

BRIEF REPORT

Preimplantation Diagnosis for Sonic Hedgehog Mutation Causing Familial Holoprosencephaly

Yury Verlinsky, Ph.D., Svetlana Rechitsky, Ph.D., Oleg Verlinsky, M.S.,
Seckin Ozen, M.D., Tatyana Sharapova, M.S., Christina Masciangelo, M.S.,
Randy Morris, M.D., and Anver Kuliev, M.D., Ph.D.

THE SONIC HEDGEHOG (*SHH*) GENE IS A HUMAN HOMOLOGUE OF THE *Drosophila* gene encoding inductive signals involved in patterning the early embryo and which is highly functionally conserved in many species.¹ The gene was mapped to chromosome 7 (7q36), the locus for the gene involved in holoprosencephaly (*HPE3*).² *SHH* mutations may cause the failure of cerebral hemispheres to separate into distinct left and right halves, leading to holoprosencephaly, which is one of the most common developmental anomalies of the forebrain and midface in humans.³ Although the majority of cases of holoprosencephaly are sporadic, familial cases are not rare, with a clear pattern of autosomal dominant inheritance.

There is great clinical variability of holoprosencephaly within families, ranging from alobar holoprosencephaly and cyclopia to cleft lip and palate, microcephaly, ocular hypotelorism, and even to a normal phenotype. This variability suggests an interaction between *SHH* and other genes expressed during craniofacial development and the possible involvement of environmental factors. Because almost one third of carriers of *SHH* mutations may be clinically unaffected, even in affected families, the prenatal detection of *SHH* mutations might not justify termination of the pregnancy. Preimplantation genetic diagnosis is thus a more attractive option for couples at risk for having a child with holoprosencephaly.

We describe the use of preimplantation genetic diagnosis for *SHH* mutation in a family with holoprosencephaly. The use of this technique, followed by confirmation of mutation-free status by amniocentesis, resulted in the birth of a healthy girl.

CASE REPORT

A couple who had had two children with clinical signs of holoprosencephaly presented for preimplantation genetic diagnosis (Fig. 1). Their second child, a girl with severe holoprosencephaly and cleft lip and palate, died shortly after birth. The results of chromosomal analysis of peripheral-blood lymphocytes from this child and the parents were normal. However, DNA analysis of samples obtained at autopsy showed that the girl had an *SHH* nonsense mutation — a change from GAG to TAG — leading to the premature termination of the protein at position 256 of exon 3 (Glu256stop)³ (Fig. 2A). *SHH* protein is an intercellular signaling molecule. This precursor is cleaved internally into a highly conserved domain (*SHH-N*) with signaling activity and a more divergent domain (*SHH-C*), which in addition to precursor processing, acts as an intramolecular cholesterol transferase crucial for proper patterning activity in animal development.¹ Although the effect on *SHH* function of the nonsense mutation identified in the child is unknown, the resulting protein may not have the expected signaling function in early morphogenesis.^{1,3}

From the Reproductive Genetics Institute, Chicago. Address reprint requests to Dr. Kuliev at 2825 N. Halsted St., Chicago, IL 60657, or at anverkuliev@hotmail.com.

N Engl J Med 2003;348:1449-54.

Copyright © 2003 Massachusetts Medical Society.

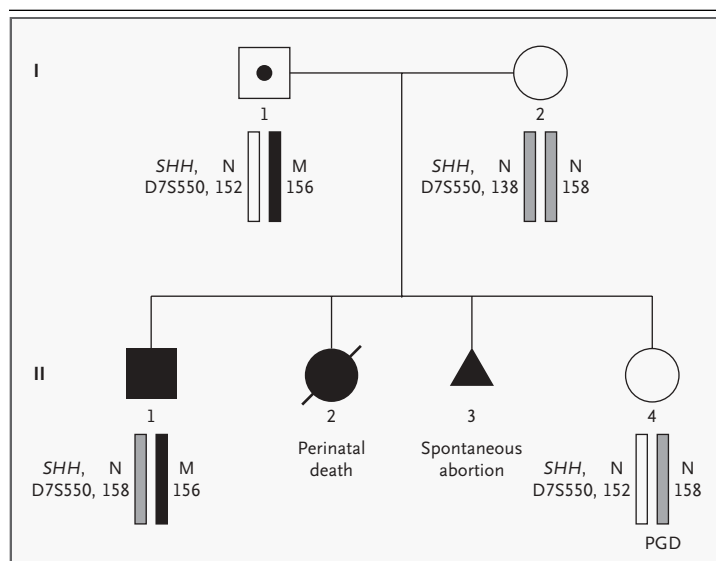


Figure 1. Pedigree.

