Uniparental and functional X disomy in Turner syndrome patients with unexplained mental retardation and X derived marker chromosomes

Tohru Yorifuji, Junko Muroi, Masahiko Kawai, Ayumi Uematsu, Hiroshi Sasaki, Toru Momoi, Masayuki Kaji, Chutaro Yamanaka, Kenshi Furusho

Abstract
We analysed parental origin and X inactivation status of X derived marker (mar(X)) or ring X (r(X)) chromosomes in six Turner syndrome patients. Two of these patients had mental retardation of unknown cause in addition to the usual Turner syndrome phenotype. By FISH analysis, the mar(X)/r(X) chromosomes of all patients retained the X centromere and the XIST locus at Xq13.2. By polymorphic marker analysis, both patients with mental retardation were shown to have uniparental X disomy while the others had both a maternal and paternal contribution of X chromosomes. By RT-PCR analysis and the androgen receptor assay, it was shown that in one of these mentally retarded patients, the XIST on the mar(X) was not transcribed and consequently the mar(X) was not inactivated, leading to functional disomy X. In the other patient, the XIST was transcribed but the r(X) appeared to be active by the androgen receptor assay. Our results suggest that uniparental disomy X may not be uncommon in mentally retarded patients with Turner syndrome. Functional disomy X seems to be the cause of mental retardation in these patients, although the underlying molecular basis could be diverse. In addition, even without unusual dysmorphic features, Turner syndrome patients with unexplained mental retardation need to be investigated for possible mosaicism including these mar(X)/r(X) chromosomes.

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Keywords: Turner syndrome; uniparental X disomy; X inactivation

Turner syndrome is one of the most common chromosomal anomaly syndromes characterised by a variety of symptoms including short stature, gonadal dysfunction, cardiac abnormalities, renal anomalies, and dysmorphic features such as neck webbing, cubitus valgus, high arched palate, and widely spaced nipples. It affects 1 in 2500-10 000 live born females and nearly half of the patients have 45,X karyotypes, while the others have structurally abnormal X chromosomes or have mosaic karyotypes. Except for short stature, which is an almost constant feature, the clinical presentation of Turner syndrome is highly variable. However, mental retardation is usually not included as a feature of this syndrome.

It has been reported that Turner syndrome patients with 45,X/46,X,r(X) karyotypes may have unusually severe phenotypes, which include mental retardation, soft tissue syndactyly, or abnormal facies. In many of these patients, it has been shown that the ring X chromosomes lack the X inactivation centre (XIC) at Xq13.2. This leads to functional disomy of the proximal part of the X chromosome, which may have contributed to the severe phenotypes observed in these patients. This "loss of XIC functional disomy theory" was challenged by the report of Migeon et al., which showed that some of the r(X) chromosomes of patients with a severe phenotype retained the XIST locus, which is a strong candidate for XIC itself. However, even in patients whose r(X) retained XIST, no or very weak expression of these XIST genes were observed, thus the "functional disomy theory" still seems valid. Migeon et al. also reported a mentally retarded, dysmorphic patient with a 45,X/46,X,del(X)(q21.3-qter)/46,X,r(X) karyotype whose del(X) retained the XIST locus but did not express XIST, which further supported the "functional disomy theory".

In this patient it was shown that the normal X, del(X), and r(X) were all derived from the maternal X chromosome. Although the causal relationship between uniparental disomy X and functional disomy X remains speculative, the proposed mechanism is that the del(X) and r(X) originated after the X counting process at the morula stage, thereby escaping the X inactivation process despite the presence of two X chromosomes in a cell.

There is, however, some evidence against the "functional disomy theory". For example, Dennis et al. reported three patients with severe phenotypes and in two of them the r(X) chromosomes were late replicating while in one of them, the XIST gene was transcribed. They also pointed out that patients with 45,X/46,X,r(Y) karyotypes had similarly severe phenotypes and concluded that the mechanism leading to a severe phenotype could be a non-specific effect of chromosomal mosaicism rather than functional disomy. In addition, in the original report of Kushnick et al., only one third of the r(X) chromosomes were early replicating while the others were late replicating. Thus the mechanism leading to mental retardation and multiple anomalies has still not been completely uncovered.
### Table 1 Summary of the results of karyotyping, FISH analysis for the XIST locus, RT-PCR analysis for XIST transcription, and polymorphic marker analysis of the patients. Alleles are arbitrarily named according to their sizes, and mar(X)/r(X) alleles are shown in bold. Since the telomeric ends of the mar(X)/r(X) chromosomes were not always shown by the Giesma banding, the second allele of the chromosome is shown in parentheses when the polymorphic marker could be absent from the mar(X)/r(X) of the patients

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### In this study, we performed molecular and cytogenetic studies on six Turner syndrome patients with X derived marker or r(X) chromosomes. Two of the patients had unexplained mental retardation and both had uniparental isodisomy of the X chromosome. The X inactivation status of these patients was also analysed.

