Preimplantation Genetic Diagnosis of Human Embryos for Marfan's Syndrome

A. BLASZCZYK,¹ Y. X. TANG,¹ H. C. DIETZ,² A. ADLER,¹ A. S. BERKELEY,¹ L. C. KREY,¹
and J. A. GRIFO^{1,3}

Submitted: January 2, 1998

Accepted: January 2, 1998

Purpose: Single-cell nested polymerase chain reaction (PCR) and DdeI endonuclease digestion were used to detect the presence of a Marfan's syndrome mutation in human preimplantation embryos derived from in vitro fertilization (IVF). These procedures were conducted to eliminate the possibility of transmission of the affected allele from the father to his offspring. The mutation on chromosome 15 is transmitted as an autosomal dominant trait, and the chance of having a child affected with the disease is 50%.

Methods: A couple presented to the Program for In Vitro Fertilization, Reproductive Surgery and Infertility for preimplantation genetic diagnosis. IVF was performed and embryo biopsy was done on day 3 embryos. Single blastomeres were removed from embryos and subjected to nested PCR analysis and endonuclease digestion to detect a Marfan's syndrome mutation located on chromosome 15 inherited from the father.

Results: Thirteen oocytes were injected with spermatozoa using intracytoplasmic sperm injection, and nine fertilized normally. Following embryo biopsy and polymerase chain reaction amplification-DdeI endonuclease digestion, five embryos were detected that were positive for the mutation. The four non-affected embryos were transferred to the uterus, resulting in a healthy and normal ongoing pregnancy.

KEY WORDS: embryo biopsy; Marfan's syndrome; PCR analysis; Preimplantation genetic diagnosis.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) is currently used in assisted reproduction laboratories to identify

the presence of specific gene mutations, mutations associated with sex-chromosomes, and aneuploidies (1). Polymerase chain reaction (PCR) and fluorescent *in situ* hybridization technology allows for the rapid detection of heritable genetic diseases. Some of these diseases, such as cystic fibrosis, Duchenne's muscular dystrophy, hemophilia, and now Marfan's syndrome can be detected in early preimplantation-stage embryos created using in vitro fertilization (IVF) techniques (2-4).

In this report we describe the detection of a mutation in the fibrillin gene located on chromosome 15. Mutations in fibrillin give rise to a autosomal dominant disease known as Marfan's syndrome (4). Originally described in the late 1800s, this connective tissue disease is caused by defects in the fibrillin protein found in microfibrils, a component of the extracellular matrix associated with elastin in tissues such as the aorta and ligaments, and some non-elastic tissues (4,5). Three major systems are affected: ocular, cardiac, and skeletal. A common phenotype is overgrowth of long bones.

The fibrillin protein is encoded by a gene, which is composed of 57 exons. It contains several critical cysteine residues found within EGF-like repeats which are responsible for stability and calcium binding. Because this gene is so large and there are so many important structural elements to the sequence, most patients have mutations unique to their pedigree (6,7).

A 26-year-old male affected with the disease carried a mutation in exon 12 in chromosome 15. The heterozygous mutation, C1585T, induces a premature stop codon which caused the patient to have severe aortic distention and root repair. He and his wife sought PGD in hopes of having a child that would not inherit the mutation. The patients were required to sign informed consent forms for the procedure,

¹ Program for In Vitro Fertilization, New York University Medical Center, 317 East 34th Street, 4th Floor, New York, New York 10016.

² Department of Pediatrics, Medicine and Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 1170 Ross Research Building, 720 Rutland Avenue, Baltimore, Maryland 21205.

³ To whom correspondence should be addressed.

which was approved by the Institutional Review Board (IRB) of New York University Medical Center (Protocol H 6321). All procedures involving embryos occurred at NYU.