The father (Subject I-1) has a gonadal mosaicism for the sonic hedgehog (*SHH*) mutation (M), which is linked to the 156-bp dinucleotide CA repeat allele of the D7S550 polymorphic marker, whereas the mother (Subject I-2) is normal (N), with one normal allele linked to a 158-bp repeat (158) and the other to a 138-bp repeat (138). Three previous pregnancies resulted in the birth of a son with holoprosencephaly (Subject II-1), who carries the mutant gene; a daughter who died soon after birth (Subject II-2), who also carried the mutant gene; and a spontaneously aborted fetus with Turner's syndrome (Subject II-3), without the *SHH* mutation. A second daughter (Subject II-4) was born after preimplantation genetic diagnosis (PGD), as described in this article. Squares indicate male subjects, circles female subjects, the triangle a spontaneous abortion, solid symbols affected subjects (the spontaneously aborted fetus was unaffected by holoprosencephaly but had Turner's syndrome), open symbols unaffected subjects, and the slash a deceased subject. The square with the dot indicates that the father was an unaffected carrier of the mutation.

The same mutation was found in the couple's five-year-old son, who had been born after a full-term, normal pregnancy, weighing 2.7 kg (6 lb), with a length of 45.7 cm (18.3 in.). This child had less severe facial dysmorphism than his sister, including microcephaly, Rathke's pouch cyst, a single central incisor, and choanal stenosis (the latter was dilated surgically after birth). He also had clinodactyly of the fifth fingers and incurved fourth toes bilaterally. His growth was slow during the first two years but subsequently has been reasonably good, and his social and cognitive development are apparently normal.

The woman had had another pregnancy, which ended in spontaneous abortion owing to Turner's syndrome (45,X). There was no evidence of inheritance of the *SHH* mutation. The observation that the

mutation was not found in either parent's genomic DNA, although paternity testing showed that the father was the biologic father of both affected children, clearly suggested a new gonadal mutation in one of the parents.

METHODS

Two cycles of preimplantation genetic diagnosis were performed with use of a standard in vitro fertilization protocol coupled with micromanipulation procedures, as described elsewhere.⁴ Single blastomeres were removed from the eight-cell embryos and tested by multiplex nested polymerase-chain-reaction (PCR) analysis, involving simultaneous testing for a specific mutation and linked marker analysis.⁵ Of 15 embryos in the first cycle, 12 were available for blastomere biopsy at the eight-cell stage. Blastomeres from four embryos failed to amplify, leaving eight with data available for mutation analysis. Seven of these eight embryos appeared to contain the mutant allele; only one embryo was free of the mutation and was transferred back to the patient, yielding no clinical pregnancy.

The second cycle of preimplantation genetic diagnosis was performed a year later. Nineteen embryos were available, 10 of which were acceptable for blastomere biopsy and DNA analysis. Of these 10 single blastomeres, only 1 failed to amplify. The remaining nine had data available for *SHH* gene and linked marker analysis, to identify the mutation-free embryos appropriate for transfer (Fig. 2C).

Before preimplantation genetic diagnosis cycles, single-sperm testing was performed that identified mosaicism for the *SHH* mutation in the father. The mutation led to the addition of an *Xba*I restriction site³; the normal allele was thus identified on the basis of the undigested PCR product, and the mutant allele was represented by two fragments, as a result of *Xba*I digestion (Fig. 2B).

To avoid misdiagnosis in mutation analysis owing to preferential amplification of certain alleles (also termed "allele dropout"), the rate of which exceeds 10 percent in single-blastomere DNA analysis,⁶ a closely linked microsatellite DNA marker D7S550 was used as an internal control. Table 1 lists the primers used in the first- and second-round PCR for mutation and linked marker analysis and the reaction conditions. A haplotype analysis showed that the mutant allele was linked to a 156-bp dinucleotide CA repeat and that the normal gene was linked to a 152-bp repeat allele in 7q36 (Fig. 1). Although

