Molecular Study of X-Chromosome Mosaicism in Turner Syndrome Patients using DNAs Extracted from Archived Cytogenetic Slides.

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To study the X chromosome mosaicism in the cytogenetically pure 45,X Turner syndrome patients, we applied PCR technique using DNAs extracted from archived cytogenetic slides. We amplified the DNAs using nested primers targeted to a highly polymorphic short tandem repeat(STR) of the human androgen receptor gene(HUMARA) for the detection of X chromosome mosaicism. This assay is a very sensitive and useful method which can be applied to the DNAs extracted from archived cytogenetic slides to detect X mosaicism. We have tested 50 normal Korean females to determine whether the HUMARA locus is highly polymorphic among Koreans. 85% of Korean population showed heterozygosity in the HUMARA locus. We analysed the 24 DNAs extracted from archived slides of patients and abortuses with Turner syndrome in cytogenetic analysis. We observed the heterozygosities of 50% from pure 45,X patients, 83% from the patients with mosaic Turner syndrome and 8.3% from the abortuses of pure 45,X. Using the PCR technique of the HUMARA locus in the archived cytogenetic slides, we detected X chromosome mosaicism which could not be detected in cytogenetic analysis.

Key words : Humara gene, Turna syndrome, mosaicism, archived cytogenetic slide, heterozygosity

INTRODUCTION

Turner syndrome is a chromosomal abnormality syndrome affecting one in 2,500-10,000 live-born females (Robinson, 1990). In about 99% of the cases where 45,X is present at the time of human conception, a natural miscarriage occurs in the first stages of embryonic development (Hook and Warburton, 1983; Hassold *et al.*, 1988). Only 1% of these fertilizations is taken successfully to term and generally displays the characteristics of Turner syndrome (Turner, 1938). Among the live-born Turner syndrome patients, only 50% are pure 45,X, whereas the rest are mosaics, mostly with normal or structurally abnormal X chromosomes, but sometimes with structurally Y chromosomes (Hook and Warburton, 1983; Hassold *et al.*, 1988). This has led to the widely held hypothesis that, in order for a 45,X conceptus to be viable, she must possess another cell line at least in some critical organs or at a critical period during embryogenesis (Hook and Warburton, 1983; Hassold *et al.*, 1988). Clinically, unlike other chromosomal abnormality syndromes, even pure 45,X patients have markedly variable phenotypes: short height, gonadal dysgenesis, and anatomical malformations such as pterygium colli and cubitus valgus (Turner, 1938). One of the possible explanations for these phenotypic variations is undetected mosaicism in these patients, since a small percentage of mosaicism can not be detected by conventional cytogenetic techniques (Tohru *et al.*, 1997).

In this study we have used PCR-based HUMARA assay, which amplifies a highly polymorphic CAG repeat in the human androgen receptor gene (HUMARA) at Xq12 (Tohru *et al.*, 1997; Allen *et al.*, 1992) to detect low-level X chromosome mosaicism among cytogenetically 45,X patients and abortuses using DNAs extracted from archived cytogenetic slides.

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MATERIALS AND METHODS

Subjects

We have tested 50 normal Korean females for the study of Korean population and studied the slides of 12 patients and 12 abortuses (1995-1999) which had been cytogenetically diagnosed as Turner syndrome. The karyotypes were determined by chromosome analysis (G-banding) of peripheral blood leukocytes (Table 1) or chorionic villus samplings. At least 30 cells were scored for karyotyping.

Extraction of DNA from 50 normal Korean females

Genomic DNAs were isolated from peripheral blood leukocytes according to the standard techniques (Sambrook *et al.*, 1989).

Extraction of DNA from archived cytogenetic slides

The archived slides were soaked in xylene to remove the immersion oil and were treated in 100% ethanol for 5 min three times. Each slide surface was added with 100 μl lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5% Tween 20; Sago *et al.*, 1996) and was sealed with a 25 mm × 55 mm rectangular piece of parafilm and left at 56℃ for 15 min. After removal of parafilm, 200 μl water was added to each slide and cellular material was scraped off by a single edge surgical steel razor blade. Suspended cellular materials was collected into a 0.6 ml microcentrifuge tube and centrifuged at 3,000g for 5 min. After discarding the supernatant, the cell pellet was resuspended in 200 μg/ml proteinase K in 50 μl lysis buffer and incubated at 56℃ for 45 min. Proteinase K was inactivated at 95℃ for 10 min (Choi *et al.*, 1999).

