

BRIEF REPORT

A Report of Dizygous Monochorionic Twins

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IT IS AN ACCEPTED MEDICAL DOCTRINE THAT MONOCHORIONIC TWINS are exclusively monozygous. This doctrine is supported by two studies that assessed placental pathological features and multiple serologic markers of zygosity in a total of almost 800 monochorionic twins.^{1,2} Although the possibility that monochorionic twins are not invariably monozygous has been raised previously, deficiencies in the cytogenetic and pathological characterization of these reports have cast doubt on their reliability.³ We report a case of sex-discordant monochorionic twins conceived by in vitro fertilization. The twins also had blood chimerism (the presence of cells derived from more than one genetically distinct zygote); in both twins, there was a predominance of male-derived lymphocytes, and as a result, the initial findings of zygosity studies performed on DNA extracted from the blood were consistent with monozygosity. Subsequent detailed investigation showed the twins to be dizygous. The evidence from this case is inconsistent with the doctrine that all monochorionic twins are monozygous.

CASE REPORT

A 48-year-old woman conceived twins by in vitro fertilization without intracytoplasmic sperm injection and with the use of donor oocytes. Three oocytes were successfully fertilized, cultured to the blastocyst stage of development, and placed in the recipient's uterus. An ultrasound scan obtained at six weeks of gestation was reported to show a monochorionic, diamniotic twin pregnancy and a third sac with a nonviable fetus. A subsequent scan obtained at 12 weeks of gestation showed a viable twin pregnancy, with findings indicative of monochorionic diamniotic twinning, including a thin intertwin membrane, a T-shaped insertion of the membrane, and absence of the lambda sign (a triangular projection of placental tissue extending into the base of the intertwin membrane, which is visible at this stage of gestation in dichorionic twins).⁴ Ultrasound examination at 20 weeks of gestation was consistent with the earlier evidence of monochorionicity, but the twins appeared to be discordant for sex. The pregnancy was otherwise uncomplicated, and at 37 weeks of gestation, a healthy boy (weighing 2114 g) and a healthy girl (weighing 2183 g) were delivered. No evidence of sexual ambiguity was present.

Both twins had type O Rh-positive blood and negative Coombs' tests. Pathological examination showed a monochorionic, diamniotic placenta. Because of this finding, blood samples were obtained from each twin at one week of age for zygosity studies and at three months for cytogenetic studies. Evaluation of the twins at five months of age showed unremarkable results on physical examination and normal, sex-appropriate external genitalia. Abdominal ultrasound examination of the female twin confirmed the presence of a uterus and ovaries. Peripheral-blood samples and skin-biopsy specimens were obtained at five months of age for additional cytogenetic and zygosity studies. Oral informed consent for clinical evaluation of the children and written consent for publication of the results were obtained from the parents. Tissue samples were not available from either of the biologic parents.

 METHODS

PLACENTAL STUDIES

Pathological examination of the placenta and membranes was undertaken. In addition, combined X and Y in situ hybridization studies were performed on paraffin-embedded sections of the placenta and the dividing membrane with the use of standard procedures modified for the double probe.⁵ The placental sections that were analyzed were taken from regions under each gestational sac, several centimeters from the dividing membrane.

CYTOGENETIC STUDIES

G-banded karyotypes of chromosomes in prometaphase from cultured peripheral-blood lymphocytes were obtained by standard techniques when the twins were three and five months old. Chromosomal studies of skin fibroblasts were also performed at five months. DNA extracted from the female twin's skin fibroblasts was tested for the presence of the sex-determining region of the Y chromosome.

INITIAL DNA ZYGOSITY STUDIES

Zygoty studies involving 26 polymorphic markers were undertaken on DNA extracted from peripheral-blood lymphocytes from each twin at one week of age. Southern analyses were performed with the use of DNA probes for the detection of alleles for five variable-number tandem-repeat regions (D2S44, D10S28, and D17S26 [Promega] and D4S139 and D5S110 [Life Technologies]). Polymerase-chain-reaction (PCR) amplification of the Polymarker + DQ α 1 Kit (Applied Biosystems) was performed by a reverse dot blot technique to evaluate six biallelic or triallelic markers (LDLR, GYPA, HBGG, D7S8, GC, and HLA-DQ α 1). Sixteen short tandem-repeat loci of the PowerPlex 16 system (Promega) were also evaluated by PCR amplification. When the twins were five months old, zygoty studies with the use of all the above markers were repeated on DNA extracted from cultured fibroblasts obtained from skin-biopsy specimens.

