cockburn, personal communication on t(X;1)(p21;p34)). The probability of this finding by chance is $(1/2)^6$ or 1·5%. A further indication that this may not be a chance finding comes from a more general analysis of de novo chromosome rearrangements in which 84% were found to be of paternal origin.[29] Duplications within the DMD gene have also been found to arise preferentially in the paternal genome.[30] Similarly, the parental origin of new germline mutations in the retinoblastoma gene have been found to be primarily paternal.[31 32] It has been suggested that the finding of preferential paternal origin of these rearrangements is a reflection of the increased opportunity for error during replication in the many mitotic divisions in spermatogenesis.[29–32]

It is tempting to suggest that this is the reason for the preferential paternal origin of the six X;autosome translocations found in DMD patients. The caveat to the argument of mitotic origin is the finding that male carriers of balanced X;autosome translocations are, with few exceptions, sterile (the father in this study would only be a carrier in some of his germ cells), resulting in the inability to pass on the translocation.[33–35] Meiotic studies done on sterile males with reciprocal X;autosome translocations have found spermatogenic arrest at meiosis I.[36 37] This phenomenon has been extensively studied in *Drosophila* and mice, and the proposal by Lifschytz and Lindsley[38] that sterility is a result of having autosomal material in immediate contact with sex chromosomal material, thereby disturbing a control mechanism for normal spermatogenesis, is still favoured.[33] If X;autosome translocations in males were unable to pass through meiosis successfully, then de novo translocations such as the one described in this study must have originated during one of the meiotic divisions. The preferential paternal origin of X;autosome translocations as shown here could be the result of a small sample size. Alternatively, if this trend continues in other studies, it suggests that there are still many questions to be answered about the inherent differences in meiosis in males and females.

Ultimately, we would like to understand the mechanism by which translocation occurs, and an analysis of sequences at or near translocation breakpoints may provide clues. The presence of a repetitive

element such as an Alu repeat or a LINE repeat might suggest a homologous exchange between these elements on non-homologous chromosomes. Another possibility is a recognition sequence for an enzyme involved in the translocation process. The CGGC tetranucleotide found in the immediate vicinity of a t(X;21)(p21;p12) translocation might be a candidate for such a recognition sequence.[39] Alternatively, variation in chromatin configuration might lead to an increased probability of rearrangement, a possibility that is supported by the fact that some DMD translocation breakpoints involve autosomal regions coincident with known fragile sites.[2] In addition, it is possible that translocation might be the result of a breakage event at a replication fork associated with a nuclear matrix anchorage site, which is repaired by reunion to a replication fork of another chromosome at a nearby anchorage point. This type of mechanism has been postulated in the generation of deletions in the β globin gene.[40] The cloning and sequencing of a number of translocation breakpoints should help to distinguish between these possibilities.

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