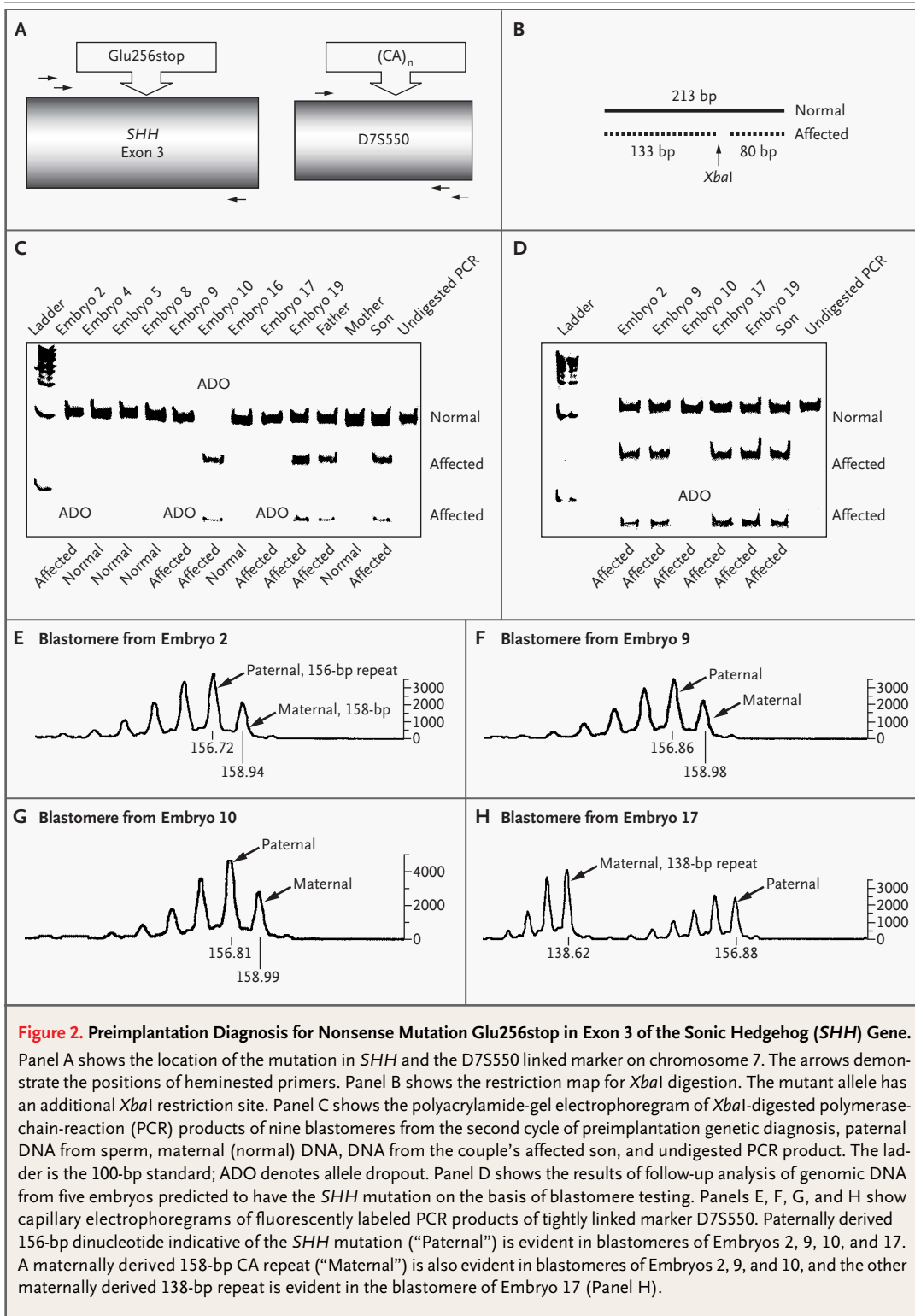


Table 1. Primers for the Detection of the Glu256stop Mutation in the Sonic Hedgehog Gene (*SHH*) and Linked Marker D7S550 in Heminested Polymerase-Chain-Reaction Analysis.

Gene or Marker	Upper Primer	Lower Primer	Annealing Temperature (°C)
<i>SHH</i>			
Outside	GAGCAGGGCGGCACCAA	GGCCGAGTCGTTGTGC	62–55
Inside	GGCACCAAGCTGGTGAAG	GGCCGAGTCGTTGTGC	56
D7S550			
Outside	ACTATCATCCACAATCCACTCC	GCAGTTGGGTTATTTCAAGTCT	62–55
Inside	ACTATCATCCACAATCCACTCC	GATGTTGTGATTAGATTGCTGTA	56

other linked markers have also been described,² they were not informative.

