Simultaneous Single-Cell Detection of Two Mutations for Cystic Fibrosis

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Purpose: A single-cell diagnosis procedure using polymerase chain reaction (PCR) technology was developed to simultaneously detect two cystic fibrosis (CF) mutations (DF-508, W1282X).

Methods: The reported test procedures made use of specific cell lines (lymphoblasts, fibroblasts) of known CF mutation status to determine the efficiency of signal generation and prevalence of allele dropout (ADO) during amplification.

Results: Using cells carrying the DF-508 mutation, the PCR signal efficiency for the affected homozygous, normal homozygous, and carrier heterozygote cell populations were 91%, 81%, and 92%, respectively. The total combined PCR efficiency was 87.7% and the ADO rate was 5.7%. For W1282X carrier heterozygote cells, the PCR signal efficiency was 82.0% and the ADO rate was 8.7%.

Conclusions: Methods have been developed to detect two common mutations simultaneously for CF in single-cell assays. The high signal efficiencies and low ADO rates obtained in these tests allow those embryos from couples wishing to avert the transmission of this serious genetic disease to their offspring to be screened by preimplantation genetic diagnosis.

KEY WORDS: Preimplantation genetic diagnosis; polymerase chain reaction; single cell diagnosis; allele dropout; assisted reproductive technologies; cystic fibrosis.

INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive disease in North American Caucasians. The incidence of the disease is 1 affected child in 2500 live births (1). The CF gene has been cloned and more than 550 mutations have been described. Inter-

estingly, only 70 of these mutations are represented in over 90% of CF alleles (2). A feature prevalent in CF is the presence of differing mutations residing on each allele (compound heterozygosity) that can complement one another, resulting in the expression of clinical disease. This situation has resulted in the misdiagnosis of embryos during preimplantation diagnosis due to the failure to detect the presence of one or the other of the mutations. Affected individuals can have several complications that may affect the respiratory, gastrointestinal, and genitourinary systems. Only 50% of affected individuals live to age 26 or longer, leading to serious emotional and financial difficulties within the family (3).

Two of the more common mutations giving rise to clinical CF in the Ashkenazi Jewish population are $\Delta F508$ and W1282X (4). The sequencing of these mutations has allowed the construction of probes that can be used for the identification of the mutations in single cells with the assistance of polymerase chain reaction (PCR) DNA amplification. This development has allowed the diagnosis of affected embryos prior to implantation. Avner *et al.* (5) reported on a system allowing the simultaneous diagnosis of the two mutations in unfertilized oocytes and polyspermic blastomeres. They reported a PCR efficiency of 51% for oocytes and 69% for blastomeres. In addition, 43% of the cells analyzed showed a single locus failure [allelic dropout (ADO)] or total lack of amplification.

We set out to develop a system of CF double mutation detection ($\Delta F508$ and W1282X) resulting in a higher efficiency and a lower ADO rate.

MATERIALS AND METHODS

Single Cells

Lymphoblast cells with CF disease gene 3 bp deletion mutation in exon 10 (GM04540A homozygous

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ΔF508), lymphoblast cells with CF disease gene intron G > C splice-site mutation in exon 20 (GM11723D compound heterozygote W1282X), and fibroblast cells with CF disease gene 3 bp deletion mutation in exon 10 (GM04346 heterozygote ΔF508) were obtained from the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Normal lymphoblast cells were used as controls.

KOH Cell Treatment

A single cell was placed in 6 μl of KOH solution (200 mM potassium hydroxide/50 mM dithiothreitol), centrifuged and heated at 65°C for 20 min. Six microliter of a neutralizing solution (900 mM Tris-HCl, pH 8.3, 300 mM potassium chloride, 200 mM hydrochloric acid) was then added and brought to 52 μl with sterile ultra-pure water (6).

PCR DNA Primer Design

Primers A new single reaction nested primer system was employed. Two pair outer primers were used for both mutations (7):

- For ΔF508:
 - 10i-5:5'-G C A G A G T A C C T G A A
A C A G G A-3'
 - 10i-3:5'-C A T T C A C A G T A G C T
T A C C C A-3'
- For W1282X:
 - 20i-5:5'-G G T C A G G A T T G A A A
G T G T G C A-3'
 - 20i-3:5'-C T A T G A G A A A A C T G
C A C T G G A-3'

Two nested primer sets were used to further amplify each mutation separately.

