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Retroactive DNA analysis for sex determination and dystrophin gene by polymerase chain reaction with archived cytogenetic slides

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Abbreviations: PCR, polymerase chain reaction; PABY, pseudoautosomal boundary region; SRY, sex-determining region of the Y chromosome; ZFY, zinc finger Y region

Abstract

We describe a rapid and efficient diagnostic method for sex determination and the dystrophin gene by the polymerase chain reaction (PCR) using archived cytogenetic slides. Archived cytogenetic slides stored for about 4 years at room temperature were used. To confirm whether DNA analysis is possible using the archived cytogenetic slides, we extracted the DNA from the slides and amplified the Y centromeric region (DYZ3), the X centromeric region (DXZ1) and the exon 46 of the dystrophin gene. Of the 50 cases, 24 were peripheral bloods, 13 were amniotic fluid cells, 5 were chorionic villus samplings and 8 were cord bloods. The PCR related sex determination in 22 females and 28 males, showed 100% concordance with the results of chromosome analysis, and all cases showed positive band for the exon 46 of the dystrophin gene. Of the 50 cases of the archived cytogenetic slides, we were fortunate enough to obtain the fresh blood sample from one fetus whose karyotype showed 45,X[34]/46,X,+mar[145] to compare the results of the gDNA with that from archived cytogenetic slide. To confirm whether the marker chromosome was derived from Y chromosome, we studied the six loci (PABY, SRY, RPS4Y (SY16, 17), ZFY, DYS14) on the short arm, one locus (DYZ3) on the centromere and one locus (DYZ1) on the long arm. Of the 8 loci studies, all PCR related Y chromosome showed positive band from both gDNA obtained from cord blood and archived cytogenetic slides. We could conclude from the above results that the marker chromosome was derived from the Y chromosome. We believe our experiment is rapid and efficient for studies of over 10 independent loci from a single slide which has been kept in storage for up to 4 years and that archival Giemsa-stained cytogenetic slide repositories represent valuable DNA resources for clinical and forensic studies.

Keywords: archived slide analysis, dystrophin gene, sex determination

Introduction

Since its introduction in 1985, the polymerase chain reaction (PCR) has been applied for DNA analysis in research and clinical fields. PCR has enormous potential for single-cell genetic diagnosis, as it allows a single gene to be amplified more than a billionfold in a matter of hours rather than days (Erlich *et al*., 1991).

Recently, the demand is increasing for molecular analysis of genetic disease using archived Giemsa banded cytogenetic slides. Previous studies reported the feasibility of amplifying DNA target sequences by PCR from biopsied tissues or fixed cells on glass slides (Fey *et al*., 1987; Grant *et al*., 1995; Sago *et al*., 1996). Jonveaux (1991) used a long extraction procedure prior to amplifying a single DNA target sequence from unstained slides. Yap and McGee (1991) developed a method for amplifying DNA by PCR directly on the microscopic glass slides with cell cultured and smeared. Fey *et al*. (1987) amplified DNA from routine bone marrow slides stored up to 10 years at room temperature, and Sago *et al*. (1996) detected the point mutation from archived cytogenetic slide using the PCR and direct DNA sequencing. Since further molecular analysis is often needed for a patient who has had previous cytogenetic analysis, but is no longer able to provide a blood sample, there is a clear need for the development of techniques that would allow retrospective study of archived cytogenetic slides. Through our study, we determinated that for the analysis of genetic abnormalities in cases where the patient had already passed away or any sampling was not available for the DNA analysis, archival samples on the cytogenetic slide can

be used. Nevertheless, it is now possible to reanalyze samples which have been stored prior to development of DNA analysis.

In this study, we took 50 archived cytogenetic slides which had been stored for 4 years at room temperature, even though cytogenetic procedure with acid treatment had been considered to depurinate the DNAs. For the ultimate purpose of applying the method clinically, we limited our PCR applied DNA analysis to X-linked recessive disorder muscular dystrophy patients who required both sex determination and detection of exon 46 deletion which is localized in the central hot spot region of the dystrophin gene. In addition, we also analyzed a fetus who was found to have marker chromosome through conventional karyotyping. To confirm whether the marker chromosome was derived from the Y chromosome, we amplified 7 specific regions on the Y chromosome with PCR to detect the deletion loci. We also performed PCR simultaneously in order to compare the results using the gDNA extracted from cord blood with that using DNA extracted from the archived cytogenetic slide.

