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ORIGINAL INVESTIGATION

A case of true hermaphroditism reveals an unusual mechanism of twinning

Vivienne L. Souter · Melissa A. Parisi · Dale R. Nyholt · Raj P. Kapur · Anjali K. Henders · Kent E. Opheim · Daniel F. Gunther · Michael E. Mitchell · Ian A. Glass · Grant W. Montgomery

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Abstract Traditionally twins are classified as dizygous or fraternal and monozygous or identical (Hall Twinning, 362, 2003 and 735–743). We report a rare case of 46,XX/46,XY twins: Twin A presented with ambiguous genitalia and Twin B was a phenotypically normal male. These twins demonstrate a third, previously unreported mechanism for twinning. The twins underwent initial investigation with 17-hydroxyprogesterone and testosterone levels, pelvic ultrasound and diagnostic laparoscopy. Cytogenetic analysis was performed on peripheral blood cells and skin fibroblasts. Histological examination and Fluorescence in situ hybridization studies on touch imprints were performed on gonadal biopsies. DNA analysis using more than 6,000 DNA markers was performed on skin fibroblast samples from the twins and on peripheral blood samples from both parents. Twin A was determined to be a true hermaphrodite and Twin B an apparently normal male. Both twins had a 46,XX/46,XY chromosome complement in peripheral lymphocytes, skin fibroblasts, and gonadal biopsies. The proportion of XX to XY cells varied between the twins and the tissues evaluated. Most significantly the twins shared 100% of maternal alleles and approximately 50% of paternal alleles in DNA analysis of skin fibroblasts. The twins are chimeric and share a single genetic contribution from their mother but have two genetic contributions from their father thus supporting the existence of a third, previously unreported type of twinning.

V. L. Souter (✉)
Department of Obstetrics and Gynecology,
Banner Good Samaritan Medical Center,
1111 E McDowell Rd, Phoenix, AZ, 85006, USA
e-mail: vsouter@yahoo.com

M. A. Parisi · D. F. Gunther · I. A. Glass
Department of Pediatrics, Childrens Hospital and Regional Medical Center, University of Washington,
Seattle, WA, USA

D. R. Nyholt · A. K. Henders · G. W. Montgomery
Genetic Epidemiology Laboratory, Queensland Institute of Medical Research, Brisbane, QLD, Australia

R. P. Kapur · K. E. Opheim
Department of Laboratories, Seattle Childrens Hospital and Regional Medical Center, Seattle, WA, USA

M. E. Mitchell
Department of Urology, Seattle Childrens Hospital and Regional Medical Center, Seattle, WA, USA

Introduction

True hermaphroditism (TH) is considered a disorder of sex development (Hughes et al. 2006) characterized by the presence of both ovarian tissue (containing follicles) and testicular tissue (with distinct seminiferous tubules) in the same individual (Krob et al. 1994). Typically, both Mullerian and Wolffian derivatives are present and the genitalia are ambiguous. However, the internal and external genitalia described in hermaphrodites encompass a spectrum of findings. TH has been associated with various chromosomal complements including 46,XX, 46,XY, 46,XX/46,XY, 47,XXY, 47,XYY, 45X, and 46,XX/47,XXY mosaicism (Krob et al. 1994). Molecular genetics can help elucidate the mechanism by which TH arises. Few cases of TH with such studies have been reported.

The twins in this report were investigated because of ambiguous external genitalia in one twin. They were

spontaneously conceived and were born at term following an uncomplicated pregnancy. Prenatal ultrasound was reported to show a dimaniotic twin pregnancy. Chorionicity was not reported. The still images were reviewed retrospectively but the views were insufficient to determine chorionicity. No amniocentesis or placental pathology was undertaken. At birth, Twin A had ambiguous external genitalia with an enlarged clitoris and separate urethral and vaginal openings without labial fusion (Fig. 1a, b). Female gender was assigned. At 3 weeks of age, a 1 cm mass was found in the left inguinal region of Twin A. Twin B had normal male external genitalia.

