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Detection of cryptic Y chromosome mosaicism by coamplification PCR with archived cytogenetic slides of suspected Turner syndrome

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Abbreviation: TS, Tuner syndrome; PCR, polymerase chain reaction; GBY, gonadoblastoma locus on the Y chromosome; FISH, fluorescent *in situ* hybridization

Abstract

Turner syndrome is one of the most common cytogenetic abnormalities. It is known that the Y chromosome or Y derived material is present in 6-9% of TS patient and it may develop a high risk of gonadoblastoma in 15-25%. So it is crucial to carry out cyto genetic analysis and Y-specific probe studies for all persons with gonadal dysgenesis to rule out mosaicism with Y-bearing cell line; eg 45,X/46,XY. In this study, 26 archival slides previously analyzed cytogenetically as 45,X, 45,X/46,X,i(X), 45,X/46,X,r(X), and 45,X/46,XX were examined. Coamplification PCR, having the advantage of providing rapid result and confirming PCR failure, was performed with the slide samples in the regions of dystrophin gene in Xp21and DYZ3 in the Y centromeric region. All of archived slides were positive for X-specific gene and one slide of 45,X was found to have the cryptic Y chromosome material. Our result suggests that the archived cytogenetic slides could be applied for the detection of Y chromosome rapidly and efficiently in TS patients.

Keywords: archived slide, coamplification PCR, Turner syndrome, cryptic Y chromosome

Introduction

Molecular analysis technique using small number of cells, paraffin embedded tissues, archived cytogenetic slides, the blood stain, and denatured aged-tissue samples has been developed recently. Especially, archived cytogenetic slides can be easily used for a retrospective study when the patient had already passed away or further sampling was not available for the DNA analysis. Sago *et al*. (1996) identified a heterozygous point mutation from archived cytogenetic slides using PCR and direct DNA sequencing. Choi *et al*. (1999) detected the origin of marker chromosome by 10 independent PCR amplification reaction from a Giemsa-stained cytogenetic slide.

Turner syndrome (TS) is a chromosomal abnormality syndrome affecting one in 2,500-10,000 live-born female. Approximately 40-50% of the chromosomal aberration associated with TS is monosomy X (45,X), and the rest of the patients have a variety of other X chromosomal anomalies including several mosaicisms (Zinn *et al*., 1993). Cytogenetic analysis detects the Y chromosome mosaicism in 6-9 % of TS patients (Ostrer and Clayton, 1989). Routine analysis by conventional number of 30 metaphases may miss if the Y chromosome material is present in only a few cells. The detection of Y chromosome sequences in TS patients is important because the risk of developing gonadoblastoma or dysgerminoma is as high as 30% (Salo *et al*., 1995).

The recent development of molecular DNA studies should provide more sensitive means for detection of Y chromosome DNA. Coamplification PCR has an advantage of obtaining rapid result and detecting PCR failure (Choi *et al*., 1998). We tried to identify previously unrecognized Y chromosome materials by coamplification PCR with X- and Y-specific primers. Because the presence of centromeric region on the Y chromosome implies the risk of gonadoblastoma development (Page, 1994), PCR targeting sites near or at the Y centromere are suitable for gonadoblastoma risk screening in TS. But a competition between the two primers for the two target regions due to different annealing temperature required by two primers need to be resolved.

This study was carried out to develop a method to assess the prognosis of gonadoblastoma development by utilization of the cytogenetic archived slide. Our findings demonstrated the usefulness of archived cytogenetic slides prepared from suspected TS patients, by coamplification PCR in detecting the presence of Y specific sequences to defined regions of the Y chromo-

some.

Materials and Methods

Archived cytogenetic slides

Twenty-six archival cytogenetic slides from the amniocytes (4 cases), lymphocytes (6 cases), chorionic villi cells (15 cases), and skin tissue (1 case) were included in this study. All of the cytogenetic slides have been stored at room temperature from 1 to 6 years. Chromosome analysis was previously performed in 30 metaphases with GTG banding. Each slide was diagnosed as 45,X (21 cases), 45,X/46,X,i(X) (2 cases), 45,X/46,X, r(X) (1 case), and 45,X/46,XX (2 cases).

Extraction of DNA from archived slides

To remove the immersion oil, the cytogenetic slides were soaked in xylene for 2 days at room temperature. Giemsa-stained slides were destained in 100% ethanol for 5 min three times. The cellular materials were hydrated by distilled water for 5 min three times. The surface of each slide was spread with 100 μl digestion buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween 20; Chan *et al*., 1996), covered with a 25 mm × 55 mm rectangular piece of parafilm and left for 15 min at 56°C incubator. After removal of parafilm, 100 μl distilled water was added to each slide and carefully scraped off softened cellular materials using a surgical steel razor blade under the clean bench**.** Suspended cellular materials were collected into a 0.6 ml microcentrifuge tube and centrifuged at 3,000 g for 5 min. After discarding the supernatant, cell pellets were resuspended in 200 μg/ml proteinase K in 50 μl digestion buffer and incubated for 3 h at 56°C. Proteinase K was inactivated at 95°C for 10 min. Extracted DNA samples were used immediately for PCR.