Materials and Methods

Extraction of DNA from archived cytogenetic slides

Dried, hardened and archived cells on the slides could be removed intact if the cellular materials were first softened. The archived slides were soaked in xylene at room temperature for 6 days to remove the immersion oil. Giemsa stain on the slides was removed by destaining in 100% ethanol for 5 min three times. The cellular materials on archived slides were hydrated by soaking in H_2O for 5 min three times. Afterwards, each slide surface was spread 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), covered with a 25 mm×55 mm rectangular piece of parafilm and left undisturbed for 15 min at 56˚C. After removal of parafilm, 200 μl water was added to each slide and followed by careful scraping off of the softened cellular materials using a single edge 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, the cell pellets were resuspended in 200 μg/ml proteinase K in 50 μl lysis buffer by vortexing and incubated for 45 min at 56˚C. Proteinase K was inactivated at 95˚C for 10 min. By this method, extracted DNA samples were either used immediately for PCR or stored at -80˚C.

PCR analysis

A 4 μl aliquot of proteinase K digested cell extracts was added to a 20 μl reaction mixture (10 mM HCl-Tris [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 400 mM dNTPs, 2 μM primers, and 1 U Taq polymerase). The DYZ3 on Y chromosome centromere, the DXZ1 on X chromosome centromere and exon 46 of the dystrophin gene on a total of 50 archived slide specimens were amplified (Table 1). In one case of a mosaic fetus, we amplified seven loci, 5 (PABY, SRY, RPS4Y, ZFY, DYS14) on the short arm, one (DYZ3) on the centromere and one (DYZ1) on the Yq11-Yq12 junction region (Table 2, Figure 1 and 2). All target DNAs were denatured for 10 min at 95˚C, then underwent 30 cycles of denaturing at 95˚C for 0.5-1 min, annealing at optimal temperatures of each loci (Table 1 and 2) for 1-2 min, and extension at 72˚C for 1 min. The final cycle of 10 min extension at 72˚C was followed. All cycles were performed in a Perkin Elmer Thermal Cycler (TaKaRa Schuzo Co). After amplification, 5 μl aliquots of amplifi-cation products were electrophoresed in 2% agarose gels and visualized under ultraviolet illumination following ethidium bromide staining. To avoid experimental errors, 50 samples were divide into 5 to 10 test groups. In all experiments, normal male and female controls and blanks were also studied as controls.

Results

Table 1. Sequences, annealing temperatures and product sizes of PCR for one region of the Y chromosome (DYZ3), X chromosome (DXZ1) and exon 46 of the dystrophin gene.

Locus	Primer sequences	Annealing temp. (˚C)	Product size (bp)
DYZ3	5' ATGATAGAACGGAAATATG 3' 5' AGTAGAATGCAAAGGGCTCC 3'	55	170
DXZ1	5' AATCATCAAATGGAGATTTG 3' 5' GTTCAGCTCTGTGAGTGAAA 3'	58	130
Exon 46	5' GCTAGAAGAACAAAAGAATATCTT 3' 5' CTTGACTTGCTCAAGCTTTTCTTT 3'	64	148

Table 2. Sequence, annealing temperature and product size of PCR primers related to 8 Y-specific loci from the fetus's slide (45,X[34]/46,X,+mar[145]).

