Journal of Assisted Reproduction and Genetics, Vol. 15, No. 5, 1998

Allele Dropout in Polar Bodies and Blastomeres

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Submitted: January 7, 1998
Accepted: January 7, 1998

Purpose: *Because allele dropout (ADO) is frequently observed in single-cell polymerase chain reaction analysis, it is important to develop a method for efficient detection of ADO, in order to avoid possible misdiagnosis in preimplantation diagnosis.*

Methods: *We introduced a simultaneous amplification of mutant genes and linked polymorphic markers, such as a 4-bp repeat (GATT) at the 3′ end of intron 6 in the cystic fibrosis (CF) gene and a short tandem repeat at the 5′ end of the β-globin gene. Three types of single heterozygous cells were studied for the amplification of both alleles, including 150 blastomeres, 1615 fibroblasts, and 170 first polar bodies, obtained from patients at risk for having children with cystic fibrosis (delta F-508 mutation) or sickle cell disease.*

Results: *ADO rates of as high as 33.3% for delta F-508 mutation and 22.8% for β-globin gene were observed in single blastomeres, compared to 7.1 and 7.7% in single fibroblasts and 5.9 and 9.6% in first polar bodies, respectively. The application of simultaneous amplification of the above linked polymorphic markers allowed detection of more than half of the cases of ADO in blastomeres (19.4% for cystic fibrosis and 12.3% for β-globin gene) and almost all ADOs in polar bodies, particularly when the two-step sequential analysis of the first and second polar body was applied in preimplantation diagnosis of single gene disorders.*

Conclusions: *Simultaneous amplification of linked polymorphic markers in single-cell DNA analysis of single-gene defects is an efficient method for avoiding the risk of misdiagnosis in preimplantation diagnosis.*

KEY WORDS: preimplantation diagnosis; PCR; allele dropout; short tandem repeats; single-gene disorders.

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INTRODUCTION

Preimplantation genetic diagnosis (PGD) of single-gene disorders is based on polymerase chain reaction (PCR) analysis of single blastomeres or polar bodies (PBs)(1–3). Allele-specific amplification failure, or allele dropout (ADO), has been shown to be one of the most important problems in single-cell PCR analysis (4–6). ADO is one of the most probable explanations for the three previously reported cases of misdiagnosis of cystic fibrosis (CF) in PGD by blastomere biopsy, as all the affected embryos were compound heterozygotes; i.e., only one of the two mutant alleles from heterozygous blastomeres analyzed by PCR might have been detected (7,8). To avoid misdiagnosis due to ADO, its frequency must be determined and minimized for each locus and each assay system, particularly when double-heterozygous status of the preimplantation embryo cannot be excluded.

The present paper describes our results on the evaluation of ADO rate in single blastomeres, PBs, and blastomeres and approaches to detecting and avoiding ADO in PGD of single-gene disorders.

MATERIALS AND METHODS

Three types of single heterozygous cells were studied for the presence of both alleles, including blastomeres, fibroblasts, and first PBs (IPB) obtained from patients at risk for having children with CF (delta F-508), hemophilia B, and sickle cell disease. We introduced the simultaneous amplification of mutant genes and their linked polymorphic markers, such as a 4-bp repeat (GATT) at the 3′ end of intron 6 in the CFTR gene (9) and a 4-bp repeat (ATTT) at the 5′ end of the β-globin gene and HUMTHO1, which is syntenic with β-globin (10,11).

Single fibroblasts were obtained by micromanipulation from cultured skin of patients and parents. PBs

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were removed from oocytes after maturation and fertilization, and blastomeres were dissociated from cleaving embryos resulting from oocytes predicted to be abnormal based on the analysis of IPB and IIPB in clinical cases (12).

Primers were synthesized on an Applied Biosystems 381A Synthesizer (with the Trityl off protocol). Primer sequences, reaction conditions, and details of the nested and heminested PCR used have been described (4,12).

In multiplex PCR experiments the first-round reaction buffer contained a mixture of outside primers for all systems. After 25 cycles, 2–5 μl was removed and placed in separate tubes containing the inside primers for each individual locus (Fig. 1). PCR products were analyzed by heteroduplex detection for delta F-508, by *Dde*1 (Promega) restriction digestion for sickle cell disease, and by fragment size assignment for short tandem repeats (STRs).

RESULTS AND DISCUSSION

ADO rates were approximately fivefold higher in blastomeres (33.3%) for delta F-508 and threefold higher in sickle cell disease (22.8%), compared to 7.1 and 7.7% in single fibroblasts and 5.9 and 9.6% in IPBs, respectively (Table I). These results were highly statistically significant, with P values of less than 10^{-6}. The statistical analysis of the β-globin results revealed a high significance ($P = 0.00055$) in the comparison of fibroblasts to blastomeres.

