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ARTICLE

Lack of expression of *XIST* from a small ring X chromosome containing the *XIST* locus in a girl with short stature, facial dysmorphism and developmental delay

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A 46,X,r(X) karyotype was found in a three and a half year old girl with short stature, facial dysmorphism and developmental delay. The clinical findings were consistent with the phenotype described in a limited number of patients with small ring X chromosomes lacking the *XIST* locus, a critical player in the process of X chromosome inactivation. Surprisingly, in our patient, fluorescent *in situ* hybridisation demonstrated that the *XIST* locus was present on the ring X. However, expression studies showed that there was no *XIST* transcript in peripheral blood cells, suggesting that the ring X had not been inactivated. This was confirmed by the demonstration that both of the patient's alleles for the androgen receptor gene were unmethylated, and that both of the patient's *ZXDA* alleles were expressed. The active nature of the ring X would presumably result in overexpression of genes that may account for the developmental delay observed for the patient. Using polymorphic markers along the X chromosome, the ring X was determined to be of paternal origin with one breakpoint in the long arm between *DXS8037* and *XIST* and one in the short arm in Xp11.2 between *DXS1126* and *DXS991*. To attempt to determine why the *XIST* gene failed to be expressed, the promoter region was sequenced and found to have a base change at the same location as a variant previously associated with nonrandom X chromosome inactivation. This mutation was not seen in over one hundred normal X chromosomes examined; however, it was observed in the paternal grandmother who did not show substantial skewing of X chromosome inactivation.
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Introduction

Ring X chromosomes have been recognised in girls with Turner syndrome, often with mosaicism for a 45,X cell line. In some instances, they are associated with mental retardation and a distinct phenotype of short stature, facial dysmorphism characterised by long palpebral fissures, a relatively broad nasal root and tip, anteverted nares, a wide mouth with a thin upper lip, soft tissue syndactyly, and mental handicap. Careful cytogenetic characterisation of the ring X chromosomes has suggested that the smaller the size of the ring X, the more likely are the findings of mental handicap and dysmorphic features in the patient. This has been attributed to failure of dosage compensation, by X chromosome inactivation, for the genes on the ring chromosome.[1–3]

A region of the proximal long arm of the X chromosome is required in *cis* for inactivation of the chromosome,[4,5] and

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contains the *XIST* gene, which is expressed exclusively from the inactive X chromosome[6] and is necessary for X inactivation in mice (reviewed in Avner and Heard[7]). Smaller ring chromosomes may lack the *XIST* locus, rendering them functionally disomic for the genes present on the ring.[8,9] The phenotype of individuals with small ring X chromosomes presumably results from the continued expression of genes in the pericentromeric region of the X chromosome due to failure of inactivation.[10]

In females, the presence of mosaicism for a ring X lacking *XIST* has been correlated with mental retardation, facial dysmorphism often described as similar to the facies of Nikkawa syndrome, limb anomalies and abnormal pigmentation.[8,11–17] Even more severe phenotypes have been reported, including prune-belly syndrome in a stillborn fetus with a ring X lacking *XIST*[18] and anencephaly and diaphragmatic hernia in a female fetus with a ring X that was not characterised for *XIST* expression.[19] In addition, there have been reports of males with a supernumary ring X chromosome, also lacking *XIST*, in some cases with a somewhat similar phenotype, including learning disabilities or mental handicap of varying severity, facial dysmorphism and digital anomalies.[8,20–22]

There are rare cases where a small ring containing the *XIST* gene has been observed associated with the ring X phenotype. Many of these patients lack expression of the inactive X-specific transcript, which presumably precludes inactivation of the chromosome.[9] We now report the characterisation of an additional case of a small ring X chromosome which includes the *XIST* locus but fails to express the gene. A mutation at a site previously associated with nonrandom X-chromosome inactivation was identified in the girl; however, the same change was found in her paternal grandmother who did not show substantial skewing of X-chromosome inactivation.

