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Original Article

Extremely skewed X-chromosome inactivation patterns in women with recurrent spontaneous abortion

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Abstract

Background: The role of extremely skewed X-chromosome inactivation (XCI) has been questioned in the pathogenesis of recurrent spontaneous abortion (RSA) but the results obtained were conflicting.

Aims: We therefore investigated the XCI patterns in peripheral blood DNA obtained from 80 patients who had RSA and 160 age-matched controls.

Methods: Pregnancy history, age, karyotype, and disease information was collected from all subjects. The methylation status of a highly polymorphic cytosine-adenine-guanine repeat in the androgen-receptor (*AR*) gene was determined by use of methylation-sensitive restriction enzyme *Hpa*II and polymerase chain reaction.

Results: Skewed XCI (> 85% skewing) was observed in 13 of the 62 patients informative for the AR polymorphism (20.9%), and eight of the 124 informative controls (6.4%) ($P = 0.0069$; χ^2 test). More importantly, extremely skewed XCI, defined as > 90% inactivation of one allele, was present in 11 (17.7%) patients, and in only two controls ($P = 0.0002$; χ^2 test).

Conclusions: These results support the interpretation that disturbances in XCI mosaicism may be involved in the pathogenesis of RSA.

Key words: androgen receptor, mosaicism, mutation, recurrent abortion; X-chromosome inactivation.

Introduction

Recurrent spontaneous abortion (RSA) is an important medical problem that affects 1–2% of couples wishing to have children. It is defined as loss of three or more consecutive pregnancies prior to 20 week of gestation.[1] The aetiologic factors that have been implicated in RSA include anatomical abnormalities (15%), infectious agents (1–2%), hormonal imbalances (20%), immunologic factors (20%) and genetic disorders (2–5%).[2,3] However, a large proportion of couples (37–79%) receive no explanation for their pregnancy losses. Based on the observation that an Xq28 deletion is associated with a high spontaneous abortion rate and familial skewed X-chromosome inactivation (XCI) in a large family,[4] male lethal X-linked mutations that cause skewed XCI in female carriers was proposed as an aetiologic factor in RSA.

X-chromosome inactivation is a physiologic event that takes place during early embryonic development and results in the transcriptional silencing of one of the two X-chromosomes in females.[5] The inactive X can be either paternally or maternally derived in different cells of the same individual, and once the decision as to which X to inactivate is made in a particular cell, all the clonal descendants of that cell abide by the decision. Therefore, females who are heterozygous for X-linked genes are mosaics of two cell types, with either the paternal or the maternal alleles active. The choice of which X to inactivate is generally a random event.[6] However, under extraordinary circumstances, exclusive or almost exclusive inactivation of one X-chromosome which leads to skewed XCI may be observed.[7,8] According to the 'male lethal X-linked mutations as a cause of recurrent abortions' model,[4]

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Table 1 Studies of skewed X-chromosome inactivation and recurrent spontaneous abortion (RSA)

Study	No. (%) observed with > 90% skewing	
	RSA	Control
Lanasa *et al.* 1999[10]	7/48 (14.6)	1/67 (1.5)
Sangha *et al.* 1999[11]	14/76 (18)	6/111 (5)
Uehara *et al.* 2001[12]	7/42 (16.7)	2/36 (5.6)
Beever *et al.* 2003[13]	25/207 (12)	7/102 (7)
Sullivan *et al.* 2003[14]	7/106 (6.6)	4/102 (3.9)
Kim *et al.* 2004[15]	1/45 (2.2)	5/54 (9.3)

X-chromosomal mutations that are cell lethal or associated with cell-growth disadvantage could be tolerated in females as a result of the XCI process. This means that in those cells in which the mutant X-chromosome is the active X, cell death eventually occurs during development, and the XCI pattern becomes skewed. However, at the phenotypic level, the carrier female lives and exhibits some (if not no) symptoms. However, in males, as there is only one X-chromosome that is always active, the putative cell lethal mutation becomes a male lethal trait, and thus contributes to the aetiology of RSA.