MATERIALS AND METHODS

In Vitro fertilization

The IVF patient, a 25-year-old nulligravida female, underwent follicle stimulation involving luteal down-regulation with leuprolide acetate and daily human menopausal gonadotropin stimulation followed by an injection of human chorionic gonadotropin (hCG). Transvaginal ultrasound-guided follicular aspiration was performed 36 hr post-hCG. This was the couple's fourth attempt at assisted reproduction coupled with PGD. Thirteen oocytes were obtained and intracytoplasmic sperm injection (ICSI) was performed 3–4 hr postretrieval. Fertilization was confirmed by the presence of two pronuclei at 16–18 hr after ICSI. Embryos were cultured in HTF medium (Irvine Scientific, Santa Ana, CA) supplemented with maternal serum until day 3 postretrieval.

Embryo Biopsy Procedure

On the morning of day 3, the embryos (five- to eight-cell stage) were biopsied using micromanipulation procedures described previously (6). In brief, a small hole was made in the zona pellucida with acid Tyrode's solution, and one blastomere was gently removed by mouth pipette suction. Each blastomere was washed and carefully placed into individual sterile PCR microcentrifuge tubes containing lysis buffer (50 mM dithiothreitol in 200 mM potassium hydroxide). Following the addition of neutralization buffer, the DNA was heat denatured (10 min at 65°C).

PCR Analysis

Single-cell nested PCR was performed using primers designed to amplify the region containing the mutation within exon 12 on chromosome 15. The primers (0.5 μ M used in Round 1 were as follows: sense, 5'-AGAATTATGAGGTATTGCTTATG-3'; and antisense, 5'-CAGTTAGCATATATGTCCCAC-3'. Thirty-five cycles (95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec) were run at a final concentration of 1.5 mM Mg²⁺ and 2.5 U/50 μ l Taq polymerase (Boehringer Mannheim, Indianapolis, IN). In Round

2 (nested), an additional 35 cycles were run using the following primers: sense, 5'-GTGCTTTGCAGATGT-TGATG-3'; and antisense, 5'-CAGTTAGCATATATG-TCCCAC-3'.

Following PCR, 5 μ l of the product was incubated for 1 hr at 37°C with 5 U/0.5 μ l of *DdeI* restriction enzyme (New England Biolabs, Beverly, MA) and then analyzed by electrophoresis on a 1.5% agarose gel. Band migration was analyzed according to the known size of expected fragments with and without the mutation. The final product was 179 base pairs in the blastomeres without the mutation, whereas blastomeres containing the mutation also presented 52- and 127-base pair fragments (Figs. 1 and 2).

RESULTS

Nine of 13 oocytes fertilized normally following ICSI. Biopsies were successfully performed and a nucleated blastomere was obtained for each of the nine embryos. PCR analysis provided migrating bands for each embryo (Fig. 2). No DNA fragments were visible in the two blank lanes. A 179-base pair fragment is detectable in all lanes; however, lanes 5, 6, 8, 13, and 14 contained two additional two bands (52 and 127 base pairs), which are the result of the introduction of the *DdeI* restriction endonuclease site in the heterozygote mutation. Given these results, we were confident of the genetic makeup of each embryo.

Embryo Transfer

On the evening of day 3, the four embryos that produced the blastomeres bearing only one population of migrating bands (lanes 2, 4, 7, and 12 in Fig. 2) were transferred to the patient's uterus using a Wallace catheter. The transfer was uneventful. Two months prior to this cycle a small endocervical

Fig. 1. PGD of Marfan's syndrome.

Fig. 2. PCR results following *DdeI* restriction endonuclease digestion. Lanes 5, 6, 8, 13, and 14 are embryos heterozygous for Marfan's syndrome. These embryos are characterized by three fragments of 179, 127, and 52 base pairs.

submucous myoma had been removed by hysteroscopic resection.

A positive β -hCG was obtained on day 12 post-transfer and the patient has an ongoing pregnancy. Subsequent amniocentesis has revealed a normal male fetus. We have also analyzed the amniocentesis products using the nested PCR procedure described above. There was no evidence of the mutation; only a 179-base pair fragment was present following incubation of the PCR product with *DdeI* restriction endonuclease.

