GENETICS

Preimplantation Genetic Diagnosis of Inherited Cancer: Familial Adenomatous Polyposis Coli

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Submitted: August 19, 1997
Accepted: September 30, 1997

Purpose: *Our purpose was to achieve preimplantation genetic diagnosis (PGD) of the dominant cancer predisposition syndrome, familial adenomatous polyposis coli (FAPC), as an alternative to prenatal diagnosis.*
Methods: *The affected patient was superovulated and oocytes were retrieved and fertilized by intracytoplasmic sperm injection (ICSI). Two cells were biopsied from each embryo and the whole genome was amplified by primer extension preamplification (PEP). Nested PCR was then used to amplify two APC fragments: one including the APC mutation site and the other an informative intragenic polymorphism. Both were detected by simultaneous single-strand conformation polymorphism and heteroduplex analysis.*
Results: *Four normally fertilized embryos were biopsied on day 3 post ICSI, and two cells were successfully removed from each embryo. Following PEP the APC mutation was successfully amplified in 7 of 8 cells, and the polymorphism in 6 of 8 cells. The APC mutation was detected in three embryos. This result was confirmed by identification of the mutation associated polymorphism in two cases. A single embryo was diagnosed as homozygous normal for the mutation and the polymorphism in both cells sampled. This unaffected embryo was transferred to the mother, but no pregnancy resulted.*
Conclusions: *We report here the first diagnosis of a cancer predisposition syndrome in human preimplantation embryos. Our results indicate that difficulties associated with single-cell PCR, allele-specific amplification failure in particular, need not prevent preimplantation diagnosis of diseases with a dominant mode of inheritance, provided appropriate strategies are applied.*

KEY WORDS: familial adenomatous polyposis coli; cancer predisposition; preimplantation diagnosis.

INTRODUCTION

The molecular genetic basis of a growing number of inherited cancer syndromes is now understood, but this in turn creates problems for clinical management because there are very few measures available to prevent the onset of cancer in such individuals (1). In general, prenatal diagnosis is offered to families at risk of having children with genetic disease and the pregnancy may be terminated if affected. However, termination of pregnancy after conventional prenatal diagnosis [amniocentesis or chorionic villus sampling (CVS)] for couples carrying a cancer predisposing gene mutation is controversial and may be unacceptable to some couples. Affected children are otherwise healthy and may remain so for many years. In vitro fertilization (IVF) techniques with preimplantation genetic diagnosis (PGD) allow the selection and transfer of only unaffected embryos to the uterus. Any pregnancy therefore will be unaffected and the possibility of termination following diagnosis at later stages of pregnancy avoided. PGD involves the generation of multiple oocytes by superovulation, IVF, or, more recently, intracytoplasmic sperm injection (ICSI), and biopsy of the embryo at the six- to eight-cell stage on day 3 postinsemination (2). Genetic diagnosis is car-

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1058-0468/98/0300-0140$15.00/0

ried out on the biopsied cells within 12 hr to allow embryos identified as unaffected to be transferred to the mother within the same ovulatory cycle. The first pregnancies achieved following PGD were for couples at risk of having children with a variety of X-linked recessive diseases (3). Since then, several other single gene defects (e.g., cystic fibrosis, Lesch–Nyhan, Tay–Sachs, Duchenne muscular dystrophy, and Marfan syndrome) have been successfully diagnosed and resulted in the birth of unaffected children (4,5).

We report here the first attempt at PGD of an inherited cancer predisposition syndrome, familial adenomatous polyposis coli (FAPC). FAPC is an autosomal dominant condition characterized by the development of hundreds or thousands of colorectal adenomas and the subsequent development of adenocarcinoma in all untreated cases. Approximately 1 in 10,000 persons in the American, British, and Japanese populations suffer from this relatively common condition. The mapping and cloning of the *APC* gene, which is responsible for FAPC, has made possible reliable genetic testing (6–8). Despite the availability of such tests there has been little interest in prenatal diagnosis amongst parents who are gene carriers. However, most patients would be keen to reduce the risk of an affected pregnancy if the option were available (9).