Methods

Both twins underwent evaluation by a multidisciplinary team including a geneticist, pediatric urologist and pediatric endocrinologist. Serum 17-hydroxyprogesterone and testosterone levels were drawn. Chromosomal evaluation was performed on G-banded metaphase cells from stimulated peripheral lymphocytes and cultured skin fibroblasts. Diagnostic laparoscopy and gonadal biopsies were performed. Histological examination was performed on biopsies from both gonads. Fluorescence in situ hybridization (FISH) studies of touch imprints from each gonadal biopsy were performed

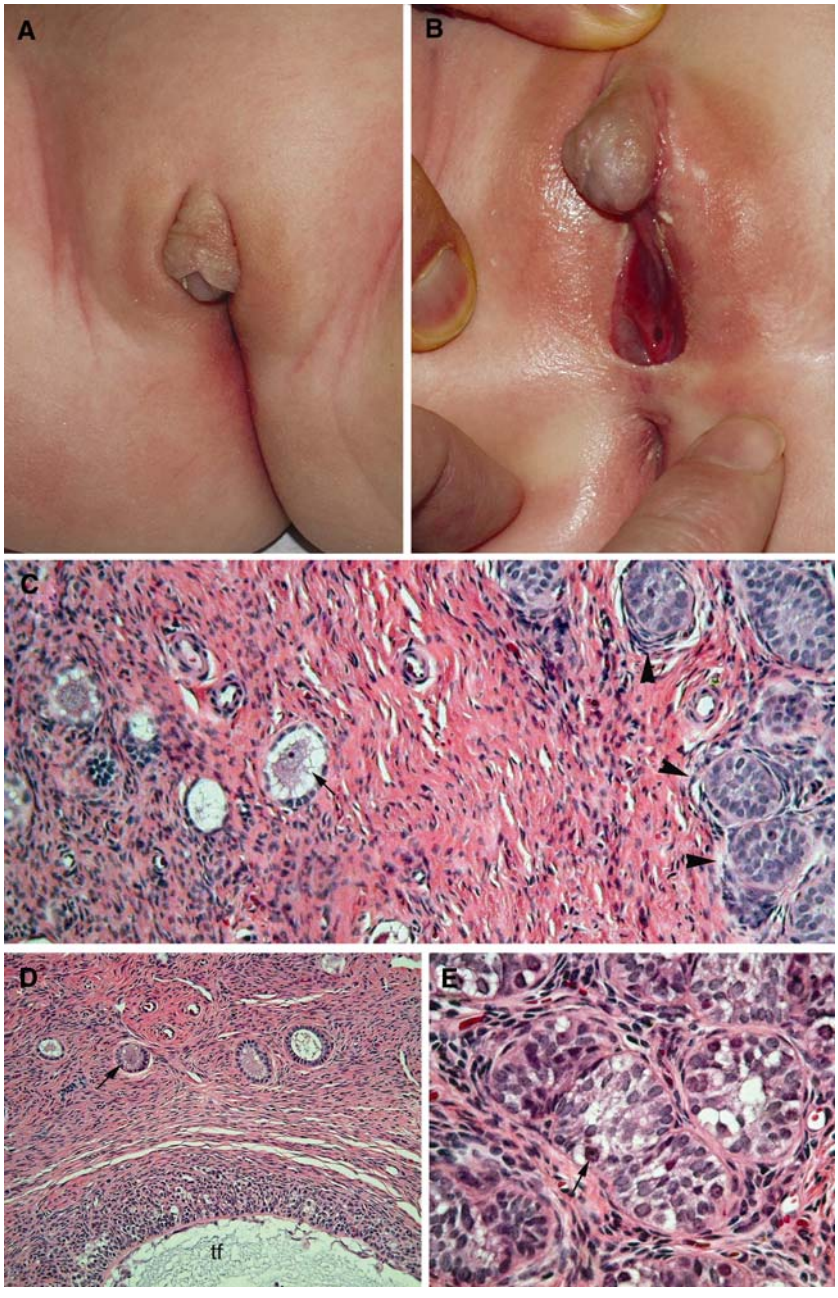

Fig. 1 Appearance of the external genitalia of Twin A at 4 months of age showing **a** enlarged clitoris and mildly hyperpigmented and rugated skin along the labial folds. **b** Separate urethral and vaginal orifices were visible. **c** Microscopic anatomy of the left ovotestis from Twin A shown at high magnification, with both ovarian (*arrow*) and testicular elements (*arrowheads*) present in the same field. **d** At low magnification, ovarian foci contain oocytes (*arrow*) in primary and tertiary (*tf*) follicles. **e** Testicular differentiation includes seminiferous tubules comprised of Sertoli cells and spermatogonia (*arrow*)

using DNA probes for the centromeric region of the X chromosome and the SRY gene of the Y chromosome (CEP X and Yp11.3, respectively; Vysis Inc., Downers Grove, IL, USA). Interphase nuclei were scored for the number of green signals (X centromere) and red signals (SRY).

