Genetics and Molecular Biology, 23, 1, 11-14 (2000)

Screening fetal losses for monosomy X with a simple PCR-based procedure

Rinaldo W. Pereira[1], Rosane Sturzeneker[2] and Sérgio D.J. Pena[1,2]

Abstract

To screen for monosomy X in spontaneous fetal losses we explored a simple molecular strategy based on loss of heterozygosity at highly polymorphic X-linked loci. We developed a multiplex fluorescent procedure that allows the simultaneous amplification of five dinucleotide repeat polymorphisms in a large low-recombination region in the long arm of the X chromosome. Analysis was performed by computer-assisted laser densitometry. We did not find any instances of homozygosity at all five loci in 30 normal females tested, nor among 37 women whose typing data were retrieved from the Fondation Jean Dausset - CEPH genotype database. In addition, all cases of monosomy X previously diagnosed by conventional cytogenetics presented the anticipated loss of heterozygosity at all loci. We studied 19 spontaneously aborted female fetuses and we found four samples homozygous for the five loci (21%), in good agreement with the expected rate of monosomy X in first trimester spontaneous abortions. We conclude that the loci have high diversity and high efficiency in PCR-amplification and that our multiplex procedure constitutes a simple and useful molecular screening test for monosomy X in abortions and stillbirths.

INTRODUCTION

Spontaneous abortions are common events, occurring in approximately 15% of all human pregnancies. At least 50% of fetal losses in the first trimester of pregnancy are caused by fetal chromosomal defects (Boué and Boué, 1973; Hassold, 1986). Monosomy X (45,X) corresponds to 20-25% of all cases, triploidy to 15-20% and the several trisomies to approximately 50%.

Whenever fetal losses occur, they are often associated with psychological trauma that may involve maternal fantasies and feelings of guilt and inadequacy (see, for instance, Neugebauer *et al.*, 1997). Showing to a couple that their miscarriage was caused by a sporadic chromosomal defect (a "genetic accident") and that continuation of the pregnancy would inevitably culminate in the birth of an abnormal child, significantly helps them in coping with the fetal loss. Moreover, establishment of the specific fetal etiology for the miscarriage dispenses with the need to search for a maternal cause of the fetal loss. For these reasons, all miscarriages should be investigated cytogenetically.

Conventional cytogenetics depends on the availability of live dividing human cells that are generally only obtained after cell cultures. Culture failures are common because tissues from abortions and stillborns are often frozen or fixed in formalin or alcohol, or are contaminated with bacteria (Hassold *et al.*, 1980). Thus, there is great need for procedures that will allow cytogenetic diagnoses in non-dividing human tissues, including specimens that have been previously processed for pathology. Moreover, it would be highly desirable that these procedures could be performed quickly and did not depend on inordinate expertise. With the utilization of computer-assisted laser densitometry it is possible to use polymerase chain reaction (PCR)-based tests to achieve the rapid, simple and inexpensive molecular diagnosis of human chromosomal disorders in non-dividing and even in non-living human tissues (Pena, 1998). Based on this, we developed a multiplex PCR procedure for the study of DNA extracted from tissues of fetal losses that effectively allows the establishment of the fetal sex and the diagnosis of triploidy and trisomies 13, 16, 18 and 21 in 100% of the cases examined (Pena, 1998). To increase the efficiency of this molecular cytogenetics procedure we needed to develop a molecular screening test for monosomy X, which is responsible for a sizeable proportion of fetal losses.

We reasoned that the simplest molecular strategy for monosomy X would be to detect loss of heterozygosity at highly polymorphic X-linked loci. For that, we utilized a multiplex procedure that permits the simultaneous amplification of five dinucleotide repeat polymorphisms in a large low-recombination region in the long arm of the X chromosome. We showed that the loci have high diversity and high efficiency in PCR amplification. Therefore, this multiplex procedure constitutes a simple and useful molecular screening test for monosomy X in abortions and stillbirths.

MATERIAL AND METHODS

DNA samples

DNA was prepared from blood samples of seven patients diagnosed as having a 45,X karyotype by conventional cytogenetics and from 30 healthy fertile females. We also extracted DNA from 19 spontaneous female fetal losses in

[1]Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, Caixa Postal 486, 30161-970 Belo Horizonte, MG, Brasil. Send correspondence to S.D.J.P. Fax: +55-31-499-2984. E-mail: spena@dcc.ufmg.br
[2]GENE - Núcleo de Genética Médica, 30130-909 Belo Horizonte, MG, Brasil.

the first (17 cases) or second (2 cases) trimester of pregnancy. These materials, received for diagnosis at GENE - Núcleo de Genética Médica, had been obtained by uterine curettage and thus contained tissues of both maternal and fetal origin. The samples were dissected previous to DNA purification by proteinase K treatment and phenol/chloroform extraction. Fetal sex was determined by PCR using the amelogenin loci in the X and Y chromosomes as described previously (Pena, 1998). Only when the sex was female did we perform further testing with the X-linked microsatellites.