### Materials and methods

#### SUBJECTS

The study subjects consisted of six Japanese Turner syndrome patients with at least one cell line with an X derived marker (mar(X)) or ring X chromosome (r(X)). Two of these patients (patients 1 and 2) had unexplained mental retardation. Another four patients (patients 3-6) were Turner syndrome patients without mental retardation. The patients regularly visited the growth clinic of one of our hospitals. All the patients were recruited after giving informed consent. The study protocol was approved by the ethical review board of the Department of Paediatrics, Kyoto University Hospital. The chromosomal origins of these small chromosomes were previously identified by fluorescent in situ hybridisation (FISH) analysis using an X centromere specific probe (DXZ-1) (data not shown).

#### KARYOTYPE DETERMINATIONS AND FISH ANALYSIS

Karyotypes on peripheral blood leucocytes were determined by conventional or high resolution trypsin-Giesma staining. The FISH analysis using X centromere (DXZ-1) and XIST specific probes was performed as recommended by the supplier of these probes (Oncor Inc, MD). Part of the karyotyping and the FISH analyses were performed at the SRL Laboratories Inc (Japan).

#### DNA ISOLATION

Genomic DNA was isolated essentially as described by Miller et al.15

#### POLYMORPHIC MARKER ANALYSIS

The types of polymorphic markers and their chromosomal locations are listed in Table 1. The sequences of the primers for DXS227, DXS441, DXS453, DXS559, DXS1003, DXS1162, and HPRT1 were obtained through the GDB Genome Data Base at http://gdbwww.gdb.org. The sequences of the primers for ALAS2, AR, and PGK are the same as those described by Cox et al., Allen et al., and Gilliland et al., respectively. One of each primer pair was end labelled with 32P; then PCR was performed in 25 μl reaction mixtures containing 50 ng of genomic DNA, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 25 mmol/l MgCl2, 0.1% Triton X-100, 200 μmol/l of each dNTP, 0.5 units of Tag DNA polymerase (Toyobo, Japan), and 25 pmol of each primer. The cycling parameters for each locus were the same as those listed in the above references.

For the analysis of di-, tri- and tetranucleotide repeat polymorphisms, 3 μl of the PCR products were electrophoresed through 6% denaturing polyacrylamide gels. Then the gels were exposed to Fuji x ray films (NewRX). For the PCR-RFLP analysis of the PGK gene, PCR was performed without 32P primer labelling. Three microlitres of the PCR products were digested with BstXI (New England Biolab, MA), then electrophoresed through a 10% polyacrylamide gel. The gel was stained with ethidium bromide and observed under a UV light.

#### RT-PCR

Mononuclear cells were isolated from heparinised peripheral blood by Ficoll/Paque gradient centrifugation, then total RNA was isolated by the method described by Chomczynski et al. The total RNA (2 μg) was reverse transcribed in 50 μl reactions containing 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2, 10 mmol/l DTT, 500 μmol/l each dNTPs, 50 μg/ml oligo(dT)12-18, and 50 units M-MLV reverse transcriptase (Gibco BRL, MD) at 37°C for one hour. Four microlitre aliquots of the reverse transcription mixtures were used to amplify segments of the transcripts from the XIST gene and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene. The reaction buffers were the same as those for the polymorphic marker.
Table 1 continued

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Analysis. For the amplification of the XIST transcripts, a pair of primers, one in exon 1 (5'-TGCCCTAAGCTCTTCGGA-3'), the other in exon 2 (5'-ACACATGCAGCGTG GTATCT-3'), were used to amplify a 188 bp segment. Cycling parameters were: denaturation at 94°C for one minute, annealing, and extension at 60°C for one minute. Similarly, a 296 bp segment of the HPRT transcript was amplified using a pair of primers, one in exon 1 (5'-TGAACTCGTCTGTGAGTG-3'), the other in exon 6 (5'-TCTGAGGTTGTGTTGCCAGTG-3'). Cycling parameters were: denaturation at 94°C for one minute, annealing, and extension at 58°C for 20 seconds. The PCR products were electrophoresed through a 10% polyacrylamide gel, stained with ethidium bromide, and observed under UV light. The amount of transcript was semiquantitated by densitometric analysis of the RT-PCR products while the amplifications were in the logarithmic phase.

