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ORIGINAL ARTICLE

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X chromosome inactivation patterns in 45,*X*/46,XX mosaics

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Abstract To investigate X chromosome inactivation (XCI) patterns in 45,X/46,XX mosaics, genomic DNA was extracted from peripheral blood samples of 15 female subjects who showed different proportions of 45,X cell clones. XCI patterns were analyzed using two assays. The first assay was the *Bst*XI restriction endonuclease detection of an X-linked phosphoglycerate kinase (*PGK*) gene polymorphism following digestion of the DNA with methylation-sensitive *Hpa*II, or with methylation-insensitive *Afa*I as a control. The second assay was the detection of a CAG triplet repeat polymorphism in the X-linked androgen receptor (*AR*) gene after sodium bisulfite treatment. Of the 15 subjects, 11 were informative due to heterozygosity for at least one of the polymorphisms (6 were heterozygous for the *PGK* polymorphism and 9 were heterozygous for the *AR* polymorphism). Four of the 11 informative subjects (36%) showed extremely skewed XCI for at least one of the polymorphisms, which was a much higher incidence than previously reported for normal females. Moreover, 3 of these 4 women had proportions of 45,X cell clones greater than 20%. Although our results may be due to several possible cytogenetic or molecular mechanisms, the most likely explanation is that cases of 45,X/46,XX that contain relatively high levels of 45,X cell clones probably arose due to structural aberrations of the X chromosome undetectable by conventional karyotyping.

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Introduction

On cytogenetic examination, mosaicisms of 45,X/46,XX (X/XX) are often identified in women who have various clinical symptoms, including Turner stigmata, primary amenorrhea, premature ovarian failure (secondary amenorrhea), and history of recurrent spontaneous abortions. These mosaicisms can be categorized according to the prevalence of the 45,X cell clones. When 45,X cells represent less than 20% of the total cells, the mosaicism is categorized as low-level, as the relatively rare 45,X cells do not result in a significant phenotype. Low-level X/XX seems to be mainly the result of premature centromere division, a phenomenon by which the two X chromosomal chromatids separate due to loss of centromere function (Fitzgerald 1983). However, as bi-parental inheritance of X chromosomes has been observed in ordinary-level X/XX that contains relatively large proportions of X monosomy clones (Robinson et al. 1995; Jacobs et al. 1997), the mosaic aneuploidy seems to result from the loss of an X chromosome from some cells during embryonic development. Nonetheless, the reasons for postzygotic X chromosome loss remain unclear.

X chromosome inactivation (XCI) was first identified by Lyon (1961), such that in mammalian females, one of the two X chromosomes is randomly inactivated to compensate for the difference in X-linked gene dosage between males and females. However, nonrandom XCI has been observed, even in some normal women without known X-linked disease (Gale et al. 1997; Plenge et al. 1997; Lanasa et al. 1999). Such skewed XCI is thought to result from several distinct mechanisms that can be categorized as either primary or secondary nonrandom XCI (Heard et al. 1997). Following studies involving a three-generation family (Naumova et al. 1996) and monozygotic twins (Richards et al. 1990;

Jorgensen et al. 1992), it was hypothesized that primary nonrandomness is induced by an X-linked gene, while secondary nonrandom XCI may be due to cell selection, such that positive or negative selection acts upon cells expressing particular alleles carried on one of the X chromosomes (Belmont 1996). Structurally aberrant X chromosomes, such as interstitial or terminal deletions, isochromosomes, ring chromosomes, or translocations, show late replication (Therman and Patau 1974) and women with these aberrant X chromosomes also exhibit secondary nonrandomness (Heard et al. 1997).

