Association of Extremely Skewed X-chromosome Inactivation with Taiwanese Women Presenting with Recurrent Pregnancy Loss

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X-chromosome inactivation (XCI) is a phenomenon that occurs in female mammals. Typically, maternally- and paternally-derived X chromosomes are inactivated at approximately the same frequency. If preferential inactivation occurs, the person is considered to have skewed XCI. Skewed XCI has been reported to occur more frequently in women who experience recurrent pregnancy loss (RPL). In this study, we sought to investigate if there is an association between skewed XCI and unexplained RPL in Taiwanese women. A total of 194 women who had experienced unexplained RPL were recruited into the study. Human androgen receptor or DXS6673E and DX15-134 loci were used in the XCI assay. The results of our study suggested that a cut-off point < 90% may not be justified for skewed XCI. Only extremely skewed (> 95%) XCI is associated with RPL. Extremely skewed XCI occurs in a subset of Taiwanese women with RPL. [*J Formos Med Assoc* 2008;107(4):340–343]

Key Words: human androgen receptor, recurrent pregnancy loss, skewed X-chromosome inactivation

Recurrent pregnancy loss (RPL) is a health problem that affects about 1–2% of couples. The etiology of RPL is extremely heterogeneous, including chromosomal abnormalities, anatomic anomalies of the uterus, endocrine dysfunction, thrombophilia, and autoimmune disorders. However, no discernible cause is uncovered by standard evaluation protocols in the majority of patients who experience RPL.[1]

X-chromosome inactivation (XCI) is a phenomenon that occurs in female mammals, in which one of two X-chromosomes is randomly inactivated during the late blastocyst stage of embryogenesis to compensate for the difference in the X-linked gene dosage between males and females. Typically, maternally- and paternally-derived X chromosomes are inactivated at approximately the same frequency. If preferential inactivation occurs, the person is considered to have skewed XCI. Skewed XCI has been reported to occur more frequently in women who experience RPL.[2,3] However, results from previous studies were based on different cut-off points to define skewed XCI (ranging from 70% to 95%) and the conclusions were not consistent.[2,3] Different cut-off values lead to different conclusions, so it is highly

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Received: July 25, 2007
Revised: August 21, 2007
Accepted: October 2, 2007

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desirable to reach a consensus on the definitive cut-off value for skewed XCI. Meanwhile, there have been no studies on the association of skewed XCI with RPL in an Asian population, except for one study by Uehara et al.[2] Therefore, in this study, we sought to investigate if there is an association between skewed XCI and unexplained RPL in Taiwanese women, as well as determine a rational cut-off value for skewed XCI.

Methods

A total of 194 women who had experienced unexplained RPL were recruited into the study. RPL was defined as two or more consecutive pregnancy losses before 24 weeks of gestation. The study was approved by the institutional review board of National Cheng Kung University Medical Center, and informed consent was obtained from each woman. All patients received comprehensive clinical evaluation, including physical examinations, karyotyping, hormonal profiles, autoimmunity, pelvic sonography and/or hysterosalpingography according to the protocol described in our previous studies.[4,5] All patients with gross karyotypic abnormalities or identifiable causes of RPL were excluded from XCI assay.

An additional 194 healthy age-matched women with normal reproductive performance were enrolled and assayed as controls for the XCI assay. The definition of normal reproductive performance was defined as: (1) delivery of at least two normal babies; (2) no history of spontaneous abortion, embryonic, or fetus with abnormal karyotypes; (3) regular menstrual periods in the past 3 years; and (4) no family history of congenital anomalies.

Genomic DNA was extracted from peripheral blood samples using a PUREGENE DNA isolation kit (Gentra, Minneapolis, MN, USA). XCI assay was performed as described previously.[6]

In brief, the human androgen receptor (AR) locus was used. This site is known to be methylated on the inactive X chromosome, so this allele would be amplified by polymerase chain reaction (PCR) after the genomic DNA is digested by the methylation-sensitive *Hpa*II restriction enzyme. The active X chromosome would not be amplified because the sequence to be amplified is cleaved by *Hpa*II (Figure). If the AR locus was uninformative, skewing was assessed using either the DXS6673E locus or DX15-134 locus.[7,8] For restriction enzyme reaction, genomic DNA (300 ng) was digested with 5 U of *Hpa*II (for the AR and DX15-134 assays) or 5 U of *Hha*I (for the DXS6673E assay) and 2 U of *Rsa*I in a total volume of 20 μL. For each sample, 300 ng of genomic DNA was also used as an undigested control. The digested and undigested samples were amplified by PCR, and the peak area for each allele was analyzed using an ABI3100 genetic analyzer and Genotyper 3.7 program (Applied Biosystems, Foster City, CA, USA).

