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Reliability of preimplantation diagnosis for single gene disorders

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Abstract

Reliability of preimplantation genetic diagnosis (PGD) depends on controlling one of the most important limitations of single cell PCR, undetected allele drop out (ADO), which may lead to misdiagnosis. To avoid this we introduced mutation analysis simultaneously with linked polymorphic markers, pre-selecting only those embryos whose unaffected status could be confirmed by at least one linked polymorphic marker. We applied this strategy for testing 1047 oocytes, from which 237 unaffected ones were pre-selected for transfer back to patients, resulting in 34 unaffected pregnancies and birth of 23 healthy children. Embryos originating from mutant oocytes and those with insufficient marker information were followed up by multiplex PCR to confirm single cell PCR diagnosis. Of 75 (8.5%) detected ADO, only seven (under 1%) were missed in the actual PGD, demonstrating high reliability of PGD (98%) based on multiplex single cell PCR. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Preimplantation genetic diagnosis; Single gene disorders; Multiplex PCR; Allele drop out; First and second polar body; Blastomeres

1. Introduction

Preimplantation genetic diagnosis (PGD) involves testing single cells biopsied from oocytes or embryos. Therefore, the reliability of PGD depends on the control of well-known limitations of single cell PCR analysis, the most important one being allele drop out (ADO). We have previously demonstrated that ADO rates in single cell PCR may vary with different lysis procedures, cell types and loci analyzed (Rechitsky et al., 1998, 1999). Therefore, reliable methods are needed to detect potential ADO, avoiding misdiagnosis in PGD of single gene disorders. Our preliminary experience demonstrated feasibility of detection of ADO by a sequential analysis of oocytes by the first and second PB (PB1 and PB2), and by simultaneous amplification of mutant genes with linked polymorphic markers (Rechitsky et al., 1999; Verlinsky et al., 1999). We have collected extensive data on this subject, which are presented below.

2. Material and methods

At least two dozen different genetic disorders have been referred to us for PGD, which are presented in Table 1. Following standard IVF protocol, PB1 and PB2 were removed from oocytes using micromanipulation technique. The biopsied single cells were placed directly into a lysis solution, consisting of 0.5 µl 10 × PCR buffer, 0.5 µl 1% Tween 20, 0.5 µl 1% Triton × 100, 3.5 µl H₂O and 0.05 µl Proteinase K (20 mg/ml in a 0.5 ml PCR tube). After spinning down at a low speed in a microfuge for a few seconds, the samples were covered with one drop of mineral oil and incubated at 45 °C for 15 min in a thermal cycler. Proteinase K was then inactivated at 96 °C for 20 min, which is also the beginning of the hot start of the first round PCR. Lower stringency and longer annealing time were used in the first round PCR, with the introduction of the mixture of all outside primers for both mutant genes and polymorphic markers. Following the first round PCR, separate aliquots were amplified in the second round PCR with specific inside primers for each site, using a higher stringency. Such a dual or multiple amplification reaction allowed detection of most of the ADO cases. To eliminate false priming to possible pseudogenes, as in PGD of Gaucher disease or long-

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Table 1

List of conditions analyzed by single cell PCR for preimplantation genetic diagnosis

Cystic fibrosis
Thalassemia
Sickle cell disease
Tay–Sachs disease
Phenylketonuria
Hemophilia A and B
Gaucher disease
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
Epidermolysis bullosa
Retinitis pigmentosa
Ornithine-transcarbamylase deficiency
Alport disease
Alpha-1-antitrypsin deficiency
Multiple epiphyseal dysplasia
X-linked hydrocephalus
Fanconi anemia
p53 tumor suppressor gene mutations
Achondroplasia
Neurofibromatosis type 1
Myotonic dystrophy

chain acyl-CoA deficiency (LCHAD), the first-round primers were designed to anneal to the regions of nonidentity with pseudogenes (Rechitsky et al., 1999). Primer sequences, reaction conditions and details of nested PCR reactions for PGD of the disorders listed in Table 1 have been described elsewhere (Kuliev et al., 1998, 1999; Rechitsky et al., 1998, 1999; Verlinsky et al., 1999). In addition to short tandem repeats (STR) linked to the genes studied, STRs located on other chromosomes were also studied for testing of a possible contamination by extraneous DNA, and identification of the origin of individual embryos in the established pregnancies. A list of STRs, their sequences and PCR reaction conditions for their analysis were also reported previously (Kuliev et al., 1998, 1999; Rechitsky et al., 1998, 1999; Verlinsky et al., 1999).

Fluorescent PCR (F-PCR) was used for a direct fragment size analysis of PCR product (Findlay et al., 1996). F-PCR was useful also for a direct sequencing of the PCR product in the detection of point mutations and for distinguishing preferential amplification from ADO.