The protocol was approved by the institutional review board, and the parents provided written informed consent. On the basis of both mutation and linked-marker analyses, unaffected embryos were identified and selected to be transferred back to the woman, whereas those predicted to have the *SHH* mutation underwent confirmatory analysis with the use of whole-embryo DNA to evaluate the accuracy of preimplantation genetic diagnosis based on analysis of a single cell.

RESULTS

Four allele dropouts were identified in analysis of the mutant allele in Embryos 2, 9, and 17 and in the normal allele in Embryo 10 (Fig. 2C and 2D and Table 2). This result was based on the marker analysis, showing that all four of these embryos were heterozygous for the mutation (Fig. 2E, 2F, 2G, and 2H). In other words, three of these four embryos (Embryos 2, 9, and 17) could have been misidentified as normal in the absence of linked marker analysis. In addition to these three embryos, Embryos 10 and 19 also contained the mutant gene.

The remaining four embryos were free of the mutant gene, as confirmed by marker analysis showing that all contained two normal alleles, the paternal one linked to the 152-bp repeat and either the normal maternal allele linked to the 138-bp repeat (Embryos 4 and 5) or the normal maternal allele linked to the 158-bp repeat (Embryos 8 and 16). Two of these embryos (Embryos 4 and 5) were transferred back to the woman, resulting in a singleton

pregnancy, followed by confirmation of the mutation-free status of the fetus by amniocentesis and the birth of a healthy girl. The other two mutation-free embryos (Embryos 8 and 16) were frozen for subsequent use by the couple.

DISCUSSION

Our data demonstrate that multiplex PCR-based blastomere analysis can accurately identify *SHH* mutations, despite the well-known high rate of allele dropout in this type of single-cell analysis.⁶ Allele-specific amplification failure is particularly common in single-blastomere analysis, as compared with analysis of single fibroblasts and polar bodies. The follow-up analysis of the mutant embryos confirmed the results of preimplantation genetic diagnosis, consistent with previous data.⁵ Although, ideally, three linked markers are needed to eliminate the risk of misdiagnosis resulting from allele dropout,⁵ we found that the use of only one linked marker was reliable, probably because the identification of mutation-free embryos was based on the presence not only of the paternally derived normal allele, but also of the maternally derived normal allele. In this case, the absence of the mutant gene together with the presence of the two normal alleles, identified by different linked markers, led to the correct identification of embryos as either normal or carrying the *SHH* mutation.

Our findings demonstrate the clinical relevance of the use of preimplantation genetic diagnosis for familial holoprosencephaly. Because of the high prevalence of congenital craniofacial anomalies, preimplantation genetic diagnosis may have prac-

Table 2. Results of Multiplex Polymerase-Chain-Reaction (PCR) Analysis, Involving Simultaneous Testing for the Sonic Hedgehog Gene (SHH) Mutation and Linked Marker Analysis and Confirmatory Embryo Analysis.

Source of DNA Embryo No.	Analysis for SHH Mutation	Linked Marker (D7S550) Analysis*	Predicted Genotype	Follow-up Analysis
2	Normal/Allele dropout	2/4	Carrier affected	Confirmed by DNA analysis
4	Normal/Normal	1/3	Normal	Confirmed by amniocentesis†
5	Normal/Normal	1/3	Normal	Confirmed by amniocentesis†
8	Normal/Normal	2/3	Normal	Frozen
9	Normal/Allele dropout	2/4	Carrier affected	Confirmed by DNA analysis
10	Allele dropout/Mutation	1/4	Carrier affected	Confirmed by DNA analysis
16	Normal/Normal	2/3	Normal	Frozen
17	Normal/Allele dropout	1/4	Carrier affected	Confirmed by DNA analysis
19	Normal/Mutation	1/4	Carrier affected	Confirmed by DNA analysis
Mother	Normal/Normal	1/2	Normal	—
Father	Normal/Mutation	3/4	Carrier mosaic	—

* The linked markers were as follows: maternal PCR product of 138 bp (1); maternal PCR product of 158 bp (2); paternal PCR product of 152 bp (3), linked to the normal gene; and paternal PCR product of 156 bp (4), linked to the mutant gene.