Peripheral blood and skin fibroblast samples from the twins and peripheral blood samples from both parents were obtained for DNA analysis to determine the parental origin of the alleles at each locus. DNA obtained from blood was amplified using the AmpF/STR-Profiler-Plus™ zygosity determination system (Applied Biosystems, Foster City, CA, USA) and an X chromosome marker (XAR). PCR products were separated on 6% acrylamide gels using the ABI 377 gene profiler. The Profiler kit includes nine DNA markers located on autosomal chromosomes and a DNA marker specific for each of the sex chromosomes (Amelogenin).

A further 95 DNA markers from 15 chromosomes were then analyzed to evaluate the degree of allele sharing between the twins using DNA extracted from skin fibroblasts.

Markers that were not 'informative' (i.e., markers where the alleles, or the specific forms for each marker that were inherited specifically from the mother or father could not be distinguished) were excluded from the analysis. Where the markers were informative, the number of times alleles inherited from the father or mother were the same (shared) or different (non-shared) for the twins was evaluated. Since many markers were used, some markers were physically close on the same chromosome and would not be inherited independently because of linkage disequilibrium. As a result, the allele counts were adjusted downward to account for such non-independence (Haseman and Elston 1972). Genotyping of the same samples was performed using the Illumina IV linkage mapping panel (Murray et al. 2004). This single nucleotide polymorphism (SNP) panel includes 6008 SNPs from across the genome. SNPs were genotyped using the Golden Gate assay according to the manufacturer's standard protocols (Illumina Inc, San Diego CA, USA).

The proportion of DNA from XY cells in the samples from lymphocyte and fibroblast DNA was estimated by real time PCR using a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) with primers for Y-specific sequences. No amplification products were detected in control DNA from normal females with these primers. Concentrations for each of the DNA samples were adjusted to 20 ng/μl and results were compared with samples constructed by creating mixtures of DNA from male and female DNA samples ranging from 5 to 75% male DNA with replicates generated from different male and female mixtures. The proportion of male DNA in samples from the twins was then estimated by regression analysis.

Results

Investigation of Twin A

Twin A's serum 17-hydroxyprogesterone level was within normal limits on day 3 of life and total testosterone was elevated at 199 ng/dl on day 12 of life (Table 1). G-banded metaphase cells from stimulated lymphocytes showed a 46,XX[60]/46,XY[40] karyotype (Table 1). Cytogenetic analysis of cultured skin fibroblasts showed a 46,XX[95]/46,XY[5] karyotype (Table 1).

Table 1 Summary of the hormone levels at 4 months of age and cytogenetic studies in tissues from each twin. *N* = the total number of metaphase cells or interphase nuclei examined

	Twin A	Twin B
Hormone studies [normal ranges below values]		
LH (mIU/ml)	2.8 [1–3.5]	1.9 [1–3.5]
FSH (mIU/ml)	4.8	0.9
Total testosterone (ng/dl)	23 [<8, female]	<4 [<165, male]
Estradiol (pg/ml)	28	9
Cytogenetic studies		
Cultured peripheral lymphocytes	46,XX[60%]/46,XY[40%] (*N* = 100)	46,XX[57%]/46,XY[43%] (*N* = 100)
Cultured skin fibroblasts	46,XX[95%]/46,XY[5%] (*N* = 60)	46,XX[45%]/46,XY[55%] (*N* = 20)
Touch imprints from right gonad	XX[42%]/XY[57%]/X[1%][a] (*N* = 200)	XX[8%]/XY[77%]/X[15%][a] (*N* = 200)
Touch imprint from left gonad	XX[35%]/XY[55%]/X[10%][a] (*N* = 200)	XX[31%]/XY[59%]/X[9%][a] (*N* = 200)

LH luteinizing hormone, *FSH* follicle stimulating hormone

[a] These nuclei exhibiting a single X chromosome green signal most likely represent artifacts because of reddish background autofluorescence that could mask the red signal of the SRY probe on the Y chromosome

Evaluation at 4 months of age showed age-appropriate growth and development, clitoromegaly, mild hyperpigmentation and rugation of the labia majora, and persistence of the left inguinal mass. Serum testosterone was mildly elevated (23 ng/dl) and gonadotrophin levels were unremarkable (Table 1). Diagnostic laparoscopy revealed a gonad, hemi-uterus and fallopian tube on the right, and a gonad in the inguinal canal on the left. Bilateral inguinal herniae were present. Vaginoscopy and cystoscopy showed a well formed vagina, a communicating cervix, a normal bladder, and normal ureteral orifices.