Methods for evaluating XCI patterns are based on the assessment of differential methylation patterns between active and inactive X chromosomes. To achieve this, X-linked genes with known nucleotide polymorphisms have been utilized, including a *Bst*XI site polymorphism in intron 1 of the phosphoglycerate kinase (*PGK*) gene and a CAG triplet repeat [(CAG)n] polymorphism in exon 1 of the androgen receptor (*AR*) gene. For the *PGK Bst*XI polymorphism assay, DNA samples are predigested with a methylation-sensitive endonuclease (Vogelstein et al. 1987; Uehara et al. 2000), while for the *AR* (CAG)n polymorphism assay, samples are first treated with a methylation-sensitive endonuclease (Sangha et al. 1999) or sodium bisulfite (Kubota et al. 1999).

Although preferential or "extremely skewed" XCI has been observed in women with various kinds of X chromosomal abnormalities, little is known about XCI patterns in X/XX mosaics. Therefore, in this study we evaluated XCI patterns in women with X/XX, and here we discuss the relationship between XCI-skewing and the etiology of X/XX.

Subjects and methods

Subjects

After cytogenetic examination by conventional G-banding on peripheral blood lymphocytes from women with symptoms such as Turner stigmata, short stature, delayed menarche, primary amenorrhea, secondary amenorrhea, or history of recurrent pregnancy loss, 15 women were diagnosed as having X/XX that contained various levels of 45,X cell clones (6 had 20% or more 45,X clones, 9 had less than 20% 45,X clones). In some subjects further karyotyping, using high resolution banding, was done, but no structural aberrations of the X chromosome were found. XCI evaluation was carried out in the 15 subjects, as well as in male (46,XY) and female (46,XX) controls. Age, clinical symptoms, and karyotypes of the subjects are shown in Table 1.

Evaluation of XCI pattern

Peripheral blood samples were taken after obtaining informed consent from the subjects and/or their parents. Genomic DNA was extracted from peripheral blood samples using SepaGene (Sanko Junyaku, Tokyo, Japan). XCI patterns were evaluated using two methods; a *PGK Bst*XI site polymorphism after digestion of the DNA with methylation-sensitive *Hpa*II, and an *AR* (CAG)n polymorphism after sodium bisulfite treatment of the DNA. The *PGK Bst*XI polymorphism method is described elsewhere

Table 1. Age, clinical symptoms, karyotypes, and results of XCI pattern analyses of subjects

Subject no.	Age (years)	Clinical symptom	Karyotype	Skewing of XCI	
				PGK	*AR*
Ordinary-level mosaicisms					
1	14	Short stature	45,X[12]/46,XX[48]	Extreme	Extreme
2	17	Delayed menarche	45,X[15]/46,XX[45]	UD	Extreme
3	5	Turner stigmata	45,X[11]/46,XX[39]	UD	Extreme
4	19	Primary amenorrhea	45,X[10]/46,XX[40]	UD	NC
5	15	Short stature	45,X[21]/46,XX[39]	UD	UD
6	34	Turner stigmata	45,X[48]/46,XX[2]	UD	UD
Low-level mosaicisms					
7	38	Secondary amenorrhea	45,X[5]/46,XX[45]	UD	Extreme
8	14	Short stature	45,X[4]/46,XX[46]	NC	UD
9	37	Turner stigmata	45,X[3]/46,XX[47]	UD	NC
10	17	Short stature	45,X[2]/46,XX[58]	UD	UD
11	22	Secondary amenorrhea	45,X[2]/46,XX[78]	NC	NC
12	32	Recurrent spontaneous abortion	45,X[1]/46,XX[49]	UD	UD
13	8	Short stature	45,X[1]/46,XX[49]	NC	NC
14	15	Turner stigmata	45,X[5]/46,XX[45]	NC	UD
15	18	Primary amenorrhea	45,X[3]/46,XX[47]	NC	NC

UD, X chromosome inactivation patterns were undetermined because of homozygosity or unclear heterozygosity; NC, skewing was not considerably different from random inactivation

