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RESEARCH LETTER

Trizygotic dichorionic triplets with 46,XX/46,XY chimerism in both fetuses of the monochorionic pair

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Monochorionic fetuses are traditionally thought of as monozygotic (MZ), arising from a single embryo that splits during early embryogenesis. With rare exception, they typically exhibit the same genotype and the same gender. However, several recent reports have documented that monochorionicity does not always indicate monozygosity (Souter *et al*., 2003). At least 14 such cases have been reported (Ekelund *et al*., 2008). We report a trizygotic triplet pregnancy conceived by *in vitro* fertilization (IVF) in which triamniotic dichorionic placentation was identified in addition to hematopoietic chimerism in the monochorionic pair.

A 29-year-old Caucasian, insulin-dependent diabetic woman gravida 2, para 1, conceived a triplet gestation with the assistance of IVF. Three embryos were transferred. An ultrasound performed at 7 weeks revealed three viable embryos in three gestational sacs. A thin membrane, with a direct, perpendicular insertion into a single placenta, was visualized between sacs B and C.

There was no evidence of a lambda sign at the placental insertion site. These findings indicated monochorionicity between the B and C sacs. Fetus A was surrounded by a single amnion and a single chorion. The sonographic findings indicated a triamniotic, dichorionic triplet pregnancy. Genotypes of the B and C embryos were assumed to be identical due to the observed monochorionicity. At 12 weeks and 5 days, the ultrasound findings were confirmed. As part of a Down syndrome risk assessment, nuchal translucency measurements were 1.9, 1.3 and 2.2 mm, for triplets A, B and C, respectively.

Ultrasound at 18 weeks revealed a gender discrepancy between the monochorionic fetuses, with triplet B demonstrating normal appearing female external genitalia and triplet C demonstrating normal appearing male external genitalia. Fetus A was noted to be female. Due to the gender discrepancy, amniocentesis was performed on all three gestational sacs. Separate needle insertions were performed for each fetus. Following withdrawal of amniotic fluid, indigo carmine dye was injected into the sacs of fetuses A and B. Clear fluid was obtained from all three sacs, documenting that each sac was successfully, individually sampled. Interphase fluorescence *in situ* hybridization (FISH) on uncultured amniotic fluid cells demonstrated a mixture of 36% XX, 64% XY cells for fetus B and 62% XX, 38% XY cells for fetus C. Amniotic fluid cell culture revealed 46,XX cells in fetus B and 46,XY cells in fetus C. Amniotic fluid cell culture of the A fetus revealed 46,XX cells. Zygosity testing was performed on cultured amniocytes from all three fetuses and compared to peripheral blood from the parents (Figure 1). Polymorphic markers from 13 chromosomes were analyzed after DNA amplification by the polymerase chain reaction. Haplotype analysis revealed informative markers between all three fetuses. This led to the conclusion that the triplets were trizygotic.

The pregnancy was complicated by premature cervical shortening. Bedrest was prescribed. Serial growth ultrasounds demonstrated concordant growth. Non-stress testing was reassuring. At 34 1/7 weeks, labor commenced. Delivery was by an uncomplicated repeat cesarean section. Baby A was a female with 1985-g weight, baby B was a female with 1917 g, and baby C was a male with 1905 g.

Cord blood chromosome analysis of triplet A demonstrated a 46,XX karyotype. Triplet B (phenotypic female) had 50% male and 50% female cells in blood taken from its umbilical cord. Chromosome analysis from peripheral blood of the Triplet B several days after birth demonstrated a normal female karyotype in 45% of cells, and a normal male karyotype in 55% of cells. Triplet B had normal female external genitalia and a normal appearing uterus and ovaries on pelvic ultrasound. Blood from the umbilical cord of Triplet C showed 50% male and 50% female cells. Chromosome analysis of peripheral blood of Triplet C several days after birth revealed 54% female cells and 46% male cells. Triplet C had normal external male genitalia and normal appearing testes on pelvic ultrasound exam. The infants had an unremarkable neonatal course and are all doing well at 1 year of age. Placental pathology revealed a dichorionic triamniotic placenta.