Microsatellites

The dinucleotide repeat microsatellites *DXS995*, *DXS8076*, *DXS1002*, *DXS8114* and *DXS1050* were chosen in a region with very low recombination of the human X chromosome (Nagaraja *et al.*, 1997). The primers used to amplify the markers were those published by Dib *et al.* (1996), with exception of the reverse primer of *DXS995* to which was added a tail of ten adenine residues in order to increase the amplicon size and avoid overlap with alleles of the locus *DXS8114* in the multiplex analysis. In all amplifications one of the primers was labeled with the fluorescent tags FITC or Cy5. The PCR conditions were optimized to simultaneously amplify the five markers in the same reaction. The PCR reactions were performed in a total volume of 12.5 µl, containing 20-50 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM dNTPs, and 0.25 U of *Taq* polymerase. The 1X amplification buffer contained 10 mM Tris base, pH 9, 50 mM KCl, 0.1% Triton X100 and 0.01% gelatin. Amplifications were carried out through 30 cycles, each one consisting of 30 s at 94°C, 30 s at 55°C and 72°C for 2 min. In the last cycle, the elongation was prolonged by 5 min.

Computer-assisted laser densitometry

After amplification, the PCR products were diluted ten-fold and 2 µl was added to 5 µl of deionized formamide containing 5 mg/ml Dextran Blue 2000 (Pharmacia) and an internal molecular size standard (300 bp). This mix was denatured at 95°C for 3 min and run in a Pharmacia A.L.F. or ALF-Express sequencer (Pharmacia, Uppsala, Sweden) using 0.5-mm spacers and standard running conditions. The fragments were analyzed using the Allele-Links program (Pharmacia). An allelic ladder was made using a DNA pool of 100 unrelated Brazilian males.

RESULTS

We chose five dinucleotide repeat microsatellite loci (*DXS995*, *DXS8076*, *DXS1002*, *DXS8114* and *DXS1050*) in a region of very low recombination rate in the human X chromosome (Nagaraja *et al.*, 1997). In the families typed for these microsatellites in the Fondation Jean Dausset - CEPH genotype database, no recombination was seen between these loci in 291 meiosis and no homozygous haplotype was seen in 37 unrelated women (Dib *et al.*, 1996). The loci amplified very efficiently in a single PCR reaction and were amenable to single-tube multiplex amplification and single-run electrophoretic analysis. As shown in the graph of the electrophoretic run the products are

