RESEARCH NOTE

May anomalous X chromosome methylation be responsible for the spontaneous abortion of a male foetus?

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Introduction

Pregnancy loss is an important reproductive problem which appears to be highly associated with genetic factors. A spontaneous abortion occurred before prenatal diagnosis could be performed, in a woman who carried a fragile X full mutation. DNA extracted from formalin-fixed paraffin-embedded chorionic villi preparations indicated that it was a male embryo with an apparently methylated X chromosome. The previous analysis of the family showed that her daughter, who also carried a full mutation, exhibited an extremely skewed X inactivation of the normal allele (100%) and a severe fragile X phenotype. Thus, we speculate that the aberrant pattern of X chromosome methylation in this family may provoke the spontaneous miscarriage of this pregnancy that could be explained by at least partial inactivation of the unique X chromosome in a male foetus.

Spontaneous abortion occurs quite frequently in humans, and recurrent pregnancy loss is a significant problem in women's health (Christiansen 2006). Many cases of spontaneous abortion defy diagnosis and genetic factors have been proposed as a major contribution (Lanasa and Hogge 2000; Sierra and Stephenson 2006). The X chromosome inactivation (XCI) is the process in which one of the two X chromosomes present in each cell of female mammals is inactivated during early embryogenesis, to achieve dosage compensation with males (Avner and Heard 2001; Heard 2004). Initial steps of XCI involve a 'counting process', which senses the X chromosome/autosome ratio that restricts XCI to female embryos and, thereafter, the choice of which chromosome is inactivated (Morey *et al*. 2004).

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Skewed XCI leads to an expression of X-linked recessive disorders in females (Plenge *et al*. 2002; Martínez *et al*. 2005; Renault *et al*. 2007). Another form of X-inactivation called meiotic sex chromosome inactivation (MSCI), takes place in males, during spermatogenesis and is a manifestation of the general meiotic-silencing mechanism (Turner 2007). In this study, we report the spontaneous abortion of a male foetus with an apparently methylated X chromosome, and propose that anomalous inactivation of its unique X chromosome could explain nonviability of the embryo and pregnancy loss. Therefore, we strongly suggest, as a follow up, that the methylation status of the X chromosome be tested in early spontaneous abortion of males in the absence of any other known genetic or nongenetic cause.

Materials and methods

Clinical information

A pregnant female, carrier of a fragile-X full mutation was previously diagnosed in our laboratory, had a spontaneous abortion before the prenatal diagnosis could be performed. She had a prior normal pregnancy and delivered a girl (patient II-1, see figure 1) who also carried a full mutation and with an extremely skewed X chromosome inactivation (Martínez *et al*. 2005). The second pregnancy was interrupted at 13 weeks following the last menstruation, although obstetric ultrasonography showed a 7-mm foetus, without pulse, indicating delayed abortion. Formalin-fixed extra embryonic tissues were sent for anatomopathologic studies. Archival formalin-fixed paraffin-embedded chorionic villi preparations were given to us for molecular analysis.

Keywords. human genetics; X-inactivation; epigenetics; *PGK* locus methylation.