Locus	Primer sequence	Annealing temp. (˚C)	Product size (bp)
PABY	5' CTGAGAGTGGAAGTGTCGCAG 3' 5' GTACTACCTTTAGAAAACTAGTATTTTCCC 3'	55	1100
SRY (XES)	5' CCCGAATTCGACAATGCAATCATATGCTTCTGC 3' 5' CTGTAGCGGTCCCGTTGCTGCGGTG 3'	60	609
RPS4Y (SY16)	5' CCTGGTGCTTCTGTGAAAAA 3' 5' TGAAAGGAGCATAGTCCTGC 3'	63	210
RPS4Y (SY17)	5' CAGACGGAACTATCTCACAGG 3' 5' GCTGAGAACAGTGCTAAGGG 3'	63	329
ZFY	5' CGAATTCATACCGGCGAGAAGCCATACC 3' 5' AAAGCTTGTAGACACATCGTTAGGG 3'	60	735
DYS14	5' CTAGACCGCAGAGGCGCCAT 3' 5' TAGTACCCACGCCTGCTCCGG 3'	62	239
DYZ3	5' ATGATAGAACGGAAATATG 3' 5' AGTAGAATGCAAAGGGCTCC 3'	55	170
DYZ1	5' TCCACTTTATTCCAGGCCTGTCC 3' 5' TTGAATGGAATGGGAACGAATGG 3'	62	149

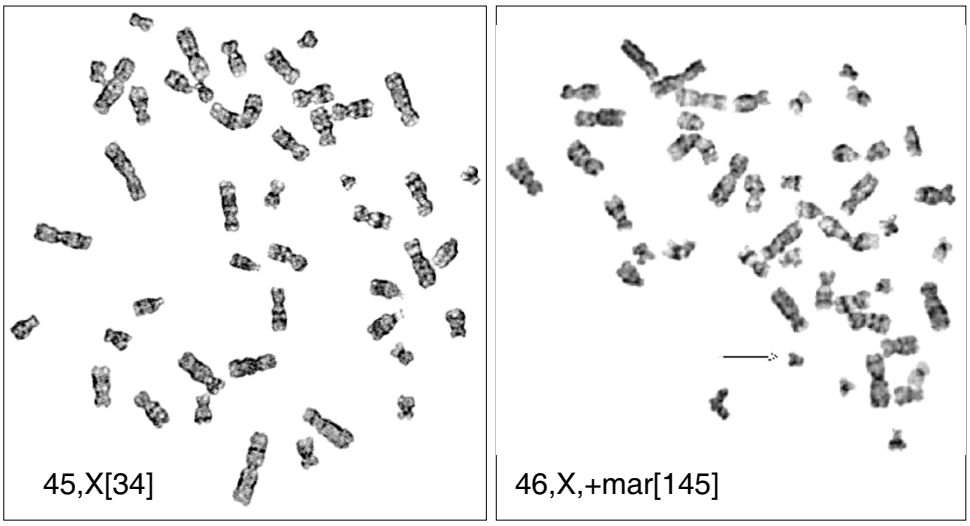

Figure 1. Giemsa stained metaphase from the fetus showing mosaicism with 45,X[34]/46,X,+mar[145]. The arrow indicates the marker chromosome.

Table 3. Breakdown of positive signals in DYZ3, DXZ1 and exon 46 of the dystrophin gene loci from cytogenetic archived slides according to sample source.

Locus	peripheral blood	amniocyte	chorionic villus	cord blood
DYZ3	12	8	3	5[a]
DXZ1	23	13	5	9
Exon 46	23	13	5	9

[a] including a fetus karyotyped 45,X[34]/46,X,+mar[145]

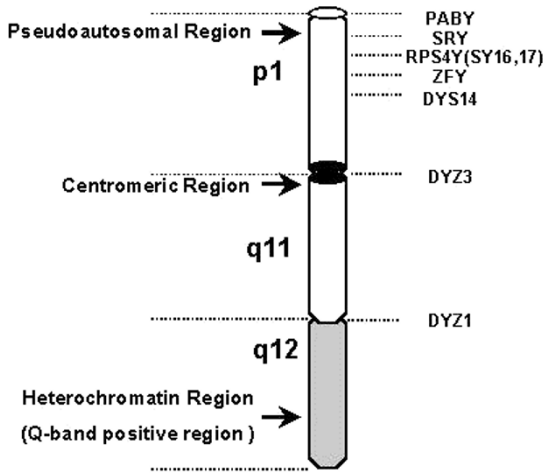

Figure 2. Diagram shows the Y chromosome. Three major regions are shown on the left and 7 regions (8 loci) used in our studying on the right.