- ΔF508 Mutation nested primer set:
 - CF1: 5'-G A A C T G G A G C C T T C
A G A G-3'
 - CF2: 5'-A T G T A G A C T A A C C G
A T T G A-3'
- W1282X Mutation nested primer set (7):
 - W1: 5'-T A C C T A T A T G T C A C A
G A A G T-3'
 - W2: 5'-G T A C A A G T A T C A A A
T A G C A G-3'

The expected amplified products are 261/258 bp for ΔF508 and 270 bp for W1282X mutation. Identity of the amplified normal products for each mutation was confirmed by DNA base pair sequencing (results not shown).

First-Run PCR

The initial 90-μl reaction volume containing DNA and consisting of PCR buffer (2.5 mM MgCl₂, 40 mM Tris-HCl, pH 8.3, 33 mM KCl, 0.01% wt/vol gelatin), 200 μM dNTP (Perkin Elmer), 0.1 μmole outer primers, and 3 U "AmpliTaQ Gold" polymerase (Perkin Elmer), was placed in a DNA thermal cycle (Perkin Elmer PCR system 9600). Following an initial denaturation step at 96°C for 12 min, two programs were followed: (i) Five cycles were carried out using 97°C denaturation for 30 sec, 50°C annealing for 45 sec, and 72°C denaturation for 45 sec; and (ii) 25 cycles were carried out using 95°C denaturation for 20 sec, 50°C annealing for 30 sec, and 72°C extension for 45 sec. Final product extension was carried out at 72°C for 12 min.

Second-Run PCR

A PCR reaction mix was used for the second-run PCR consisting of 1.5 mM MgCl, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% wt/vol gelatin, 200 μM dNTP (Perkin Elmer), 0.3 μmole inner primers and 0.5 U "AmpliTaQ Gold" polymerase (Perkin Elmer). Five microliters of first-run PCR product was removed and added to 45 μl of the reaction mix. After an initial denaturation of 96°C for 12 min, 35 cycles were performed as follows: 95°C of denaturation for 20 sec, 55°C annealing for 30 sec, and 72°C extensions for 45 sec, followed by 72°C extension for 12 min.

Controls

In the first and second PCR runs, reaction tubes containing identical reaction volumes of each PCR buffer but without added DNA from the single cells were run simultaneously with the tubes containing the samples. These served as negative controls for the possible contamination of extraneous DNA. No amplification products were expected or observed from control tubes. Consistent contamination-free processing of samples is averted by adhering to strict isolation protocols during the preparation and analysis of specimens. Separate facilities and isolation hoods were used during processing steps.

Analysis of PCR Products

The analysis of the amplified PCR products from the heterozygous, unaffected homozygous and affected homozygous cell populations is based on the

formation of a heteroduplex artifact pattern on the electrophoresis gel (see Fig. 1, 2 bands). The pattern produced does not always match the pattern expected from the known cell genotype because of the phenomenon referred to as "allelic dropout" (ADO). When gene sequences are amplified from a given cell containing two alleles, one or both of the alleles either may not be amplified or may not be detected during electrophoretic separation. Analysis of the heteroduplex pattern formation, from gene sequences amplified from cell populations with known genotypes, produce accurate measurements of the ADO rate. Knowledge of these ADO rates is crucial during optimization of PCR conditions in order to lessen the chance of transferring embryos incorrectly considered to be unaffected.

ΔF508 Mutation

The expected heteroduplex patterns produced by affected homozygous (A), heterozygous (C-carrier), and unaffected homozygous (N-normal) cell populations are shown in Fig. 1. Lanes 1, 3, 5, and 7 contain 5 μ l of the unmixed products amplified during the second PCR run. Lanes 2, 4, 6, and 8 contain 10 μ l of the following mix. (i) 6.5 μ l of the amplified products for the second PCR run, and (ii) 6.5 μ l of the amplified known normal DNA from the second PCR run. The mixture is denatured at 95°C for 5 min. Reannealing is allowed to take place at 65°C for 5 min. Ten percent polyacrylamide gels were used for electrophoresis and fluorescent SYBR® green I (molecular probes) was used to stain the separated products (8).

