Severe phenotype resulting from an active ring X chromosome in a female with a complex karyotype: characterisation and replication study

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Abstract

We report on the characterisation of a complex chromosome rearrangement, 46,X,del(Xq)/47,X,del(Xq),+r(X), in a female newborn with multiple malformations. Cytogenetic and molecular methods showed that the del(Xq) contains the XIST locus and is non-randomly inactivated in all metaphases. The tiny r(X) chromosome gave a positive FISH signal with UBE1, ZXDA, and MSN cosmid probes, but not with a XIST cosmid probe. Moreover, it has an active status, as shown by a very short (three hour) terminal BrdU pulse followed by fluorescent anti-BrdU antibody staining. The normal X is of paternal origin and both rearranged chromosomes originate from the same maternal chromosome. We suggest that both abnormal chromosomes result from the three point breakage of a maternal isodicentric idic(X)(q21.1). Finally, the phenotype of our patient is compared to other published cases and, despite the absence of any 45,X clone, it appears very similar to those with a 45,X/46,X,r(X) karyotype where the tiny r(X) is active.

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Small ring X chromosomes are most frequently described in mosaic 45,X/46,X,r(X) karyotypes of females with the Turner syndrome (TS) phenotype, characterised by gonadal dys-

genesis, short stature, and minor anomalies such as widely spaced nipples, narrow palate, small mandible, and webbed neck. In such cases, the r(X) chromosome is usually inactivated and the TS phenotype results from the X monosomy. Some females have been shown to have the same karyotype associated with a more severe phenotype, including mental retardation, syndactyly, and dysmorphic features, which are not characteristic of TS.¹⁻² The severe phenotype of these females has been attributed to the inability of their ring X chromosome to inactivate, often resulting from the lack of the X inactivation centre.³⁻¹¹

We report on a complex mosaic rearrangement, 46,X,del(Xq)/47,X,del(Xq),+r(X), in a female newborn with multiple malformations. Unlike previously reported cases, our proband does not show characteristic features of Turner syndrome, which is consistent with the absence of any 45,X clone in her karyotype. The coexistence of an apparent interstitial deletion and a ring chromosome, originating from the same chromosomal pair, may have arisen from a single event.¹²⁻¹⁶ In such a rearrangement, one of the breakpoints would be located within the

Figure 2 Three partial karyotypes from the proband showing the X chromosomes after RBG banding. The del(Xq) chromosome is present in all metaphases and seems to result from an interstitial deletion lying between Xq21.1 and Xq24. The r(X) is present in 20% of metaphases.

Figure 1 Facial dysmorphism of the proband at 12 months of age.

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Figure 3 Metaphases from the proband illustrating replication (A) and FISH (B, C, D) studies: the arrowhead indicates the r(X) when present in a metaphase. (A) Metaphase showing the activity status of the X chromosomes after a short (three hour) terminal BrdU pulse and FITC anti-BrdU antibody staining. The X chromosomes are identifiable by the centromeric alphoid signal (red). Only the del(Xq) has incorporated BrdU (late replicating) as shown by FITC antibody (green). Chromosomes are counterstained with DAPI. (B) Metaphase after XIST cosmid hybridisation: the r(X) does not show the XIST signal (red). Chromosomes are counterstained with DAPI. (C) Metaphase after ZXDA cosmid hybridisation: the normal X, del(Xq), and r(X) all give a positive green signal. Chromosomes are counterstained with propidium iodide. (D) Metaphase after cohybridisation of digoxigenin labelled STS cosmid and X alphoid probes: the normal X shows two red signals, one at the centromere, the other at the telomere Xp, as expected. However, there are three red signals on the del(Xq), one at the centromere and one at each telomere of the chromosome, indicating the presence of two STS loci, that is, two Xp telomeres.

centromere and the ring chromosome derived from the excised segment. We therefore hypothesised that the abnormal phenotype of our patient results from the non-inactivation of either the del(X) or the r(X) chromosome, the r(X) containing loci excised from the del(X).

Standard and molecular cytogenetics, as well as other molecular techniques, were used in order to characterise the composition of both rearranged chromosomes. In support of our hypothesis, we have shown by FISH that the del(Xq) chromosome contains the XIST locus and is late replicating, while the r(X) does not contain the XIST locus and is early replicating. Nevertheless, the r(X) chromosome does not seem to be the counterpart of the del(Xq), as FISH analysis showed that both rearranged chromosomes contain the pericentric loci UBE1, ZXDA, and MSN.