The application of simultaneous amplification of linked polymorphic markers allowed the detection of

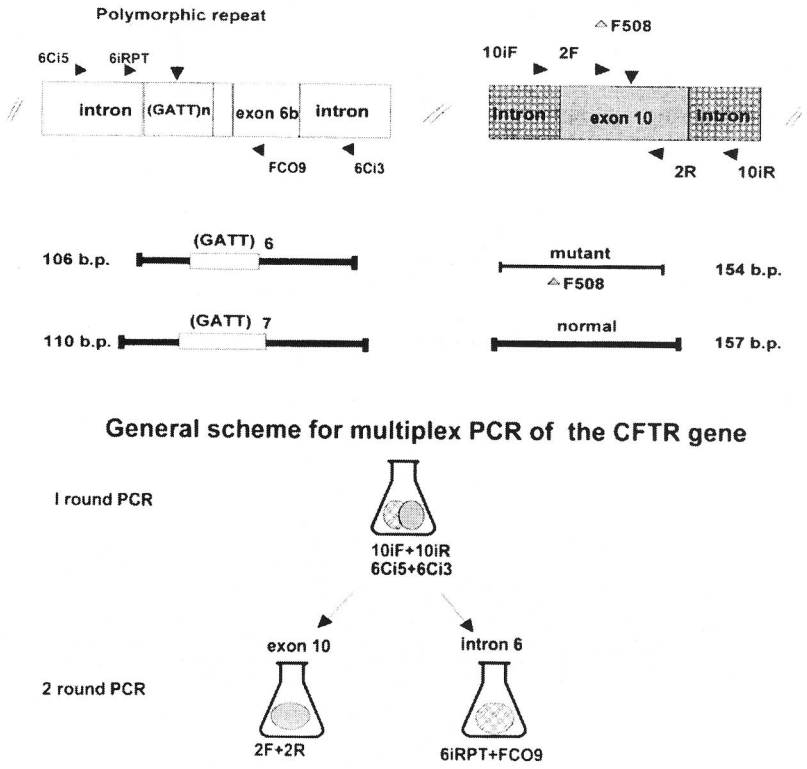

Fig. 1. Schematic diagram of multiplex PCR for the delta F-508 mutation and intron 6 polymorphism marker in the CFTR gene. Top left: Intron 6, 4-bp polymorphism (six or seven repeats). Primer sequences described in Ref. 4. Top right: Exon 10 delta-F508 mutation. Primer sequences described in Ref. 4. Middle: Expected sizes of the second-round PCR products for both loci. Bottom: Diagram of scheme. Initially four outside primers are added to the single cell and 25 rounds of PCR performed. Then 2- to 5-μ aliquots are distributed into two separate tubes. In one tube the inside primers for the intron 6 polymorphism are added prior to performing 25 additional PCR cycles (second-round PCR). In the second tube, inside primers for the delta F-508 mutation are added prior to performing 25 additional PCR cycles second-round PCR).

Table I. Allele Dropout Rate in Different Types of Heterozygous Single Cells

Cell type	Heterozygous cystic fibrosis cells (CFTR delta-F508/N)			Heterzygous cells with sickle cell mutation (S/N)		
	Total amplified	Both alleles	One allele (ADO)	Total amplified	Both alleles	One allele (ADO)
Single blastomeres	93	62	31 (33.3%)	57	44	13 (22.8%)
Single fibroblasts	1126	1046	80 (7.1%)	489	451	38 (7.7%)
1st polar bodies	118	111	7 (5.9%)	52	47	5 (9.6%)

more than half of the cases of ADO in blastomeres (19.4% for CF and 12.3% for the β-globin gene) (Table II) and most of the cases of ADO in PBs (Table III).

The method was applied in clinical cases of PB diagnosis of single-gene disorders, demonstrating the efficiency of the method in avoiding the risk of misdiagnosis in PGD.

The results of our experiments on the efficiency of multiplex PCR for nine loci are presented in Table IV. In addition to CFTR, the β-globin gene, and hemophilia B, the data set includes STR loci on chromosome 21 and the Von-Willibrands disease gene on chromosome 12 (13,14). As shown in Table IV, ADO occurred in 7 (4%) of 153 oocytes studied for the three disease mutations and 18 (6.5%) of 291 cells analyzed for STRs. Overall, the ADO rate in IPB was as low as 5.5% for all loci studied. However, as can be seen from the follow-up study of the embryos resulting from the oocytes predicted to contain the affected allele, most ADOs were picked up by multiplex PCR: 97.1% of the predicted genotypes were confirmed (98.5% for mutations and 96.3% for STRs) (Table IV). The application of simultaneous amplification of as many as five loci in the follow-up study of an embryo, from which 13 dissociated blastomeres were analyzed, is shown in Fig. 2: in one blastomere only one locus showed ADO, whereas in another cell the other allele showed ADO; in some blastomeres more than one loci showed ADO (Fig. 2).