Polymerase chain reaction

PCR was performed in 20 μl reaction mixture containing 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1,5 mM $MgCl_2$, 250 μM each dNTP, 10 pmol of each primer and 0.5 units Taq DNA polymerase (Amplitaq Gold, 5 U/μl, PE). Each 1 μl archival slide DNA and 1 ng genomic DNA were used to amplify the CAG repeats in the HUMARA gene. For the detection of low-level X chromosome mosaicism, we used nested PCR. The sequences of the primers were as follows: forward primer (HUMARA-1) 5'-TCCAGAAT CTGTTCCAGAGCGTGC-3', reverse primer (HUMARA-2) 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'. The polymerase was activated at 95℃ for 10 min. PCR was done under the following conditions: 94℃ for 45 sec, 60℃ for 30 sec and 72℃ 30 sec for 30 cycles. The 1 μl aliquots of PCR products were used for the second cycle of nested PCR. The primer sequences for nested PCR were 5'-GTGCGCGAAGTGATCCAGAA-3' (HUMARA-3) and 5'-CCAGGACCAGGTAGCCTGTG-3' (HUMARA-4). Nested PCR was performed under the same conditions as for the first PCR, except that the annealing temperature was 62℃. The products of second PCR were analysed by electrophoresis through 10% polyacrylamide gels and staining with ethidium bromide.

Table 1. Karyotypes of Turner syndrome patients

No. of patients	Karyotype
1	45,X/46,Xdel(Xp)
2	45,X/46,X,i(X)(q10)
3	45,X/46,X,r(X)
4	45,X
5	45,X/46,X,i(Xq)
6	45,X
7	45,X
8	45,X
9	45,X/46,XX
10	45,X
11	45,X/46,X,i(Xq)
12	45,X

RESULTS

We first tested 50 normal Korean females to determine whether the HUMARA locus is highly polymorphic among the Koreans. The locus was highly polymorphic among

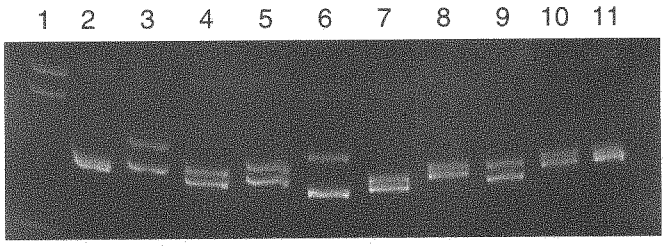

Figure 1. Example of HUMARA genotypes.
Lane 1: 100bp DNA ladder,
Lane 2 to 11: ten different Korean women

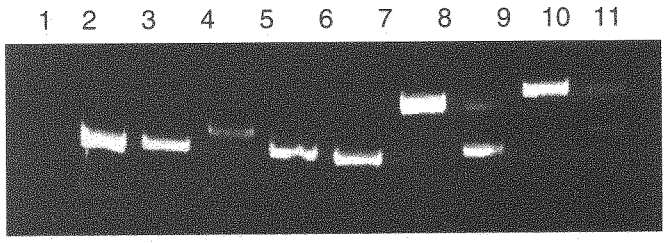

Figure 2. DNA analysis of Turner patients and abortuses.
Lane 1: 100bp DNA ladder, Lane 2, 3, 4, 7, 8, 9 and 10 : Turner patients, Lane 5, 6: abortuses.

these Korean females with an heterozygosity of 85%. When the HUMARA locus was amplified with DNA from a normal female subject, two bands of similar intensity were obtained (Fig. 1).

In the PCR study of DNAs extracted from archived cytogenetic slides, we observed the heterozygosities of 50% (3/6) from the patients with pure 45,X karyotype and 83% (5/6) from the patients with mosaic Turner syndrome. And 8.3%(1/12) heterozygosity was detected from abortuses of pure 45,X karyotype (Fig. 2).

