Analysis of haplotype and coamplification PCR of dystrophin gene and Y-specific gene using PEP-PCR in single fetal cells

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Duchenne/Becker muscular dystrophy are the major neuromuscular disorders with X-linked recessive inheritance. Preimplantation diagnosis of sex determination has been generally applied to avoid male pregnancies with these diseases. However, in order to determine if the embryo is normal, carrier or affected regardless of the sex, there is a need for a combined analysis of specific exon on dystrophin gene as well as sex determination of embryo using the same biopsied blastomere. If the exon deletion is not determinable, further diagnosis of carrier or patient can be performed by haplotype analysis. In this study, we applied the primer extension preamplification (PEP) method, which amplifies the whole genome, in 40 cases of single amniocyte and 40 cases of chorionic villus cell. We analysed haplotypes using two (CA)n dinucleotide polymorphic markers located at the end of 5' and 3' region of the dystrophin gene. Exon 46 of dystrophin gene and DYZ3 on chromosome Y were chosen as a target sequence for coamplification PCR. Upon optimizing the conditions, the amplification rates were 91.25% (73/80) for haplotypes (92.5% in amniocyte, 90% in chorionic villus cell) and 88.75% (71/80) for coamplification (85% in amniocyte, 92.5% in chorionic villus cell). The result of the study indicates that haplotypes analysis and coamplification of dystrophin and Y-specific gene using PEP can be applied to prenatal and preimplantation diagnosis in Duchenne/Becker muscular dystrophy making it possible to determine if the fetus is a carrier or an affected one.

Keywords : Coamplification-PCR, Muscular dystrophy, Exon & Haplotype analysis

INTRODUCTION

The goal of preimplantation genetic diagnosis PGD in IVF is to implant healthy embryo by selecting normal embryos to transfer after the examination of chromosome abnormalities and genetic disease. Currently, there are two methods which are used for this purpose: generally PCR method is used for the single gene disorder and FISH method is used for the chromosomal aneuploids. PGD was first tried on rabbit's embryo (Edwards and Gardner, 1967). And Handyside *et al.* (1990) applied this method to human embryos.

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In PGD, there have been limitations in analysing the genetic defects in one or two gene loci by PCR because only one or two blastomeres could be obtained. Zhang *et al.* (1992) and Xu *et al.* (1993), with the 15-random oligonucleotide primer, first amplified the small amount of whole genome DNA which is contained in a single cell. Then, amplified products were used for multilocus analysis applying PCR method. This primer extension preamplification (PEP-PCR) can solve the limitation of PGD. Duchenne/Becher muscular dystrophy (DMD/BMD) are X-linked neuromuscular disorder with an incidence of about 1 in 3500 live born males. The responsible gene named dystrophin has been localized to Xp21 and contains 79 exons spans about 2.4 Mb of DNA which encodes different mRNA size. More than 65% of affected males have partial dystrophin gene deletion, including several exons (Chamberlain *et al.*, 1988; Beggs *et al.*, 1990). Recently, in the case of muscular dystrophy disease resulting from exon deletion, the deletion can be detected by multiplex-PCR, and the results can be applied to prenatal and preimplantation genetic diagnosis. For sex determination and detection of exon deletion simultaneously, we have reported application of PEP-PCR for PGD in DMD/BMD families (Choi *et al.*, 1996). Futhermore, in order to

determine whether female is a carrier or not, polymorphic microsatellite markers which is located close to the dystrophin gene can be used for linkage analysis (Choi *et al.*, 1997).

For majority of DMD/BMD patients, 70-80% of deletion in exon 42-53 is observed (Florentin *et al.*. 1995; Singh *et al.*, 1997). When two or more deletions of dystrophin's exon are present, deletions occur consecutively. These deletions have been found in a male patient, the segregation of the deletion can be followed in his family. When applying the PGD, it is possible to diagnose a patient through analysing one exon deletion. And along with the above, sex determination is also done because it is important in the X-linked recessive inheritance diseases. Therefore, in this study, we applied coamplification-PCR method to exon 46 and Y-specific region which has been observed as the hot spot with high frequency of mutations. Coamplification-PCR has an advantage of providing rapid results and confirming PCR failure. As such, coamplification-PCR is an appropriate method for PGD (Storm *et al.*, 1991; Katagiri and Katayama, 1996).