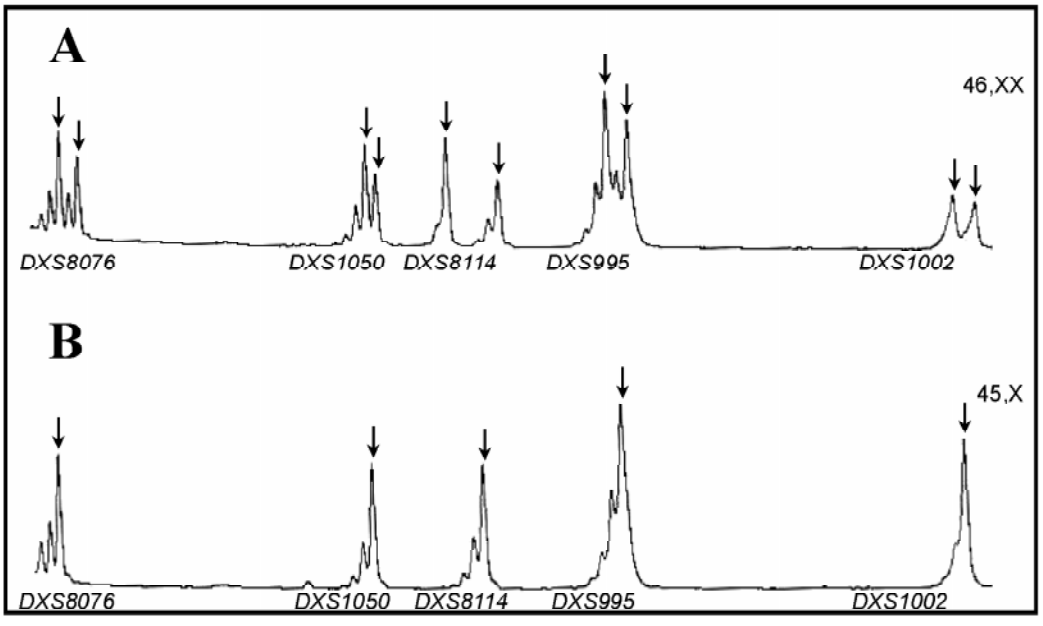

Figure 1 - Diagnosis of monosomy X using dinucleotide microsatellites from a low recombination region of the X chromosome. **A**, Normal female heterozygous at all 5 microsatellites. **B**, 45,X patient with loss of heterozygosity. The arrows indicate the alleles at each locus.

well spaced and can be easily typed individually without ambiguity (Figure 1).

To test the variability of the multiplex procedure we typed 30 unrelated normal women and all of them were heterozygous in at least one of the loci. Elsewhere we will also present data that demonstrate that these five loci have a haplotypic diversity larger than 99.5% in Caucasian, African, Asian and Amerindian populations (Pereira, R.W. and Pena, S.D.J., unpublished results). In contrast, seven samples from Turner syndrome patients previously diagnosed as 45,X by conventional cytogenetics consistently showed homozygosity at all loci (Figure 1B).

Among 19 spontaneously aborted fetuses, we found four samples homozygous for the five loci (21%). We expect to see monosomy X in 20-24% of all female first trimester abortions (this is because 50% are chromosomically abnormal and of these, since we were dealing exclusively with female fetuses, we would expect 40-48% to have monosomy X; Hassold, 1986). Thus, we could conclude that our results were in good agreement with general expectations.

DISCUSSION

The simplest and most obvious strategy for the molecular diagnosis of monosomy X is to use polymorphic loci on the X chromosome and to look for loss of heterozygosity. For instance, Gicquel *et al.* (1992) used Southern blotting with the M27β probe, which recognizes a hypervariable (88% heterozygosity) minisatellite region mapping to Xcen-p11.22 (Fraser *et al.*, 1989), to diagnose Turner syndrome. The advantage of microsatellites over minisatellites is their greater simplicity of study using the PCR and applicability to degraded DNA samples, which is especially critical when studying fetal loss specimens. However, in comparison with minisatellites, microsatellites have lower informativeness. Thus, we need to type several polymorphic microsatellites to obtain the necessary discrimination power to replace the more informative minisatellites. Since undertaking several separate microsatellite PCR amplifications is a cumbersome, time-consuming endeavor, we decided to use a multiplex procedure based on the simultaneous PCR amplification and electrophoretic analysis of five microsatellite loci. The development of highly informative multiplex systems has been greatly facilitated by the availability of fluorescent DNA sequencers that detect allele sizes on real time as they migrate through a laser beam. We report here the use of a single-tube multiplex PCR set of five polymorphic microsatellites for the diagnosis of monosomy X in fetal losses.

Application of this microsatellite multiplex set for detection of monosomy X in spontaneous fetal losses provides a reliable molecular screening test. In theory, false positives could occur whenever a 45,XX female was by chance homozygous at all five loci, but this situation seems to be rare. We did not see any uniformly homozygous females in 30 tested normal women nor among 37 females whose typing data could be retrieved from the CEPH database (Dib *et al.*, 1996). Moreover, in a large population study with these five loci in Caucasian, African, Asian and Amerindian populations we found a haplotypic diversity exceeding 99.5% (Pereira, R.W. and Pena, S.D.J., unpublished results). Let us assume that 50% of first trimester fetal losses have chromosomal etiology and that monosomy X is responsible for 24% of them (Hassold *et al.*, 1980), together with a false positive rate of 0.5%. Straightforward application of Bayes' theorem allows us to calculate that, given homozygosity at all five loci, the probability of a first trimester spontaneously aborted female fetus having monosomy X is 98.4%. Confirmation of diagnosis, if wished, can be achieved with the use of more X-linked polymorphic loci, or the genotypes of the parents can be established at the five microsatellites used in this study.

We tested 7 known cases of monosomy X and diagnosed correctly 100% of them. False negative cases could occur in cases of mosaicism or whenever maternal tissues were present in the tested material. The former situation is very rare. Among the 112 cases of monosomy X diagnosed by Hassold *et al.* (1980) in first trimester spontaneous abortions, only 1 was a mosaic and it was 45,X/46,XY. However, contamination of fetal material by maternal blood or decidua might represent a potential cause of false-negative results.