Histological examination revealed both gonads to be ovotestes (Fig. 1c–e). The biopsy of the left gonad was predominantly testicular with seminiferous tubules that contained Sertoli cells and spermatogonia. Areas of ovarian differentiation including follicles, oocytes, and ovarian stroma were also present. A vas deferens was present on this side. The right gonadal biopsy showed predominantly ovarian differentiation and some seminiferous tubules.

Fluorescence in situ hybridization studies of touch imprints showed that each gonad was composed of both XX and XY cell lines with the following distributions: XX[42%]/XY[57%]/X[1%] in the right gonad and XX[35%]/XY[55%]/X[10%] in the left gonad (Table 1).

Investigation of Twin B

Physical examination was normal at 4 months of age. Pelvic and scrotal ultrasound examinations showed apparently normal testes, small bilateral hydroceles, and no female reproductive organs. Serum testosterone and gonadotrophin levels were consistent with values expected for a prepubertal male (Table 1). Cytogenetic studies on peripheral lymphocytes showed a 46,XX[57%]/46,XY[43%] karyotype (Table 1). Cytogenetic analysis of fibroblast metaphases from a skin biopsy showed a 46,XX[45%]/46,XY[55%] karyotype (Table 1). Cystoscopy of the lower urogenital tract was normal. Histological examination of bilateral gonadal biopsies was consistent with normal testes. FISH studies of touch imprints from the gonadal biopsies showed that each gonad was composed of both XX and XY cell lines with the following distributions: XX[8%]/XY[77%]/X[15%] in the right gonad and XX[31%]/XY[59%]/X[9%] in the left gonad (Table 1).

Molecular studies

The twins were concordant for all of the DNA markers in peripheral blood, with identical alleles for each marker. However, three different alleles were detected for three of the nine autosomal markers suggesting the samples contained a mixture of cells from both twins. The Y chromosome allele for the Amelogenin marker was detected in DNA samples from peripheral blood for both twins. Two alleles were present for the XAR marker for Twin A as was expected, but two XAR alleles were also present in the sample for Twin B.

The same markers were typed in DNA samples from skin fibroblasts for both twins. Two predominant alleles were detected for each marker in each twin. In skin, the twins were discordant for four of the ten markers excluding the possibility of monozygosity. A minor peak for the Y allele for the Amelogenin marker was still detected in the fibroblast sample from Twin A and two alleles for the XAR marker were detected for both Twin A and Twin B.

DNA analysis using a further 95 markers was complicated by the presence of two cell lines in the twins and three alleles were detected for some markers. No markers with four alleles were observed. In each case, the additional allele was inherited from the father. For remaining markers, the twins were found to share the same allele inherited from the mother for all informative chromosome markers.

After adjusting for non-independence, alleles could be scored from the father and the mother 47.1 times out of the total of 104 markers (Table 2). Dizygous (DZ) twins should share 50% of their alleles. So the expected number of alleles shared and non-shared (after adjustment for non-independence) was $0.5 \times 47.1 = 23.55$. The twins shared all alleles inherited from the mother (Table 2). This single maternal contribution to the twins was significantly higher than the expected 50:50 proportion of markers shared for DZ twins ($P < 10^{-11}$; Table 2). Consistent with the Profiler results, the twins were discordant for paternal alleles at the equivalent of 16.8 independent markers demonstrating two paternal contributions to the twins.

Analysis of the Illumina linkage panel markers identified 779 SNPs where the father was heterozygous (carried two different alleles) and the alleles inherited from both parents could be unambiguously assigned for both twins. There were 675 similarly informative SNP markers for the mother. The twins shared alleles for 52.1% of 779 informative markers from the father. In contrast, the twins shared 100% of alleles for 675 informative markers from the mother. The linkage panel data, including more markers located toward chromosome telomeres confirms results from the microsatellite markers demonstrating the twins share 100% of maternal alleles.