Table 2. Primer sequences

Primer sets	Sequence (5′-3′)
PGK	
Forward[a]	AGCTGGACGTTAAAGGGAAGC
Reverse	TAGTCCTGAAGTTAAATCAAC.
AR	
Methylated allele	
Forward[a]	GCGAGCGTAGTATTTTTCGGC
Reverse	AACCAAATAACCTATAAAACCTCTACG
Unmethylated allele	
Forward[a]	GTTGTGAGTGTAGTATTTTTTGGT
Reverse	CAAATAACCTATAAAACCTCTACA

[a] Fluorescently labeled with 6-FAM (carboxy-fluorescein)

(Uehara et al. 2000). Briefly, DNA samples were incubated with methylation-sensitive *Hpa*II (Takara Shuzo, Otsu, Japan) to completely digest nonmethylated (active) alleles. Duplicate samples were incubated with *Afa*I (Takara Shuzo) as a control. DNA samples were then amplified by polymerase chain reaction (PCR), using primer sets, as shown in Table 2, with the forward primer fluorescently labeled with 6-FAM (carboxy-fluorescein). Aliquots of the PCR products were digested with *Bst*XI (Toyobo, Tokyo, Japan) and subjected to denaturing polyacrylamide gel electrophoresis. Heterozygous band patterns (530- and 433-bp bands) were detected using an ABI Prism 377 DNA sequencer (Perkin-Elmer, Foster City, CA, USA), and band-peak patterns were analyzed using GeneScan Analysis 2.0.2. software (Perkin-Elmer).

The *AR* (CAG)n polymorphism method was performed according to Kubota et al. (1999). Briefly, DNA samples were denatured with 0.3 M NaOH, and then incubated with 0.5 M hydroquinone (Sigma, St. Louis, MO, USA) and 3.1 M sodium bisulfite (Sigma). Modified DNA samples were purified using the Wizard DNA clean-up system (Promega, Madison, WI, USA), and the chemical reaction was stopped by the addition of 0.3 M NaOH. After ethanol precipitation, DNA samples were resuspended in distilled water, and amplified by PCR. Primer sets were designed to amplify either methylated or unmethylated alleles (Table 2), with forward primers fluorescently labeled with 6-FAM. PCR products were subjected to electrophoresis, and heterozygous band patterns (approximately 200 bp) evaluated as for the *PGK Bst*XI polymorphism.

Data interpretation

Allelic methylation leads to the alteration of cytosine to methylcytosine in CpG dinucleotides, and plays an important role in XCI (Razin and Riggs 1980). Therefore, patterns of XCI can be evaluated by assessing the differential methylation patterns between active and inactive X chromosomes, as *Hpa*II sites of *PGK* alleles are methylated in inactive X chromosomes (Vogelstein et al. 1987; Uehara et al. 2000). As cytosines are altered to uracil by sodium bisulfite, but methylcytosines are not, PCR products can be amplified using primers specific for methylated versus unmethylated *AR* alleles following sodium bisulfite treatment, and methylation status can be determined (Herman et al. 1996; Kubota et al. 1999).

In random XCI, there is an equal chance that either the paternal or maternal X chromosome is inactivated by allelic methylation in any given cell. Thus, bands from cells heterozygous for a polymorphism show similar intensities after *Hpa*II predigestion or sodium bisulfite modification. However, differences in heterozygote peak sizes indicate unequal methylation of the bi-parental X chromosomes, suggesting nonrandom or preferential XCI. When peak sizes differed considerably (more than 90:10 large peak:small peak size), we determined the XCI pattern to be extremely skewed.

For XCI pattern evaluation using *Hpa*II, DNA derived from 45,X cell clones is cleaved and cannot be amplified because the X chromosome is unmethylated (i.e., not inactivated). Thus, the presence of 45,X cells can be ignored in XCI pattern analyses. In contrast, in experiments using sodium bisulfite treatment, DNA from 45,X cells influences peak sizes in PCR of unmethylated alleles. Therefore, we compared peak sizes between the PCR products of methylated and unmethylated alleles.