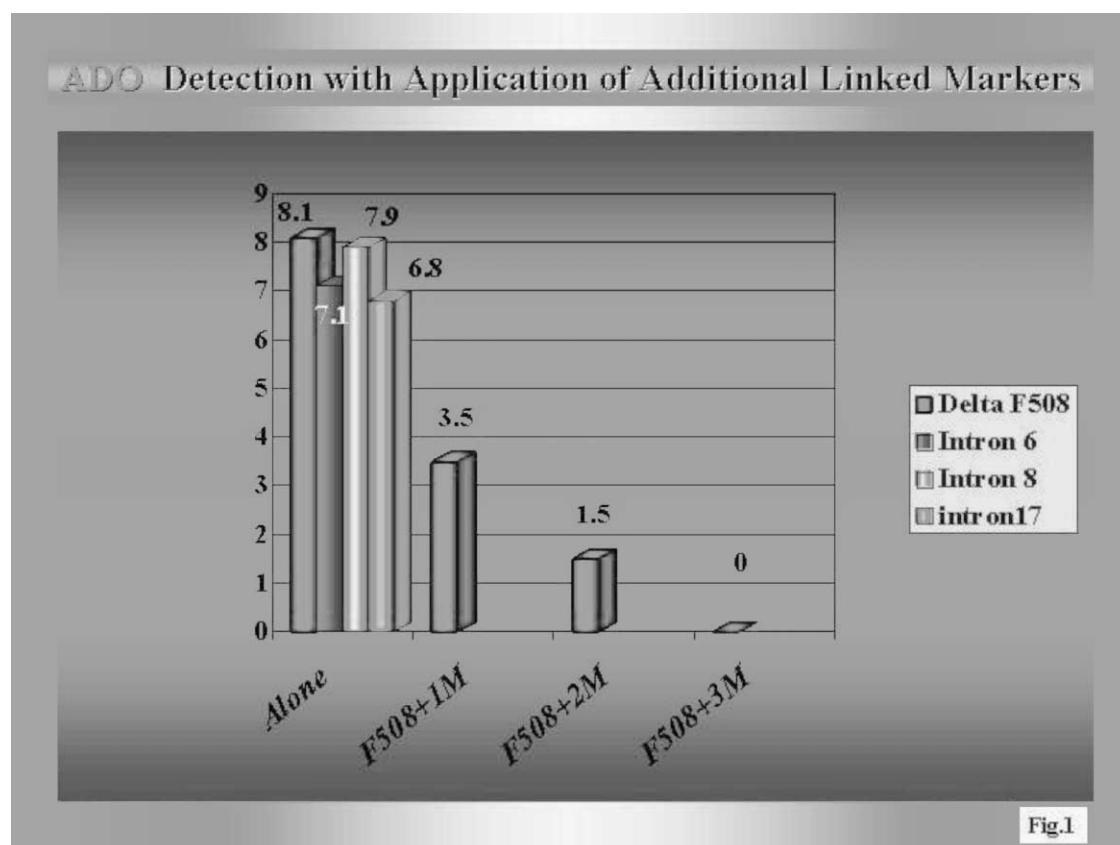


Fig. 1. ADO detection with application of additional linked markers. ADO rates are shown for single and multiplex PCR reactions with single cultured human fibroblasts heterozygous for delta F508 mutation in the CFTR gene. Left panel shows ADO rates for each four single PCR reactions alone, while the right panel demonstrates the decrease of the ADO rates with addition to mutation analysis of one, two and three markers.

The reliability of PGD was tested by the follow up study of the embryos resulting from the oocytes predicted to contain a mutant maternal allele or those with insufficient polymorphic marker information.

3. Results and discussion

A considerable proportion of ADO was detected by sequential analysis of PB1 and PB2. Data on sequential PCR analysis of 26 alleles in PB1 and PB2 obtained from 1047 oocytes showed that 32 of 53 of all ADOs in mutation analysis are detected simply by sequential analysis of PB1 and PB2, avoiding misdiagnosis due to ADO when no informative polymorphic markers are available. A high rate of ADO is observed especially in blastomeres, leading to an obvious misdiagnosis in compound heterozygous embryos, which was the case at the initial stage of application of PGD for single gene disorders when using blastomere biopsy obtained from apparently compound heterozygous embryos (Handyside et al., 1993; Verlinsky et al., 1993; Harper et al., 1996). This may now be avoided by testing two or more linked polymorphic markers if available, mak-

ing DNA testing in PB or single blastomeres a highly reliable procedure (Fig. 1). Contrary to expectation, the application of F-PCR did not sufficiently improve detection of potential misdiagnoses in PGD of single gene disorders (Fig. 2). Testing 148 single fibroblasts by both conventional and F-PCR provided a minor contribution to misdiagnosis (1% for Delta F-508 mutation and 0.9% for intron 6 polymorphism). Simultaneous amplification of single cells for CF Delta F-508 mutation together with one linked polymorphic marker reduced the ADO rate by more than half, irrespective from the use of conventional or F-PCR. With the additional second marker in multiplex PCR the ADO rate was further reduced by half, being completely absent if three markers were simultaneously amplified, as shown above in Fig. 1.