† Embryos 4 and 5 were transferred, but it is not known which one resulted in the singleton pregnancy and thus was confirmed by amniocentesis as having a normal genotype.

tical implications for couples at risk. Initiated first for severe mendelian disorders,⁷⁻¹² preimplantation genetic diagnosis was then used to exclude aneuploidy¹³⁻¹⁶ and has recently been used for diseases for which there is a genetic predisposi-

tion^{17,18} and for congenital malformations.¹⁹ These data suggest that preimplantation genetic diagnosis may be an option for a large group of couples at risk for having children with congenital malformations.

REFERENCES

1. Online Mendelian inheritance in man, OMIM. Baltimore: McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University, 2001. (Accessed February 19, 2003, at <http://www.ncbi.nlm.nih.gov/Omim>.)
2. Muenke M, Gurrieri F, Bay C, et al. Linkage of a human brain malformation, familial holoprosencephaly, to chromosome 7 and evidence for genetic heterogeneity. *Proc Natl Acad Sci U S A* 1994;91:8102-6.
3. Nanni L, Ming JE, Bocian M, et al. The mutational spectrum of the sonic hedgehog gene in holoprosencephaly: SHH mutations cause a significant proportion of autosomal dominant holoprosencephaly. *Hum Mol Genet* 1999;8:2479-88.
4. Verlinsky Y, Kuliev A. Atlas of preimplantation genetic diagnosis. Pearl River, N.Y.: Parthenon, 2000.
5. Rechitsky S, Verlinsky O, Strom C, et al. Experience with single-cell PCR in preimplantation genetic diagnosis: how to avoid pitfalls. In: Hahn S, Holzgreve W, eds. *Fetal cells in maternal blood: new developments for a new millennium*. Basel, Switzerland: Karger, 2000:8-15.
6. Rechitsky S, Strom C, Verlinsky O, et al. Allele drop out in polar bodies and blastomeres. *J Assist Reprod Genet* 1998;15:253-7.
7. Handyside AH, Kontogianni EH, Hardy K, Winston RML. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* 1990;344:768-70.
8. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM. Analysis of the first polar body: preconception genetic diagnosis. *Hum Reprod* 1990;5:826-9.
9. Handyside AH, Lesko JG, Tarin JJ, Winston RML, Hughes MR. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *N Engl J Med* 1992;327:905-9.
10. Sermon K, Lissens W, Joris H, et al. Clinical application of preimplantation diagnosis for myotonic dystrophy. *Prenat Diagn* 1997;17:925-32.
11. Kuliev A, Rechitsky S, Verlinsky O, et al. Preimplantation diagnosis of thalassemia. *J Assist Reprod Genet* 1998;15:219-25.
12. Xu K, Shi ZM, Veeck LL, Hughes MR, Rosenwaks Z. First unaffected pregnancy using preimplantation genetic diagnosis for sickle cell anemia. *JAMA* 1999;281:1701-6.
13. Munne S, Magli C, Cohen J, et al. Positive outcome after preimplantation diagnosis of aneuploidy in human embryos. *Hum Reprod* 1999;14:2191-9.
14. Gianaroli L, Magli MC, Ferraretti AP, Munne S. Preimplantation diagnosis for aneuploidies in patients undergoing in vitro

BRIEF REPORT

- fertilization with poor prognosis: identification of the categories for which it should be proposed. *Fertil Steril* 1999;72:837-44.
15. Wilton L, Williamson R, McBain J, Edgar D, Voullaire L. Birth of a healthy infant after preimplantation confirmation of euploidy by comparative genomic hybridization. *N Engl J Med* 2001;345:1537-41.
16. Kuliev A, Verlinsky Y. Current features of preimplantation genetic diagnosis. *Reprod Biomed Online* 2002;5:294-9.
17. Verlinsky Y, Rechitsky S, Verlinsky O, Masciangelo C, Lederer K, Kuliev A. Preimplantation diagnosis for early-onset Alzheimer disease caused by V717L mutation. *JAMA* 2002;287:1018-21.
18. Rechitsky S, Verlinsky O, Chistokhina A, et al. Preimplantation genetic diagnosis for cancer predisposition. *Reprod Biomed Online* 2002;5:148-55.
19. Abou-Sleiman PM, Apessos A, Harper JC, Serhal P, Delhanty JDA. Pregnancy following preimplantation genetic diagnosis for Crouzon syndrome. *Mol Hum Reprod* 2002;8:304-9.

Copyright © 2003 Massachusetts Medical Society.