Patient and Methods

Patient

This girl presented with dysmorphic features, short stature and developmental delay. She was the first child of healthy nonconsanguineous, young, white parents. She was born at 37 weeks of gestation following an uncomplicated pregnancy. Birth weight was 2895 g (>25th percentile). Her neonatal course was complicated by hypoglycemia with no recognised specific cause. This resolved spontaneously. Milestones were somewhat delayed. She walked at 16 months, had a pincer grasp at 2 years, and used two to three word phrases by 3 years. When assessed using the Bailey Scales of Infant Development at 3 years, 7 months, level of function approximated that of a child of 2 years, 3 months. At 5.5 years, language skills were significantly delayed. She did not consistently recognise colors or letters of the alphabet. Social skills were immature. She was not able to pedal a tricycle.

When examined at 3.5 years, height of 89 cm and weight of 12.8 kg were just above the 3rd percentile. OFC of 50.5 cm was between the 50th and 75th percentiles. Inner canthal distance of 3 cm was proportionate to the OFC. Total hand and middle finger lengths were below the 3rd percentile, consistent with the clinical impression of brachydactyly. Facial appearance was distinctly dysmorphic (Figure 1). It was slightly asymmetric with left eye position lower than the right, and there was mild malar flattening. Ears were normal. Alveolar ridges were moderately thickened, although teeth were normal. Philtrum was poorly defined. Lower jaw was normal, although in infancy it had been described as being small. Hair pattern and neck shape were normal, as was chest shape. There was a clinical impression of short limbs but she did not cooperate with measuring this. Mild soft tissue syndactyly of right middle and index finger was seen, and there was significant soft cutaneous syndactyly of left 4th and 5th toes. One large diffuse café au lait spot was seen on the right buttock, and a small one was present on the right knee. Otherwise, the examination was unremarkable.

Investigations showed a normal brain CT scan. Brachydactyly was seen on a radiograph of the upper limb at 9 months of age, but no other bony anomalies were noted. Renal ultrasound was normal at age 4 years of age, but a hypoplastic uterus was described. Echocardiogram was normal. TSH was normal. Cytogenetic testing was done at 3.5 and 4.5 years of age.

Cytogenetic methods

Peripheral blood specimens were set up for chromosomal analysis using routine methods, and metaphases were examined with GTG-banding.[23] Fluorescent *in situ* hybridisation (FISH) was carried out according to supplier's protocol (Oncor, Gaithersburg, MD, USA), using probes for centromeric alphoid DNA sequences from X (*DXZ1*) or Y (*DYZ3*) chromosomes, and for the *XIST* locus. Fixed cells were saved for preparation of RNA to evaluate expression of the *XIST* gene.

Molecular methods

DNA was prepared by salt/SDS precipitation,[24] while RNA was prepared by acid-guanidinium extraction.[25] DNA and RNA was isolated from cells stored in fixative after cytogenetic analysis, blood, or cultured control cell lines, GM07348 (female), GM07033 (male), GM06563 (female with large ring X) and GM07059 (female with non-random X inactivation), obtained from the Coriell Cell Repository. cDNA was synthesised as previously described.[6] PCR amplification of DNA and cDNA used primers and conditions listed in Table 1 with 1 μM primer, 20 μM dNTPs and 10 U Taq polymerase (Gibco-BRL) unless otherwise noted. Methylation of the androgen receptor (*AR*) locus was analysed as has been previously described.[26] For microsatellite analysis, PCR products were resolved on polyacrylamide gels and observed after staining with silver, while other PCR products were

resolved on 2% agarose gels stained with ethidium bromide. The promoter region of *XIST* was amplified from the patient using XIST primers, U2 and H3, and purified product was sequenced using U2 and PM primers by the NAPS facility, UBC. A primer was designed with a mismatch (PM2, Table 1) to generate a *Ban*I restriction enzyme site in the presence of the −43A variant base. After amplification with primers PM and PM2, PCR products were digested with 15 U of *Ban*I, and resolved on polyacrylamide gels. In the presence of the −43A, 16 bp were removed from the end of the 145 bp fragment. To examine methylation in this region, 1 μg of DNA was digested with 15 U of *Hha*I in a 20 μl reaction. The reaction was then heated to 95°C for 5 min and then 1 μl was amplified with primers PM and PM2. The PCR product was digested with *Ban*I to distinguish between the two alleles.