Results

Peak patterns in control subjects

For the *PGK Bst*XI polymorphism, a markedly smaller peak was observed in male control samples after *Hpa*II predigestion, while a predominant single peak (either 530 or 433 bp) was observed in samples after *Afa*I predigestion. For the female control samples heterozygous for the *PGK* polymorphism, two predominant peaks (530 and 433 bp) were observed in both the *Hpa*II and *Afa*I predigested samples.

For the *AR* polymorphism, male controls showed no peak representing PCR products from methylated alleles, but showed a single peak representing products from unmethylated alleles. Female controls heterozygous for the *AR* polymorphism showed two peaks of similar sizes for both PCR products.

The results are shown in Fig. 1.

*PGK Bst*XI polymorphism XCI patterns

Peak patterns after *Afa*I predigestion revealed that 6 of the 15 subjects were informative due to heterozygosity for the *PGK Bst*XI polymorphism. Of the six heterozygotes, five showed two band peaks after *Hpa*II predigestion, and one showed a predominant 530-bp peak and a markedly smaller 433-bp peak (Fig. 2). Because the peak sizes of the two heterozygous bands differed considerably after *Hpa*II predigestion compared with findings in female controls (i.e., the allele represented by the 530-bp peak was not digested

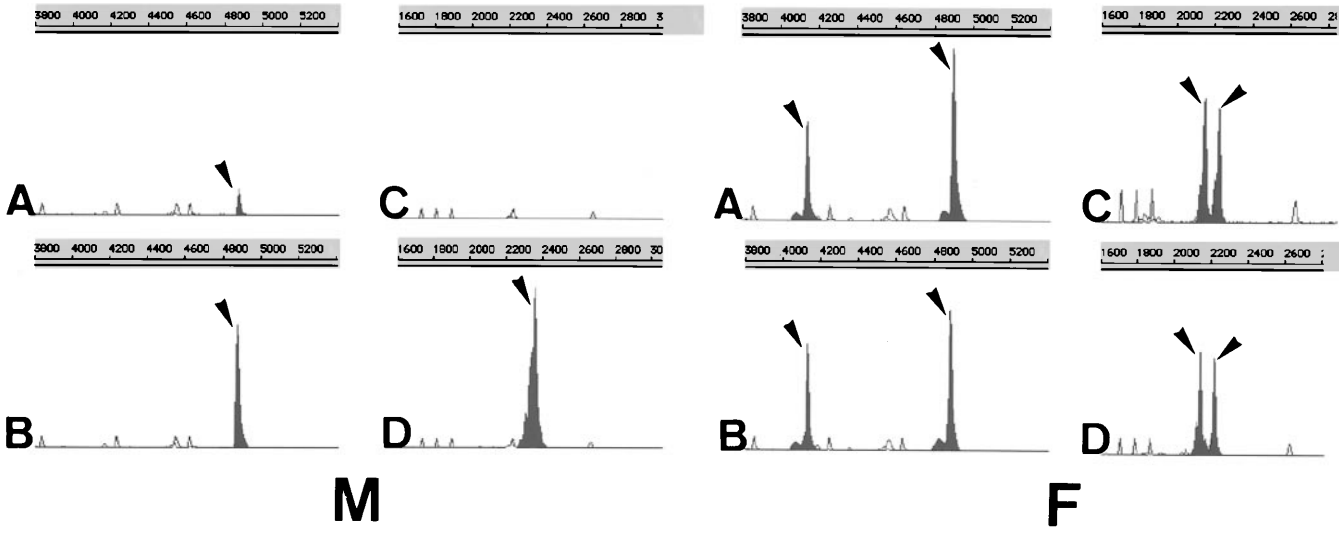

Fig. 1. Peak patterns of control subjects (46,XY and 46,XX). *Panels* (*A*) show *PGK Bst*XI assay patterns after predigestion with methylation-sensitive *Hpa*II; *panels* (*B*) show patterns after predigestion with *Afa*I. *Panels* (*C*) show patterns of *AR* assay products derived using primers specific for methylated alleles; *panels* (*D*) show patterns of products derived using primers specific for unmethylated alleles. Panels of male controls are indicated by *M*, those of female controls by *F*. In the *PGK Bst*XI assay of a male control, a markedly smaller peak (530 bp, indicated by *arrowhead*) is observed in the *upper panel*, while a predominant single peak (530 bp, indicated by *arrowhead*) is observed in the *lower panel*. In the *AR* assay of a male control, no peak is observed in the upper panel, while a single peak (indicated by *arrowhead*) is observed in the lower panel. For female controls, two heterozygous peaks (indicated by *arrowheads*), with similar peak sizes, are observed in both the *PGK Bst*XI and *AR* assays. Peaks that are *not shadowed* represent size markers