The other approach to detecting ADO and avoiding misdiagnosis in IPB diagnosis is sequential analysis of IPB and IIPB. A combined application of the two-step analysis of IPB and IIPB with multiplex PCR is the most effective method to avoid undetected ADO, as demonstrated in Fig. 3, which shows examples of ADO in IPB, which could potentially lead to misdiagnosis without using this combined approach for detecting of ADO in IPB analysis. As shown in Fig. 3, some ADOs are obvious even without multiplex PCR: the situation in which both IPB and IIPB are tested, having the same single band. Such results are interpreted as ADO in a heterozygous IPB and were confirmed by multiplex PCR using linked polymorphisms (also shown in Fig. 3). However, in cases where IPB and IIPB have different single signals, in the absence of the linked polymorphic markers, the ADO in IPB will not be detected, and this could lead to a transfer of an affected embryo. The actual example of such a case is demonstrated in Fig. 3: because of ADO in the heterozygous IPB, proven by the heterozygosity

Table II. Value of Linked Markers to Detect ADO in Blastomeres

Locus	No. of blastomeres amplified	Both alleles amplified	One allele amplified	ADO rate (%)
CFTR				
Exon 10	93	62	31	33.3
Intron 6	93	74	19	20.4
Both loci[a]	93	80	13	13.9
β-Globin gene				
SCA mutation	57	44	13	22.8
5′ STR	57	49	8	14.0
Both loci[a]	57	51	6	10.5

[a] At least one locus indicated the heterozygote genotype.

Table III. Value of Linked Markers to Detect ADO in Heterozygote IPBs

Locus	No. of heterozygote I PBs amplified	Both alleles amplified	One allele amplified	ADO rate (%)
CFTR				
Exon 10	46	43	3	6.8
Intron 6	46	44	2	4.3
Both loci[a]	46	46	0	0
β-Globin gene				
SCA mutation	22	20	2	9
5′ STR	22	19	3	13.6
Both loci[a]	22	21	1	4.5
β-Globin gene				
SCA mutation	28	25	3	10.7
HumTHO 1	28	24	4	14.28
Both loci[a]	28	27	1	3.5

[a] At least one locus indicated the heterozygote genotype.

Table IV. Results of Two-Step Polar Body Analysis in Predicting the Genotype of Oocytes and Follow-up of the Diagnosis in the Resulting Embryos

Locus	Total oocytes obtained	Oocytes with IPB		Oocytes with IIPB	Oocytes with IPB and IIPB		Resulting embryos	
		Heterozygote	Homozygote		Total	ADO	Studied	Confirmed
Mutations (3)[a]	308	166 (59.9%)	101 (36.5%)	201 (72.7%)	153 (55.2%)	7 (4%)	130	128 (98.5%)
STRs (6)[b]	472[c]	259 (60.1%)	165 (38.9%)	314 (74.0%)	291 (68.6%)	18 (6.5%)	219	211 (96.3%)
Total	780[c]	425 (60.6%)	266 (37.9%)	515 (73.5%)	444 (63.3%)	25 (5.5%)	349	339 (97.1%)

[a] CFTR delta F-508, sickle cell anemia, and hemophilia B mutations.
[b] HumvWf, Hum21S11, HumTHOI, HumFES/FPS, HumF13A1, and 5β-globin STRs.
[c] These numbers do not correspond to the number of oocytes, because STRs and mutations were studied simultaneously in the same oocytes.

of the linked polymorphic marker, the actual genotype of the resulting oocyte is opposite to the predicted genotype from the mutation analysis alone.

A combination of both approaches (linked markers and sequential PB analysis) for the detection of ADO has been applied in 24 clinical cycles of PGD for CF,