Figure 1 Facial appearance of the patient at 3.5 years.

Results

Cytogenetic analysis

GTG-banding analysis of the initial peripheral blood specimen showed mosaicism for two cell lines: 28% of the cells had 45,X; 72% had 46 chromosomes including one normal X chromosome and a small ring with bands consistent with an X chromosome origin (Figure 2A). A second blood sample 13 months later had the ring X chromosome in all metaphases examined. FISH with *DXZ1* and *DYZ3* showed that the marker was of X chromosome origin (not shown). FISH with a DNA probe for the *XIST* locus demonstrated fluorescent signals on both the normal X and the ring X chromosomes (Figure 2B). The karyotype was designated 46,X,r(X)(p11.3q13).ish r(X)(XIST+).

Activity of the X chromosomes

As shown in Figure 3A, cDNA from the patient was amplified with primers for *PGK1*, but no product was detected with primers for *XIST*. This result was similar to the amplification observed in males who do not have an inactive X chromosome, while cDNA from female cells with a normal karyotype or containing a large ring X chromosome (GM06563) showed amplification for *XIST* as well as the *PGK1* control gene.

Table 1 Primers used in these analyses

Marker/Gene	*Primer sequence*	*PCR conditions*
DXS1126	F: TTCTAGAAAGGTGCGTGTCGTCTGG R: GACCATTCCCTCTCAAACACAAACG	(94^0-1′; 58^0-1′; 72^0-2′) × 30
DXS991	A: CTTCAACCACAGAAGCCTC B: ATCATTTGAGCCAATTCTCC	(95^0-1′; 56^0-31″; 72^0-1′) × 30
AR	A: TCCAGAATCTGTTCCAGAGCGTGC B: GCTGTGAAGGTTGCTGTTCCTCAT	(95^0-1′; 62^0-30″; 72^0-1′) × 30
DXS1162	1: GTGGGAGGGACAGGCT 2: GAGTGTAGATTCTGCTCAGGG	(95^0-1′; 58^0-1′; 72^0-1′) × 30
XIST	PM: ATAAAGGGTGTTGGGGGAC PM2: GCTCTCTCCGCCCTCGGC* U2: AACACATCAAAGCTCTACC H3: ATCAGCAGGTATCCGATACC	(95^0-1′; 58^0-1′; 72^0-1′) × 30
	C7B2rev: CTCCAGATAGCTGGCAACC C9-4: AGCTCCTCGGACAGCTGTAA	(94^0-1′; 54^0-1′; 72^0-2′) × 30
DXS8037	A: GAGGCAAGACATCCATTCC B: TGACTTTGAGCGAGCAGGT	(95^0-1′; 58^0-1′; 72^0-1′) × 30
FMR1	FRAXA-2: AGCCCCGCACTTCCACCA FRAXA-7: GCTCAGCTCCGTTTCGGTTTCACTTCCGGT	as in Carrel and Willard[27]
PGK	R1: TCGGCTCCCTCGTTGACCGA R2: AGCTGGGTTGGCACAGGCTT	(94^0-1′; 54^0-1′; 72^0-2′) × 30
ZXDA	F: TCAATTAAGGTGGGAGGCAG R: TGTGAGGTAATTATGGCAAAGT	(95^0-1′; 60^0-1′; 72^0-1′) × 30

*Underlined nucleotide is changed in the primer to create a restriction enzyme recognition site.

The patient was heterozygous for the polymorphic trinucleotide repeat in the androgen receptor (AR) gene,[26] as shown by the presence of two bands in the uncut DNA in Figure 3B. After digestion with the methylation-sensitive restriction enzyme, *Hha*I, DNA from active (unmethylated) X chromosomes should be digested, eliminating the template for amplification. Amplification of both alleles in the patient was eliminated, suggesting that both alleles were unmethylated and active. In a control female cell line (GM07059) with nonrandom X-chromosome inactivation, digestion with *Hha*I resulted in loss of only one allele, that from the active X chromosome (Figure 3B).