At the present time we have performed PGD in 114 cycles for couples at high risk for having children with single gene disorders, providing the possibility to pre-select and transfer a sufficient number of mutation-free oocytes in almost all cycles. Of 1047 oocytes with DNA results, 672 (64.1%) had to heterozygous PB1, i.e. with both normal and mutant genes amplified, which is ideal for further testing, although their potential transfer

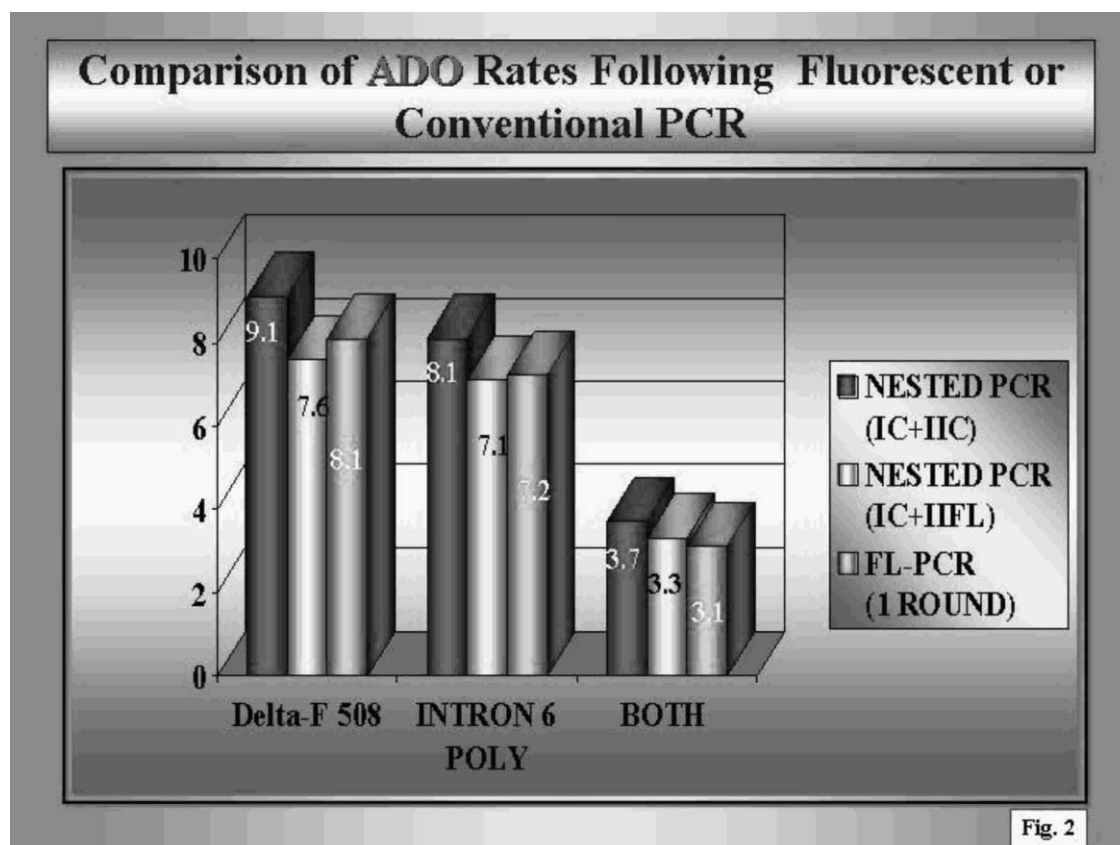


Fig. 2

Fig. 2. Comparison of ADO rates following fluorescent or conventional PCR analysis. ADO rates were analyzed in single cultured human fibroblasts heterozygous for delta F508 mutation in the CFTR gene. First columns show ADO rates observed following both first and second rounds conventional PCR. Second columns show ADO following first conventional and second fluorescent PCR. Third columns show ADO following one round fluorescent PCR (see explanation in text).

depended entirely on the identification of the mutant gene in the sequential analysis of PB2. Thus, a priority in pre-selection of embryos for transfer were given to the embryos resulting from the oocytes with heterozygous PB1 because in the absence of DNA contamination this indicates the absence of ADO of either normal or mutant allele. Although most of the transferred embryos were pre-selected using this particular strategy, some pre-selected embryos still originated from homozygous normal secondary oocytes, inferred from homozygous mutant status of PB1 and hemizygous normal status of PB2. These embryos were accepted for transfer only if ADO could have been excluded using linked polymorphic marker analysis. Otherwise, such embryos were excluded from transfer and exposed to follow up confirmation analysis of the resulting embryos.