**ANDROGEN RECEPTOR ASSAY**

Human androgen receptor assay was performed basically as described previously. Briefly, genomic DNA was digested either with Rsal alone or with Rsal plus HpaII. Then, 100 ng of the digested DNA was used as the template for amplification. The sequences of the primers were 5'-CTCGAGAATCT GTCCACGGCTGTC-3' (HUMARA-1) and 5'-GCTGTGAAAGTTGTGTTCCCC TCTA-3' (HUMARA-2). One of the primer pair (HUMARA-1) was labelled with 32P, then PCR was performed in the same buffer as for polymorphic marker analysis. The cycling parameters were as follows: initial denaturation at 94°C for five minutes then 30 cycles of denaturation at 94°C for one minute, annealing, and extension at 60°C for 1 minute. Three microlitre aliquots of the PCR products were electrophoresed on a 6% denaturing polyacrylamide gel and autoradiographed on Fuji x ray films. In order to show the presence of DNA in the HpaII/Rsal digests, a 204 bp genomic segment (exon 5 of the TBG gene) at Xq21-q22 was amplified using a pair of primers, 5'-GCTGCCATAGGCTG-3' and 5'-CTAC GCTTCGATGTGTTCCC-3'. The cycling parameters were the same as those for the androgen receptor assay. The PCR products were electrophoresed through a 10% polyacrylamide gel, stained with ethidium bromide, and observed under a UV light.

**Results**

**Patient Profiles**

Patient 1 was born after an uneventful pregnancy and delivery with a birth weight of 3210 g. Shortly after birth, muscular hypotonia and feeding difficulty were noted. Muscle biopsy on day 10 showed normal histology. Her hypotonia improved gradually although her developmental milestones were delayed with head control at 4 months, sitting at 10 months, and walking alone at 1 year 10 months. Her developmental delay persisted with DQs around 60-65. At the age of 3, she came to our clinic because of short stature. At this time, chromosome analysis of peripheral blood leucocytes showed a 45, X/46, X, + mar (50:50) karyotype. Growth hormone treatment was started at the age of 6. Currently she is 16 years of age and still on growth hormone therapy. Her height is 145 cm (mean -2.45 SD) and she shows no signs of puberty. She has Turner syndrome characteristics such as increased number of naevi, widely spaced nipples, and chronic otitis media. Except for mental retardation, she does not have any other features unusual for Turner syndrome. Karyotype analysis was repeated at the age of 16 and found to be 45, X/46, X, + mar (X) (89:11).

Patient 2 was born at 38 weeks' gestation with a birth weight of 2420 g. The pregnancy and delivery were uneventful. At 1 year 3 months, she had white spots over her left thigh and left abdomen which disappeared gradually over several years. Her mental development...
was moderately delayed. At the age of 15 years 11 months, she came to our clinic because of short stature (141.9 cm, mean –3.64 SD), obesity, and mental retardation. Apart from short stature, she had cubitus valgus, a low hair line, chronic otitis media, and impaired glucose tolerance. She had breast development at Tanner stage 3 and she did not have other dysmorphic features unusual for Turner syndrome. Chromosome analysis on peripheral blood leukocytes showed a 45,X/46,X,r(X) (88:12) karyotype. Although she did not have formal IQ testing, her mental delay was in the educable range.

Patients 3 to 6 were control patients without mental retardation. Karyotyping was performed at ages 18 (patient 3), 4 (patient 4), 11 (patient 5), and 14 (patient 6). These patients had features of Turner syndrome, although, generally, the degree of dysmorphism was milder than typical Turner syndrome patients.

