Accuracy of Preimplantation Diagnosis of Single-Gene Disorders by Polar Body Analysis of Oocytes

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Purpose: A number of pitfalls in single-cell DNA analysis, including undetected DNA contamination, undetected allele drop out, and preferential amplification, may lead to misdiagnosis in preimplantation genetic diagnosis of single-gene disorders.

Methods: Preimplantation genetic diagnosis was performed by sequential first and second polar body analysis of oocytes in 26 couples at risk for having children with various single-gene disorders. Mutant genes were amplified simultaneously with linked polymorphic markers, and only embryos resulting from the mutation-free oocytes predicted by polar body analysis with confirmation by polymorphic marker testing were transferred back to patients.

Results: Overall 529 oocytes from 48 clinical cycles (26 patients) were tested, resulting in the transfer of 106 embryos in 44 clinical cycles. As many as 46 (9.6%) instances of allele dropout were observed, the majority (96%) of which were detected. Seventeen unaffected pregnancies were established, of which nine resulted in the birth of an unaffected child, and the rest are ongoing.

Conclusions: A high accuracy of preimplantation genetic diagnosis of single-gene disorders is achieved by application of sequential analysis of the first and second polar body and multiplex polymerase chain reaction.

KEY WORDS: preimplantation diagnosis; single-gene disorders; first and second polar bodies; allele dropout; multiplex PCR.

INTRODUCTION

Because preimplantation genetic diagnosis (PGD) involves genetic analysis in single cells, its accuracy depends on the controlling the limitations of single-cell

polymerase chain reaction (PCR) analysis, which include allele dropout (ADO). It has been demonstrated that single-cell ADO rates vary with different cell types and loci analyzed (1–4). This makes the development of methods for efficient detection of ADO desirable, to minimize misdiagnosis in PGD of single-gene disorders. We have recently introduced a sequential analysis of the first (I) and second (II) polar body (PB) for preselection of mutation-free oocytes, in addition to simultaneous amplification of mutant genes and linked polymorphic markers, to detect ADO and avoid misdiagnosis (4,5). In the present paper, the impact of the application of both of these approaches for PGD of different single-gene disorders was studied, demonstrating their accuracy in detecting ADO and avoiding misdiagnosis in PGD of single-gene disorders.

MATERIALS AND METHODS

Forty-eight in vitro fertilization cycles in 26 couples were performed for PGD of the following single-gene disorders: cystic fibrosis (CF)(Δ F-508 and G551D), thalassemia (IVSI-110, IVSI-6, and IVSII-745), sickle cell disease, Gaucher disease, hemophilia B, and long-chain acyl-CoA dehydrogenase deficiency (LCHAD). This includes some data from the PGD cycles described elsewhere (4,6,7). PGD was performed by genetic analysis of the IPB and IIPB, removed from oocytes after maturation and fertilization, using micromanipulation techniques. Genetic analysis was based on multiplex nested PCR analysis, with the introduction of primers for the mutant genes and polymorphic markers in the first round (4). Primer sequences, reaction conditions, and details of the nested PCR reaction for PGD of the majority of the above single-gene disorders have been described (1,4,6). The strategies for PGD for Gaucher disease, CFTR G551D, and LCHAD

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are presented in Figs. 1–3 and Table I. In addition to short tandem repeats (STRs) linked to the genes studied, STRs located on other chromosomes were also studied to test possible contamination by extraneous DNA. The following STRs were tested: THO 1,

F13A1, FES/FPS, vWF, D21S11, and CSFIR. To investigate the accuracy of PB PGD diagnosis, the embryos resulting from oocytes predicted to contain a mutant maternal allele were exposed to confirmatory PCR analysis.

Fig. 1. Schematic diagram of nested PCR for the 1448c/444 mutation in the glycerol-3-phosphate dehydrogenase gene (Gaucher disease). To eliminate false priming to the pseudogene, first-round primers GP-1, GP-4, GA-1, and GA-5 are designed to anneal to the regions of nonidentity with the pseudogene. To detect potential ADO in the first polar body, two linked polymorphic markers were analyzed. The *PvuII* polymorphism is located in intron 6 of the glycerol-3-phosphate dehydrogenase gene. The *BspHI* polymorphism is located outside the boundaries of the glycerol-3-phosphate dehydrogenase gene and displays 100% disequilibrium with the mutation. The maternal mutation is linked to the presence of the *BspHI* restriction site and to the absence of the *PvuII* site. (A) Relative positions of the mutation site (+*NciI*), linked restriction polymorphisms on chromosome 1 (*BspHI*, *PvuII*), and PCR primers used in the analysis. The pseudogene is shown as a bar directly below the gene. The shaded areas represent regions of identity with the Gaucher gene. The outer primers lie outside of these identity regions. (B) Restriction maps of the mutation and the polymorphic sites, showing band lengths and nucleotide substitutions. (C) Primer sequences and lengths used for analysis.