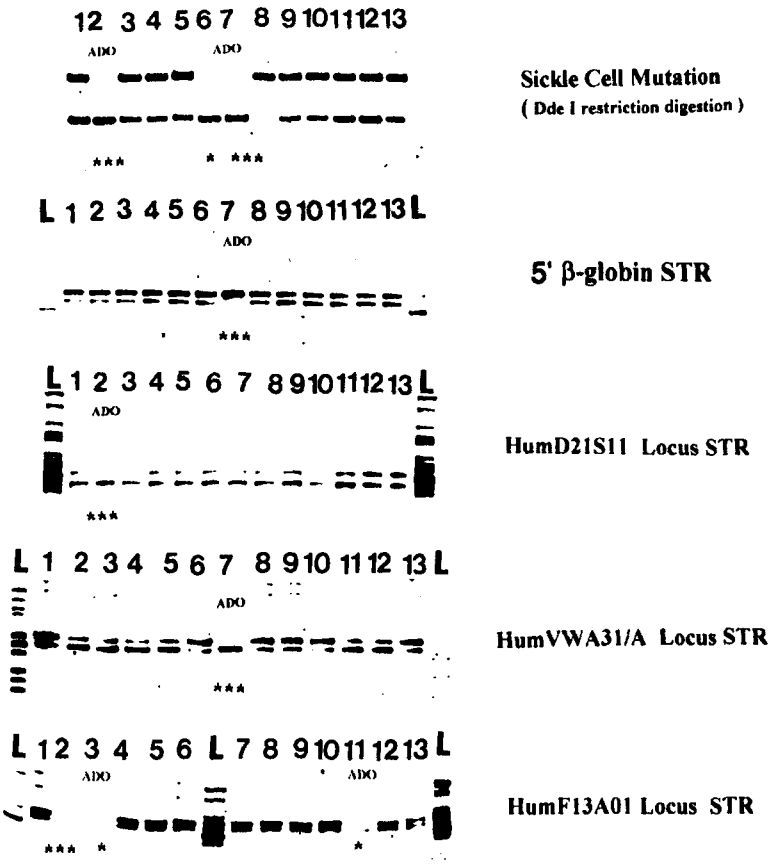

Fig. 2. Multiplex PCR analysis of dissociated blastomeres at five loci from a single embryo. The loci analyzed are noted at the right. L, allele-specific ladder (differs for each system analyzed). Numbers 1–13 correspond to blastomeres 1–13 throughout. (*) ADO observed in one system. (***) ADO observed in three of five systems from a single blastomere.

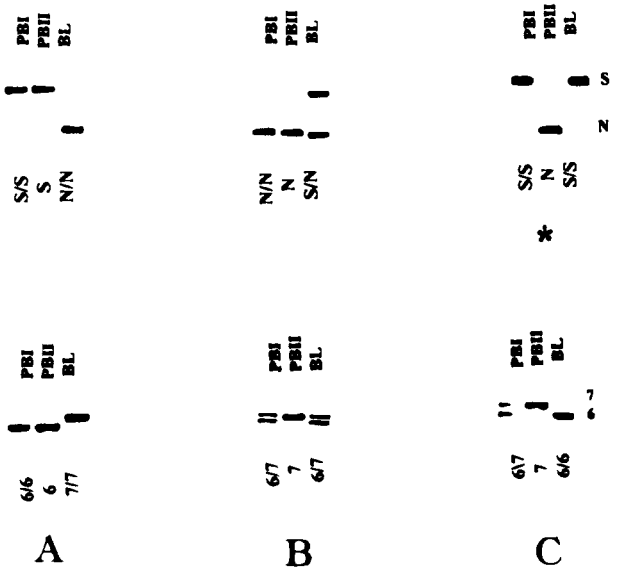

Fig. 3. Examples of detection of ADO that could lead to misdiagnosis in a two-step polar body analysis at the β-globin locus using linked marker analysis. Top: *Dde*1 restriction digestion of PCR products to assay the sickle cell mutation. Bottom: 5′ Globin STR polymorphism (either six or seven repeats). A, Embryo A; B, Embryo B; C, Embryo C. PBI, first polar body; PBII, second polar body; BL, blastomere. S, sickle cell allele; N, unaffected allele; SS, homozygous sickle cell; SN, heterozygous sickle cell/unaffected; NN, homozygous unaffected, 6, six-repeat polymorphism; 7, seven-repeat polymorphism. (*) ADO detected by use of linked markers. Without the use of this linked marker, this affected embryo could have been transferred.

sickle cell disease, hemophilia B, and thalassemia, resulting in the birth of three unaffected children (12). The follow-up study of the embryos resulting from the oocytes diagnosed as affected (Table IV) confirmed the diagnosis in 128 of 130 embryos (in 211 of 219 for polymorphic markers), demonstrating the reliability and accuracy of the above approaches in avoiding misdiagnosis due to ADO in PGD of single-gene defects. This establishes a misdiagnosis rate of approximately 1% when linked markers are combined with sequencial PB analysis. However, when the IPB is heterozygous, there is no potential misdiagnosis due to ADO, because both alleles successfully amplified.

The five-fold higher rate of ADO in blastomeres with respect to both PBs and fibroblasts demonstrates that blastomere biopsy should not be used for PGD in recessive diseases when the patients carry different mutations, X-linked diseases, and dominant diseases. Other groups have also described high ADO rates in blastomere analysis for CF (5,6).

These data demonstrate that sequential PB analysis is the method of choice for PGD of Mendelian recessive and X-linked disorders and in dominant diseases when the mother has the affected allele.

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