

# Cryptic mosaicism involving a second chromosome X in patients with Turner syndrome

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The high abortion rate of 45,X embryos indicates that patients with Turner syndrome and 45,X karyotype could be mosaics, in at least one phase of embryo development or cellular lineage, due to the need for the other sex chromosome presence for conceptus to be compatible with life. In cases of structural chromosomal aberrations or hidden mosaicism, conventional cytogenetic techniques can be ineffective and molecular investigation is indicated. Two hundred and fifty patients with Turner syndrome stigmata were studied and 36 who had female genitalia and had been cytogenetically diagnosed as having "pure" 45,X karyotype were selected after 100 metaphases were analyzed in order to exclude mosaicism and the presence of genomic Y-specific sequences (*SRY*, *TSPY*, and *DAZ*) was excluded by PCR. Genomic DNA was extracted from peripheral blood and screened by the human androgen receptor (*HUMARA*) assay. The *HUMARA* gene has a polymorphic CAG repeat and, in the presence of a second chromosome with a different *HUMARA* allele, a second band will be amplified by PCR. Additionally, the CAG repeats contain two methylation-sensitive *HpaII* enzyme restriction sites, which can be used to verify skewed inactivation. Twenty-five percent (9/36) of the cases showed a cryptic mosaicism involving a second X and approximately 14% (5/36), or 55% (5/9) of the patients with cryptic mosaicism, also presented skewed inactivation. The laboratory identification of the second X chromosome and its inactivation pattern are important for the clinical management (hormone replacement therapy, and inclusion in an oocyte donation program) and prognostic counseling of patients with Turner syndrome.

Key words: Turner syndrome; X-inactivation; *HUMARA* assay; DNA methylation; Cryptic mosaicism

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## Introduction

The Ullrich-Turner syndrome or Turner syndrome (TS) is defined as the combination of phenotypic features and complete or partial absence of one of the X chromosomes, frequently accompanied by cell line mosaicism, in women. About 99% of the cases where 45,X is present at the time of human conception, a natural miscarriage occurs in the first stages of embryonic development (1). This has led to the widely held hypothesis that, in order to be viable, a 45,X conceptus must possess another cell line, at least in some

critical organs or at a critical period during embryogenesis (1,2). For this reason, many females with TS stigmata who have been ascribed a non-mosaic 45,X karyotype after cytogenetic analysis of a limited number of cells may, in fact, be mosaics (3). In addition to occult mosaicism, other factors affecting the phenotype have not yet been fully elucidated, including genomic imprinting or anomalous X inactivation, leading to difficulties in diagnosis and genetic counseling (4).

In mammals, X-inactivation (XI) silences one of the two female X chromosomes and is associated with a series of

epigenetic modifications in the inactive X, including DNA methylation, and histone modifications (5,6). In addition, the inactive X chromosome condenses into the Barr body and becomes late replicated during the S phase. At the initiation of XI in early embryogenesis, *XIST* RNA (*XIST* in human and *Xist* in mouse) becomes stable and coats the inactive X in *cis* (7,8). Adding further to the curiosities of XI is the discovery that *Xist* is regulated in *cis* by an antisense gene (*Tsix*) expression, which blocks the accumulation of *Xist* RNA along the future active X (Xa). Studies have shown the presence of an insulator and transcription factor, CTCF, as a candidate *trans*-acting factor for X chromosome selection in the mouse (9). If both X chromosomes are intact in a female, the choice of which one becomes inactivated is usually random. However, if one chromosome is structurally abnormal, it is typically inactivated in a majority of cells in the adult. If one X containing *XIST* is involved in a balanced translocation with an autosome, the normal X is usually inactivated, whereas the abnormal X is inactivated in some unbalanced translocations (10). Structural defects that delete *XIST* result in the failure of X inactivation (7).