The purpose of this study was to establish a better method of prenatal/preimplantation genetic diagnosis for the detection of patients or carriers in DMD/BMD families. We performed haplotyping for the detection of carrier, and coamplification-PCR for the detection of sex and deletion of exon 46 on single fetal cells obtained from amniocentesis or chorionic villi sampling by PEP-PCR.

MATERIALS AND METHODS

Preparation of single fetal cells

A total of 80 cases of human fetal cells were obtained from pregnant women undergoing 40 cases of amniocentesis and 40 cases of chorionic villi sampling. The single fetal cells were separated using micropipet in the Dullbecco's phosphate buffer saline (D-PBS) under the stereomicroscope and conducted three separate washings of the cells. After the washing, it was placed into a prepared PCR tube with 20 μl of distilled water and covered 50ul of mineral oil. A control was kept for each tube without a fetal cell and both the tests and the controls were kept for one hour at -70°C. After preservation at -70°C, the cells were thawed at room temperature for 10 min. This procedure was repeated three times. For the PEP-PCR, DNA denaturation was done at 95°C for 20 min.

PEP-PCR

The fetal cells were added to a mixture containing the final concentration as follows : Tris-HCl 10 mM, pH 8.3, KCl 50 mM, $MgCl_2$ 2.5 mM, dNTPs 100 mM of each, 35 μM 15-base random oligomers. Immediately after, 1.0 μl of Taq polymerase (5 U/μl, Perkin Elmer) was added to the tube and short spin downs were conducted to mix the contents thoroughly. For the PCR, thermal cycler system (TP 3000, TAKARA; 480, Perkin Elmer) was used and was increased to 45 cycles under the following conditions: 90 sec at 92°C, 150 sec at 37°C, and 4 min at 55°C.

Coamplification and haplotype analysis by PEP-PCR

By placing the 5 μl of the PEP products on separate templates, 5'dysIII and 3'(CA)n markers were used for the determination of the haplotype analysis of dystrophin gene. PCR was done under the following conditions: for the

Table 1. Sequences of primers, size of target region and time of PCR for linkage analysis (5'dysIII, 3' (CA)n), dystrophin (exon 46) and Y-specific region (DYZ3).

Primers	Sequences	Sizes (bp)	Time[b]
5'dys	5'-TTTTTTAGGTATAACTTACATACAATAAACC 5'-GTGACAATAAGCATATCAGTGGCTGCC	≒220[a]	2h 20m
3'CA	5'-GAAAGATTGTAAACTAAAGTGTGC 5'-GGATGCAAAACAATGCGCTGCCTC	≒134[a]	2h 20m
Exon 46	5'-GCTAGAAGAACAAAAGAATATCTT 5'-CTTGACTTGCTCAAGCTTTTCTTT	148	3h 30m
DYZ3	5'-ATGATAGAACGGAAATATG 5'-AGTAGAATGCAAAGGGCTCC	170	

[a] approximation is due to polymorphism of people
[b] timed with thermal cycler 480 (Perkin Elmer) & MP TP 3000 (TaKaRa)

5'dysIII, 94°C for 30 sec, 53°C for 30 sec, 65°C for 4 min for 35 cycles: for the 3'(CA)n , 94°C for 30 sec, 58°C for 4 min for 35 cycles. In all experiments, a series of control tubes was run alongside experimental samples, consisted of negative controls with D-PBS and left over from the washing of the fetal cells. For the determination of sex and deletion of the dystrophin gene, coamplification-PCR was accomplished using the primers of alphoid repeat sequence (DYZ3) on the Y chromosome and exon 46 of dystrophin gene (Table 1). The PCR for the Y-specific region was amplified by 40 cycles under the following conditions: 30 sec at 95°C, 20 sec at 54°C, and 30 sec at 72°C. All PCR products were detected by electrophoresis with 2.0 % agarose gel contained ethidium bromide (0.2 μg/ml) and directly visualized by the UV transilluminator.

