SHORT COMMUNICATION

MONTPELLIER, FRANCE

Rapid Detection of the $\Delta F508$ Mutation in Single Cells Using DHPLC: Implications for Preimplantation Genetic Diagnosis

Submitted August 1, 2002; accepted November 19, 2002

Purpose: Practice of preimplantation genetic diagnosis (PGD) requires efficient amplification and analysis techniques. We have tested Denaturing High Performance Liquid Chromatography (DHPLC) to screen the $\Delta F508$ mutation in heterozygous single cells in order to assess its usefulness for PGD of cystic fibrosis.

Methods: One hundred and two single lymphocytes—including N/N and N/ $\Delta F508$ —were studied. F508 locus was amplified by nested PCR followed by the analysis of PCR products by DHPLC in non-denaturing conditions.

Results: On the basis of PCR-amplified product analysis, total efficiency of amplification was 98.78% (101/102), and allele dropout (ADO) rate was 3.7% (3/81). For each sample, results were obtained in less than 4 min with high resolution.

Conclusions: DHPLC is a rapid and efficient technique to detect the $\Delta F508$ mutation in single cells and is therefore appropriate for clinical application of preimplantation genetic diagnosis of cystic fibrosis.

KEY WORDS: Cystic fibrosis; DHPLC; $\Delta F508$; preimplantation genetic diagnosis.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) was developed to detect and transfer unaffected embryos, in couples at high risk of transmitting a genetic disorder to their children, thus avoiding the need for termination of pregnancies following prenatal diagnosis (1). PGD involves the screening of one or two biopsied blastomeres from in vitro generated embryos, on the 3rd day following insemination at about the 6–10 cell stage. The difficulties of PGD mainly rely on the extremely small amount of DNA template available in each of the studied preimplantation embryos (about

7 picograms of DNA in a single cell) and on the short period allowed to the analysis, as unaffected embryos have to be transferred to the maternal uterus within the day of biopsy to optimize the chances of implantation. Therefore, in addition to efficient and optimal amplification conditions, accurate and rapid analysis techniques are required in clinical PGD to ensure quality control and quality insurance measures.

There is a continuing need for improved mutation detection technology in PGD. We have tested Denaturing High Performance Liquid Chromatography (DHPLC) (2) to detect the most common mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene ($\Delta F508$) in single heterozygous cells (N/ $\Delta F508$) in order to evaluate whether the technique fulfils the criterias of efficiency, reliability, and rapidity for a potential use in a PGD procedure.

MATERIALS AND METHODS

Collection of Single Lymphocytes

Single lymphocytes were obtained from a $\Delta F508$ heterozygous patient. Lymphocytes from an unaffected homozygous subject were also used as controls. Individual cells were collected under an inverted microscope and transferred by mouth-controlled pipetting to microtubes containing 3 μ l alkaline lysis buffer (200 mM KOH, 50 mM DTT). The single cells were lysed by incubation at 65°C for 10 min, then stored at –20°C until PCR was performed.

Single Cell PCR

A nested PCR approach was used for the amplification of the region surrounding the $\Delta F508$ region. In the first-round, the F508 locus was amplified in a total volume of 30 μ l by adding 27 μ l of a reaction mix containing neutralization solution (900 mM Tris-HCl pH 8.3, 300 mM KCl, 200 mM HCl), potassium-free amplification buffer (100 mM Tris-HCl pH 8.3, 15 mM MgCl₂, 0.1% (w/v) gelatin), 2 mM dNTPs (Amersham Pharmacia), 0.3 μ M primers 10i5 and 10i3 (3), and 1 U Amplitaq DNA polymerase (Applied Biosystems). Thermal cycling was performed in a Gene Amp PCR System 9700 (Applied Biosystems) at 96°C for 4 min, 25 cycles of 96°C for 20 s, 56°C for 20 s, 72°C for 40 s, and one cycle of 72°C for 10 min. Two microliters from each first-round PCR product was then transferred to 28 μ l reaction mixtures and was reamplified with inner primers C16B and C16D (4) for 25 cycles (20 s at 94°C, 20 s at 62°C, 40 s at 72°C). Several negative

Fig. 1. DHPLC elution profiles of $\Delta F508$ region obtained after amplification of genomic DNA extracted from homozygous mutant (lane 1) and normal (lane 2) individuals, and from a heterozygous subject (lane 3). Analysis was performed under non-denaturing conditions (50°C).

controls (microtubes containing identical reaction mixes but without added DNA) were run simultaneously with samples tubes to check absence of contamination.

DHPLC Analysis

PCR efficiency was checked by running $10\ \mu\text{l}$ of the second-round products on 1% agarose gels prestained with ethidium bromide. Eight microliters of the second-round positive amplification products were then analyzed by DHPLC (Wave DNA fragment analysis system, Transgenomic). Separation of the alleles was performed in nondenaturing conditions (i.e. 50°C). Buffers gradient was calculated by

the Wavemaker[®] software (version 4.1.40) to allow quick separation of the 95- (mutant allele) and 98-bp (wild-type allele) amplified DNA fragments. Chromatograms were recorded at 260 nm using a UV detector.

PCR products from 50 ng genomic DNA (N/N, N/ $\Delta F508$, and $\Delta F508/\Delta F508$) were also subjected to DHPLC analysis to be used as pattern controls.