Understanding the inactivation process makes it possible to study the existence or absence of a second X

chromosome in patients with 45,X karyotype (11). The human androgen receptor (*HUMARA*) gene, mapped on Xq12, has a polymorphic CAG repeat with 87% heterozygosity. In the presence of a second chromosome with a different *HUMARA* allele, the second band will be amplified by PCR. The CAG repeats contain two methylation-sensitive *HpaII* enzyme restriction sites. In the *HUMARA* assay, prior to PCR amplification, genomic DNAs are digested with *HpaII* (Figure 1) (11). The *HUMARA* assay was used to detect low-level mosaicism involving X chromosomes in 45,X patients.

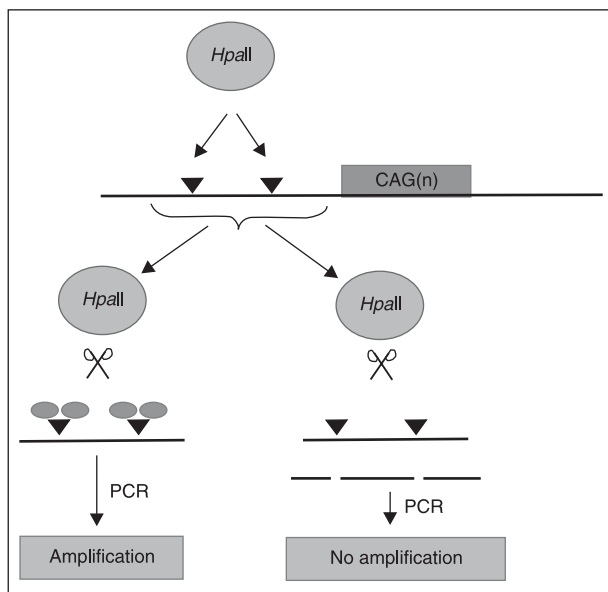
## Patients and Methods

### Patients

The study was approved by the Research Ethics Committee of the Clinical Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo. The medical records from 1991 to 2005 of 250 patients with TS stigmata and female genitalia were reviewed, and the cases with 45,X karyotype were selected, after 100 metaphases were analyzed in order to exclude mosaicism (12). These patients were investigated for Y-specific sequences (*SRY*, *TSPY* and *DAZ*) in peripheral blood by Bartmann et al. (13) and by us (data not shown) and 36 patients were considered 45,X and Y-negative. In addition, four normal men (negative controls) and four normal women (positive controls) were included.

### Molecular genetic analysis

Genomic DNA was isolated from peripheral blood (14). A modified *HUMARA* assay from Yorifuji et al. (11) was carried out to detect X chromosome mosaicism. Prior to PCR, the DNA samples were digested with *HpaII* in 10  $\mu$ L, overnight at 37°C. Aliquots (1  $\mu$ L) of the digested DNA were used to amplify the segment spanning the CAG repeats in the *HUMARA* gene. The sequences of the primers were as follows: forward primer (*HUMARA*-1) 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'; reverse primer (*HUMARA*-2) 5'-GCTGTGAAGGTTGCTGTTCTCAT-3'. The PCR products were diluted 1000-fold in distilled water and 1- $\mu$ L aliquots were used for the second cycle of nested PCR. The second set of primers was: 5'-GTGCGGAAGT GATCCAGAA-3' (*HUMARA*-3) and 5'-CCAGGACCAGG TAGCCTGTG-3' (*HUMARA*-4). For band pattern comparison, the results with and without *HpaII* digestion were run side by side on electrophoresis gel for each patient. The DNA of normal men and women was also used. PCR was performed in 25  $\mu$ L and the products were analyzed by electrophoresis through 10% polyacrylamide gels and stained with silver nitrate.



**Figure 1.** *HUMARA* assay. When an X chromosome is inactive, these *HpaII* sites are methylated (oblong circles) and are resistant to digestion, and subsequent PCR yields products of the expected size (left side of the figure). However, when an X chromosome is active, these *HpaII* sites are not methylated (inverted triangles without oblong circles) and are susceptible to *HpaII* digestion and, consequently, PCR amplification fails to yield products (right side of the figure) (Modified from Ref. 11).

## Results

The data for mosaicism involving a second X chromosome indicate that 25% (9/36) had a second X chromosome and 14% (5/36), or 55% (5/9) of the patients with cryptic mosaicism, also presented skewed inactivation (Table 1).