RESULTS

We obtained the 91.25% amplification using the microsatellite marker primers, 5'dysIII and 3' (CA)n, 37 out of 40 amniocyte cells, 36 out of 40 chorionic villi cells. In homozygote females, single band was observed; in heterozygote females, double bands were observed; in hemizygote males, single band was observed (Fig. 1). In comparing the sex determination results of the coamplification-PCR and chromosome analysis results, 88.75% concurrence rate was found: 34 out of 40 amniocyte cells and 37 out of 40 chorionic villi cells (Fig. 2) (Table 2). To rule out contamination during experimental process, for each of the 80 samples, the D-PBS, left over from the washing of the fetal cells, was used as controls and all showed negative results.

Table 2. Successful rates of PCR amplification in single fetal cells

	Amniocyte cells	Chorionic villus cells	Total
PEP-PCR	37/40	37/40	74/80
haplotype (3'(CA)n, 5'dysIII)	37/40	36/40[a]	73/80
coamplification (exon 46, DYZ3)	34/40[b]	37/40	71/80

[a] 1 CVS, proven male, was not seen less than one band.
[b] The sex determination of 3 amniocyte cells were not equal to chromosome study as well as exon 46 region of dystrophin gene were not amplified.

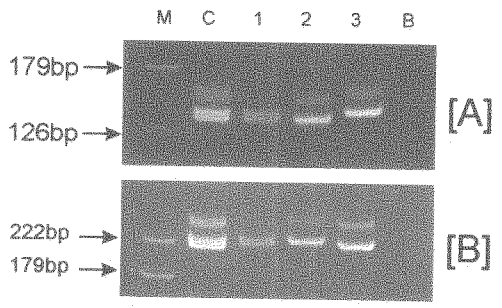

Fig. 1. Haplotype analysis of dystrophin 3' (CA)n region (A) & 5'dys III region (B) from fetal cells and genomic DNA. M, pGEM marker; C, genomic DNA (female); 1-3, single fetal cells (1, heterozygote female; 2, homozygote female; 3, hemizygotemale); B, blank.

DISCUSSION

Allele drop-out (ADO) refers to a partial failure in the PCR amplification for genetic analysis. Some of the causes of ADO can be attributed to deletion of the gene, lysis of cell membranes, denaturation temperature of DNA and separation of DNA strand, which result from misstep during or before the PCR process. From the above, complete lysis of cells is an important factor in the success of PCR or the occurrence of ADO (Ray and Handyside, 1996). Currently, lysis of cells is done by boiling, freeze-thawing, add the dithiotreitol or proteinase K and alkaline lysis method (Sermon *et al.*, 1995; Wu *et al.*, 1993; Gitlin *et al.*, 1996; Kontogianni *et al.*, 1996). The frequency of ADO can be controlled by DNA denaturation temperature: DNA denatured at 96°C shows 5% ADO, 90°C shows 21% and 93°C shows 13%. Denaturation temperature of 96°C seems to be appropriate, and decreasing temperatures less than 96°C show increasing frequency of ADO (Lissens and Sermon, 1997). After lysing the cells through freeze-thawing process, keeping the denaturation temperature at 95°C, using 4 primers and amplifying the PCR, we observed 8.75% ADO for amnio-cyte cells and 11.25% ADO for chorionic villi cells. In comparison to other studies, we achieved greater

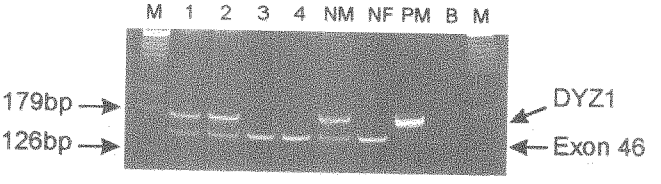

Fig. 2. Coamplification PCR of dystrophin gene (Exon 46) and Y-specific region (DYZ1) from fetal cells and genomic DNA. M, pGEM marker; 1-4, single fetal cells (1and 2, normal; 3 and 4, normal female); NM, normal male; NF, normal female; PM, male DMD patient; B, blank.