Moreover, using microsatellite markers, we have shown that no locus distal to Xq21.1 is contained within the del(Xq), which is discordant with the standard cytogenetic data and suggests a more complex rearrangement. In-

deed, additional studies showed that the del(Xq) and the r(X) chromosome, which are both of maternal origin, probably resulted from a three point breakage of an isodicentric chromosome, idic(X)(q21.1), the del(Xq) being in fact a recombinant X chromosome as follows: der(X)(pter→q21.1::p21→pter).¹⁷

Finally, we correlate the karyotype of our patient with her phenotype, in the light of related published observations.

Case report

The proband was the second child of a healthy, 27 year old woman. Cerebral ventricular dilatation was detected on ultrasound at 35 weeks of gestation. The delivery, after spontaneous labour, was at 39 weeks of gestation. Birth weight was 2900 g (around the 30th centile), length was 47 cm (10th centile), and head circumference 33 cm (25th centile). Some malformations were noted on initial physical examination, such as syndactyly of the right third and fourth fingers, syndactyly of the left

Figure 4 Results from FISH and marker analyses. All the hybridised probes, but only informative markers, are listed in relative order from the Xp telomere to the Xq telomere. (Left) + or - means presence or absence of the locus on the corresponding chromosome. (Right) F=father, C=child, M=mother. Alleles are denoted a, b, and c.

fourth and fifth toes, and cardiac murmur. Echocardiography showed ventricular and atrial septal defects, patent ductus arteriosus, and pulmonary stenosis. Cranial ultrasound showed agenesis of the corpus callosum and mild cerebral ventricular dilatation.

On examination at the age of 12 months, she was severely handicapped, with no speech, generalised hypotonia, and some dysmorphic features, including a broad face, short neck, prominent forehead, apparent telecanthus and convergent strabismus, a small nose with a broad, flat nasal bridge and anteverted nostrils, prominent and inverted V shaped upper lip, short philtrum, and low set and mildly posteriorly rotated ears (fig 1). The cardiac malformations were well supported with medical treatment, and surgical intervention was scheduled at a later date.

Material and methods

CONVENTIONAL AND MOLECULAR CYTOGENETICS
Routine chromosome analysis of the proband and her parents was performed on RHG banded metaphases from 72 hour PHA stimulated peripheral blood lymphocytes. RBG banding was also performed on metaphases of the proband using terminal 5-bromo-2'-deoxyuridine (BrdU) pulse for the last seven hours of culture (60 µg/ml). A lymphoblastoid cell line is available.

Three different FISH approaches were used to characterise the rearranged chromosomes. First, a biotinylated α satellite DNA probe specific for DXZ1 (Oncor, Gaithersburg, MD) was in situ hybridised on metaphase chromosomes to determine whether the supernumerary ring chromosome originated from the X chromosome. Second, a whole chromosome X library

(Oncor) was used to detect any non-X chromosome specific segment present on the rearranged chromosomes. Third, various X specific sequences, labelled by nick translation with bio-16-dUTP or dig-11-dUTP, were used to characterise the abnormal chromosomes further. They were, in map order from Xp to Xq telomere, cosmids STS and KAL (Xp22.3) (from Oncor), cosmids UBE1 (Xp11.23) and ZXDA (Xp11.21),¹⁰ cosmid MSN (Xq11.2-q12),¹⁸ and cosmid XIST (Xq13.2) (from ICRF).¹⁹

In all FISH experiments, the hybridisation biotin signals were made visible with fluorescein-labelled avidin and the digoxigenin signals with rhodamin labelled antibody, following standard protocols.²⁰ Chromosomes were counterstained either with propidium iodide or with DAPI, diluted in antifade.

REPLICATION STUDIES

Replication studies of the X chromosomes were performed using a very late terminal BrdU pulse for the last three hours of culture. As it is often difficult to determine the BrdU incorporation in a small ring because of its condensed structure, we used a highly sensitive immunochemical staining with FITC conjugated anti-BrdU antibody (Boehringer Mannheim Cat No 1202 693).²¹ Briefly, chromosomal DNA was denatured in 70% formamide for two minutes at 70°C, then diluted with fluorescein antibody to a final concentration of 25 µg/ml in PBS, placed on slides, and incubated for 60 minutes at room temperature. The slides were then washed 3 × 5 minutes in PBS and the chromosomes were counterstained with DAPI as hybridisation with an X aliphoid probe was performed simultaneously.