Fig. 2. Schematic diagram of hemi-nested PCR for the **G1528C** mutation in the **LCHAD** gene on chromosome 2p24.1. To eliminate false priming to the pseudogene, first-round primers LC-1 and LC-2 are designed to span the intron 14/exon 15 boundary and intron 15/exon 16 boundary, respectively (8). The G1528C mutation causes the formation of a *Pst*I restriction site in exon 15. Restriction digestion of the second-round PCR product produces two fragments (60 and 42 bp) demonstrating the presence of the mutant allele, while a normal allele results in a single fragment (102 bp). (A) Positions of the mutation site and of the primers involved in the analysis. The synthetic pseudogene is shown as a shaded bar directly below exon 5. The outer primers lie outside of the pseudogene. (B) Restriction map of first- and second-round PCR products, showing band lengths and the nucleotide substitution. (C) Sequences and melting temperatures (T_m) of the primers involved. Primers LC-1 and LC-2 were obtained from Ref. 8.

RESULTS AND DISCUSSION

Five hundred twenty-nine oocytes were obtained from 48 clinical cycles of 26 patients at risk for having children with single-gene disorders, including 17 cycles for thalassemia, 6 for sickle cell disease, 21 for CF, 2 for LCHAD, 1 for hemophilia B, and 1 for Gaucher disease. Of these 529 oocytes, 331 (62.6%) were heterozygous after IPB genetic analysis, so their potential transfer depended on the sequential

analysis of the IIPB. No heterozygous oocytes were observed in PGD of Gaucher disease. This is not surprising, as the gene is mapped close to the centromere of chromosome 1. Of all six oocytes studied whose IPBs were homozygous, three were predicted normal and transferred, while the other three, predicted to be abnormal, were confirmed to contain a mutant gene, based on the follow-up study of the embryos resulting from these oocytes (Fig. 4).

The data from both the IPB and the IIPB were

Fig. 3. Schematic diagram of nested PCR for the G551D mutation and linked polymorphisms in the CFTR gene. To increase the accuracy and reliability of the mutation analysis, several linked polymorphic markers were tested along with the mutation. The patient was informative for three restriction polymorphisms in exons 10, 19, and 21, and an STR in intron 6. (A) Relative positions of the mutation site (+MboI), linked restriction polymorphisms (poly [GATT] repeat, TUB 18, TUB 20), and PCR primers used in the analysis. (B) Sequences and melting temperatures (T_m) of the primers used to amplify the mutation segment.

available in 318 of 529 oocytes, making it possible to predict the genotype of 285 oocytes, including 203 derived from those with heterozygous IPBs, of which 142 were mutation-free and 153 were affected (Table II). Of 142 mutation-free oocytes, the genotype of only 42 (29.6%) could have been predicted normal following meiosis I (based on IPB analysis). However,

in the absence of polymorphic marker analysis, the homozygous status of such oocytes cannot be distinguished from ADO in oocytes with a heterozygous IPB. In fact, ADO was detected in 24 (7.2%) of 318 oocytes studied, of which 18 (5.4%) were detected solely by the sequential IPB and IIPB analysis, and 6 (1.8%) by the use of polymorphic markers (Table III).

Table I. Optimal Conditions for Nested PCR of Single Cells in Preimplantation Diagnosis of LCHAD, Gaucher Disease, and CFG551D

	Round I PCR, multiplex	Round II PCR, separate reactions	
		Exon 10 mutation (1448C/444) ^a	PKLR polymorphism (BspHI)
MgCl ₂	1.5 mM	1.5mM	1.5 mM
Acetamide (Sigma)	5%	5%	5%
dNTPs (4)	100 μM (each)	50 μM (each)	50 μM (each)
PCR buffer	600 mM Tris-HCl, 150 mM (NH ₄) ₂ SO ₄		
Outside primers	0.2 μM (each)		
Inside primers		0.4 μM (each)	0.4 μM (each)
Lysis buffer ^b	0.1% Triton X-10, 0.1% Tween 20, 0.2 ng/μl proteinase K		
Taq polymerase	1.25 U	1 U	1 U
No. of cycles	28	25	25
Annealing temp.	45°C	53°C	50°C
Denaturing temp.	90°C	95°C	95°C

^a Conditions for the second-round PCR for the Intron 6 PvuII polymorphism and mutation 1528 of LCHAD were the same as for the exon 10 (1448C) mutation.

^b Cells were lysed in 5 μl of lysis buffer at 45°C for 15 min and then proteinase K was destroyed by incubation at 95°C for 20 min.