**KARYOTYPE AND FISH ANALYSIS**

Peripheral blood karyotypes of the patients and the results of FISH analysis using an XIST specific probe are summarised in table 1. Where available, estimated breakpoints of the mar(X)/r(X) chromosomes are also shown. The mar(X)/r(X) chromosomes of all patients retained the XIST locus on FISH analysis (data not shown).

**PARENTAL ORIGIN OF MARKER CHROMOSOMES**

Since marker chromosomes of all patients retained the X chromosome and the XIST locus at Xq13.2, polymorphic markers within this interval were used to determine the parental origins of the mar(X)/r(X) chromosomes. In addition, several other polymorphic markers outside the region were also examined. In order to examine if the assays could detect 1 to 10 ratio mosaicism, mixtures of two different male DNAs at 1 to 20 ratios were used as sensitivity controls.

As shown in table 1, mar(X)/r(X) chromosomes were maternal in patients 1, 5, and 6 and paternal in patients 2, 3, and 4. Interestingly, patient 1 had only a maternal contribution of X chromosomes, while patient 2 had only a paternal contribution, showing that patient 1 had maternal isodisomy X and patient 2 had paternal isodisomy X. Paternity of patient 1 was confirmed by using three other highly polymorphic markers on autosomes (D7S440, D15S11, and APOB, data not shown). All other patients had both a maternal and paternal contribution of X chromosomes. Sensitivity test PCR showed that the allele of a male DNA could be detected in the presence of a 20-fold larger amount of a second male DNA except when the minor allele was two base pairs longer in dinucleotide repeat polymorphisms, in which case the second fainter band was often obscured in the ghost band of the major band (data not shown).

**XIST EXPRESSION**

The expression of the XIST gene was analysed by RT-PCR analysis of peripheral blood leukocytes. RT-PCR for the HPRT transcripts was used as an internal control. The results are summarised in table 1 and fig 1 shows the representative results. Although HPRT expression was similar in all subjects, XIST expression was not observed in patient 1 indicating that the mar(X) of patient 1 is not expressing XIST. As expected, XIST expression was not observed in a male control. For other patients, when the RT-PCR products for XIST were semiquantitated by the densitometric analysis, the intensity of the bands correlated well with the expected percentage of XIST genes on mar(X)/r(X) chromosomes. Interestingly, the intensity of bands from patients 2 and 3 was similar. Since both of these patients had a similar ratio of mosaicism (table 1), these results suggest that the expression of the XIST gene on the r(X) of patient 2 is comparable to that of patient 3.

**X INACTIVATION STATUS OF THE MARKER/RING CHROMOSOMES**

We then analysed the X inactivation status of the mar(X)/r(X) chromosomes. Since replication is sometimes hard to interpret for a very small marker chromosome, we used the androgen receptor assay originally described by Allen et al.11 This assay is based on the fact that the methylation status around the polymorphic CAG repeat in the androgen receptor gene (Xq12) correlates well with the inactivation status of the chromosome. When the X chromosome is inactive, the HpaII sites adjacent to the CAG repeat are methylated. Therefore, if the genomic DNA is digested with the methylation sensitive HpaII before amplification of a DNA segment spanning the HpaII sites and the CAG repeat, only the allele from the inactive X chromosomes is amplified. By this assay, when undigested male DNA was mixed with a 40-fold excess of HpaII digested male DNA, the allele of the undigested DNA could be easily amplified, showing that the method could detect very low level mosaicism (data not shown).

As shown in fig 2A, the mar(X) of patient 1 was not inactivated as expected from the results of RT-PCR analysis. Interestingly, when the DNA of patient 2 was digested with HpaII, amplification was very poor (fig 2B). This suggests that the r(X) in patient 2 was almost entirely active despite the fact that the XIST gene on r(X) appeared to be expressed by the RT-PCR analysis. For both of these patients, an unrelated genomic segment (exon 5 of the TBG gene) could be easily amplified from HpaII/RsaI digested DNA showing the presence of DNA in the digested samples (data not shown). All other patients with normal intelligence showed obvious bands derived from inactive mar(X)/r(X) chromosomes (fig 2C, D).