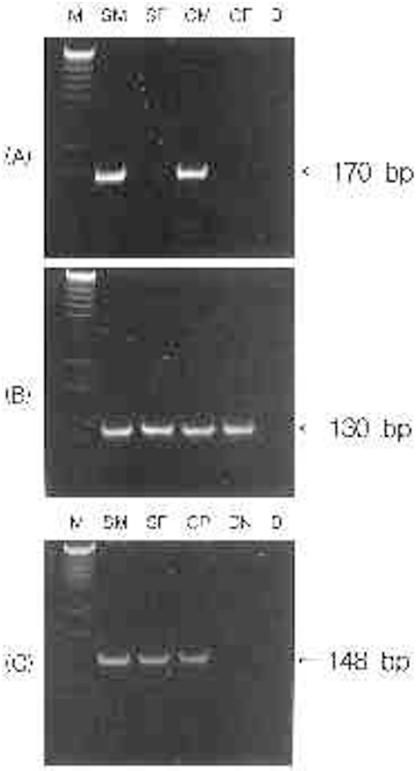

Figure 3. Electrophoretic analysis of PCR products for (A) DYZ3, (B) DXZ1 and (C) exon 46 of the dystrophin gene. [M: pGEM marker, SM: slide male sample, SF: slide female sample CM: control male, CF: control female, CP: control positive, CN: control negative, and B: blank]

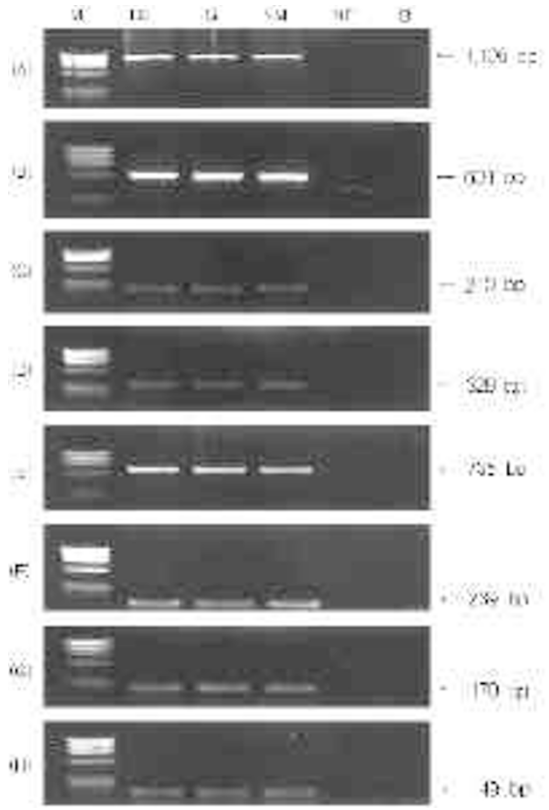

Figure 4. Electrophoretic analysis of PCR products for (A) PABY, (B) SRY, (C) RPS4Y: SY16, (D) RPS4Y: SY17, (E) ZFY, (F) DSY14, (G) DYZ3, and (H) DYZ1. [M: ϕX174-HaeIII marker, FS: fetal slide, FG: fetal gDNA, NM: normal male, NF: normal female, B: blank]

PCR analysis was performed on the DNA extracted from 50 archived cytogenetic slides which were stored for about 4 years at room temperature. Of the 50 cases, 24 were peripheral blood, 13 were amniotic fluid cell, 5 were chorionic villus sell and 8 were cord blood. The reliability of our experiments for the amplification of the exon 46 of the dystrophin gene and X-specific locus (DXZ1) was obtained. Also, the Y centromeric locus (DYZ3) was amplified for comparison with the chromosome study. The PCR results, 22 females and 28 males, were judged 100% concordance with the results of the chromosome analysis, and all the cases showed normal positive band for the exon 46 of the dystrophin gene (Table 3, Figure 3).

In case of a mosaic cord blood specimen which showed positive for the Y centromeric region and revealed a mosaic karyotype 45,X[34]/46,X,+mar[145] cytogenetically, the marker chromosome was strongly suspected to be derived from Y chromosome. To investigate this possibility, 7 loci on Y chromosome were observed in both PCR reaction using the gDNA from cord blood and

Table 4. Comparison of PCR analysis results in the archived slides and gDNA from fetus with 45,X[34]/46,X,+mar[145]. All loci on the Y chromosome were represented for both.