The *ZXDA* gene in Xp11 contains a polymorphic CA repeat in the transcribed but untranslated region of the gene. This gene is subject to X-chromosome inactivation, so expression of only the allele on the active X chromosome should be detected.[27] In the patient, expression of both alleles was observed (Figure 3C), although one allele was fainter in both DNA and cDNA, presumably due to mosaicism for the ring chromosome.

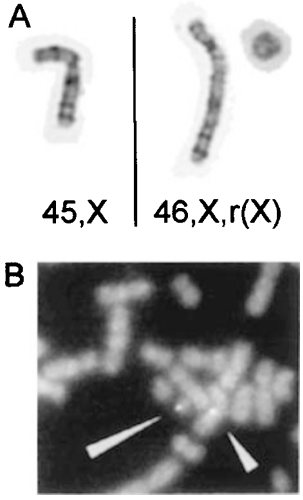

Figure 2 Cytogenetic analysis. (**A**) Partial karyotypes of GTG-banded chromosomes from the patient showing the single X chromosome found in one cell line on the left, and the X chromosome with the ring X chromosome on the right. (**B**) A metaphase following fluorescent *in situ* hybridisation with the *XIST* locus probe showing fluorescent signals on both the intact X chromosome (short arrow) and the ring X chromosome (long arrow).

Molecular characterisation of the ring X chromosome

DNA isolated from the mother, the father and the patient was amplified with primers for polymorphic X-linked markers. Informative markers are shown in Figure 4A. For all loci, the patient retained a maternal allele, whereas paternal alleles were retained only in the pericentromeric region, demonstrating that the ring X is paternal in origin and spans from a breakpoint between *DXS1126* and *DXS991* in Xp11 to a breakpoint between *DXS8037* and *XIST* in Xq13.

To examine possible causes for the failure of expression of the *XIST* locus on the ring X chromosome, the promoter region of *XIST* in the patient was sequenced. Only one basepair difference was observed in comparison to previously described *XIST* minimal promoter sequence.[28] As shown in Figure 4B, there was a small 'A' peak beneath the 'C' peak at basepair-43 of *XIST*. This change was confirmed by creating a primer with a mismatch (PM2, as shown in Figure 4B) that would generate a *Ban*I restriction site in the presence of the base change. Amplified product from the patient's DNA and the patient's father's DNA was digested with *Ban*I, suggesting that the variant *XIST* sequence was paternally inherited. The band corresponding to the variant sequence (digested product, Figure 4C) was fainter in the patient, consistent

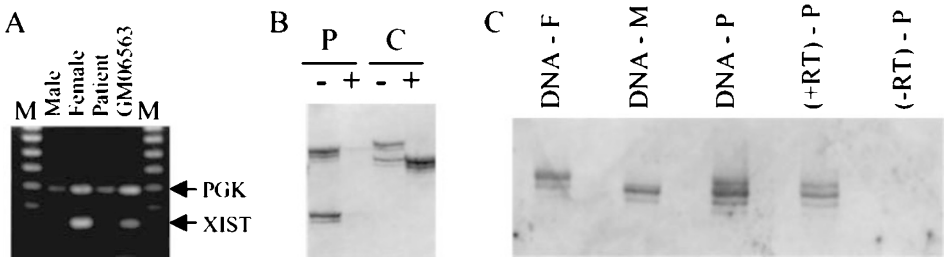

Figure 3 Activity of the r(X). (**A**) Expression of *XIST*. cDNA from the patient as well as from a male, female, or large ring X chromosome-containing cell line (GM06563) was amplified with primers for the *PGK1* (R1 : R2) and *XIST* (C9-4:C7B2rev) genes. The PGK1 product is larger and serves as a positive control for the presence of amplifiable cDNA. Both primer pairs flank exon/intron junctions and therefore amplify only cDNA, not DNA. Products were run on a 2% agarose gel with 100 bp ladder as a size standard (M). (**B**) Methylation of androgen receptor (*AR*). DNA from the patient (P) or a control female cell line (C; GM07059) with non-random X chromosome inactivation was amplified with primers for *AR* with (+) or without (−) predigestion with the methylation-sensitive restriction enzyme *Hha*I. (**C**) Expression of *ZXDA*. DNA from the patient (P), her mother (M) and father (F) was amplified with primers flanking a CA repeat in the *ZXDA* gene. The paternal allele in the patient's DNA is fainter, consistent with the mosaic ring X chromosome being paternally derived. cDNA from the patient showed a similar pattern of expression (+RT), while the control without reverse transcriptase (−RT) showed no amplification at all, demonstrating that the product was derived from amplification of cDNA, not contaminating DNA.