In the studies conducted to test the hypothesis that skewed XCI may be involved in the pathogenesis of RSA, both supporting[9–13] and refuting[14,15] results have been obtained (Table 1). We therefore investigated the peripheral blood XCI patterns of controls and females who experience recurrent pregnancy loss to delineate the molecular basis of RSA in the Turkish population.

Materials and methods

Patients

Eighty Caucasian women diagnosed with RSA, and 160 apparently healthy female controls, were genotyped to determine whether women with a history of RSA have a greater frequency of extremely skewed XCI than control subjects. The ethics review board of the participating institutions approved the study protocol. Pregnancy history, age, and disease information, accompanied by informed consent, was obtained from all subjects. We included those cases with three or more clinically recognised spontaneous abortions of unknown cause. A positive urine pregnancy test or serum βhCG and evidence of the loss of conception products accompanied by vaginal bleeding, or ultrasonographic findings of fetal demise with subsequent reduction in serum βhCG, were used to confirm spontaneous abortion. While ectopic or molar pregnancies were excluded, only those abortions that have occurred before week 20 of gestation were included in the study. Clinical evaluation of the cases excluded anatomic, cytogenetic, infectious, immunologic, hormonal, and social/exposure as causes of spontaneous abortion.[2,3] Also abnormal hysterosalpingogram, antiphospholipid antibodies, or connective tissue disorder were included in the exclusion criteria. While all of the RSA cases were karyotyped using peripheral blood samples, this was not done in the control group because of the lack of an indication for cytogenetic studies. Gravida 2, para 2 women with no history of pregnancy loss comprised the control group. The mean age of the cases and controls were 30.4 ± 5 (mean ± SD; range = 22–42 years), and 32.4 ± 4.9 years (range = 21–47 years), respectively.

X-chromosome inactivation study

DNA was extracted from 10 mL venous blood samples of patients and controls using Nucleospin DNA isolation kit (Macherey-Nagel, Dueren, Germany). Genotyping of a polymorphic site in the androgen receptor gene (*AR*) was performed and quantified to assess the XCI patterns as described.[16] The degree of skewing was estimated by an assay based on a methylation-sensitive *Hpa*II restriction site located near the *AR* gene. This site is methylated on the inactive X, and unmethylated on the active X-chromosomes. When the genomic DNA is cleaved with *Hpa*II prior to polymerase chain reaction (PCR), only the methylated *AR* allele, which represents the inactive X-chromosome, is amplified. A trinucleotide cytosine-adenine-guanine (CAG) repeat polymorphism located within the amplified region is used to distinguish between the two alleles. For each patient and control two separate PCRs, with or without *Hpa*II treatment, were performed using the same set of primers. Male DNA with cytogenetically verified 46XY karyotype was used as control for complete digestion. All of the PCR products, before and after digestion, were separated by employing two independent detection methods. One is electrophoretic separation of the alleles in 4% MetaPhor agarose (FMC Bioproducts, Rockland, ME, USA) and ethidium bromide staining. The other is separation of radioactive PCR products (α-[^{33}P]-dCTP) (PerkinElmer, Wellesley, MA, USA) on 8% sequencing gels. Densitometric analysis of the alleles was performed using the Multi-Analyst software version 1.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results of the densitometric analyses included normalisation of the ratios based on the non-digested samples by dividing the allele ratio of the digested sample by the ratio of the non-digested sample from the same specimen. The use of this ratio corrects for preferential amplification of one allele, which often occurs for the shorter microsatellite allele. The results from control and test groups were compared by the χ^2 test with Yates' correction.

Results

X-chromosome inactivation status was found to be informative in 77.5% of both the cases (62/80) and the controls (124/160). Only those individuals whose alleles resolve adequately for densitometric analysis were included in the study. Extremely skewed XCI was present in 11 (17.7%) cases and in two (1.6%) controls ($P = 0.0002$) (Table 2). Extremely skewed XCI, defined as > 90% inactivation of one allele, is observed in ~1–7% of females aged 25 or younger, and in ~2–16% of women aged 60 years or older.[17,18] When XCI values between 85 and 89%

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Table 2 Proportion of the cases and controls with skewed X-chromosome inactivation