	Y-specific locus							
	PABY	SRY	RPS4Y (ST16)	RPS4Y (ST17)	ZFY	DYS14	DYZ3	DYZ1
archived slide	+	+	+	+	+	+	+	+
gDNA	+	+	+	+	+	+	+	+

the DNA from the archived cytogenetic slide. The amplification of gDNA and slide DNA showed the same results that a fetus had 7 loci each on the Y chromosome (Table 4, Figure 4). With PCR study, analysis was proposed that in this patient the marker chromosome that was derived from the Y chromosome. To exclude the possibility of false positive and negative cases, we included male, female and blank controls to each of our test groups. In the male control, we obtained the expected size of the amplificated products. And in the female and the blank, we could not, as expected. In conclusion, the results of the controls confirmed that our experiment was free of contamination and is reliable.

Discussion

Recently, with the development of better molecular analysis techniques, we were able to analyze DNA using a very small number of cells, and small amounts of paraffin embedded tissues, old stained archival slides, the blood stain, and the even the denatured aged-tissue samples. Especially among them, archival samples of the cytogenetic slide can be useful for the purpose of retrospective study for genetic abnormalities in cases where the patient had already passed away or further sampling was not available for the DNA analysis. Such researches have been previously done for genetic analysis from a biopsied tissues or cells fixed to glass slides (Fey *et al*., 1987; Grant *et al*., 1995; Sago *et al*., 1996). Attempts at isolating high molecular weight DNA from older material have been less successful than low molecular DNA (Higuchi *et al*., 1984).

Generally, to obtain successful PCR results, the primer sequence, the target size, and PCR amplification con-ditions are important for reliable amplification from old stained slides. In this experiment, target regions from 130 to 1,100 base pairs were amplified successfully using extracted DNA from slides which has been stored up to 4 years. Our experiment was limited due to the fact that no slides beyond the 4 years were available. Were older slides available, we would still have been able to obtain successful amplification regardless of age. The procedures of preparing the cytogenetic slides by fixing, staining, and archiving can minimize the contamination more effectively than others, fixed cells stick tenaciously to glass slides. In our experimental procedures, we have seen that none of our results were due to contamination and thus leading to false positive results.

Another use of cytogenetic archival slides for the similar purpose of our experiment is termed **"**slide PCR**"**. The slide PCR amplifies the DNA directly on archival slides. Slide PCR provides an alternative for analysis of cell samples that are already on slides without the need for scrapping, and minimizing risk of contamination by manipulation (Yap and McGee, 1991). However, multi-target region analysis is not possible simultaneously with this method. To get around this limitation, we extracted the DNA from archival slides and performed 10 independent target-region analysis.

DNA fragments can be minimized with the treatment of the proteinase K with Tween-20 detergent in lysis buffer until cellular materials on the slide are loose (Sago *et al*., 1996). During the proteinase K digestion, we used parafilm in place of glass coverslips to prevent binding of DNA and evaporation. Also, parafilm is easily removable during the extraction of DNA from slides. Through the above the protocols, our PCR results for the sex determination were exactly the same as those of cytogenetic results. As for dystrophin gene, all samples were found to have the gene.

In case of one mosaic fetus whose karyotype showed 45,X[34]/46,X,+mar[145], we were able to obtain the cord blood gDNA for analysis and compare the results with the DNA from archival slides. As such, we could provide a DNA analysis using the archival slides to support the clinical diagnosis for a patient.

In our experiment, we were able to study over 10 independent target regions of the dystrophin and X, Y-specific regions. Although we used archival slides from abortues, fetuses, newborns, leukemic patients and adult samples which had been kept in storage for an extended period, we successfully amplified the archival DNA for sex determination and dystrophin gene study both rapidly

and reliably with the DNA extraction. We believe that this method can provide a rapid and efficient genetic analysis when it is not possible to obtain the fresh samples, but have access to only archival cytogenetic slides from family members or the patient with genetic disease.

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