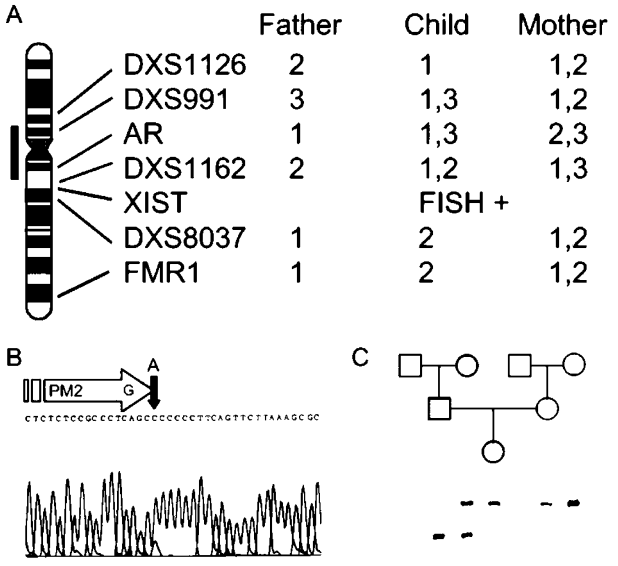

Figure 4 Molecular characterisation of the r(X) chromosome. (**A**) Summary of polymorphic markers examined in the patient and her parents. Alleles are arbitrarily designated with the smallest allele observed in the family as '1'. Only informative markers are shown. The heavy line to the left of the X chromosome schematic delineates the presumed extent of the ring (X) chromosome based on the loss of paternal alleles. (**B**) Sequencing of DNA amplified from the patient for the promoter of the *XIST* gene showed a base change as marked by the vertical arrow. A primer, PM2, was designed with a sequence change (G as shown in large arrow rather than A) that would generate a *Ban*I restriction enzyme site in conjunction with the observed variant. (**C**) Detection of the change in family members (shaded) after amplification with primer PM2 and PM followed by digestion with *Ban*I. The digested product (lower band) is 16 bp smaller than products that are unable to be digested.

with the variant sequence being on the paternally inherited ring chromosome that is not present in all of her cells. The patient's paternal grandmother was heterozygous for the variant (Figure 4C). Over 100 X chromosomes from male and female controls were examined for this variant using the *Ban*I assay, and no other individuals were identified with the same change.

To determine the effect of this sequence alteration we examined the X inactivation status of the chromosome carrying the variant. The primers that recognise the variant basepair flank two methylation-sensitive enzyme sites, which are known to be differentially methylated on the active and inactive X chromosome[29] (Figure 5A), making it possible to analyse methylation of the two *XIST* alleles individually. As shown in Figure 5B, predigestion with *Hha*I did not reduce the intensity of either the *Ban*I cut (lower=variant) or uncut (upper=normal) alleles, suggesting that both *XIST* promoter region alleles are methylated. DNA from the patient's paternal grandmother (carrier, Figure 5B) amplified both alleles after predigestion, demonstrating that both the variant and normal alleles are unmethylated in a proportion of the grandmother's cells.

To confirm the inactivation of each X chromosome in a subset of the grandmother's cells, we examined methylation at the *AR* locus. Both alleles were amplified after predigestion, with a slight alteration in intensity from the uncut sample (Figure 5C). Quantitation performed using the NIH IMAGE program with scanned gels for both *AR* and *XIST*, suggest that the grandmother has 60% of cells with the variant on the active X chromosome.