Unmixed amplified products from homozygous cells, either affected or normal (lanes 1, 3, 5 of Fig. 1), are expected to yield a single product during electrophoresis because reannealing of two identical complementary strands takes place. In contrast, unmixed amplified products from heterozygous cells are expected to display heteroduplex patterns since two different alleles are amplified. When reannealing occurs, different double-strand species are created. Each species has a different conformation, and therefore migrates to a different position in the gel (see lanes 2, 4, 7, 8, Fig. 1).

The ADO rate is calculated from the proportion of carrier (heterozygote) cells that fail to yield a heteroduplex gel pattern expected from heterozygote cells. If both alleles are amplified, then the expected pattern is obtained. However, if the normal allele is not amplified, the resulting gel pattern will be that

of an affected homozygous cell, thus creating a false-positive report. Conversely, if the affected allele is not amplified, the resulting gel pattern will be that of a normal homozygous cell thus creating a false negative result. During preimplantation genetic diagnosis (PGD), a false-positive result would end in no embryo transfer, since affected embryos are not transferred. However, a false-negative result could cause the transfer of an embryo that carries an affected allele because the embryo would be falsely considered "normal." ADO of normal or affected homozygous cells are of little clinical consequence, since the electrophoretic patterns will continue to reflect the allelic status of the embryo whether ADO does or does not occur. Of course, the lack of total amplification would yield no band pattern, and thus invalidate the test and disqualify an embryo for transfer. The extent to which lack of amplification occurs also is a reflection of the efficiency of the system employed for diagnosis.

W1282X Mutation

No homozygous affected cells were available for analysis. Only homozygous normal and heterozygous affected cells were analyzed. The analysis was based on the band pattern produced by electrophoresis following digestion of the PCR products with the restriction enzyme MnlI. The enzymatic digestion was performed as follows. Five microliters of the second PCR product were incubated with 5 U of MnlI (New England BioLabs) at 37°C for 1 hr in a total volume of 20 μ l. The products were then run on 10% polyacrylamide gels and stained with SYBR® green I. For a normal allele, the 270 bp PCR product yielded two bands: 123 and 60 bp. In contrast, the mutated allele yielded two bands: 210 and 60 bp. Carrier heterozygotes yield three bands: 210, 123, and 60 bp (Fig. 2).

RESULTS

ΔF508 Mutation

The PCR signal efficiency for the affected homozygous, normal homozygous, and carrier heterozygote cell populations were 91%, 81%, and 92%, respectively. The total combined PCR efficiency was 87.7% (Table I).

The ADO rate could not be determined for the homozygous cell populations because the electropho-

Gel patterns obtained from single cell PCR amplification using primers for $\Delta F508$ CF mutation.

Lane 1, 3, 5, 7: PCR product only

Lane 2, 4, 6, 8: equal volume of known normal cell PCR product mixture with 1, 3, 5, 7 PCR product respectively

Lane M: 50 bp DNA marker

Fig. 1. Heteroduplex formation of PCR product in 10% polyacrylamide gel.

Gel patterns obtained from single cell PCR amplification using primers for W1282X CF mutation following digestion with MnII

Lane 1 to 3: Normal cell

Lane 4 to 7: W1282X mutation carrier

Lane M: 50 bp DNA marker

Fig. 2. PCR product cut by MnII in 10% polyacrylamide gel.

Table I. ΔF508 Mutation

Cell type	Genotype	PCR signal efficiency	ADO	
			Affected allele	Normal allele
Lymphoblast Mg04540A	ΔF508 homozygous	64/70 (91%)	N/A ^a	N/A
Fibroblast GM11853	Normal homozygous	46/57 (81%)	N/A	N/A
Fibroblast GM04346	ΔF508 heterozygote	112/122 (92%)	3/122 (2.4%)	4/122 (3.3%)
Total		321/366 (88%)	7/122 (5.7%)	

^a N/A, Not assessable.

retic pattern produced when ADO occurs is no different from that of the pattern seen when ADO does not occur. Nine percent of homozygous affected cells and 19% of homozygous normal cells gave no electrophoretic signals.

The ADO rate for the affected allele in heterozygote cells was 2.4% and an additional 3.3% for the normal allele. The total combined ADO rate was 5.7% (Table I).