**Discussion**

In our series of six patients with mar(X)/r(X) chromosomes, two had unexplained mental retardation and both of these had uniparental isodisomy X, while none of the patients without mental retardation had uniparental disomy X. Although the number of patients

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**Figure 2.** Androgen receptor assay of the X inactivation status. Results on patients 1 (A), 2 (B), 3 (C), and 4 (D) are shown. M and P are maternal and paternal DNA, respectively, which are amplified without HpaII digestion. Each patient’s DNA was amplified with (+) or without (−) previous HpaII digestion. With HpaII digestion, an inactivated allele is preferentially amplified, while in the absence of digestion, both alleles are equally amplified.
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was small, these results suggest that uniparental origin of these small X chromosomes is not a rare phenomenon. Our cases are similar to the case described by Migeon et al. with a 45,X/46,X,del(X)(q21.3-pter)/46,X,r(X) karyotype in whom del(X) and r(X) were derived from maternal uniparental disomy. However, unlike their case, our patients presented only with moderate mental retardation in addition to the usual Turner syndrome phenotype and did not have unusual dysmorphic features. The degree of mental retardation also appeared less severe in our patients. The reason for the difference in clinical presentation is unclear although the difference in the length of X chromosomes which are functionally disomic or the difference in the ratio of mosomies in various organs could be possible explanations. Although it is not clear at present whether uniparental disomy X is specific for mentally retarded patients, our results showed that this type of chromosomal abnormality should be taken into account even in Turner syndrome patients with only mild mental retardation. In this regard, it is interesting that in patient 1 aged 3, the mar(X) constituted 50% in the peripheral blood leucocytes, whereas at the age of 16 the percentage fell to 11%. This suggests that at an older age the chromosomal mosaicism could be undetectable by the usual karyotyping of peripheral blood. Turner syndrome patients with some degree of mental retardation probably require karyotyping of a large number of peripheral blood leucocytes to detect mar(X)/r(X) chromosomes and, when detectable, parental origin and X inactivation status of these chromosomes need to be analysed.

As a mechanism leading to a severe phenotype in patients with mar(X)/r(X), "loss of XIC functional disomy theory" has been accepted since the initial description of the patients. However, our results indicate that the situation is far more complicated.

The mar(X) in patient 1 retained the XIST locus, but did not express XIST transcripts as shown by the RT-PCR experiment. It was also shown that this patient had maternal uniparental isodisomy X. The mechanism leading to shutdown of the expression of XIST on the mar(X) remains unclear. One possible mechanism was proposed by Migeon et al., that is, the mar(X) originated after the X chromosome counting at the morula stage. Another possibility is that XIST expression requires additional factors, which could be an unknown sequence in the XIC region or factors supplied by other chromosomes. Given the difference between patients 1 and 2, these additional factors could be paternal in origin. It is well known that the paternal X chromosome is preferentially inactivated in marsupials and in extraembryonic tissues of mice. Similar phenomena have been reported in humans. In addition, a recent report by Skuse et al. showed that, in humans, maternal and paternal X chromosomes are imprinted differently.

The transcription of XIST is known to be necessary for the XCI inactivation process to start normally. However, whether the expression of XIST is sufficient for the initiation of the X inactivation is still unknown. In our patient 2, the clinical phenotype was similar to that of patient 1, although the patient had paternal isodisomy. Like patient 1, her r(X) retained the XIST locus, but, unlike patient 1, this XIST appeared to be transcribed at a similar level to the normally inactivated X chromosomes. However, by the androgen receptor assay, the methylation status of the r(X) is similar to that of the active X chromosome. The reason for the discrepancy between the expression of the XIST gene and the X inactivation status of r(X) is currently unknown. It could be that the expression of XIST is necessary for an X chromosome to be inactivated, but not sufficient. There could be other factors which cooperate with XIST to cis inactivate the X chromosome. Another possibility is that there are mixed populations of r(X) in patient 2 although they all appeared the same on cytogenetic examination. It is possible that a larger population of r(X) is not expressing XIST and is not inactivated while other populations of r(X) are expressing XIST and are inactivated. Detailed examination of each of the individual r(X) might answer this question.

Overall, our results support the idea that functional X disomy is a cause of mental retardation in Turner syndrome patients. However, the mechanism leading to functional disomy is not as simple as the loss of the XIST locus. It appears that there are multiple pathways leading to functional X disomy. Furthermore, how it affects the intellectual development remains completely unknown. Detailed structural and functional examination of mar(X)/r(X) chromosomes on a number of patients for karyotype-phenotype correlation might answer some of these questions.

We thank our patients and their families who kindly participated in this research.


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