Discussion

Chromosomal analysis of the patient was initiated because of short stature and mental handicap. The initial chromosome

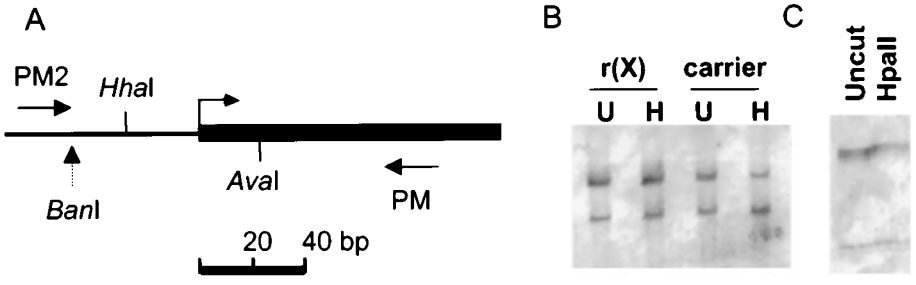

Figure 5 X chromosome inactivation in females with the *XIST* promoter polymorphism. (**A**) Diagram of the promoter region of XIST showing the location of the variant identified by *Ban*I, the transcription start site (bold line with arrow), and the location of two sites previously demonstrated to be differentially methylated on the active and inactive X chromosomes (*Hha*I and *Ava*I). (**B**) Methylation of the *XIST* promoter was determined by comparing amplification of DNA before (U), or after (H), digestion with the methylation-sensitive restriction enzyme *Hha*I. The allele with the variant base was identified by digestion of the PCR product with *Ban*I, which will cut the variant sequence (in conjunction with a base change introduced into primer PM2). The assay was performed with DNA from the patient with the ring(X) chromosome (r(X)) or her paternal grandmother (carrier). (**C**) Methylation near the polymorphic trinucleotide repeat in *AR* was examined by amplification of DNA from the paternal grandmother before (Uncut) or after (*Hpa*II) digestion with the methylation-sensitive restriction enzyme *Hpa*II.

result of 45,X/46,X,r(X)(p11.3q13) suggested the girl's phenotype might be explained by functional disomy of genes on the small ring X chromosome; however, FISH revealed that the *XIST* locus was present on the ring. Investigation of the expression of the *XIST* gene demonstrated no expression. The activity of the ring X chromosome was confirmed by the lack of methylation at the *AR* locus and by the expression of both *ZXDA* alleles. The direct examination of gene activity on the ring chromosome using the expressed polymorphism in *ZXDA* should be valuable to resolve conflicts that have occasionally arisen in determining ring X activity using AR methylation or replication timing.[30–32]

There appears to be a general phenotype associated with active small ring X chromosomes that includes short stature and mental handicap. The short stature, which is also found in Turner syndrome, may be due to the absence of genes regulating stature such as SHOX (short stature homeobox-containing gene) in the pseudoautosomal region of the short arm of the X chromosome.[33] The observed mental handicap specifically associated with the small active ring X chromosome is likely due to functional disomy for genes that are normally inactivated. Some of the considerable variation observed between individuals with ring X chromosomes might be related to the parental origin of the ring X, mosaicism or the size of the ring X chromosome.

Parental origin does not seem to correlate with outcome,[34] although uniparental disomy (UPD) has been associated with a more severe outcome.[30] This could reflect the duplication of an X chromosome after the time at which marking of an X chromosome to remain active occurs, thereby resulting in derivative chromosomes which are unable to inactivate.[35] In the case reported here, the normal X chromosome was from the mother and the ring chromosome was derived from the father, which is not uncommon for rearranged X chromosomes (rings or isochromosomes).[36] Thus, both chromosomes were present throughout early development and would be present at the time of inactivation.

Mosaicism is very large confounding factor in attempting phenotype-genotype correlations to define critical regions for different aspects of the 'syndrome'. Cytogenetic testing in our patient at different times showed different percentages of ring chromosome, demonstrating the difficulty in predicting levels of mosaicism from single samples of a single tissue. Rare cases where *XIST* expression has been detected with the more severe phenotype generally associated with an active ring chromosome[12,30,37] may reflect the presence of active ring (X) chromosome at a critical stage in development.