success of amplification by lowering ADO (Table 2). Of the failed samples, 3 from amniocyte cells and 3 from chorionic villi cell were ADO cases resulted from failed PEP-PCR, failure of the remaining 3 amniocyte cells and 1 chorionic villi cells resulted from failure of amplification in specific gene region. Microsatellite regions have polymorphism in each individual, which were passed down from parents to offsprings. We observed 2 bands in heterozygote female, 1 band in homozygote female and 1 band in hemizygote male. For heterozygote females with two different alleles, this genetic information can be used for the purpose of prenatal/ preimplantation genetic diagnosis in determining the responsible allele of the diseases. With single coamplification-PCR, we can easily determine if the fetus is affected or not through the amplification of exon 46 of dystrophin gene and Y-specific region.

Comparing the two methods, coamplification of two target regions and general PCR for a single region, in the case of coamplification, we observed weaker band due to insufficiency of PCR products. We believe that there is competition between the two primers for the two target regions due to different annealing temperature required by two primers.

Using the minute amount of DNA from single blastomere, PCR method lead to 4-21% failure rate (Strom *et al.*, 1991). However, with the application of PEP-PCR, we obtained 91.25% success rate. This will make it possible for clinical application of PGD. It has been reported by Munne *et al.* (1994) that concerning use of the FISH method with PCR will allow observation of both chromosomal aneuploid and abnormalities of the gene, overcoming the limitation of applying PCR. Futhermore, Thornhill *et al.* (1994) and Rechitsky *et al.* (1996) reported of the cell recycling method which facilitates application of PCR and FISH simultaneously.

Building on this, we expect that with the application of multi-color FISH method, we may be able to prevent fetus with chromosomal aneuploid which result from common trisomy of chromosome 13, 18, 21, X and Y in elder pregnant women. In addition, fluorescence-PCR method which also facilitate abnormalities of the gene from single blastomere has been reported by Hattory *et al.* (1992). With the fluorescence-PCR, by using fluorescent labeled primers, laser can be used to detect the genetic abnormalities, which provides accuracy, sensitivity and low ADO rates. Such advantages make it a good method for PGD (Findlay *et al.*, 1996).

In our experiment, we amplified the whole genome using the PEP-PCR to obtain greater amount of DNA allowing simultaneous analysis of multi loci genes and we increased the reliabilities of the results with repeat test. The PEP-PCR process took 7 h and the gene analysis took 3-4 h spending total of 10-11 h for the experiment. Thus the time duration of our experimental process was within 8-12 h which was reported as the optimal time for the transfer of embryo to the uterus (Dawson *et al.*, 1995). In this study, we obtained a total of 90% amplification for the genetic analysis by PEP-PCR. Therefore, the coamplification PCR method for sex determination and the detection of deletion of dystrophin genes and the haplotype analysis for the diagnosis of carrier or patients will be useful methods for the clinical application of prenatal and preimplantation genetic diagnosis.

REFERENCES

Beggs, A. H., Koenig, M., Boyce, F. M. and Kunkel, L. M. (1990) Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. *Hum Genet* 86: 45-48

Chamberlain, J., Gibbs, R., Ranier, J., Nguyen, P. N. and Caskey, T. (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res* 16: 11141-11156

Choi, S. K., Lee, E. H., Lee, H. J., Jun, J. H., Kang, I. S., Paik, E. C., Ryu, H. M. and Jun, J. Y. (1996) Preimplantation genetic diagnosis using Primer Extension Preamplification in Duchenne/ Becker muscular dystrophy (DMD/BMD) families. *Kor J Fert Steril* 23: 109-114

Choi, S. K., Kim, J. W., Cho, E. H., Ryu, H. M. and Kang, I. S. (1997) Analyses of dystrophin gene and determination using PEP-PCR in single fetal cells. *Kor J Fert Steril* 24: 51-56

Dawson, K. J., Conaghan, J., Ostera, G. R., Winston, R. M. L. and Hardy, K. (1995) Delaying transfer to the third day post-insemination, to select non-arrested embryos, increasers development to the fetal heart stage. *Hum Reprod* 10: 177-182