MATERIALS AND METHODS

Patient History

The patient was 34 years old, parity 0. Her two affected sisters died in their early twenties from extracolonic malignancies despite earlier colectomies. The patient had a total colectomy, which resulted in blocked fallopian tubes and consequently required IVF treatment to conceive.

The causative mutation in the family was determined to be a T insertion at codon 764, a site normally correlated with a classical FAPC phenotype (10). The basis for the severe manifestation in this family remains to be elucidated fully (11).

Strategy

Apart from the general difficulties of DNA amplification in single cells, the specific risk when dealing with a dominantly inherited condition is that one allele will fail to amplify, a phenomenon known as allele dropout (ADO) (12,13). This can lead to a misdiagnosis if it is the mutant allele that is not represented. To avoid this problem a fail-safe strategy for the detection of a mutant *APC* allele in preimplantation embryos was devised. The approach was to biopsy two cells from the same embryo and to analyze the DNA of each for the specific *APC* mutation and also a second marker, an intragenic polymorphism (14). The patient is heterozygous (allele *A/B*), and her husband is homozygous (allele *B/B*) at the polymorphic site. Allele *A* has been shown to segregate with the *APC* mutation in the patient's family (data not shown), and consequently any embryo with a heterozygous compliment (*A/B*) at this locus is predicted to be affected and would not be considered for transfer. This approach was made possible by amplifying the whole genome using primer extension preamplification (PEP) (15) followed by independent nested PCR for these two loci.

IVF and Embryo Biopsy

After counseling regarding PGD and the possible outcome, the patient underwent routine downregulation and ovarian stimulation IVF treatment (16). Oocytes were retrieved by vaginal ultrasound-guided aspiration, and ICSI was performed with her husband's sperm (day 0) to avoid possible contamination by extraneous sperm. Oocytes were examined for two pronuclei 15–19 hr post ICSI. The embryos were biopsied as described previously (2). Two blastomeres were removed from each embryo for genetic analysis on day 3 post ICSI. The blastomeres were washed two or three times in fresh droplets of handling medium and immediately lysed in lysis buffer (17). After the biopsy the embryos were immediately returned to culture until the time of transfer.

Genetic Analysis of Single Blastomeres

PEP was performed for each blastomere as described earlier (15). A 5-μl aliquot of the PEP product was used to provide DNA templates for subsequent amplification of the *APC* mutation site. An equal volume was also taken for amplification of the polymorphic sequence. To amplify DNA from PEP products, a nested PCR protocol was used to increase the sensitivity and specificity. Two sets of primer pairs were designed for two PCR assays: PCR assay I, an outer primer pair, 5′ GGAATCTCATGGCAAATAGGCCT 3′ and 5′ TGGTGAAAGGACAGTCATGTTGC 3′ was used to amplify a 293-bp DNA fragment encompassing the mutation in exon 15, and an inner (nested) pair of primers, 5′ TGTCTCCTGGCTCAAGCTTGCCA 3′ and 5′ TACGATGAGAT-

GCCTTGGGACTT 3′, was used to amplify a 132-bp DNA fragment; PCR assay II, the outer primer pair encompassing the polymorphic locus was 5′ CCCAGACTGCTTCAAAATTACC 3′ and 5′ GAGCCTCATCTGTACTTCTGC 3′, and the inner primer pair was 5′ AGCTGCCTGTGTACAAACTTCT 3′ and 5′ AGGAATGGTATCTCGTTTTTCA 3′, which amplified 318- and 242-bp DNA fragments, respectively. The nested PCR procedure was as described previously (18) except for a few modifications. The annealing temperatures for PCR assay I were 61 and 56°C and the annealing temperatures for PCR assay II were 55 and 65°C. The PCR cycles were increased to 28 for the primary PCR and 32 for the nested PCR for both the assays.

The PCR products were analyzed by a combined approach of single-strand conformation polymorphism (SSCP) and heteroduplex analysis on the Pharmacia Phastsystem by automated electrophoresis and silver staining. The gel conditions were essentially as described previously (10) with the following modifications: the *APC* mutation was detected at 4°C on a gel run for a total of 216-V hr (Vh) (50-Vh prerun); the polymorphism was best visualized at 15°C on a gel run for a total of 200-Vh (10-Vh prerun).