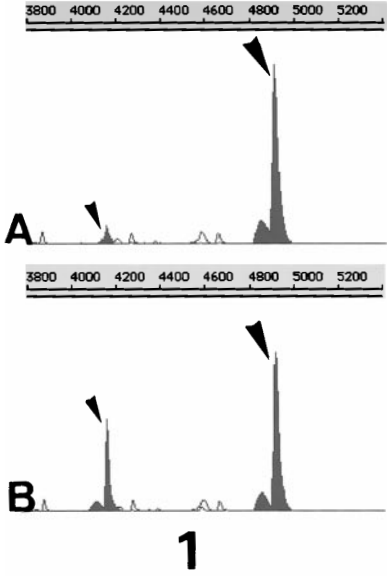

Fig. 2. *PGK Bst*XI assay patterns for a subject showing extremely skewed XCI. *Panel A* shows the band pattern after predigestion with *Hpa*II; *panel B* shows the pattern after predigestion with *Afa*I. *The number below the panels* corresponds to the subject number in Table 1. Definite heterozygous-band peaks (433 and 530 bp, indicated by *small* and *large arrowheads*) are observed in the panel after *Afa*I predigestion, while the 433-bp peak (indicated by a *small arrowhead*) is much smaller than the 530-bp peak (indicated by a *large arrowhead*) after *Hpa*II predigestion. These peak patterns demonstrate extremely skewed XCI due to differential methylation of the paternally and maternally derived X chromosomes. Peaks that are *not shadowed* represent size markers

by *Hpa*II due to unequal methylation), the peak pattern of the subject demonstrated extremely skewed XCI. Results for the 15 subjects are summarized in Table 1.

AR polymorphism XCI patterns

For the *AR* polymorphism, 9 of the 15 subjects were informative heterozygotes. In four of the nine heterozygotes, a single peak was observed for the two PCR products representing methylated and unmethylated alleles (Fig. 3). As the molecular sizes clearly differed, the peak patterns observed in these four women demonstrated extremely skewed XCI. Results are summarized in Table 1.

Relationship between extremely skewed XCI and karyotype

Karyotypes of the four subjects that showed extremely skewed XCI for the *PGK Bst*XI or *AR* (CAG)n polymorphisms were 45,X[12]/46,XX[48], 45,X[15]/46,XX[45], 45,X[11]/46,XX[39], and 45,X[5]/46,XX[45]; i.e., three ordinary-level mosaicisms and one low-level mosaicism. Partial karyograms of the women with 45,X[12]/46,XX[48] and 45,X[15]/46,XX[45] are shown in Fig. 4. Three of four heterozygotes with ordinary-level mosaicisms showed extremely skewed XCI, whereas only one of seven heterozygotes with low-level mosaicisms showed extremely skewed XCI.