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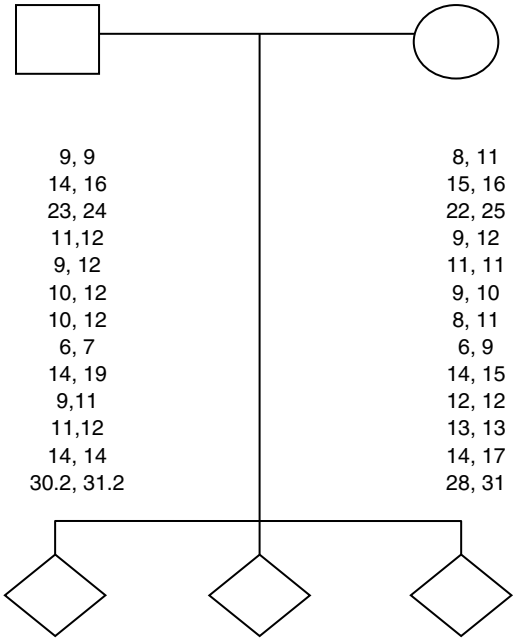

CHROMOSOME	Locus			
Chromosome 2	TPOX	9, 9		8, 11
Chromosome 3	D3S1358	14, 16		15, 16
Chromosome 4	FGA	23, 24		22, 25
Chromosome 5	D5S818	11,12		9, 12
Chromosome 5	CSF1PO	9, 12		11, 11
Chromosome 7	D7S820	10, 12		9, 10
Chromosome 8	D8S1179	10, 12		8, 11
Chromosome 11	TH01	6, 7		6, 9
Chromosome 12	vWA	14, 19		14, 15
Chromosome 13	D13S317	9,11		12, 12
Chromosome 16	D16S539	11,12		13, 13
Chromosome 18	D18S51	14, 14		14, 17
Chromosome 21	D21S11	30.2, 31.2		28, 31

CHROMOSOME	Locus	Triplet A	Triplet B	Triplet C
Chromosome 2	TPOX	9, 11	8, 9	8, 9
Chromosome 3	D3S1358	15, 16	15, 16	14, 15
Chromosome 4	FGA	24, 25	24, 25	22, 24
Chromosome 5	D5S818	11, 12	12, 12	12, 12
Chromosome 5	CSF1PO	9, 11	11, 12	11, 12
Chromosome 7	D7S820	10, 10	9, 10	9, 12
Chromosome 8	D8S1179	8, 10	10, 11	11, 12
Chromosome 11	TH01	6, 7	6, 7	7, 9
Chromosome 12	vWA	14, 14	14, 15	15, 19
Chromosome 13	D13S317	9, 12	9, 12	11, 12
Chromosome 16	D16S539	12, 13	12, 13	12, 13
Chromosome 18	D18S51	14, 17	14, 17	14, 14
Chromosome 21	D21S11	28, 30.2	28, 31.2	28, 30.2

Figure 1—Zygosity determination using polymorphic DNA markers from 13 chromosomes revealing that these triplet fetuses are trizygotic

We report a case of trizygotic dichorionic triamniotic triplets where the monochorionic fetuses were surprisingly found by ultrasound to have discordant genders. Amniocentesis results revealed conflicting results, in which the interphase FISH on uncultured amniotic fluid cells identified a mixture of XY and XX cells in each fetus of the monochorionic pair, but amniotic fluid cell culture demonstrated only 46,XX cells in the female (fetus B) and only 46,XY cells in the male (fetus C). Post delivery chromosome analysis of the B and C triplets demonstrated 46,XX/46,XY chimerism in each.

Several different mechanisms have been reported to explain discordance of fetal gender between monochorionic twins. The most common explanation is that chorionicity assignment by ultrasound has been incorrect. In our case, several first trimester ultrasounds revealed triamniotic dichorionic triplets. Monochorionicity between triplets B and C was confirmed pathologically after birth. Congenital adrenal hyperplasia could explain the findings. There was no evidence of congenital adrenal hyperplasia after birth. Zech *et al*. (2008) reported that discordant MZ twins may occur due to post-zygotic loss of an X chromosome from a 47,XXY zygote resulting in a normal appearing 46,XY male fetus and loss of a Y chromosome from this same 47,XXY conceptus resulting in a normal appearing 46,XX female.