RESULTS

Ten oocytes were collected from the patient but only four embryos were normally fertilized with two pronuclei after ICSI. On day 3 at embryo biopsy two embryos had reached the eight-cell stage, while the remaining two had reached seven cells. Two blastomeres were biopsied from all four embryos. Following PEP the *APC* mutation site was successfully amplified in seven of eight blastomeres, and only one embryo (embryo 2) was considered to be normal, both cells showing only the normal allele by SSCP and heteroduplex analysis (Table I). One blastomere from embryo 3 appeared to be anucleate under the dissecting microscope and, not surprisingly, displayed no amplification at either locus. Amplification of the polymorphism failed with the other blastomere from the same embryo, and analysis of the mutation revealed only an affected allele. As it is not possible for these parents to produce a mutant homozygote, this result indicates that ADO of the normal allele had occurred. The polymorphic site was successfully amplified in six of eight cells (Table I) and provided confirmatory results regarding the affected status of embryos 1 and 4 and the unaffected status of embryo 2 (Table I). Two examples of ADO were seen to affect the polymorphic site. In both cases the mutant allele failed to amplify in one blastomere but was detected in the other biopsied cell. Embryo 2 was transferred on day 4 but no pregnancy resulted.

DISCUSSION

The patient in this case is infertile due to surgical treatment for cancer and requires assisted reproduction to conceive. Of 10 oocytes collected, 4 were normally fertilized after ICSI. Following embryo biopsy and genetic analysis, only one embryo was diagnosed as unaffected; this was transferred but did not result in a pregnancy. The pregnancy rate following single embryo transfer is reported to be only 10%, disproportionately lower than if two embryos are available (19). The failure to obtain more embryos for the procedure is due to the low normal fertilization rate. In a recent study, the numbers of oocytes collected and normally fertilized (56%) in these mostly fertile couples undergoing PGD were similar to the numbers after IVF with infertile couples (20).

The accuracy of the genetic test was assessed by analyzing the remaining embryos that were not transferred. The results were concordant with the previous

Table I. Genetic Analysis of Preimplantation Embryos Tested for FAPC[a]

Embryo No.	Cell 1: Diagnosis of		Cell 2: Diagnosis of:		Final diagnosis (number of PCR results supporting diagnosis)
	15J polymorphism	APC mutation	15J polymorphism	APC mutation	
1	A	A	N*	A	Affected (3/4)
2	N	N	N	N	Normal (4/4)
3	F	A* (hom)	F	F	Affected (1/1)
4	N*	A	A	A	Affected (3/4)

[a]Results gained from the four biopsied embryos. Two cells were assessed per embryo at an intragenic *APC* polymorphic site (15J) and at the *APC* mutation site. Successful PCR allowed embryos to be diagnosed as affected (A) or normal (N). Failure of PCR amplification (F) was observed in three tests, and ADO in three (designated by superscript asterisks).