Screening for Y chromosome mosaicism

All of the 26 archived cytogenetic slides were screened for the presence of Y chromosome using the coamplification PCR with two primers, exon 46 of dystrophin gene in Xp21 (Prior *et al*., 1995) and DYZ3 in the Y centromeric region (Witt and Erickson, 1989) (Table 1). In the first step, a master mixture was prepared containing PCR buffer, 2.5 mM $MgCl_2$, 400 mM dNTPs, 200 nM of each primers and 1U of Gold AmpliTaq (Perkin Elmer Cetus). The coamplification PCR was performed by 40 cycles under the following conditions: 30 s at 95°C, 20 s at 54°C and 30 s at 72°C. After PCR amplification, all PCR products were run on a 8.0% polyacryamide gel and detected. In all experiments, PCR assays were carried out at least twice and performed simultaneously with DNA extracted from normal male slide, normal female slide, and blank as controls.

Table 1. Sequence, locus, and product size of the oligonucleotide primers

primer	sequence	locus	product size
Ya1.1	5'-ATGATAGAACGGAAATATG-3'	Ycen	170 bp
Ya1.2	5'-AGTAGAATGCAAAGGGCTCC-3'		
Ex46f	5'-GCTAGAAGAACAAAAGAATATCTT-3'	Xp21	148 bp
Ex46r	5'-CTTGACTTGCTCAAGCTTTTCTTT-3'		

Results

By coamplifing the centromere (DYZ3) gene for Y chromosome and dystrophin gene (exon 46) for the X chromosome, all 26 archived slides from 1 to 6 years old were successfully determined to have 148 base pair X-specific fragments and 25 of them could be detected to the absence of Y chromosome materials simultaneously. However, one archived slide (case 8) prepared from chorionic villi in 1996, proven to be pure 45,X in cytogenetic analysis, was identified to have the Y-centromere region (lane 4 in Figure 1). The cytogenetic and molecular findings of 26 archived slides are summarized in Table 2. These results revealed approximately 4% frequency of Y-derived sequence from 26 archived slides which were analyzed as pure 45,X, mosaic 45,X/46,X, i(X), 45,X/46,X,r(X), or 45,X/46,XX karyotype. We included cytogenetically normal male (46,XY) and female (46,XX) slide as controls to each of our test groups to exclude the possibility of false positive and negative findings. In the slides of normal male and female, we obtained two bands of the amplified products and one band fragment respectively. No band was visualized in the blank control (Figure 1).

Discussion

We detected the presence of Y chromosome material by coamplification PCR from cytogenetic slides that were

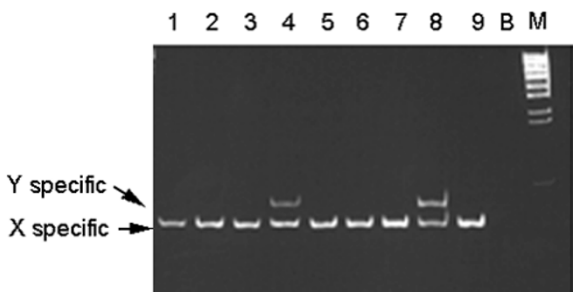

Figure 1. DNA analysis of archival cytogenetic slides by coamplification PCR (X and Y specific region). The 170 and 148 base pair DNA fragments were detected in one case of 45,X slide (lane 4) and 46,XY male control. Only 148 base pair was visualized in six cases of 45,X slides and 46,XX female control. Lane 1-7: seven 45,X slides, lane 8: control male slide, lane 9: control female slide, lane B: blank and lane M: 100bp DNA marker.

Table 2. Summary of specimen, date, karyotype, and DNA analysis of the 26 archived cytogenetic slide. Each slide was diagnosed by 30 G-banding metaphases. A = amniocytes; B = lymphocytes; C = chorionic villi; S = skin; + = presence; - = absence