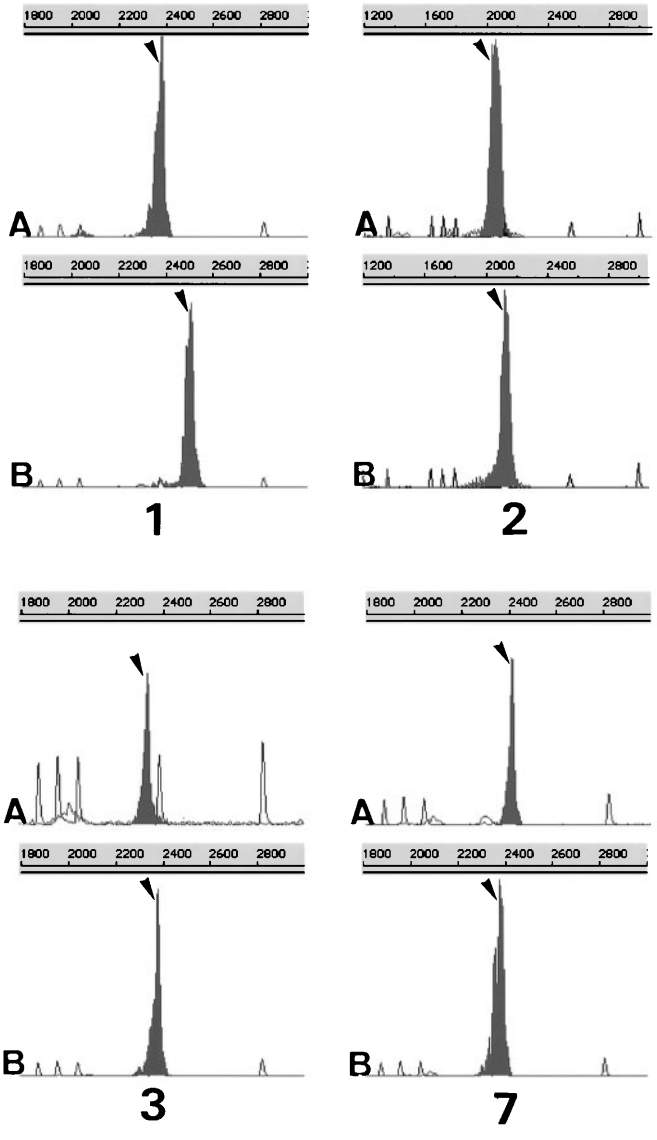

Fig. 3. *AR* assay patterns for four subjects who showed extremely skewed XCI. The *A* panels show peak patterns derived using primers specific for methylated alleles, and the *B* panels show patterns derived using primers specific for unmethylated alleles. *Numbers below the panels* correspond to the subject numbers in Table 1. Single peaks (indicated by *arrowheads*) of different molecular sizes are observed in the *upper* and *lower panels* of the four subjects. These peak patterns indicate extremely skewed XCI. *Peaks not shadowed* represent size markers

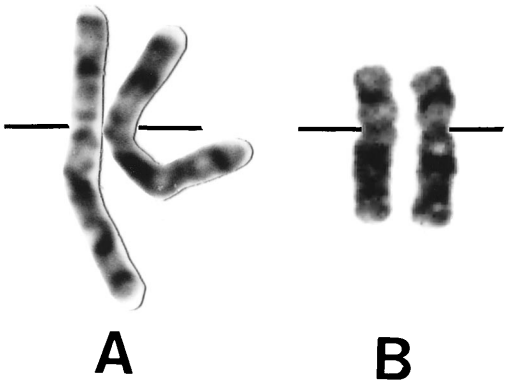

Fig. 4. X Chromosomes of representative subjects with *A* 45,X[12]/46,XX[48] (high-resolution banding) and *B* 45,X[15]/46,XX[45] (G-banding). No structural aberrations were detectable by conventional karyotyping

Discussion

In both the *PGK Bst*XI and *AR* polymorphism assays, peak patterns of the male controls (46,XY) indicated that the X chromosomes were unmethylated, while those of female controls (46,XX) demonstrated equal methylation of the biparental X chromosomes. Therefore, it was determined that these two methods could reliably detect XCI.