MOLECULAR INVESTIGATIONS

Genomic DNA from the proband and her parents was extracted from whole blood samples using standard methods. DNA polymorphisms were analysed by polymerase chain reaction (PCR) amplification of polymorphic short sequence repeats. We used the following markers: DXS996, DXS1003, DXS1001, DXS1062, DXS 1192, DXS984, DXS996 and DXS8063 from Genethon²²; F8C (intron 13)²³; DXS1236 and DXS1238²⁴; ALAS2²⁵; DXS424²⁶; DXS52²⁷; DXS1684²⁸; AR²⁹; DXS1108.³⁰ Amplification of DNA sequences was carried out using published cycle parameters in a 50 µl reaction volume containing 100 ng of genomic DNA, 1 × PCR buffer, 1.5 mmol/l MgCl₂, 200 µmol/l dATP, dGTP, dTTP, 20 µmol/l dCTP, 6 nmol/l α -³²P-dCTP, 0.6 µmol/l primers, and 0.024 unit/µl of Taq polymerase. PCR products (3 µl) were mixed with loading buffer (6 µl) (formamide 80% xylene cyanol 0.1%, bromophenol blue 0.1%) and were loaded onto a 0.4 mm thick 6% acrylamide (19:1 bis)/42% urea gel. Gels were fixed onto Whatman 3MM paper and exposed to ray film for 17 hours at -80°C.

Results

Conventional cytogenetics showed a complex mosaic karyotype in the proband:

Figure 5 Polymorphism studies. (A, B, C) PCR analyses at three markers proximal to Xq21.1, DXS1236, DXS1003, and AR, respectively. Two distinct alleles, one of paternal origin, the other of maternal origin, are identifiable in the proband. (D, E, F) PCR analyses at three markers distal to Xq21.1, DXS8063, DXS1001, and DXS984, respectively. Only one allele, of paternal origin, is detectable in the proband.

46,X,del(Xq)/47,X,del(Xq),+r. A normal X and a deleted (Xq) chromosome were present in all 100 metaphases analysed. A small supernumerary ring chromosome was detectable in about 20% of the metaphases. RHG and RBG banding suggested that the del(Xq) resulted from the interstitial deletion of a region lying between Xq21.1 and Xq24 (fig 2).

The karyotypes of the proband's parents were normal.

The X specific α satellite probe gave a positive fluorescent signal on the ring chromosome, showing it to be derived from the X chromosome. The whole X chromosome paint resulted in a homogeneous staining of both del(Xq) and r(X) chromosomes, confirming that they are composed exclusively of material from the X chromosome (data not shown).

Inactivation studies using a very late BrdU pulse followed by detection with fluorescent anti-BrdU antibody gave the following results. Sixty metaphases showing detectable BrdU incorporation were analysed and in 11 of these the r(X) chromosome was present and an identical replication pattern was observed. The del(Xq) was brightly stained with fluorescent antibody, indicating its inactive status, while

the normal and r(X) chromosomes were not labelled, suggesting that they replicate synchronously with the other chromosomes and have an active status (fig 3A).

Results obtained by FISH with various probes are shown in fig 4. They show first that the r(X) chromosome does not contain the XIST locus, which is consistent with the inactivation studies (fig 3B). They secondly show that the r(X) contains several loci from the pericentric region, UBE1 and ZXDA on the short arm and MSN on the long arm (fig 3C).

Data obtained from microsatellite marker analyses are also summarised in fig 4: only the informative loci are listed, and their relative order is based on the consensus map from X Chromosome Workshop 6.³¹ These results show that no marker of maternal origin, distal to Xq21.1, is present in the proband (fig 5), suggesting a paternal origin of the normal X chromosome. They also indicated that the del(Xq) chromosome had arisen by a more complex rearrangement than the interstitial Xq21.1-q24 deletion diagnosed by standard cytogenetics. In order to define the del(Xq) further, we performed FISH with cosmids specific for distal Xp (STS and KAL); each of

Figure 6 Diagram illustrating the possible mechanism of formation of both rearranged chromosomes in the maternal meiosis. Normal X chromosome (A), breakage at Xq21.1 (B), and U type reunion of sister chromatids generate an idic(X)(q21.1) (C). At the next anaphase, this unstable isodicentric is broken at several random breakpoints (bp) such as bp1: Xq21.1, bp2: Xq11-q12, and bp3: Xp21 (D). They give rise to the rearranged del(Xq) and r(X) chromosomes and the acentric fragment containing XIST is lost (E).

them was cohybridised with an X alphoid DNA probe (Oncor) on metaphases of the proband and gave the following results. There were two FISH signals on the normal X, one at the centromere corresponding to the X alphoid DNA and one at the Xp telomere, as expected, indicating the specific cosmid locus. However, the del(Xq) showed three fluorescent signals, one at the centromere and one at each telomeric end, indicating the presence of two Xp telomeres on this chromosome (fig 3D). These results allowed a more precise description of the del(Xq) as der(X)(pter→q21.1::p21→pter).

