Characterisation of an inverted X chromosome (p11.2q21.3) associated with mental retardation using FISH

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Abstract

We report on a patient with a pericentric inversion of the X chromosome, 46,Y,inv(X)(p11.2q21.3), who was referred for cytogenetic analysis because of mild mental retardation, short stature, prepubescent macro-orchidism, and submucous cleft palate. The same chromosomal abnormality was found in the proband's mother. The inverted X chromosome was late replicating in all the mother's lymphocytes studied, indicative of a likely unbalanced inversion. We show, by fluorescence in situ hybridisation (FISH) using a panel of ordered yeast artificial chromosome (YAC) clones, that the Xp breakpoint is localised in Xp11.23 between DXS146 and DXS255 and that the Xq breakpoint is assigned to the X-Y homologous region in Xq21.3. YACs crossing the Xp and Xq breakpoints have been identified. One of these two breakpoints could be linked to the mental retardation in this patient as many non-specific mental retardation (MRX) loci have previously been located in the pericentromeric region of the X chromosome. Moreover, the elucidation at the molecular level of this rearrangement will also indicate if cleft palate or prepubescent macro-orchidism, or both, in this boy are related to one of the two X breakpoints.

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To date, as many as 147 X linked clinical entities with mental retardation (XLMR) have been described.¹ The overall incidence of XLMR has been estimated to be 1.8 per 1000 males whereas nearly 2.8 per 1000 females are expected to be carriers of at least one mutated gene responsible for XLMR.^{2,3} XLMR can be classified as either syndromal or non-specific, depending on the presence or absence of phenotypic characteristics that define a syndrome. The lack of distinctive clinical criteria within the non-specific XLMR group (MRX) means that the only approach to differentiate between them is through non-overlapping regional localisation of the associated gene.⁴ Genetic heterogeneity of MRX has been largely shown by mapping studies¹ and as many as 42 MRX families with a lod score of ≥ 2.0 (including

FRAXE) have been reported so far.⁵ In addition, there is evidence for a clustering of such genes in the pericentromeric region of the X chromosome.⁶⁻⁸ Numerous disease loci have been mapped precisely through the study of chromosome aberrations⁹ and progress has been made in the identification of possible specific sites for MRX from patients with structural abnormalities of the X chromosome (X;autosome translocations and small deletions).¹⁰⁻¹⁸ The results of these two approaches (linkage and cytogenetic analysis) indicate that at least 10-12 non-overlapping genes must be involved in MRX.¹⁵

We have identified a male with an inverted X chromosome, inv(X)(p11.2q21.3), associated with mild mental retardation and minor anomalies. Here we report on the fine mapping of X chromosomal breakpoints as the first step towards the identification of gene(s) involved in this MRX.

Case report

The proband, an 11 year old boy, was the first born child to healthy, non-consanguineous parents. Subsequently, the couple had three pregnancies of which two were electively terminated early and one led to a stillborn infant as a result of eclampsia. From a second union, his mother had a normal daughter. The proband was born at term after an uneventful pregnancy. Birth weight was 2850 g and birth length 46 cm. Early development was reported as normal. He was first seen at the age of 11 years for decreased height and mental retardation. On physical examination, his height was 128.5 cm (-2.5 SD) and weight 26 kg (-1.5 SD for chronological age). Testicular volumes were increased (30 mm in length, +2 SD for age and pubertal stage I). There was also a slight submucous cleft palate without any palatal dysfunction or velar insufficiency. There were no other dysmorphic features. He had mild mental retardation with a two year delay in school progression. On endocrinological evaluation partial growth hormone insufficiency was shown after two pharmacological stimulations. The thyroid function was normal. There was a low testosterone level, in the normal range for prepubescent boys and in contrast to the testicular size. On magnetic resonance imaging, the hypothalamus and pituitary glands were reported as normal. Fragile X (FRAXA) syndrome was ruled out by molecular analysis.

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Figure 1 Partial GTG banded karyotype and idiogram of the inversion *inv(X)(p11.2q21.3)* from the mother (A) and her son (B).

Cytogenetic analysis

Cytogenetic analysis was performed on thymidine synchronised cultures in order to obtain chromosomes suitable for high resolution studies.¹⁹ R banding was achieved by BrdU incorporation for at least six hours of culture, followed by fluorescence-photolysis-Giemsa (FPG) staining. G banding was achieved by the trypsin-Giemsa method. Late replication studies were performed from synchronised and standard 72 hours lymphocyte cultures by adding BrdU for the last six hours of culture and FPG staining.