EXTENDED DNA ZYGOSITY STUDIES

A further 98 random microsatellite markers covering 17 chromosomes from the Applied Biosystems ABI PRISM Linkage Mapping Set (version 2.5) were genotyped from fibroblast cultures from the twins at the Australian Genome Research Facility.⁶

STATISTICAL ANALYSIS

Genotypes for the 119 markers with four or more potential alleles were analyzed by the GeneHunter program⁷ and a program for the graphic representation of relationship errors.⁸ Using population allele frequencies, GeneHunter estimates the probability that shared alleles are identical by descent (i.e., the probability that the two siblings have inherited the same parental allele) for each marker in the twin pair. The program for the graphic representation of relationship errors calculates the mean and variance of the sharing of alleles that are "identical by state" (i.e., occurrences of shared alleles that may or may not originate from the same parent) over a number of polymorphic loci for the twin pair, independently of population allele frequencies. Zygoty was determined from the results obtained from the GeneHunter program and the program for the graphic representation of relationship errors, according to the characteristic pattern of allele sharing.

 RESULTS

PLACENTAL STUDIES

Histopathological examination confirmed that the placenta was monochorionic and diamniotic (Fig. 1A). Fluid injection revealed fine arterial-to-arterial anastomoses on the fetal surface of the placenta.

The in situ hybridization studies of placental sections taken deep in the male twin's sac demonstrated Y signals in most nuclei in the trophoblast, amnion, villous mesenchyme, and circulatory system. Two X signals were not convincingly demonstrated in any nuclei from these sections (Fig. 1C), except for very rare circulating leukocytes and Hofbauer cells. In a placental section from under the girl's gestational sac, the trophoblast, amnion, and most of the nuclei in the villous mesenchyme showed only X-specific signals. However, Y signals were present in a large fraction of the circulating leukocytes and in villous Hofbauer cells (Fig. 1D). In sections of the dividing membrane, one amnion showed signals for X only and the other amnion showed signals for the X and Y in situ hybridization probes in all cells (Fig. 1B).

CYTOGENETIC STUDIES

Cytogenetic studies of blood lymphocytes obtained when the twins were three months old showed 46,XY[96]/46,XX[4] in the girl and 46,XY[46]/

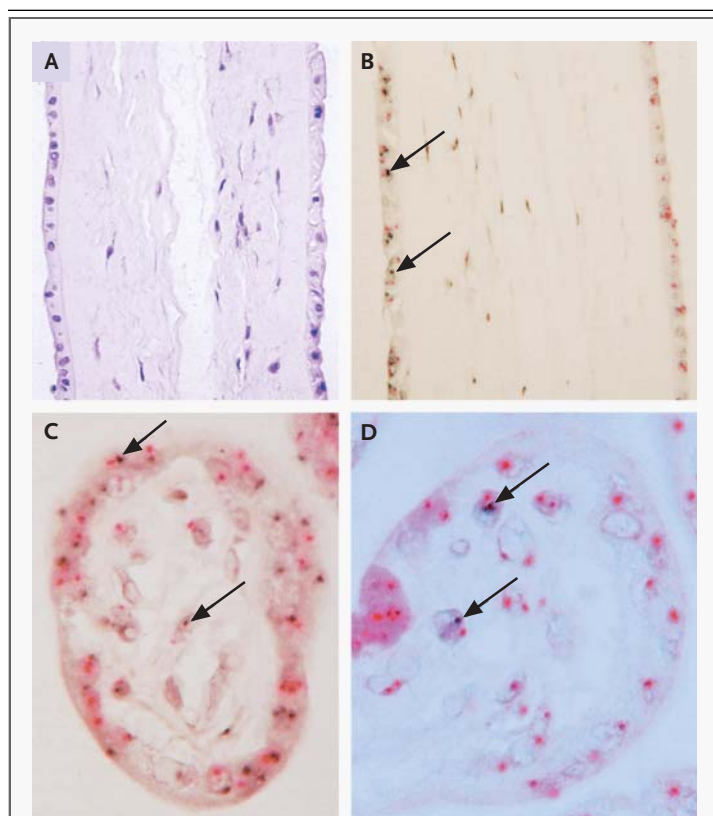


Figure 1. Results of Histologic Studies (Panel A) and X,Y in Situ Hybridization (Panels B, C, and D) of Placental Sections.

In Panel A, a hematoxylin-and-eosin-stained section of the dividing membrane that separated the two gestational sacs shows two layers of amnion with no intervening chorion ($\times 400$). In Panel B, in situ hybridization with probes specific for the X (red) and Y (black) chromosomes indicates that nuclei in the left-sided amnion contain both sex chromosomes and that nuclei in the right-sided amnion contain only X chromosomes ($\times 400$). In Panel C, a cross section of a villus from the disk underlying the phenotypically male twin shows X and Y chromosomes in trophoblast and stromal cells ($\times 1600$). In Panel D, in contrast, Y chromosomes are found only in a subgroup of stromal cells and circulating blood cells of villi underlying the phenotypically female twin ($\times 1600$). Arrows in Panels B, C, and D show examples of the Y-chromosome signal.