Edwards, R. G. and Gardner, R. L. (1967) Sexing of live rabbit blastocysts. *Nature* 214: 576-577

Findly, I., Quirke, P., Hall, F. and Rutherford, A. (1996) Fluorescent PCR; a new technique for PGD of sex and single-gene defects. *J Assist Reprod Genet* 13: 96-103

Florentin, L., Mavrou, A., Kekou, K. and Metaxotou, C. (1995) Deletion patterns of Duchenne and Becker Muscular dystrophies in Greece. *J Med Genet* 32: 48-51

Giltin, S. A., Lanzendorf, S. E. and Gibbons, W. E. (1996) Polymerase chain reaction amplification specificity: incidence of allele dropout using different DNA preparation methods for heterozygous single cells. *J Assist Reprod Genet* 13: 107-111

Handyside, A. H., Kontogianni, E. H., Hardy, K. and Winston, R. M. L. (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* 344: 768-780.

Hattory, M., Yoshioka, K. and Sakaki, Y. (1992) Highly-sensiive fluorescent DNA sequencing and its application for detection and mass-screening of point mutations. *Electrophoresis* 13: 560-565

Katagiri, Y. and Katayama, S. (1996) Influence of mosicism of sexing of human preembryos detected by the polymerase chain

reaction. *J Assist Reprod Genet* 13: 586-591

Kontogianni, E. H., Griffin, D. K. and Handyside, A. H. (1996) Identifying the sex of human preimplantation embryos in X-linked disease: amplification efficiency of a Y-specific alphoid repeat from single blastomeres with two lysis protocols. *J Assist Reprod Genet* 13: 125-132

Lissens, W. and Sermon, K. (1997) Preimplantation genetic diagnosis, current status and new developments. *Human Reproduction* 12: 1756-1761

Munne, S., Grifo, J., Cohen, J. and Weier, H. U. G. (1994) Chromosome abnormalities in human arrested preimplantation embryos: a multiple-probe FISH study. *Am J Hum Genet* 55: 150-159

Ray, P. F. and Handyside, A. H. (1996) Increasing the denaturation temperature during the first cycles of amplification reduces allele dropout from single cells for preimplantation diagnosis. *Mol Hum Reprod* 2: 213-218

Rechitsky, S., Freidine, M., Verlinsky, Y. and Strom, C. M. (1996) Allele dropout in sequential PCR and FISH analysis of single cells (cell recycling). *J Assist Reprod Genet* 13: 115-124

Sermon, K., Lissems, W., Nagy, Z. P. (1995) Simultaneous amplification of the two most frequent mutations of infantile Tay-Sachs disease in single blastomeres. *Hum Reprod* 10: 2214-2217

Singh, V., Sinha, S., Mishra, S., Chatuvedi, L. S., Pradhan, S., Mittal, R. D. and Mittal, B. (1997) Proportion and pattern of dystrophin gene deletions in North Indian Duchenne and Becker muscular dystrophy patients. *Hum Genet* 99: 206-208

Strom, C. M., Rechitsky, S. and Verlinsky, Y. (1991) Reliability of gender determination using the polymerase chain reaction (PCR) for single cells. *J Vitro Fert Embryo Transfer* 8: 225-229

Thornhill, A., Holding, C. and Monk, M. (1994) Recycling the single cell to detect specific chromosome and to investigate specific gene sequences. *Hum Reprod* 9: 2150-2155

Wu, R., Cuppens, H., Buyse, I. (1993) Co-amplification of the cystic fibrosis ΔF508 mutation with the Hla DQA1 sequence in single cell PCR: implications for improved assesment of polar bodies and blastomeres in preimplantation diagnosis. *Prenat Diagn* 13: 1111-1122

Xu, K., Tang, Y., Grifo, J. A., Rosenwaks, Z. and Cohen, J. (1993) Primer extension preamplification for detection of multiple genetic loci from single human blastomeres. *Hum Reprod* 8: 2206-2210

Zhang, L., Cui, X., Schmitt, K., Hubert, R., Navidi, W. and Arnheim, N. (1992) Whole genome amplification from a single cell: implications for genetic analysis. *Proc Natl Acad Sci USA* 89: 5847-5851