MZ twins with discordant genders has also been reported to occur from an original 46,XY embryo in which anaphase lag results in the loss of a Y chromosome in one embryo after twinning resulting in a normal male 46,XY fetus and a female 45,X fetus with Turner syndrome (Edwards *et al*., 1966). Zech *et al*. (2008) also suggested that a post-zygotic somatic mutation may be responsible for gender discordance in MZ twins. Ginsberg *et al*. (2005) hypothesized that this situation may arise if two oocytes within a single zona pellucida are fertilized (with one Y-bearing sperm and one X-bearing sperm) with subsequent fusion of the embryos. Miura and Niikawa (2005) have hypothesized that fusion of the outer cell mass of two separate embryos at a late morula stage (before day 4 post-conception) may explain the findings. They further speculate that artificial reproductive technologies that result in cell surface changes (assisted egg hatching, cell culture conditions and implantation of several embryos at adjacent uterine sites) may predispose to this phenomenon. In order to

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rule out these possible etiologies, extensive cytogenetic investigations of the children would be required. The parents declined further testing of the babies.

The use of indigo carmine dye to confirm successful sampling of each fetus separately as well as the 46,XX/46,XY chimerism identified both in cord blood and peripheral lymphocytes after birth eliminates amniotic fluid contamination as the explanation for the interphase FISH on uncultured amniotic fluid cell findings. The possibility that these cells are placental in origin cannot be ruled out.

Ekelund *et al*. (2008) commented that vascular anastomoses in a monochorionic placenta would permit exchange of blood stem cells through a common placenta. This would allow for exchange of hematopoietic material and result in blood chimerism between the fetuses. In all other cases we are aware of in which chimerism has been identified among dizygous monochorionic twins, it has been found exclusively in blood. Chimerism has not been observed in amniotic fluid fibroblasts, skin, hair root or buccal swabs (Souter *et al*., 2003; Williams *et al*., 2004; Miura and Niikawa, 2005; Walker *et al*., 2007).

We believe the findings in our case are explained by understanding the cells analyzed by each technique. The interphase FISH on uncultured amniotic fluid cells evaluates both fibroblastic and lymphocytic cells. This is evidenced by the fact that interphase FISH on amniotic fluid contaminated with maternal blood will identify both amniotic fluid fibroblast cells and maternal peripheral blood lymphocytes (Hockstein *et al*., 1998). Amniotic fluid cell cultures analyze only fibroblasts. Cord blood and peripheral blood cultures analyze lymphocytes. Using Ekelund's vascular anastomoses theory (Ekelund *et al*., 2008), we believe the vascular anastomoses allowed for mixture of blood between the monochorionic fetuses. Thus the interphase FISH on uncultured amniotic fluid cells are consistent with mixing of blood between the fetuses due to placental vascular anastomoses akin to twin–twin transfusion. Cell culture from amniocentesis specimens are believed to be derived predominantly from skin fibroblasts—non-hematopoietic tissue. This explains why lymphocyte chromosome analysis revealed chimersim in our case, whereas cultured fibroblasts from amniotic fluid cells did not.

A mixture of 46,XY and 46,XX lymphocytes could be due to chimerism of the bone marrow or could be a transient phenomenon due to twin–twin transfusion with admixture of cells from one fetus into the circulation of the other. Repeat blood chromosome studies would be expected to normalize over time if the chimerism is transient due to twin–twin transfusion. Bone marrow chromosome analysis would also help clarify this issue but the parents declined further testing because of its invasiveness.

In our opinion, the sonographic, cytogenetic, zygosity analysis and interphase FISH on uncultured amniotic fluid cells observations suggest that the best mechanism to explain the findings in this case is early placental fusion of separate embryos (triplets B and C) resulting in a diamniotic monochorionic placenta with vascular anastomoses between these fetuses resulting in hematopoietic chimerism.

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