Approximately half of all clinically recognized postnatal cases of Turner syndrome have an apparently nonmosaic 45,X constitution while the remainder are mosaics with a 45,X line and/or a structurally abnormal X chromosome (Jacobs *et al.*, 1997). The screening test developed by us is based on loss of heterozygosity and thus is not ideally suited for diagnosis of Turner syndrome after birth, since we could predict false negatives, even if we performed quantification of the microsatellite peaks by computer-assisted densitometry (Pena, 1998). However, due to its simplicity and quick results (less than 12 h when necessary), the multiplex X chromosome microsatellite test could be used as a preliminary screening when a rapid diagnosis of monosomy X is needed or desired, especially in the neonatal period.

ACKNOWLEDGMENTS

This research was supported by grants from FAPEMIG and CNPq. R.W.P. is the recipient of a Ph.D. fellowship from CAPES.

RESUMO

Para a detecção molecular de monossomia X em perdas fetais espontâneas, nós exploramos uma estratégia baseada em perda de heterozigosidade, desenvolvendo um sistema multiplex fluorescente que permite a amplificação simultânea de cinco microssatélites em uma região com baixa recombinação no cromossomo X. A análise

foi então feita por densitometria a laser assistida por computador. Nenhum caso de homozigosidade em todos os cinco locos foi encontrado em 30 mulheres normais estudadas, nem em 37 mulheres cuja tipagem foi extraída do banco de dados do CEPH. Além disso, todos os casos de monossomia X previamente diagnosticados por citogenética convencional apresentaram a prevista perda de heterozigosidade. Quando estudamos 19 casos de perdas fetais femininas do primeiro trimestre da gravidez, encontramos quatro amostras uniformemente homozigotas em todos os locos (21%), de acordo com a proporção esperada de casos de monossomia X em perdas fetais do primeiro trimestre. Concluímos que o sistema multiplex que nós desenvolvemos apresenta alta diversidade e alta eficiência de amplificação pela PCR e constitui um método simples e útil de triagem para monossomia X em abortamentos e natimortos.

REFERENCES

Boué, A. and **Boué, J.** (1973). Evaluation des erreurs chromosomiques au moment de la conception. *Biomedicine 18*: 372-377.

Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J. and **Weissenbach, J.** (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature 380*: 152-154.

Fraser, N.J., Boyd, Y. and **Craig, I.** (1989). Isolation and characterization of a human variable copy number tandem repeat at Xcen-p11.22. *Genomics 5*: 144-148.

Gicquel, C., Cabrol, S., Schneid, H., Girard, F. and **Le Bouc, Y.** (1992). Molecular diagnosis of Turner's syndrome. *J. Med. Genet. 29*: 547-551.

Hassold, T. (1986). Chromosome abnormalities in human reproductive wastage. *Trends Genet. 2*: 105-110.

Hassold, T., Chen, N., Funkhouser, J., Jooss, T., Manuel, B., Matsuura, J., Matsuyama, A., Wilson, C., Yamane, J.A. and **Jacobs, P.A.** (1980). A cytogenetics study of 1000 spontaneous abortions. *Ann. Hum. Genet. 44*: 151-178.

Jacobs, P., Dalton, P., James, R., Mosse, K., Power, M., Robinson, D. and **Skuse, D.** (1997). Turner syndrome: a cytogenetic and molecular study. *Ann. Hum. Genet. 61*: 471-483.

Nagaraja, R., MacMillan, S., Kere, J., Jones, C., Griffin, S., Schmatz, M., Terrell, J., Shomaker, M., Jermak, C., Hott, C., Masisi, M., Mumm, S., Srivastava, A., Pilia, G., Featherstone, T., Mazzarella, R., Kesterson, S., McCauley, B., Railey, B., Burough, F., Nowotny, V., D'Urso, M., States, D., Brownstein, B. and **Schlessinger, D.** (1997). X chromosome map at 75-kb STS resolution, revealing extremes of recombination and GC content. *Genome Res. 7*: 210-222.

Neugebauer, R., Kline, J., Shrout, P., Skodol, A., O'Connor, P., Geller, P.A., Stein, Z. and **Susser, M.** (1997). Major depressive disorder in the 6 months after miscarriage. *J. Am. Med. Assoc. 277:* 383-388.

Pena, S.D.J. (1998). Molecular cytogenetics I: PCR-based diagnosis of human trisomies using computer-assisted laser densitometry. *Genet. Mol. Biol. 3:* 317-322.

(Received February 25, 2000)