DISCUSSION

Although nearly 99% of 45,X conceptuses abort spontaneously, among the live-born Turner syndrome patients, about 50% are pure 45,X (Hook and Warburton, 1983; Hassold *et al.*, 1988). The high percentage of fetal and embryonic miscarriage for karyotype 45,X points to the necessity of mosaicism for survival (Held *et al.*, 1991, 1992). Many authors have suggested that 45,X monosomy is lethal and that most surviving 45,X individuals are actually mosaics undetected by cytogenetic analysis (Held *et al.*, 1991, 1992; Hassold *et al.*, 1992). However, a small percentage of mosaicism can not be detected by conventional cytogenetic techniques, since this type of analysis would require a very large number of cells.

We detected the heterozygosity of 50% using the PCR from the slides of pure 45,X patients who had been analysed by conventional cytogenetic method. We can suggest several reasons for this high rate of our result. First, we had analysed about 30-50 cells of metaphase of the peripheral blood leukocytes routinely. However, rare mosaicism can not be detected precisely by 30-50 chromosomal analysis of metaphase cells. There is a report that repeated chromosomal analysis of a large number of metaphases of all 6 patients in the 45,X group has confirmed the presence of low-level 46,XX cell line in lymphocytes of 5 patients and repeated chromosomal analysis of 155 metaphase cells of a patient has confirmed the presence of a low-level 45,X cell line in 9% of cells (Nazarenko *et al.*, 1999). Therefore, it is possible that reproductive loss and abnormalities of sexual development in some females may be caused by undetected low-level X monosomy mosaicism since standard cytogenetic analysis missed these patients, owing to its insensitivity to detect cell lines with a proportion of 45,X cells less than 10% (Nazarenko *et al.*, 1999). Thus our results indicate that in cases when mosaicism with a low-level frequency is suspected (less than 10%), the results of standard metaphase analysis should be supplemented with additional studies such as PCR or FISH. Second, the prevalence of mosaicism for Turner syndrome patients depends of the methods used and the number of tissues screened (Nazarenko *et al.*, 1999; Lespinasse *et al.*, 1998). The application of molecular techniques, such as fluorescence in situ hybridization (FISH) and the polymerase chain reaction (PCR), substantially improves the detection of low-frequency cell lines and possible structural alterations (Rosa *et al.*, 1996). There is a report that the use of FISH analysis of interphase nuclei of tissues from different germ layers (lymphocytes from mesoderm and buccal epithelial cells from ectoderm) improves the accuracy of detection of low-level mosaicism. FISH studies on interphase nuclei reveled that 29% of patients with a pure form of monosomy X detected by metaphase analysis are, in fact mosaics (Nazarenko *et al.*, 1999). The majority of patients (92%) with mosaic form of Turner syndrome have considerable tissue-specific differences in level of X aneuploidy (Nazarenko *et al.*, 1999) and the phenotypic variation (growth and pubertal development) can be explained by tissue specific mosaicism (Lespinasse *et al.*, 1998).

As we observed 85% of heterozygosity in normal Korean female population at the HUMARA locus using PCR, we applied this method for the detection of low-level X chromosome mosaicism. In the presence of a second X chromosome with a different HUMARA allele, the second band is detected by PCR more sensitively than other molecular techniques. Since the locus is highly polymorphic (Tohru *et al.*, 1997; Desmarais *et al.*, 1998; Edwards *et al.*, 1991), the chance of the second X chromosome having a different allele is high. Since HUMARA locus is located at Xq12 between the centromere and the X-inactivation center at Xq13, even the second, structurally abnormal X chromosome would probably retain the locus. Indeed, Xq11.2-q21 is retained in almost all structurally abnormal X chromosomes (Pettigrew *et al.*, 1991). In the analysis of abortuses, we observed a second X chromosome allele in only one abortus. Therefore, most surviving 45,X individuals might be actually mosaics undetected by cytogenetic analysis.

In this study, we extracted DNAs from archived cytogenetic slides for the PCR analysis. With the continuous development and improvements in the genetic analysis method, the archival slides have been found to be a great source for genetic analysis (Choi *et al.*, 1998, 1999). Generally, there are reports which assert that the DNA quality is comparable for fresh slides and archival slides which are about 5 years old. However, there are

also reports which assert that for older stained slides, choice of PCR primers, target DNA size and amplification condition of PCR are important factors (Sago *et al.*, 1996). Therefore, HUMARA assay is a very sensitive and useful method which can be applied to the DNAs extracted from archived cytogenetic slides to detect low-level X chromosome mosaicism. We have shown that archival slides can be used effectively and efficiently requiring only low costs maintenance with great benefits.

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