Fluorescence in situ hybridisation

Mapping of the X chromosome breakpoints was achieved by using YAC clones from well characterised YAC contigs in both Xp11¹⁸⁻²⁰⁻²² and Xq21.3.²³⁻²⁴ YAC clones expected to be located between the genetic markers DXS1000 and OATL1 in Xp11.2 and between DXS1196 and DXS214 in Xq21.3 were used as probes for FISH on the patient's chromosomes. YAC clones were obtained from the CEPH MegaYAC library,²⁵ the ICRF YAC library,²⁶ and the ICI YAC library.²⁷ Total yeast DNA was isolated and then labelled with biotin by nick translation using standard methods.²⁸ In order to distinguish between the p and q arms of the

submetacentric *inv(X)* chromosome in our patient, a digoxigenin labelled probe specific for the steroid sulphatase (STS) locus on Xp22.3 (Oncor) was used as a marker for the p arm. Approximately 50 ng biotin labelled total yeast DNA was mixed with 2.5 µg human Cot-1 DNA to suppress repetitive sequences and 10 µg sheared salmon sperm DNA as carrier, precipitated in ethanol, and redissolved in a 25 µl hybridisation mix (2 × SSC, 50% deionised formamide, and 10% dextran sulphate). After denaturation for 15 minutes at 75°C, the probe mix was allowed to anneal for 30 minutes at 37°C and was then transferred to microscope slides with metaphase chromosome spreads from the patient. Metaphase chromosomes were denatured in 70% formamide at 75°C for two minutes and dehydrated in an ethanol series. After hybridisation for 16 hours at 37°C, the slides were washed in 50% formamide, 2 × SSC (3 × 5 minutes, 45°C) followed by washes in 0.1 × SSC (3 × 5 minutes, 60°C). The hybridisation signals were coloured by Texas Red and avidin-fluorescein isothiocyanate (FITC), so that the STS probe signal appeared red-purple and the specific YAC clone signal appeared green on metaphase chromosomes that were DAPI (4',6-diamidino-2-phenylindole) counterstained.

Results

A pericentric *inv(X)(p11.2q21.3)* (fig 1) was diagnosed in the proband and his mother. Other relatives refused to cooperate. However, no increased miscarriage rate or liveborn unbalanced offspring were observed in two generations. No difference was observed between the mother's and son's inverted X chromosome. One hundred metaphases were scored on the mother for late replication studies. The inverted X chromosome was late replicating in all lymphocytes examined (data not shown).

An idiogram of the pericentric inversion is shown in fig 1. A painting probe from the X chromosome was tested on the mother's and the proband's metaphases. We did not detect any other gross chromosomal rearrangements (data not shown). YACs from previously established YAC contigs in Xp11 (fig 2A) and Xq21 (fig 2B) were used to map by FISH the inversion breakpoints on both the mother's metaphases (fig 3A, C) and the son's metaphases (fig 3B, D). In the mother, the normal X homologue served as a control for the result obtained for the aberrant chromosome. In the Xp11 region (fig 2A), YACs positive with OATL1 (ICRF y900F501) and OATL2 (ICRF OATL2-1) both gave the same hybridisation pattern on the inverted X chromosome with one signal on both p and q arms, indicating that they cross the Xp11 breakpoint. FISH analysis of cosmids derived from ICRF YAC y900F501, positive with either the left or the right arm of the pYAC4 vector, gave the same result on the proband's metaphase with only one signal distal to the Xp11 breakpoint. From this result we can conclude that the YAC signals resulted from cross hybridisation between sequences reiterated several times at

Figure 2 (A) Molecular localisation of the Xp breakpoint by FISH using YACs between OATL1 and DXS1000. (B) Molecular localisation of the Xq breakpoint by FISH inside the X-Y homologous region in Xq21.3.

each non-adjacent OATL cluster in Xp11.²⁹ Only one signal was observed on a normal X chromosome because the physical length between the two clusters is only a few Mb. YAC clone ICRF y900C0191, positive with DXS6851 (fig 2A), gave two hybridisation signals on the inverted X chromosome (fig 3A, B) indicating that this YAC spans the Xp11.2 breakpoint. Localisation of the Xq breakpoint (fig 2B) showed that ICI YAC 25FD6 crosses

the Xq breakpoint (fig 3C, D), locating the Xq21.3 breakpoint in the X-Y homologous region within a 220 kb region between DXYS114 and DXYS118. These results are consistent with all the YACs tested and shown to map both proximally and distally according to their positions on the map (fig 2A, B).