Fig. 4. Preimplantation genetic diagnosis for Gaucher's disease and confirmation of results. Polyacrylamide gel electrophoresis of PCR products: PB1, first polar body; PB2, second polar body; B, blastomere; ET, embryo transferred; normal, 202-bp undigested band representing the normal allele; mutant, cleavage products of the mutant allele. The absence of bands in a particular lane signifies amplification failure (108 and 84 bp). *Pvu II* -, 311-bp undigested PCR product; *Pvu II* +, 237- and 74-bp cleavage products of *PvuII* digestion. *BspH I* -, 121-bp undigested PCR product; *BspH I* +, 54 and 67-bp cleavage fragments of *BspHI* digestion. For this particular patient, the normal allele was linked to *Pvu II* + and *BspH I* -. Left panels: Diagnoses of oocytes 1-6 based on first and second polar body analysis. In all left panels the first lane represents the first polar body of oocyte number 1, the second lane represents the second polar body of oocyte number 1, etc. Right panels: Confirmation of polar body diagnoses of affected oocytes and their corresponding, nontransferred embryos.

For example, ADO in 2 of 15 oocytes was detected by sequential analysis of the IPB and IIPB in the case of PGD of LCHAD, which is obvious from the homozygous mutant state for both the IPB and the IIPB, suggesting ADO of a normal allele in a heterozygous IPB (Fig. 5). The follow-up study of the embryos

resulting from these oocytes, as well as in similar cases of PGD of other conditions, confirmed the PB diagnosis in 157 (98%) of 160 embryos for which confirmation analysis was available (Table IV).

Similar results were obtained for analysis of polymorphic markers, which was performed in 459

Table II. Results of Two-Step Polar Body Analysis in Predicting the Genotype of Oocytes

Loci	Total No. of oocytes amplified	Oocytes with IPB + IIPB data	Homozygotes after IPB analysis		Hemizygotes after IIPB analysis		Oocytes with predicted genotypes		
			N (A1)	M (A2)	N (A1)	M (A2)	N (A1)	M (A2)	Total
Mutations (9)	529	318	42	50	100	103	142	153	285
STRs (6)	459	314	54	40	95	83	149	123	272
Total	988	632	96	90	195	186	291	276	557

Table III. Follow-Up of PGD of Single-Gene Disorders Based on Sequential Analysis of Polar Bodies

Loci	Total oocytes amplified	Heterozygous IPB	ADO detected by	
			2-step PB analysis	Linked markers
Mutations (9)	529	331	18	6
STR's (7)	459	289	17	2
			35	8
Total	988	620	(5.3%)	(1.2%)

oocytes. Two hundred eighty-nine (63%) oocytes were heterozygous after IPB analysis, with ADO observed in 22 oocytes (7%), of which 19 (6.5%) were detected, including 17 (5.9%) detected by sequential analysis of the IPB and IIPB and 2 (0.6%) by the use of STRs (Table III). The follow-up analysis of 147 embryos for the polymorphic markers studied confirmed the PB diagnosis in 139 cases (96%). Overall, the follow-up analysis for all 16 loci, including 9 mutant alleles and

7 STRs, confirmed the PB PGD in 296 of 307 embryos studied, suggesting a 96% accuracy of PGD (Table IV).

Of 48 clinical cycles performed for PGD of single-gene disorders listed, normal embryos for transfer were preselected in 44 of them. Of a total of 529 oocytes tested, 318 had data for both the IPB and the IIPB, indicating the mutation-free status in 106 of these oocytes. The transfer of corresponding embryos resulted in 17 (36.6%) pregnancies, of which 6 children have already been born. The availability of approximately six or seven oocytes per cycle with data for both the IPB and the IIPB made it possible to preselect at least two or three mutation-free oocytes for transfer per cycle. The method, therefore, may be acceptable for clinical practice, as it allows preselection of a sufficient number of mutation-free embryos for transfer and provides the possibility to minimize misdiagnosis due to ADO. Further optimization of the method may completely avoid the risk of misdiagnosis due to ADO and other limitations of single-cell PCR analysis for PGD of single-gene disorders.

Fig. 5. Preimplantation genetic diagnosis of LCHAD deficiency. Polyacrylamide gel electrophoresis of *Pst*I digested PCR products of the first and second polar bodies from 12 oocytes. PB1, first polar body; PB2, second polar body; ET, embryo transferred; ADO, detected allele dropout; normal, 102-bp undigested PCR product; mutant, 60- and 42-bp *Pst*I digestion products; 3H, LCHAD mutation allele; N, normal allele; FA, failed amplification.

Table IV. Results of Confirmation of PGD of Single-Gene Disorders by Polar Body Analysis

Loci	Total oocytes amplified	Heterozygous IPB	Total ADO detected by 2-step PB analysis or linked markers	Resulting embryos	
				Studied	Confirmed
Mutations (9)	529	331	29	160	157 (98%)
STRs (7)	459	289	19	147	139 (95%)
Total	988	620	43	307	296 (96%)

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