Case	Specimen	Date	Cytogenetic Result	DNA analysis	
				X specific	Y specific
1	C	1997	45,X	+	-
2	C	1997	45,X	+	-
3	C	1995	45,X	+	-
4	A	1997	45,X	+	-
5	S	1997	45,X	+	-
6	B	1998	45,X/46X,i(X)	+	-
7	C	1997	45,X	+	-
8	**C**	**1996**	**45,X**	**+**	**+**
9	C	1997	45,X	+	-
10	C	1996	45,X	+	-
11	A	1998	45,X/46,XX	+	-
12	B	1995	45,X/46,X,r(X)	+	-
13	B	1995	45,X/46,X,i(X)	+	-
14	B	1996	45,X	+	-
15	B	1998	45,X	+	-
16	C	1994	45,X	+	-
17	C	1998	45,X	+	-
18	B	1995	45,X/46,XX	+	-
19	C	1997	45,X	+	-
20	A	1995	45,X	+	-
21	C	1998	45,X	+	-
22	C	1996	45,X	+	-
23	C	1999	45,X	+	-
24	C	1997	45,X	+	-
25	A	1999	45,X	+	-
26	C	1999	45,X	+	-

fixed, baked, Giemsa stained, and stored for varying lengths of time ranging from a few weeks to 6 years (Table 2). Our study indicates that existing archived cytogenetic slides prepared from amniocytes, chorionic villi, lymphocytes and skin tissues could be maintained indefinitely as a valuable resource for forensic, scientific, and medical studies. Storing cells on cytogenetic slides would be less expensive than storing them in liquid nitrogen or extracting DNAs. Fixed cells stick tenaciously to glass slides, minimizing an anticipated cross contamination. And at least 10 PCR reactions with unequalled primers can be completed with a single DNA slide (Sago *et al*., 1996; Choi *et al*., 1999).

The current results confirm that it is possible to diagnose the presence of Y chromosome materials using simultaneous amplification of X- and Y-chromosome specific DNA by PCR. Coamplification PCR is a powerful tool used to avoid misdiagnosis, detect PCR failure, and acquire correct results rapidly.

The study of fragments from Y chromosome in females with gonadal dysgenesis is important, since the risk of developing gonadal cancers is increased by 30% (Wolman *et al*., 1985; Verp and Simpson, 1987). It is known that gonadoblastoma is caused by a gene, GBY, present on the Y chromosome. In normal males this gene may play a role in spermatogenesis but predisposes to tumor formation in dysgenetic gonads. Although numerous studies have attempted to localize GBY, the exact location of GBY is unknown at present. Page (1994) reported that the presence of the centromeric region of Y chromosome implies the risk of gonadoblastoma development, and Tsuchiya *et al*. (1995) have presumed that the GBY gene laid around the centromeric region or within interval three on the Y chromosome short arm.

Using the Y centromere probe, DYZ3, which the detection limit is close to 1 in 10,000 cells (Yorifuji *et al*., 1997), we identified from one archived slide the differences between the initial diagnosis based on cytogenetic study and the diagnosis determined by the application of coamplification PCR. We have assumed that the possibility of cryptic Y mosaicism is very high in our case. Because a segregating Y chromosome should retain the centromere, except on rare translocations to another chromosome, and each slide should detect 10% mosaicism with 95% confidence if thirty metaphase spreads were routinely analysed (Hook, 1977).

Recent reports have shown that 4-30% of 45,X patients are positive for some parts of Y chromosome. Some groups have reported a high percentage of patients to be positive for the Y chromosome sequence. Other studies showed that a few patients were positive for the Y chromosome sequences. For example, Coto *et al*. (1995) detected the Y sequence in 11 out of 18 patients (61%) with a 45,X or 46,X, +mar karyotype. But Medlej *et al*. (1992), Binder *et al*. (1995), and Quilter *et al*. (1998) identified karyotypically unrecognized Y chromosome materials in 1/40 (2.5%), 2/53 (3.8%), and 1/50 (2%) TS patients, respectively. Our result shows the Y chromosome material is present in 1 of 26 (3.8%) archived slides analyzed as 45,X or mosaic 45,X/46,XX cytogenetically. The frqency of detection of Y chromosome mosaicism in TS varied widely because of higher sensitivity of the methodology.

Fluorescent *in situ* hybridization (FISH) analysis has been used to confirm the initial positive DNA findings by Y-specific PCR (Patsalis *et al*., 1998; Robinson *et al*., 1999). This reliable and sensitive methdology enables to identify the presence of Y chromosome material in TS patients. Thus it is able to detect that the markers were of chromosome origin and helped to elucidate their structure. But we have not performed FISH analysis because a archived slide with the Y chromosome material is not enough to perform the other investigation.

In conclusion, this technique using archived cytogenetic slide solves many problems in clinical cytogenetics and can provide further extension of molecular genetic studies. We have been able to demonstrate that the application

of molecular techniques, such as FISH and PCR, improves the detection of low-frequency cell lines and possible structural alterations when a small percentage of mosaicism cannot be detected by conventional cytogenetic techniques. Especially, coamplification PCR is a powerful tool for screening 45,X patients with low level mosaicism of Y chromosome sequences and it can be adapted for rapid use in a diagnostic laboratory.

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