## Discussion

Several studies have been carried out to detect X cryptic mosaicism in cytogenetically 45,X cases. The frequency reported varies from 0-75% (Table 2) (1,3,11,15-23) and in most cases a second X chromosome was detected, but skewed inactivation is not. Although peripheral blood may not represent the rest of the tissues of the body, analysis of samples of other tissues usually depends on invasive procedures. In the study of Yorifuji et al. (11), 11% (2/18) of the patients with 45,X karyotype were positive for X chromosome mosaicism using the HUMARA assay. For these authors, the detection limit was one in 960 cells (11), and we have used the same detection limit.

Different numbers of metaphases have been analyzed to identify the karyotype as a "pure" 45,X (Table 2). It should be noted that in our study 100 metaphases from each patient were analyzed by conventional cytogenetics, in order to detect chromosomal mosaicism equal or superior to 3% with a confidence interval of 0.90 or 5% with a confidence interval of 0.99 (12). The number of cells ana-

lyzed by us was greater than reported for other studies, in which the maximum number of metaphases analyzed was usually up to 50 cells. Therefore, the sample used in the present study was very carefully selected and a very small number of informative cases were considered. Nevertheless, in 25% (9/36) of these cases the HUMARA assay revealed the presence of X cryptic mosaicism (Figure 2).

The choice of which X chromosome becomes inactivated is usually a random process. However, if one X chromosome is structurally abnormal it is skewed inacti-

**Table 1.** Results of the molecular analysis of 36 patients with karyotype 45,X [100].

Number of patients	X molecular analysis		
	Without <i>Hpa</i> II	With <i>Hpa</i> II	Results
20	1	1	NI
7	1	0	NI
4	1	2 (=)	XM
3	1*	1*	XM/SI
1	1	2 (>/<)	XM/SI
1	2 (</>)	1 (>)	XM/SI

One hundred metaphases of each patient were analyzed. NI = not informative for mosaicism and skewed inactivation; SI = skewed inactivation; XM = X chromosome mosaicism; \*with and without digestion giving different band patterns; 0, 1, and 2 in the columns *Hpa*II show the number of bands; (>) = band of greater intensity; (<) = band of lower intensity; (=) = bands of equal intensity.

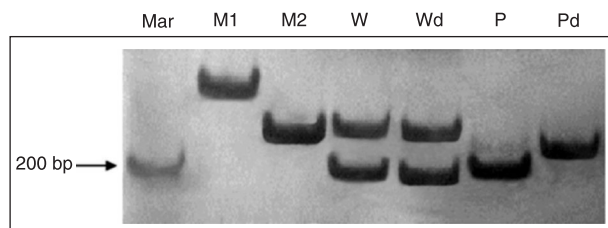
**Table 2.** Reports of cryptic mosaicism involving a second X chromosome in patients with Turner syndrome.

Reference	Method	Cells analyzed/patient (N)	Subjects with 45,X studied	Second X (%)
Hassold et al. (1)	Cytog/PCR	4 to 120	31 abortions, 5 liveborns	5.5
Mathur et al. (15)	Cytog/Southern blot	≥25	25 patients	0
Gicquel et al. (16)	Cytog/Southern blot	4 to 50	29 patients	3.5
Hassold et al. (17)	Cytog/Southern blot	3 to 80	27 abortions, 10 liveborns	10.8
Larsen et al. (3)	Cytog/Southern blot	≥50	40 patients	15
Jacobs et al. (18)	Cytog/PCR	100	84 patients	2.4
Yorifuji et al. (11)	Cytog/HUMARA	>20	18 patients	11
Nazarenko et al. (19)	Cytog/FISH	≥29 (Cytog) 200 to 1007 (FISH)	21 patients	42.8
Fernández-García et al. (20)	Cytog/FISH	30 (Cytog) 254 to 1326 (FISH)	16 patients	75
Hanson et al. (21)	Cytog/FISH	10 to 68 (Cytog) 161 to 313 (FISH)	23 patients	43
Monroy et al. (22)	Cytog/PCR	100	10 patients	0
Wiktor and Van Dyke (23)	Cytog/FISH	≥30 (Cytog) 500 (FISH)	22 patients	14
Present study	Cytog/HUMARA	100	36 patients	25