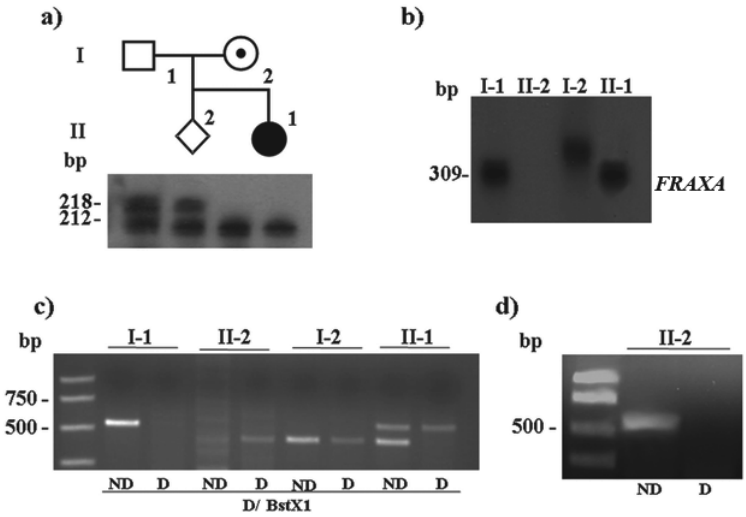

Figure 1. (a) Pedigree of the family under study and PCR of amelogenin locus. The index (II-2) is a male foetus that shows a 212 bp amplicon from the X chromosome and a 218 bp amplicon from the Y chromosome, and the same pattern was observed in his father (I-1). Both females (I-2 and II-1) carried a full mutation at the *FRAXA* locus and presented only the 212 bp amplicon corresponding to the X chromosome. (b) PCR of the *FRAXA* locus showed a normal allele present in subject I-1 that was inherited by his daughter (II-1). Patient I-2 and patient II-1 amplified only the normal allele and PCR from the foetus was unproductive (II-2), suggesting a fragile X male. (c) X-chromosome inactivation analysis. Samples were either digested (D) or nondigested (ND) with methylation sensitive restriction enzyme *Hpa*II. After PCR of the *PGK* locus, all samples (D and ND) were digested with the restriction enzyme *Bst*XI to show polymorphism. This digestion indicates that subject I-1 have a 530-bp allele and patient I-2 is homozygous for 433-bp allele. Accordingly, patient II-1 is heterozygous with both alleles, 530 bp inherited from her father and 433 bp inherited from her mother. The foetus (II-2) inherited one of the two 433 bp maternal alleles. Samples previously digested with *Hpa*II showed complete disappearance of the maternal 433 bp allele in patient II-1. As expected, the 530 bp allele of subject I-1 also disappeared in *Hpa*II digested sample. Patient I-2 is homozygous for this locus and a detectable decrease in PCR product was observed after *Hpa*II digestion. The foetus inherited either one of 433-bp alleles from his mother that was not digested by *Hpa*II enzyme. (d) DNA from the foetus was fully digested with *Msp*I restriction enzyme.

DNA analysis

DNA was extracted from peripheral blood samples in adult patients and from the archival formalin-fixed paraffin-embedded chorionic villi using the methods of Miller *et al*. (1988) and Robino *et al*. (2006), respectively, with minor modifications. The sex of the index was determined by studying the amelogenin locus using a kit (GenetPrint Sex Identification System-Amelogenin) from Promega (Promega, Madison, USA) (Buel *et al*. 1995). Inactivation of the X chromosome was performed by analysing the phosphoglycerate kinase locus (*PGK*; Uehara *et al*. 2000) previously reported as informative in this family (Martínez *et al*. 2005). To obtain methylation status, DNA samples (0.2 μg) were incubated for 12 h at 37°C in a total volume of 20 μl using 30 U of methylation-sensitive restriction endonuclease *Hpa*II to completely digest nonmethylated (active) alleles. After digestion, 5 μl of DNA samples were amplified by PCR in a total volume of 20 μl containing 1× PCR buffer, including 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM forward primer (5′-AGC TGG ACG TTA AAG GGA AGC- 3′), 0.5 μM reverse primer (5′-TAG TCC TGA AGT TAA ATC AAC- 3′), and 1 U *Taq* DNA polymerase (Roche Applied Science, New Jersey, USA). After an initial incubation of the sample for 5 min at 94°C, reactions were cycled for 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C for 33 cycles. Aliquots of the PCR product (5 μl) were digested with 30 U of restriction endonuclease *Bst*XI for 12 h at 37°C to show polymorphism. Resultant DNA fragments were electrophoresed in 2% agarose gels and stained with ethidium bromide. DNA from the foetus was also digested with the *Msp*I restriction enzyme which is insensitive to methylation. In addition, we performed a PCR from the *FRAXA* locus as previously described (Pintado *et al*. 1995).