Discussion

We described a new familial pericentric inversion (X)(p11.2q21.3) associated with mild non-specific mental retardation. Rearrangements of the X chromosome are relatively frequent and include deletions, translocations, and isochromosomes. However, pericentric inversions of the X chromosome are very rare, occurring at a frequency of 1/28 000–1/30 000.³⁰ The cytogenetic abnormality was found in an 11 year old boy with mild mental retardation, short stature, submucous cleft palate, and prepubescent macroorchidism. The proband's mother, a carrier of the same inv(X), is phenotypically normal. X inactivation study in the mother shows a preferential skewed inactivation of the inverted X chromosome. This non-random inactivation pattern suggests an unbalanced inversion. However, another possibility could be that the "skewed" X inactivation reflects a selection for cells with the inverted X inactivated. As a direct approach towards positioning the X chromosome breakpoints, we have used YAC clones as probes for FISH analysis. Our results show that YAC clones ICRF y900C0191 and ICI 25FD6 span the Xp and Xq breakpoints, respectively. This suggests that the chromosome segments inserted in these YAC clones might include an MRX gene. Two cases with X chromosome aberrations and breakpoints similar to those reported in our study have been published: a moderately mentally retarded adolescent female with general hypotrophy associated with a de novo apparently balanced

Figure 3 FISH analysis of biotinylated ICRF YAC y900C0191 to a mother's metaphase (A) and a proband's metaphase (B) showing two signals on the inverted X chromosome. The red signals correspond to Oncor STS probe on Xp22.3, which is supplied together with a centromeric X probe. FISH analysis of biotinylated ICI YAC 25FD6 on a mother's metaphase (C) and a proband's metaphase (D) showing two signals on the inverted X chromosome and one signal on the Y chromosome (X-Y homologous region) (arrow). The red signal corresponds to the STS locus on Xp22.3

t(X;20)(p11.2;q13.3), and a mentally retarded boy with a paracentric inversion of the long arm of the X chromosome, inv(X)(q21q24), that cosegregated with mental retardation.³¹ In this family, carrier females were asymptomatic. Deafness was also observed but did not seem to cosegregate with the X inversion. In addition, by linkage analysis, a large number of syndromic and isolated XLMR were mapped to the region including Xp11 and Xq21.^{3 5 7 8} In our family, the affected boy suffers from borderline mental retardation (MR) with other clinical signs, but because he is the only affected male we must consider the MR, if genetically determined, as a non-specific mental retardation which could be linked to one of the two X breakpoints both located in the pericentromeric region. The Xp11.2 breakpoint seems to be a better candidate for disruption of an XLMR gene as it is crossed by many MRX genetic intervals.⁵

The clinical features also include short stature, submucous cleft palate (CP), and prepubescent macro-orchidism. The short stature is probably unrelated to the inv(X) as the father who does not carry the inversion is only 165 cm tall. The occurrence of cleft palate is more striking as a gene for an X linked CP (CPX, MIM 303400) is located in band Xq21 between DXS1196 and DXS1217.²³ FISH analysis has enabled us to map the Xq21 breakpoint in the X-Y homologous region (fig 3C, D), a few Mb distal to the telomeric end of the CPX interval as determined by linkage study in an Icelandic pedigree.²³ This location seems to rule out the involvement of the Xq breakpoint in the CP in this boy. Nevertheless, the X inactivation pattern in the proband's mother could indicate a more complex rearrangement with a deletion of part of the CPX region. It is noteworthy that an intensive STS PCR screening with markers from DXS95 to DXYS1X did not detect any deletion (data not shown). A transgenic mouse model of X linked cleft palate has been reported.³² The disrupted cellular sequences have been mapped to the proximal region of the mouse X chromosome, just proximal to the *Araf* locus. This mouse locus may represent the analogue of a human locus mutated in a sex linked, heritable form of CP. The human *ARAF1* gene is localised in Xp11.23, several Mb distal to the location of our Xp11.23 breakpoint,³³ excluding the involvement of this locus in the CP in this boy. The inv(X) was also associated with prepubescent macro-orchidism at the age of 11. Molecular examination excluded a mutation in the *FMR1* gene and endocrinological evaluation showed normal thyroid function. In the case reported by German *et al.*,³⁴ the male carriers of inversion inv(X)(p11q26) have ambiguous genitalia and Madan³⁵ suggested that "some sort of position effect in the X affecting the sexual phenotype may also be operating in the male". In view of the fact that the Xq breakpoint in our patient occurs in the X-Y homologous region, one could speculate that it disrupts expressed sequences involved in testicular development. The Xq breakpoint is located inside the so-called critical region (CR)

(q13-q26) which has to be intact on both X chromosomes for proper ovarian development and function.³⁶ Our case is another exception to this rule and molecular cloning of breakpoints inside the CR with or without ovarian dysgenesis should clarify this complex concept of CR.

Further studies will involve cosmid cloning of both breakpoints and search for expressed sequences at or near Xp and Xq breakpoints. Elucidation at the molecular level of this rearrangement will indicate if any of the clinical symptoms in this patient are the result of the inverted X chromosome.

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