W1282X Mutation

For normal homozygous cells, the PCR signal efficiency was 87%. The ADO rates could not be assessed. No signal was observed in 13.1% of cells analyzed. For the carrier heterozygote cells, the PCR signal efficiency was 82.0%. The affected allele ADO rate was 4.3% and the normal allele ADO rate was 4.3%. No signal was observed in 16.6% of the cells analyzed. The total combined PCR efficiency was 85% and the total combined ADO rate was 8.7% (Table II).

No differences were detected between ADO rates of normal or mutated alleles for either mutation.

DISCUSSION

We report a new amplification system to detect two common mutations of the CFTR gene: ΔF508 and W1282X. The system uses novel nested primers that can be employed simultaneously for PCR amplification. The system is more robust than previously reported systems (5, 9) and the stronger signals produced during amplification can be used for single-cell PGD in couples who are carriers of either one or both mutations. Others have in fact reported on the efficiency and accuracy of PCR reactions identifying a single mutation of cystic fibrosis (ΔF508) in single cells (9, 10). It was the PCR diagnosis of the ΔF-508 mutation for cystic fibrosis that led to the first report of successful PGD due to exclusion of a single locus mutation (11).

Avner *et al.* (5), using primers differing from those used in the present study, reported a total PCR signal efficiency of 51% for the ΔF508 allele and 69% for W1282X allele in unfertilized human oocytes and single polyspermic blastomeres. We report a total combined efficiency of 87.7% for the ΔF508 mutation and 81.8% efficiency for the W1282X mutation. The

Table II. W1282X Mutation

Cell type	Genotype	PCR signal efficiency	ADO	
			Affected allele	Normal allele
Fibroblast GM11853	Normal homozygous	53/61 (87%)	N/A ^a	N/A
Fibroblast GM11723D	W1282X heterozygote	46/56 (82%)	2/46 (4.3%)	2/46 (4.3%)
Total		99/117 (85%)	4/46 (8.7%)	

^a N/A, Not assessable.

problem of ADO during amplification of heterozygote (carrier) single cells, as well as situations where compound mutations reside, is of paramount importance (see ref. 12), since it can result in the misdiagnosis of an embryo and result in the transmission of clinical CF. Using the present amplification system, a heterozygote cell, which fails to amplify the normal allele, will show a heteroduplex pattern consistent with an affected cell and the embryo will consequently not be transferred. Conversely, a heterozygote cell that does not amplify the affected allele will show an electrophoretic pattern consistent with a normal diagnosis, and as a result potentially cause expression of the CFTR mutation. In our system, the affected ADO rates are 2.4% for $\Delta F508$ and 4.3% for the W1282X mutation. A mechanism to minimize the risk of transferring an embryo affected with a compound mutation because of misdiagnosis as a carrier due to ADO of one of the alleles, or normal as a result of double dropout, is to analyze more than one blastomere during PGD. The number of blastomeres available for diagnosis, however, is restricted in the early (8 cell) embryo to two without negatively impacting developmental potential. The probability of a double dropout is already low in most cases ($0.024 \times 0.043 = 0.001$ or 0.1%) even with one blastomere. However, the single allele ADO rate will be additive so that an affected embryo will result as an observed carrier on average 3.4% of the time ($0.024 + 0.043 = 0.067/2$ or 3.4%). Using two blastomeres will reduce this rate to 0.12% [$(0.024 \times 0.024) + (0.043 \times 0.043) = 0.0024/2$ or 0.12%] if both blastomeres present as identical carriers. This will lower the potential misdiagnosis rate to acceptable clinical levels well below 1%.

Factors that may have improved the sensitivity of our system over those previously reported include: (i) newly designed nested primers that most likely improved the specificity of amplification of the respective alleles; (ii) "hot start" polymerase (AmpliTaq Gold) that has been shown to enhance the sensitivity of PCR reactions; and (iii) use of SYBR green I fluorescent staining of products separated during electrophoresis, which enhances detection of weaker bands by 10 $\times$ over the more common ethidium bromide staining method. It remains to be seen (work in progress) whether or not the described procedure will provide comparable results when using actual blastomeres from early-stage human embryos. Embryos containing the genetic makeup necessary to test this particular PCR primer system and allow the

assessment of ADO are difficult to obtain in large numbers.

The procedure for determining the presence of two common mutations for CF described in this article combines both high-level signal generation with low ADO outcome, which together help to ensure reliable diagnosis of preimplantation embryos. The time required to accomplish diagnosis (5–8 hr) is compatible with the transfer of selected embryos in a clinical setting.

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