Results and discussion

This research was performed with protocols approved by the local institutional review board and informed consent of participants and/or their guardians. The pedigree of the family under study is shown in figure 1,a. Amelogenin locus indicated that the foetus was a male with corresponding X (212 bp) and Y (218 bp) bands (figure 1,a). PCR from the *FRAXA* locus was unproductive for the foetus suggesting a fragile X male (figure 1,b); patients I-2 and II-1 showed only the normal allele.

Our previous knowledge of the anomalous X chromosome inactivation in this family (Martínez *et al*. 2005) motivated us to test the hypothesis of a possible aberrant inactivation of the unique X chromosome in the foetus as a cause for the early spontaneous abortion. The X-inactivation was studied by methylation analysis of the *PGK* locus. As indicated, all samples were digested with *Bst*XI to illustrate the polymorphism and to differentiate maternal and paternal alleles (figure 1,c). Extremely skewed X-inactivation in patient II-1 is clearly demonstrated, since after digestion with *Hpa*II, the allele inherited from the mother completely disappeared. The male foetus (II-2) inherited a maternal allele that appeared to be methylated because *Hpa*II was unable to digest DNA. Enzyme activity was confirmed by complete digestion of DNA from the father (I-1) and maternal allele of the sister (II-1) of the index. Further, foetal DNA was completely digested with *Msp*I enzyme (figure 1,d). Although the mother (I-2) was homozygous for *PGK* locus, we observed a lower productivity of PCR after *Hpa*II digestion than with the undigested sample, corresponding with the loss of half the DNA (figure 1,c). However, the same productivity was observed from the foetal sample either digested or undigested with *Hpa*II. DNA was extracted from chorionic villi supporting foetal origin, and the inability to amplify *FRAXA* locus argue against significant maternal DNA contamination. These results would also eliminate the possibility of an extra X chromosome that could explain the methylation pattern observed at the *PGK* locus and strongly suggest that this male foetus may carry a methylated and probably inactivated X chromosome that could be responsible for the early spontaneous abortion.

Pregnancy miscarriage is an important health problem and recent research has focussed on genetic markers for recurrent pregnancy loss (Sierra and Stephenson 2006). It is assumed that the process of X chromosome inactivation is stochastic, affecting only females (Carrel and Villard 1998; Boumil and Lee 2001). Nevertheless, disturbance in any step of this important epigenetic phenomenon is likely to have pathophysiological consequences in males as well. The mechanism by which the number of X chromosomes are counted, keeping only one active, and the signal that triggers silencing, remain unanswered questions (Heard 2005; Yang and Kuroda 2007). Another form of X-inactivation takes place in male spermatogenesis, which is not restricted to meiosis (Turner 2007).

Aberrant XCI in the index case (II-2) may have produced a nonviable embryo and the extreme skewed X inactivation in patient II-1 could account for her severe fragile X phenotype previously reported (Martínez *et al*. 2005). However, XCI in patient II-1 and in the foetus (II-2) are from different parental origin suggesting either two independent events, or the existence of a locus outside the X chromosome involved in the aberrant X-inactivation in this family.

It has been proposed that along with the X-inactivation centre (XIC) other loci not yet identified on the X chromosome, or on autosomes, may contribute to the correct function of the whole X-inactivation process (Carrel and Villard 1998; Clerc and Avner 2006). Anomalous cross-talk between the X and Y chromosome after fertilization, or an unbalanced X-autosome translocation may underlie the X-silencing in this male foetus (II-2). We propose that aberrant methylation of the X chromosome in males may be considered as a possible cause of spontaneous abortion that would explain pregnancy loss in the absence of other genetic or nongenetic causes. To further support this conclusion, we strongly suggest that it would be of interest to determine the activation status of the X chromosome in a series of male early spontaneous abortions.

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