DISCUSSION

As a result of the many advances made by scientists in the human genome project and, by molecular biologists in the industry of DNA sequence analysis, we will soon be able to identify many deleterious gene defects in human embryos by coupling either PCR analysis or fluorescent *in situ* hybridization (FISH) with assisted reproduction technology. Many centers currently use this technology to identify sex chromosome linked mutations by detecting the sex of the embryos using specific repeats in the X and Y chromosomes (7, 8). A simpler method than PCR, FISH allows the investigator to view the entire DNA spread in either metaphase or interphase chromosomes and count the

fluorescent signals corresponding to an individual chromosome or mutation (9). Although FISH is ideal for sex determination and aneuploidy detection, many heritable gene defects are single amino acid substitutions that are not suitable for FISH detection. The use of combined PCR and FISH on the same blastomere to confirm diagnosis has also been developed but it has not been used to the same extent as either PCR or FISH (11,12).

Although single-cell PCR has been used for PGD, there are many potential problems associated with this technique. Single-cell PCR has been erroneous in the past, the result of amplification failure or the amplification of only one allele (13). Such misdiagnoses are not acceptable and are terribly painful for the parents. We have previously reported transcript-targeted PGD for a patient with a different heterozygous mutation resulting in Marfan's syndrome which attempts to diminish the possibility of misdiagnosis (14). One should take great care when pursuing such technology in the lab. The fibrillin gene is extremely large and has many exons. Like cystic fibrosis, Marfan's syndrome can be caused by a variety of different mutations which must be screened for very carefully. Once the appropriate mutation is identified in a patient, procedures such as that presented here can be used with confidence to ensure that the mutation is not passed on to their children.

REFERENCES

1. Handyside AH, Penketh RJA, Winston RML, Pattinson JK, Delhanty JDA, Tuddenham EGD: Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet* 1989;1:347-349
2. Verlinsky Y, *et al.*: Preconception and preimplantation diagnosis for cystic fibrosis. *Prenatal Diagn* 1992;12:103-110
3. Kristjansson K, Chong SS, Van den Veyver IB, Subramanian S, Snabes MC, Hughes MR: Preimplantation single cell analyses of dystrophin gene deletions using whole genome amplification. *Nature (Genetics)* 1994;6:19-23
4. Kainulainen K, Pulkkinen L, Savollainen A, *et al.*: Location on chromosome 15 of the gene defect causing Marfan's syndrome. *N Eng J Med* 1990;323:935-939
5. Sakai LY, Keene DR, Engvall E, *et al.*: Fibrillin, a new 350 kD glycoprotein is a component of extracellular microfibrils. *J Cell Biol* 1986;103:2499-2509
6. Child AH: Marfan's syndrome—current medical and genetic knowledge: How to treat and when. *J Card Surg* 1997;12:131-135
7. Gray JR, Davies SJ: Marfan's syndrome. *J Med Genet* 1996;33(5):403-408
8. Cohen J, Malter HE, Talansky BE, Grifo J: *Micromanipulation of Human Gametes and Embryos*. New York, Raven Press, 1992
9. Grifo JA, Tang Y, Cohen J, Gilbert F, Sanyal M, Rosenwaks Z: Pregnancy after embryo biopsy and coamplification of DNA from X and Y chromosomes. *JAMA* 1992;268:727-729
10. Grifo JA, Boyle A, Tang Y, Ward D: Preimplantation genetic diagnosis. *Arch Pathol Lab Med* 1992;116:393-397
11. Verlinsky Y, Rechitsky S, Freidine M, Cieslk J, Strom C, Lifehez A: Birth of a healthy girl after preimplantation gender determination using a combination of polymerase chain reaction and fluorescent in situ hybridization analysis. *Fertil Steril* 1996;65:358-360
12. Munne S, Tang Y, Grifo JA, Rosenwaks Z, Cohen J: Sex determination of human embryos using the polymerase chain reaction and confirmation by fluorescence in situ hybridization. *Fertil Steril* 1994;61:111-117
13. Grifo JA, Tang YX, Munne S, Krey L: Update in preimplantation genetic diagnosis: Successes, advances and problems. *Curr Opin Obstet Gynecol* 1996;8:135-140
14. Eldaidah ZA, Grifo JA, Dietz HC: Marfan's syndrome as a paradigm for transcript-targeted preimplantation diagnosis of heterozygous mutations. *Nature Med* 1995;1(8):798-803