Cytog = conventional cytogenetics analysis; HUMARA = human androgen receptor assay; FISH = fluorescence *in situ* hybridization analysis; PCR = polymerase chain reaction with other X chromosome markers; Second X (%) = cases with a second X chromosome.

vated in the majority of cells in the adult (19). Although it is a semi-quantitative test in the way that it was carried out here (an automated sequencer for the analysis was not used), 55% (5/9) of the cases of X cryptic mosaicism also revealed the presence of skewed inactivation, showing the possible presence of a second structurally altered X chromosome that was selectively inactivated and amplified (Figure 2).

We used the *HUMARA* assay for the detection of low-level mosaicism in TS for several reasons. First, the locus is located at Xq12 (between the centromere and the X-inactivation center at Xq13) and a structurally abnormal X chromosome would probably retain the locus. Second, since the locus is highly polymorphic (percentage heterozygosity is about 87-90%), the chance of the second X chromosome having a different allele is high. Third, it has been shown in TS that the structurally abnormal X chromosome is inactivated selectively and will be amplified selectively. Even in patients with 45,X/46,XX karyotype, the allele of the second X will be more efficiently amplified, since X chromosomes in 46,XX cells are randomly inactivated; therefore, both alleles will be equally amplified, even in the presence of a large excess of 45,X cells (11).



**Figure 2.** Results of *HUMARA* nested PCR amplification visualized on a 10% polyacrylamide gel electrophoresis stained with silver nitrate. M1 and M2 = Normal men DNA without prior digestion with *HpaII* (only one band corresponding to the presence of one X chromosome); W = normal woman DNA without prior digestion with *HpaII* (two bands corresponding to the presence of two X chromosomes); Wd = DNA from a normal woman with prior digestion with *HpaII* (two bands corresponding to the presence of two X chromosomes inactivated, showing the female natural mosaicism for the X-inactivation); P = DNA from a patient with karyotype 45,X [100] (a strong and a very weak band, showing a positive result for cryptic X chromosome mosaicism); Pd = DNA from a patient (P) treated with *HpaII* (only one band showing a skewed inactivation); Mar = molecular marker (100 bp). One hundred metaphases of each patient were analyzed.

In the remaining patients (27/36), the allele of a second X chromosome was not detected by the *HUMARA* assay. In seven (7/27) of these cases no band was visualized after enzyme digestion, possibly due to the complete digestion of the material by the restriction enzyme. This indicated that these patients could be “pure” 45,X in peripheral blood, with the probability of other lineages in other tissues, or of being mosaics 45,X/46,XY. The last hypothesis was excluded (Araújo A, Ramos ES, unpublished data) and Bartmann et al. (13), at least in peripheral blood, using PCR for Y-specific sequences. Twenty cases (20/27) were not informative, because they all presented the same band after digestion with *HpaII*. The reason for this phenomenon could be due to incomplete digestion with the enzyme *HpaII* (11). Another explanation is the presence of homozygote individuals in the sample studied or the presence of alleles with a very close band pattern, due to a small difference in the number of repetitive CAG between them. However, these samples could be “pure” 45,X in peripheral blood, with other lineages in different tissues, or 45,X/46,XY, excluded in the blood by PCR using Y-specific sequences. Some other cases which are not informative could be explained by uniparental isodisomy of the original X chromosome (both of the X chromosomes would have the same allele and therefore would not be identified) (11).

The reagents and equipment used in the present study can be found in the majority of hospitals and the *HUMARA* test could be included in routine clinical investigation. The sensitivity was high and the *HUMARA* assay, as used here, could be used in association with the PCR (using Y-specific sequences) and cytogenetic analysis (conventional and molecular). The laboratory identification of the presence of the second X chromosome and its pattern of inactivation, associated with the exclusion of the Y chromosome, are important for the clinical management and prognostic counseling of patients with TS.

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