46,XX[4] in the boy. Repeated karyotyping at five months showed 46,XY[91]/46,XX[9] in the girl and 46,XY[92]/46,XX[8] in the boy. The skin fibroblasts showed a normal 46,XX[100] karyotype in all 100 cells in metaphases examined from the girl's skin and 46,XY[100] in all of those from the boy's skin. The sex-determining region of the Y chromosome (SRY) was not detected by PCR of DNA extracted from the girl's skin fibroblasts, a result that effectively rules out low-level XY mosaicism.

INITIAL DNA ZYGOSITY STUDIES

Initial zygosity studies performed on DNA from peripheral-blood lymphocytes showed sharing of all 26 autosomal DNA markers, which was interpreted as equivalent to a 99.9 percent probability that the twins were monozygous (Table 1). However, zygosity studies on skin found that the twins differed at 16 of the 26 markers, indicating dizygosity (Table 1). Given this discrepancy, we retrospectively reviewed the Southern blots from peripheral-blood lymphocytes, and after prolonged exposure times on autoradiography, minor bands of 1.75, 8.1, and 2.3 kb were identified at the D10S28, D4S139, and D5S110 loci, respectively. These bands corresponded exactly to the girl's alleles at these loci in skin fibroblasts (Table 1), indicating that each of the twins carried two populations of lymphocytes, a major subgroup derived from the male and a minor subgroup derived from the female.

EXTENDED DNA ZYGOSITY STUDIES

Of the 21 markers with the potential for manifesting four or more alleles, all but one (D17S26) produced three alleles or fewer (Table 1). To investigate this apparent high degree of allele sharing, 98 additional multiallelic markers were genotyped. When the nonindependence of linked markers was taken into account, these 119 markers were equivalent to 92 completely independent markers. The estimated mean observed likelihoods of sharing zero, one, or two alleles that are identical by descent (0.24, 0.52, and 0.24, respectively) were as expected for normal dizygous twins (0.25, 0.50, and 0.25). Similarly, the mean number of alleles that were identical by state, estimated to be 1.15 (57.5 percent shared alleles), did not depart significantly from 1.0 (50 percent shared alleles), the expected sharing for dizygous twins, and was consistent with the twins' being tetragametic.

DISCUSSION

Prenatal determination of monochorionicity and, by implication, monozygosity is crucial for the assessment of the fetal risk of chromosomal abnormalities and for decision making about selective reduction in twin pregnancies.⁹ Postnatally, knowledge of zygosity affects decisions about organ donation between twins, assessment of the risk of inherited conditions when one twin is affected, and interpretation of twin-based genetic and epidemiologic studies.¹⁰

This case report therefore has important implications. First, it contravenes the doctrine that monochorionic twins are invariably monozygotic. Second, it demonstrates that high levels of blood chimerism in dizygous twins can produce DNA zygosity results in peripheral blood that may lead to erroneous assignment of monozygosity. Blood chimerism is a well-documented phenomenon, detectable in a minority of dichorionic twins.^{11,12} In this case, a predominance of male-derived lymphocytes in the circulation of both twins explains the results of the zygosity study, which were initially interpreted as showing monozygosity. The female alleles for at least some of the markers were visible retrospectively in DNA extracted from blood but appeared as minor bands on Southern blotting because of their much lower concentrations. The PCR-based zygosity tests did not detect the female twin's alleles in the blood. This raises the possibility of false assignment of zygosity for same-sex dizygous twins when zygosity is based on testing of peripheral blood. Testing of other tissues, such as buccal cells, may circumvent this problem in future clinical practice and twin research.

We can only speculate about the embryologic events that resulted in monochorionic placentation for these dizygous twins. Nylander and Osunkoya proposed in 1970 that the existence of a set of twins who were phenotypically discordant for sex but with monochorionic, diamniotic membranes on pathological examination might be explained by fusion of the chorions early in pregnancy, with subsequent degeneration of the fused chorion within the dividing membrane.³ However, no cytogenetic or zygosity studies were available. Although this mechanism cannot be ruled out, in the present case both *in situ* hybridization and histologic examination of the dividing membrane showed close apposition of the amnions, with no evidence of intervening chorionic tissue.