Group	No. (%) observed with 85–89% skewing	> 90% skewing	Total
RSA patients ($n = 62$)	2 (3.2)	11 (17.7)	13 (20.9)
Control females ($n = 124$)	6 (4.8)	2 (1.6)	8 (6.4)

For comparison by χ^2, $P = 0.0002$. RSA, recurrent spontaneous abortion.

were also considered, skewed XCI was observed in 13 of the 62 (20.9%) informative cases, and eight of the 124 (6.4%) controls ($P = 0.0069$). These results indicate that extremely skewed XCI is more frequent in RSA cases than in controls, and raises the important question as to what causes this observation.

Discussion

Skewed XCI could originate from primary (bias in the initial choice) or secondary (selection following random XCI) causes.[8] Two different hypotheses have been proposed to explain the aetiology of skewed XCI in women who experience RSA: (i) male lethal X-linked mutations[4,19] and (ii) a reduction of the size of the follicular pool[13] associated with skewed XCI and trisomic pregnancies. A gender bias towards female offspring among liveborn children and maternal inheritance of skewed X-inactivation was documented in conjunction with the first hypothesis. However, a significant excess of boys among live births was observed in females with skewed XCI who were ascertained on the basis of trisomic pregnancies. Among our 11 patients with extreme skewing (> 90%), only three patients had live births and the observed female : male ratio is 3 : 1 (Table 3). Deviation from the expected female : male ratio of 1 : 1 raises the possibility that male lethal X-linked mutations could be an aetiologic factor in our RSA cases, at least in the group of patients with extreme skewing. In addition to deleterious X-linked mutations, X-autosome translocations, normal ageing, twining, confined placental mosaicism, reduction of the size of the follicular pool, monoclonal expansion of peripheral blood cells, and environmental insults, such as chemotherapy, are examples of secondary causes. Some of these factors are unlikely to be involved in our patients: for example only those patients with a normal karyotype were included in the study, excluding the potential contribution of X-autosome translocations. Twining, because of negative history, and ageing, because of the mean age of diagnosis at 30.4 ± 5 years, are also highly unlikely. A history or present diagnosis of haematologic malignancies, which may be associated with monoclonal expansion of peripheral blood cells, or exposure to environmental insults, such as chemotherapy, was not documented in any study subject. With respect to the primary causes of skewed XCI, we do not think dysfunction of gene(s) that regulates XCI[20] could be implicated in the aetiology of RSA as dosage compensation for X-linked genes is not physiologically required in XY cells and therefore is not expected to be lethal in male fetuses. Our findings also leave us with the question of why some studies did not show a statistically significant association between RSAs and skewed XCI patterns.[14,15] It is important to note that a heterogeneous group of aetiologic factors is known to contribute to the development of RSAs,[2,3] and that small sample size[15] may influence the outcome of statistical analyses.

We plan to direct our future efforts towards the ascertainment of extended pedigrees to assess the parental origin of the inactive X-chromosome. In addition, detailed high-resolution genomic analysis by newer molecular techniques, such as comparative genomic hybridisation microarray analysis,[21] could prove to be very valuable at least in a subgroup of patients who may harbour X-chromosomal submicroscopic deletions.

Table 3 Characteristics of the cases with extremely skewed X-chromosome inactivation

Patient	X-inactivation	Date of birth	Pregnancy history	Sex of children
1 (03–358)	> 95	1976	G6,P2	F2
2 (03–384)	> 95	1970	G6,P1	F1
3 (03–697)	> 95	1975	G3,P0	–
4 (03–759)	> 95	1977	G3,P0	–
5 (03–811)	> 95	1976	G3,P0	–
6 (04–009)	> 95	1979	G5,P0	–
7 (04–010)	> 95	1977	G3,P0	–
8 (03–405)	90–95	1972	G4,P1	M1
9 (03–687)	90–95	1978	G3,P0	–
10 (03–914)	90–95	1975	G4,P0	–
11 (04–011)	90–95	1975	G5,P0	–
12 (03–915)	85–89	1968	G5,P1	M1
13 (03–916)	85–89	1962	G6,P0	–

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