In the study of 15 X/XX mosaics, we detected four cases of extremely skewed XCI. Of the 15 subjects, allelic heterozygosity was confirmed in 6 for the *PGK Bst*XI polymorphism, and in 9 for the *AR* (CAG)n polymorphism. As 4 women were homozygotes or undetectable heterozygotes for both polymorphisms, 11 women were heterozygous for at least one of the two polymorphisms. Therefore, the incidence of extremely skewed XCI was 36% (4/11) in our X/XX mosaics. Extremely skewed XCI has been reported to have an incidence of 1.5%–3.5% in the general female population (Gale et al. 1997; Plenge et al. 1997; Lanasa et al. 1999) and it increases with age (Busque et al. 1996). However, the subjects in the present study were all relatively young, with the oldest being aged 38 years (no. 7 in Table 1). This suggests a much higher incidence of extremely skewed XCI than that in the general female population. Our study revealed only one woman with low-level X/XX that exhibited extremely skewed XCI (no. 7). In contrast, the three subjects (nos. 1, 2, and 3 in Table 1) who showed extremely skewed XCI had ordinary-level X/XX (i.e., the proportion of 45,X cell clones was more than 20%). Thus, our results suggested that extremely skewed XCI may be related to mosaic level. However, one *AR* (CAG)n heterozygote with ordinary-level X/XX (45,X[10]/46,XX[40]) showed random XCI (no. 4 in Table 1), which suggested that not all cases of ordinary-level X/XX are associated with nonrandom XCI.

Although we had initially expected random XCI patterns in X/XX mosaics, due to the large proportion of 46,XX cells, we observed instances of nonrandom XCI. Given these nonrandom XCI patterns in four of our subjects, it is possible to speculate as to the etiology of X/XX. Cryptic X chromosomal aberrations and gene mutations can be proposed as possible etiologies. As structurally aberrant X chromosomes are unstable and are likely to give rise to 45,X cell lines due to replication errors during mitosis (Kuznetzova et al. 1995; Fernandez et al. 1996), it is possible that ordinary-level X/XX is the direct result of structural aberrations of the X chromosome. Extremely skewed XCI, due to positive or negative cell selection, has been observed in women with structurally aberrant X chromosomes (Therman and Patau 1974; Heard et al. 1997). Pegoraro et al. (1997) found a relatively large deletion (around 500 kb) in distal Xq28 in a family that included many females who exhibited extremely skewed XCI. Therefore, one explana-

tion for extreme XCI skewing in ordinary-level X/XX is that one of the X chromosomes contains a cryptic structural aberration not identified by conventional G-banding or even by high-resolution banding. Such cryptic aberrations induce chromosomal instability and give rise to 45,X cell lines, along with other X chromosome aberrations, such as microdeletions and fragmentary marker chromosomes. However, gene mutations as a mechanism of extremely skewed XCI cannot be ruled out. For example, patients with X-linked dominant incontinentia pigmenti type II, and nonpenetrant carriers of X-linked dominant Rett syndrome show extremely skewed XCI (Migeon et al. 1989; Schanen et al. 1997; Sirianni et al. 1998). Moreover, familial cases showing a mutation in the X inactivation-specific transcript (*XIST*) gene have been reported (Plenge et al. 1997). Based on these reports, it is possible that there may be undiscovered genes related to both XCI-skewing and X chromosome loss. To confirm the etiology of ordinary-level X/XX, further molecular cytogenetic analyses are necessary. In contrast to ordinary-level X/XX, low-level X/XX is often detected in the normal female population (Horsman et al. 1987; Nowinsky et al. 1990), and seems to result from the premature centromere division of the X chromosome (Fitzgerald 1983), or may be an artifact of the cytogenetic analysis. Therefore, women with low-level X/XX would be expected to show random XCI. However, one woman with low-level X/XX in our study (no. 7, aged 38 years) showed extremely skewed XCI. Because the incidence of extremely skewed XCI increases with age as a consequence of hematopoietic stem cell selection (Busque et al. 1996), the extremely skewed XCI observed in the middle-aged woman may be due to hematopoietic stem cell selection rather than other etiologies.

In conclusion, although further studies are necessary, it appears that skewed XCI may have cytogenetic or molecular significance in the etiology of X/XX mosaics.

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