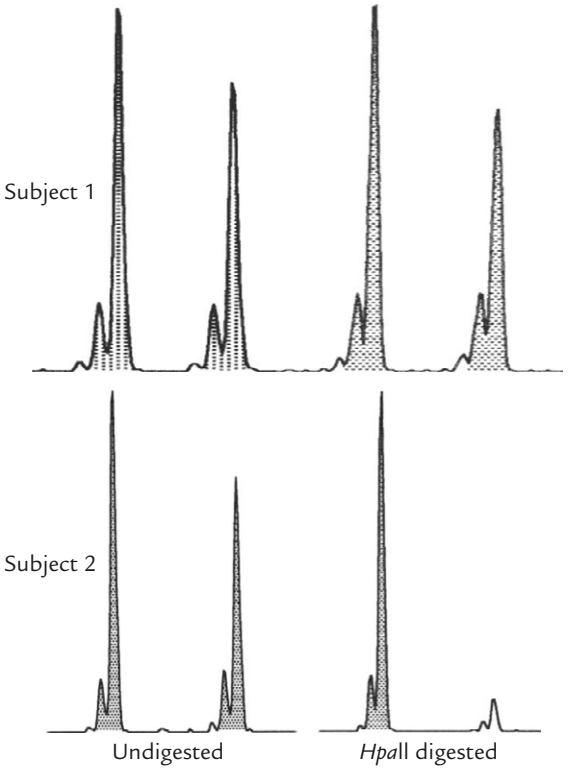

Figure. X chromosome inactivation (XCI) assay by genotyping of the CAG repeat in the human androgen receptor gene after digestion by the *Hpa*II methylation-sensitive enzyme. Subject 2 has skewed XCI and the peak heights of inactive (methylated, undigested) and active (unmethylated, digested) X chromosome differed significantly. Subject 1 had no skewed XCI and the peak heights of digested and undigested alleles were almost equal.

To account for preferential amplification, the areas of the peaks of the digested sample were normalized in relation to measurements from the undigested sample. The degree of skewing was calculated as (d1/u1)/(d1/u1 + d2/u2), where d1 and d2 represent the peak areas of the more intense allele and the less intense allele, respectively, from the digested sample, whereas u1 and u2 are the corresponding bands from the undigested sample.[8] Every test was duplicated and an average of the results was obtained. To ensure the quality of each assay, we incorporated a sample without skewed XCI as an internal control. Fisher's exact test was used to compare the proportion of skewed XCI in the patient and control populations. All statistical tests were conducted at the significant level of 0.05.

Results

After excluding uninformative cases, the study group comprised 155 women (mean age, 30.42 ± 3.822 years; range, 20–40 years) with idiopathic PRL. There were 151 women in the control group (mean age, 31.38 ± 4.139 years; range, 20–40 years). There was no significant difference in age between the two groups. The coefficient of variation of internal controls from 10 repeated assays and five independent experiments were less than 3.2% and between 2.0% and 5.1%, respectively. These data revealed that variability for both intra- and inter-assay was small.

There was no significant difference by using 75% or 90% as the cut-off value for skewed XCI. When a more stringent criterion for skewing (≥ 95%) was used, a 5.92-fold increase in skewing was seen in women with RPL (7.7%) as compared with the control group (1.3%) ($p = 0.0111$, Fisher's exact test).

Patients were divided into three categories: those who had experienced two or three or more than three consecutive pregnancy losses, respectively. We found that patients who had experienced two and those who had experienced three consecutive losses were associated with extremely skewed (≥ 95%) XCI ($p = 0.0183$ and 0.0297, respectively). However, the difference from patients who had experienced at least four consecutive pregnancy losses did not reach statistical significance ($p = 0.2062$), probably due to the extremely small sample size of this category ($n = 12$).

Discussion

Several mechanisms have been proposed to explain non-random choice in XCI. Promoter mutations in the X inaction specific transcript (*XIST*) gene have been identified in families with extremely skewed XCI.[9] However, mutations in the X inactivation pathways are thought to be rare and these subjects should be without abnormal phenotypes. Other potential explanations include reduction in embryonic precursor pool size and selection against cells with a growth disadvantage in chromosomal rearrangements involving one of the X chromosomes or carriers of X-linked disorders.[10,11] None of the cases in this study had X-chromosome abnormalities. If it was the X-chromosome genes that contributed to embryonic lethality, then most, if not all, abortus should be male. However, there was no sexual preference in the abortus in our cases (unpublished data). Therefore, the model of reduced embryonic precursor pool size is more appealing than others. Presumably, women with reduced embryonic pool size are more likely to have reduced ovarian reserve and early menopause.[12] It would be worthwhile to observe the age of menopause in women with skewed XCI, considering the conflicting data with regard to the association between skewed XCI and premature ovarian failure.[13]

So far, the biggest population-based study for the degree of XCI involved 415 adult women and 500 newborns. Of the 415 DNA samples from the women, 14.2% of samples had a degree of skewing that was > 80%, 3.6% was > 90%, and only 1.7 % was > 95%.[14] Due to the limited number of cells present in the embryo when XCI occurs, there may be bias in the proportion of cells with a preferentially inactivated parental X chromosome. Given the rarity of extremely skewed XCI in

the normal population, only cases with skewed inactivation as extreme as 90% or 95% deserve scrutiny. The results of our study also suggest that a cut-off point <90% may not be justified for skewed XCI.

Acknowledgments

This study was supported by a research grant from Chi Mei Medical Center (CMFHR9555).

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