Finally, both polymorphic markers and FISH data showed that the del(Xq) and the r(X) chromosome came from the same maternal chromosome, and we propose a mechanism of formation involving a maternal isodicentric idic(X)(q21.1).

Discussion

We report a female newborn with multiple malformations and a complex chromosomal rearrangement: 46,X,del(Xq)/47,X,del(Xq),+r(X). Cytogenetic and molecular studies showed that the del(Xq) is the result of both deletion and duplication of various parts of the X chromosome, but that it is non-randomly inactivated in all metaphases. In contrast, the r(X) chromosome, which contains loci (but not XIST) from the X juxtacentromeric region, has an active status. We have shown that the normal X originates from the father and both abnormal chromosomes from the same maternal X. We propose the following reconstruction of the events that led to the formation of these rearranged X chromosomes (fig 6).

Since the del(Xq) chromosome is found in all cells of the patient, a postzygotic origin of

this chromosome can be excluded. It therefore follows that the rearrangement occurred in the germline of the patient's mother. We postulate that the initial event was a breakage at Xq21.1 in a maternal X chromosome (fig 6B), followed by a U type reunion of sister chromatids. This mechanism generates an idic(X)(q21.1) chromosome (fig 6C). Such an isodicentric is unstable as it contains two functional daughter centromeres which will, in the next anaphase, go to opposite poles, forming a bridge. Bridges can break at several random points and here we propose that the breakpoints (bp) were bp1 at Xq21.1, bp2 at Xq11-12, and bp3 at Xp21 (fig 6D), giving rise to the rearranged del(Xq) and r(X). The acentric fragment generated, which contained one of the two XIST loci present on the idic(X), would be lost. The del(Xq) can therefore be described as follows: der(X)(pter→q21.1::p21→pter). Such a mechanism of stabilisation of a dicentric by excision of a centromere has been hypothesised by Therman *et al*³² and by Wolff *et al*.³³ Therefore, our report underlines the need to search for a low percentage of cells with a tiny supernumerary active r(X) chromosome in patients with an apparent i(Xq), del(Xq), or del(Xp), which have inactivated status but are nevertheless associated with a severe phenotype.

From a cytogenetic point of view, this study shows that the conjunction of a very short (three hour) terminal BrdU pulse and immunohistochemical staining with fluorescent anti-BrdU antibody is a highly sensitive and easy method for studying the inactivation status of abnormal X chromosomes. Such a study is often difficult because of the small size of r(X) chromosomes and the fact that the presence of

the XIST locus may not always be correlated with XIST expression.¹¹

From a clinical point of view, the abnormal phenotype of our patient results from the presence of three aneuploid X segments and from their inactivation status. (1) There is a Xq21.1→qter deletion in the non-randomly inactivated del(Xq). Similar published observations have not been associated with a severe phenotype.³⁴⁻³⁵ (2) The karyotype of our patient also has an Xp21→pter duplication in the inactivated del(Xq). Published data show that females with 46,X,dup(X)(p21→pter) and non-random inactivation of the abnormal X are phenotypically normal.³⁶⁻³⁷ (3) Finally, there is an Xp11.3→q11-q12 duplication (including the UBE1 and AR loci) on the r(X) chromosome which has been shown to remain active. Much evidence now exists that the presence of functional disomy of sequences on the X, albeit in only a proportion of cells, can confer a severe phenotype in females. 45,X/46,X,r(X) patients with active r(X) show increased frequency of mental retardation/developmental delay. Such mentally retarded ring(X) patients also have facial dysmorphism and cutaneous syndactyly as described in our patient.^{1-2,4-11,38} Moreover, our patient has agenesis of the corpus callosum and we found this malformation in two observations with active r(X) chromosomes, that of El Abd *et al*³⁹ with a 45,X/46,X,r(X) karyotype and that of Migeon *et al*⁴⁰ with a 45,X/46,X,del(Xq)/46,X,r(X) karyotype.

We conclude that the severe phenotype of our patient, who had the complex karyotype 46,X,der(X)(pter→q21.1::p21→pter)/47,X,der(X),+r(X), essentially results from the presence, in 20% of metaphases, of a tiny active r(X) chromosome. This r(X) chromosome has been shown to contain the UBE1 and ZXDA loci from the X short arm and the MSN locus from the X long arm. This observation underlines the need to perform precise characterisation of other small r(X) chromosomes, in order to establish phenotype-genotype correlations as a first step towards the identification of the genes responsible for this severe phenotype.

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