Other Mutation Detection Techniques

In order to compare the results obtained using DHPLC with alternative techniques commonly used in PGD procedures, some single cell PCR products were resolved on 10% polyacrylamide gels (PAGE

Fig. 2. Genotyping of single cells after DHPLC analysis. Lanes 1–5: single lymphocytes heterozygous for $\Delta F508$ mutation. Lanes 1 and 4 show equivalent amplification from both alleles (normal : 98 bp and mutant : 95 bp). Lanes 2 and 3 demonstrate preferential amplification of the mutant (lane 2) or normal (lane 3) alleles. Lane 5 shows allele dropout in which the mutant allele has failed to amplify to detectable levels. Lane 6: single lymphocyte from a homozygous normal individual.

Table I. Amplification Efficiency and Allele Dropout (ADO) Rates in Single Lymphocytes from One Individual Heterozygote N/ Δ F and One Individual Homozygote N/N, Obtained by PAGE 10%, Fluorescent PCR, and DHPLC Analysis

	PAGE 10%	Fluorescent PCR	DHPLC
Amplification efficiency (%)			
N/N	26/27 (96.3)	54/56 (96.43)	20/20 (100)
N/ Δ F	97/101 (96.04)	121/127 (95.28)	81/82 (98.78)
Total	123/128 (96.09)	175/183 (95.63)	101/102 (99.02)
ADO (%) ^a	12/97 (12.37)	5/121 (4.13)	3/81 (3.7)

^a On the basis of heterozygous cells showing an amplification product.

10%) then visualized after ethidium bromide staining, while others were analyzed on an automatic DNA sequencer (ABI 377, Applied Biosystems). PCR conditions were as described earlier except for C16B primer that was fluorescently labelled with FAM dye to detect amplified products on a DNA sequencer.

RESULTS AND DISCUSSION

In the present study, we have tested the DHPLC system to analyze the Δ F508 mutation. For all samples tested, results were obtained in less than 4 min with high resolution. Elution profiles obtained after amplification of genomic DNA from three different CF genotypes (N/N, N/ Δ F, and Δ F/ Δ F) are shown in Fig. 1. Two peaks are clearly visible for the heterozygous sample; the presence of an additional peak eluting immediately before the two homoduplex signals corresponds to the heteroduplexes that elute earlier than homoduplexes. A single peak of undenatured homoduplex DNA is visualized for the homozygous DNA samples: the single peak corresponding to the mutant allele elutes first, whereas the peak corresponding to the normal one elutes approximately 15 s later.

DHPLC-single cell analyses were carried out on 82 isolated lymphocytes from a Δ F508 heterozygous individual and 20 lymphocytes from a control individual homozygous for the normal CFTR sequence. Chromatogram patterns (Fig. 2) are similar to those obtained after amplification of genomic DNA. Results obtained using three different detection methods are summarized in Table I. Using DHPLC, even in cases of extreme preferential amplification of one allele, strong evidence of heterozygosity is given by the presence of a peak eluting immediately before homoduplexes, thus allowing differentiation with true allele dropout (ADO) or with homozygous normal samples. Although we observed a low ADO rate in this study, it may be higher in PGD procedures, as

Rechitsky *et al.* (5) have reported that human blastomeres exhibit a greater ADO rate than other cell types; however, this phenomenon has not been unanimously observed (6). None of the 12 blank controls showed any signal on agarose gels, which is consistent with the absence of contamination.

Compared to fluorescent PCR which is known to be an accurate technique to screen single cells for genetic mutations and although sensitivity seems to be similar between both techniques, DHPLC has the convenience that PCR products analysis is carried out in the microtubes themselves without addition of any reagent, thus reducing the handling of samples and the cost of the technique. The recent development of the WAVE fluorescence detector should allow labelled nucleic acids to be separated with an increased sensitivity and much more rapidly as PCR products from a single fluorescent round PCR would be analyzed.

CONCLUSION

In summary, in view of the time-constraints related to PGD, DHPLC is an appropriate analysis technique, highly sensitive and specific, compatible with the transfer to the uterus of selected embryos a few hours after blastomere biopsy.

ACKNOWLEDGMENTS

This study was financially supported by Vaincre la Mucoviscidose (VLM), l'Association Française contre les Myopathies (AFM), and the CHU of Montpellier.

REFERENCES

1. Handyside AH, Kontogianni EH, Hardy K, Winston RM: Pregnancies from biopsied human preimplantation embryos

- sexed by Y-specific DNA amplification. *Nature* 1990;344:768–770
2. Oefner PJ, Underhill PA: Comparative DNA sequencing by denaturing high-performance liquid chromatography (DHPLC). *Am J Hum Genet* 1995;57:A266
 3. Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, Rommens J, Tsui LC: Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* 1991;10:214–228
 4. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm M, Iannuzzi M, Collins F, Tsui LC: Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* 1989;8:1066–1073
 5. Rechitsky S, Strom C, Verlinsky O, Amet T, Ivakhnenko V, Kukhareno V, Kuliev A, Verlinsky Y: Allele dropout in polar bodies and blastomeres. *J Assist Reprod Genet* 1998;5:253–257
 6. Sermon K, Seneca S, Vanderfaeillie A, Lissens W, Joris H, Vandervorst M, Van Steirteghem A, Liebaers I: Preimplantation diagnosis for fragile X syndrome based on the detection of the non-expanded paternal and maternal CGG. *Prenat Diagn* 1999;19:1223–1230
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