Alternatively, the trophoblasts from two embryos might have fused before implantation so that two genetically distinct, nonchimeric, inner cell masses came to reside in a chimeric trophoblast "shell." This possibility is supported by data indicating that under certain conditions, fusion of preimplantation mammalian embryos can be induced *in vitro*.¹³ Factors that brought the embryos into close physical proximity with one another could conceivably predispose them to such fusion. Binovular follicles have been reported, in which two oocytes are present within a single zona pellucida, and *in vitro* fertiliza-

Table 1. Results of DNA Zygosity Studies in Peripheral-Blood Lymphocytes and Skin Fibroblasts from Each Twin.*

| DNA Marker | DNA from Peripheral-Blood Lymphocytes | | | DNA from Skin Fibroblasts | |
|-------------------|---------------------------------------|----------------|-----------------------|---------------------------|----------------|
| | Male Alleles | Female Alleles | Minor Bands in Female | Male Alleles | Female Alleles |
| D2S44 | 3.42/2.04 | 3.42/2.04 | — | 3.42/2.04 | 3.42/2.04 |
| D10S28 | 2.60/0.95 | 2.60/0.95 | 1.75 | 2.60/0.95 | 2.60/1.75 |
| D4S139 | 11.09/4.84 | 11.09/4.84 | 8.10 | 11.09/4.84 | 11.09/8.10 |
| D5S110 | 2.60/1.99 | 2.60/1.99 | 2.3 | 2.60/1.99 | 2.30/1.99 |
| D17S26 | 5.57/4.71 | 5.57/4.71 | 3.59 | 5.57/4.71 | 3.59/1.37 |
| LDLR | A/B | A/B | — | A/B | A/B |
| GYP A | A/A | A/A | — | A/A | A/A |
| HBGG | B/B | B/B | — | B/B | B/B |
| D7S8 | A/B | A/B | — | A/B | A/B |
| GC | A/B | A/B | — | A/B | A/B |
| HLA-DQ α 1 | 2/4.1 | 2/4.1 | — | 2/4.1 | 4.1 |
| D3S1358 | 14/18 | 14/18 | — | 14/18 | 15/18 |
| Tho1 | 9.3 | 9.3 | — | 9.3 | 9.3 |
| D21S11 | 30.2 | 30.2 | — | 30.2 | 30.2/31 |
| D18S51 | 15/17 | 15/17 | — | 15/17 | 14/17 |
| PentaE | 5/15 | 5/15 | — | 5/15 | 15 |
| D5S818 | 11/13 | 11/13 | — | 11/13 | 12/13 |
| D13S317 | 12 | 12 | — | 12 | 12 |
| D7S820 | 8/10 | 8/10 | — | 8/10 | 8/9 |
| D16S539 | 11/13 | 11/13 | — | 11/13 | 11/13 |
| CSF1PO | 11/13 | 11/13 | — | 11/13 | 11 |
| PentaD | 9/14 | 9/14 | — | 9/14 | 7/9 |
| VWA | 15/17 | 15/17 | — | 15/17 | 15/16 |
| D8S1179 | 12/14 | 12/14 | — | 12/14 | 12/13 |
| TPOX | 8/11 | 8/11 | — | 8/11 | 8/11 |
| FGA | 20/22 | 20/22 | — | 20/22 | 20/21 |

* Allele sizes are given in kilobases for the markers analyzed by Southern blotting and according to allele designation appropriate for the polymerase-chain-reaction system.

tion of one or both of a pair of oocytes has been demonstrated.^{14,15} Whether both zygotes could result in a viable pregnancy is not known.

Another theoretical explanation for the production of monochorionic, dizygotic twins is double fertilization of the meiotic products of a single

oocyte with two sperm, leading to “sesquizygotic” twins, with each twin having distinct paternal and identical maternal genomes (except for homologous recombination in the first meiotic division).¹⁶ Parthenogenetic division of human oocytes can be induced experimentally in vitro,¹⁷ and analysis of DNA markers in persons with complete chimerism provides indirect evidence to support the existence of oocyte division before fertilization by two sperm.^{18,19} If this were the explanation, the twins would be expected to have a higher degree of allele sharing than that seen between dizygous twins or siblings. However, our analysis of allele sharing from the genotyping of 119 multiallelic markers effectively ruled out sesquizygosity.

Whatever the underlying mechanism, the possibility that in vitro fertilization could have promoted

this event cannot be ruled out. In vitro fertilization is associated with an increase in embryo splitting and monozygotic twinning, and blastocyst culture may further increase this risk.²⁰⁻²³ In vitro fertilization may also be associated with an increased risk of embryonic fusion before implantation; this mechanism may explain a previous report of true hermaphroditism in a chimeric infant who was conceived by in vitro fertilization.²⁴ Our case suggests that the influence of in vitro fertilization on early embryonic development merits further investigation.

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