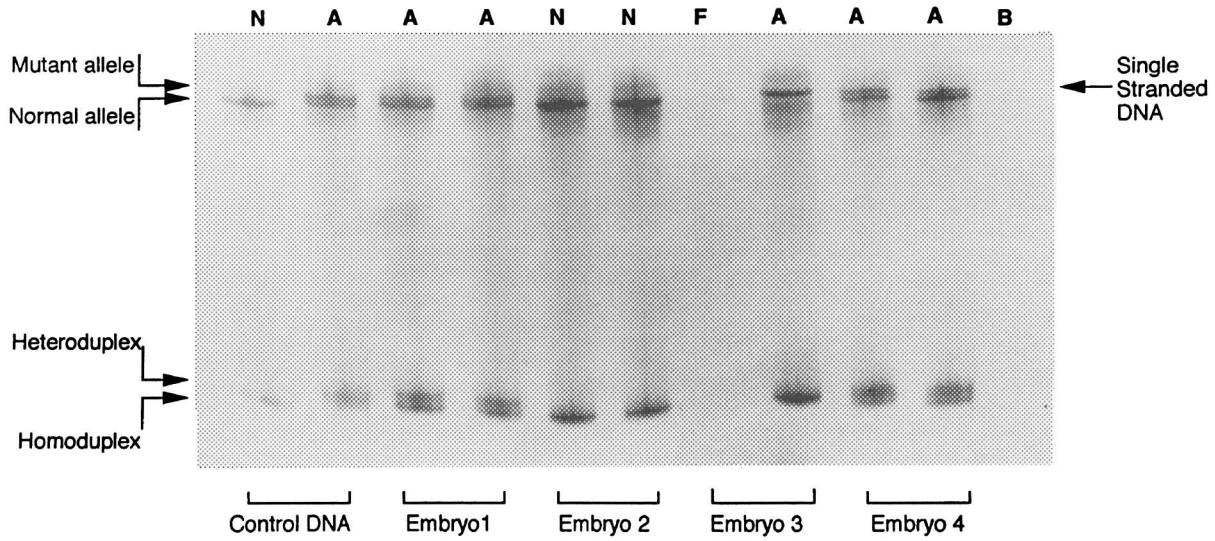

Fig. 1. Diagnosis of FAPC by gel analysis of blastomeres from four cleavage-stage embryos. Embryos 1 and 4 showed both mutant and normal alleles in three of four tests using SSCP and heteroduplex formation. Embryo 2 showed only the normal allele and no heteroduplex formation. Embryo 3 had ADO of the normal allele, and one blastomere failed to amplify. N denotes a homozygous normal embryo; A, an affected embryo; and F, failure of amplification. Lane B is a control blank.

findings; all three embryos not transferred were carrying the mutant gene. The advantage of using PEP in PGD is that more than one locus can be analyzed from the same cell and repeated aliquots may be taken for independent nested PCR reactions in the event of an ambiguous result. Preliminary work using the same strategy showed a 96% mutation detection rate in single carrier lymphocytes obtained from the patient's blood (data not shown). Thus, it was predicted that sampling two cells from each embryo should reduce the risk of misdiagnosis to considerably less than 1%.

Comparison of the results from two cells from the same embryo revealed a significant incidence of ADO (Table I). In an autosomal dominant disorder, such as FAPC, all heterozygotes are affected. However, should the mutant allele fail to amplify the cell would be wrongly diagnosed as homozygous normal. In this clinical application, using the strategy described above we could compare results for a single embryo and eliminate the embryo if any one of four assays revealed the affected allele. The design of similar strategies should be possible for most dominant genetic disorders.

At present a few drastic surgical measures, which may have a significant impact on the quality of life, are available to prevent cancers from occurring in predisposed individuals. Oophorectomy together with prophylactic bilateral mastectomy may be used for carriers of a *BRCA 1* gene mutation, and colectomy may increase life expectancy for carriers of a mutated *APC* gene, but their relative risk of dying early is still increased threefold over that of the general population (9,21).

We have shown that, technically, it is possible to detect mutations in cancer predisposing genes at the single-cell level with confidence. However, the ethical issues concerning embryo selection for diseases that are late-onset and are not immediately life-threatening remain controversial. The severity and age at onset of many cancer predisposition syndromes can vary dramatically from family to family, making it difficult to formulate universally applicable guidelines. The family history and severity of symptoms experienced will undoubtedly influence the individual patient's attitude to prenatal/preimplantation testing. We believe that there are compelling medical grounds for strategies such as that described here because surgical intervention does not completely eliminate the risk of cancer and because there is patient demand for measures that prevent the inheritance of cancer-predisposing mutations (9).

ACKNOWLEDGMENTS

The authors thank Debbie Taylor, Drs. Jonathan Skull, Amir Lass, and Karin Dawson, and all the IVF team at the Hammersmith Hospital for their contribu-

tion. This study was carried out under license from the Human Fertilization and Embryology authority of United Kingdom with the approval of the research ethics committee of the Royal Postgraduate Medical School, Hammersmith Hospital. We thank Quest Cancer Test for covering the costs of consumables at the Galton Laboratory. D.W. was supported by the Medical Research Council.

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