This approach made it possible to detect ADO in 53 of 1047 oocytes tested for mutations, which could have contributed to misdiagnosis had they been used for transfer. The follow up analysis of the embryos excluded from transfer either because they were affected or there was insufficient information to pre-select them for transfer, provides the data for evaluating the proportion of undetected ADO. Overall, 82 (7.8%) ADOs were observed, which included 75 detected and seven undetected ones, suggesting that not 970 but actually 1052 oocytes were heterozygous. Genotype of seven embryos appeared to be different from that predicted: six by mutation analysis and one by STR. These differing genotypes were the result of ADO in PB1, these embryos having been diagnosed as homozygous instead of their actual heterozygous status. The data indicate to 98% accuracy, which is quite acceptable for clinical use of PGD for single gene disorders.

The work has presently resulted in pre-selection and transfer of mutation-free embryos in almost all cycles, with 34 established pregnancies, resulting in the birth of 23 healthy children. Further optimization of the method will allow us to completely avoid the risk for misdiagnosis due to ADO and other limitations of single cell PCR analysis in PGD for single gene disorders.

In conclusion, present reliability of PGD for single gene disorders makes it not only an alternative but also an important complement to prenatal diagnosis. PGD gives couples at risk the possibility of avoiding the birth of a child affected with genetic disease without facing prenatal diagnosis and termination of pregnancy.

References

- Findlay, I., Quirke, P., 1996. Fluorescent polymerase chain reaction: Part I. A new method allowing genetic diagnosis and DNA fingerprinting of single cells. *Hum. Reprod. Update* 2, 137–152.
- Handyside, A.H., Delhanty, J.D.A., 1993. Cleavage stage biopsy of human embryos and diagnosis of X-linked recessive disease. In: Edwards, R.G. (Ed.), *Preconception and Preimplantation Diagnosis of Human Genetic Disease*. Cambridge University Press, Cambridge, p. 239.
- Harper, J., 1996. Preimplantation diagnosis of inherited disease by embryo biopsy. An update of the world figures. *J. Assist. Reprod. Genet.* 13, 90–95.
- Kuliev, A., Rechitsky, S., Verlinsky, O., Ivakhnenko, V., Evsikov, G., Wolf, G., Angastiniotis, M., Georgiou, D., Kukhareno, V., Strom, C., Verlinsky, Y., 1998. Preimplantation diagnosis of Thalassemias. *J. Assist. Reprod. Genet.* 15 (5), 219–225.
- Kuliev, A., Rechitsky, S., Verlinsky, O., Ivakhnenko, V., Cieslak, J., Evsikov, S., Wolf, G., Angastiniotis, M., Kalakoutis, G., Strom, C., Verlinsky, Y., 1999. Birth of healthy children after preimplantation diagnosis of Thalassemias. *J. Assist. Reprod. Genet.* (2).
- Rechitsky, S., Strom, C., Verlinsky, O., Amet, T., Ivakhnenko, V., Kukhareno, V., Kuliev, A., Verlinsky, Y., 1998. Allele drop out in polar bodies and blastomeres. *J. Assist. Reprod. Genet.* 15, 253–257.
- Rechitsky, S., Strom, C., Verlinsky, O., Amet, T., Ivakhnenko, V., Kukhareno, V., Kuliev, A., Verlinsky, Y., 1999. Accuracy of preimplantation diagnosis of single-gene disorders by polar body analysis of oocytes. *J. Assist. Reprod. Genet.* 16, 169–175.
- Verlinsky, Y., Kuliev, A.M. (Eds.), 1993. *Preimplantation Diagnosis of Genetic Diseases: A New Technique in Assisted Reproduction*. New York: Wiley-Liss.
- Verlinsky, Y., Rechitsky, S., Verlinsky, O., Ivakhnenko, V., Lifchez, A., Kaplan, B., Moise, J., Valle, J., Borkowski, A., Nefedova, J., Goltsman, E., Strom, C., Kuliev, A., 1999. Prepregnancy testing for single-gene disorders by polar body analysis. *Genet. Test* 3, 185–190.