The extent of DNA missing from the patient's ring chromosome was determined by allelotyping DNA from the patient and her parents. The breakpoint in Xp11.2 was between the *DXS1126* and *DXS991*. Four of the five ring X chromosomes reported by Wolff *et al*[8] had breakpoints between *UBE1* and *DXS423E*, an approximately 10 Mb region which would overlap the region defined for our proband. Other rings retaining the *XIST* gene, but not expressing it, showed variable Xp breaks[9] so the location of the short arm breakpoint does not seem to be related to inability to express *XIST*. The breakpoint in the long arm in our case occurred between *XIST* and the nearby *DXS8037* locus. Both loci are found within a single YAC, suggesting that they might be less than 1 Mb apart.[38] Two of the previous *XIST*-containing ring X chromosomes failing to express *XIST* also had breakpoints within 1 Mb of *XIST*. The *XIST* gene is apparently intact in our patient, as a polymorphism was identified in the promoter that is telomeric to the 3′ end of the gene.[39] While it is possible that more distal elements are required for proper *XIST* expression, two other *XIST*-positive non-expressing chromosomes contained substantially more distal DNA, even more than other rings expressing *XIST*.[9]

A sequence change was detected in the promoter of the XIST gene in the patient at position −43 bp. Intriguingly, this is the same site associated with nonrandom X-chromosome inactivation.[40] The change in our patient was from C to A (C-43A) while the previously reported change was to a G (C-43G). This base is not evolutionarily conserved, as a G is present in the mouse sequence and an A in rabbits.[28] The C-43G variant was detected in two families, with skewing of inactivation ranging from more than 95% inactivation of the chromosome with the variant in several individuals to only 55% skewing in others. The C-43A variant failed to undergo inactivation in the patient reported here, and was also associated with the active X chromosome in the grandmother, although she showed only 60 : 40 skewing of inactivation. Furthermore, it has been reported that skewing increases with age.[41] Thus, the extent of skewing in this individual is not different from that seen in the general population.

It is, however, clear that these variant sequences are not common in the population. We have examined over 100 X chromosomes using the *Ban*I assay described, which will detect both the C-43A and C-43G variants, and have not detected any additional cases. The two families were the only instances of the C-43G change in 1166 independent chromosomes.[40] A recent analysis of 32 females with skewed inactivation also did not detect additional cases.[42] Thus, it is surprising that these variants were detected in individuals with peculiarities in their X inactivation. Variability in the extent of inactivation might reflect interaction with other variable components of the inactivation pathway. It is also possible that the variability seen for the inactivation of the X chromosome carrying the C-43A variant was due to the chromosome being unable to lose the mark to remain active, in a manner analogous to imprinting center mutations.[43] The *Hha*I assay used to search for the C-43G variant would not detect the C-43A variant, so it will be interesting to examine other females with nonrandom patterns of X inactivation for this variant. It will also be interesting to examine the promoter sequence of other ring X chromosomes failing to express *XIST* for this, or other, variants.

In conclusion, we report a new case of a ring X chromosome containing *XIST*, but failing to express it, resulting in the derivative chromosome remaining active. Functional disomy is presumed to account for the characteristic phenotype of dysmorphic features, brachydactyly and developmental delay. The presence of *XIST* on the small, active ring X chromosome underscores the need to combine molecular analyses along with FISH for *XIST* in the analysis of such chromosomes. Detection of a variant sequence in promoter of *XIST* at the same position as another variant associated with nonrandom X chromosome inactivation is intriguing. However, the essentially random inactivation in grandmother with the same variant suggests that, if this change leads to failure to express *XIST*, the mechanism is complex.

Electronic database information

URLs for data in this article are as follows:

http://locus.umdnj.edu/higms/ for the Coriell Cell Repository.

http://rsb.info.nih.gov/nih-image/Default.html for the NIH IMAGE program.

http://www.ncbi/nlm/nih.gov/genemap/map.cgi?CHR=X for Genemap'99.

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