Table 2 The observed numbers of parental alleles shared identical by descent (IBD) in the twins in DNA samples from skin fibroblasts

Comparisons		Observed alleles corrected for non-independence		Chi Square	*df*	*P*-value
		Shared	Non-shared			
Father's alleles	Observed	30.34	16.77	20.40	1	6.23×10^{-6}
Mother's alleles	Observed	47.10	0.00			
Father's alleles	Observed	30.34	16.77	3.91	1	0.048
Frequencies for DZ twins	Expected	23.55	23.55			
Mother's allele	Observed	47.1	0.00	47.1	1	6.73×10^{-12}
Frequencies for DZ twins	Expected	23.55	23.55			

Allele counts were corrected for non-independence between markers located in the same chromosomal regions. The corrected allele counts shared from the father were compared to corrected allele counts shared from the mother. Corrected allele counts shared from the father and the mother were both tested against corrected allele counts expected in dizygotic twins. Chi-square tests of homogeneity were used to test hypotheses concerning the distribution of alleles shared and non-shared

df degrees of freedom

The proportions of DNA from XY cells in the samples from lymphocyte and fibroblast were consistent with results from the karyotype analysis. The percentage of male DNA in fibroblast DNA from the Twin A and Twin B was estimated to be 6.3 and 47%, respectively.

Discussion

Twins are broadly classified as either DZ or monozygotic (MZ). Hall (2003) ~75% of twins are believed to be DZ or fraternal and 25% to be MZ or 'identical.' The existence of twins who do not meet the traditional classification has been previously hypothesized (Boklage 1987; Golubovsky 2003). Indeed the possibility of twins with the same maternal but different paternal genetic contributions has been proposed (Golubovsky 2003). However, to our knowledge the current case provides the first evidence to support this hypothesis.

The mechanism by which the current twins arose is uncertain. Previous authors have reported human cases supporting the occurrence of parthenogenetic or premature activation of an egg. Strain et al. (1995) reported a child with a 46,XY/46,XX chromosome complement on whom DNA studies showed only one maternal genetic and one paternal genetic contribution which the authors explained by 'parthenogenetic' activation of the oocyte prior to fertilization. Giltay et al. (1998) reported a 46,XX/46,XY child with TH whose DNA markers were consistent with double paternal and single maternal genetic contributions. The authors postulated a three gamete mechanism for this dependent upon immediate cleavage secondary to parthenogenetic activation of the egg followed by fertilization of the identical cells thus formed, by two different sperm containing different sex chromosomes (Fig. 2) (Giltay et al. 1998). This is also a possible explanation in the current case. Dispermic fertilization of an ovum followed by 'diploidization' is another (Fig. 2) Golubovsky (2003) proposed this concept ('postzygotic diploidization of triploids') to explain a number of unusual cytogenetic events and in fact predicted that it might result in chimeric twins with one maternal and two paternal genomes. The possibility of fertilization of the

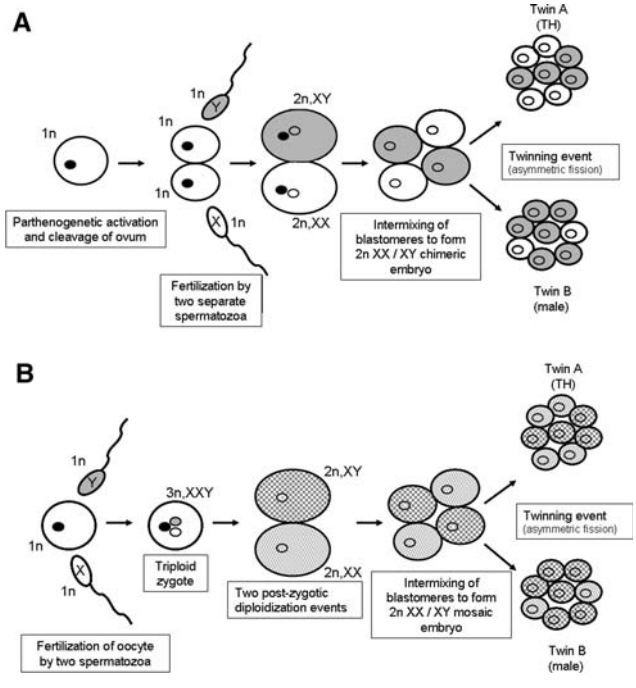

Fig. 2 Possible mechanisms for the embryological events leading to the development of the twins. **a** represents the three gamete mechanism with immediate cleavage secondary to parthenogenetic activation of the egg followed by fertilization of the identical cells thus formed, by two different sperm containing different sex chromosomes. **b** represents the scenario of dispermic fertilization of an ovum followed by the 'postzygotic diploidization of triploids' concept as postulated by Golubovsky (2003)

ovum and the second polar body by two different sperm cannot be completely excluded but based on the results of DNA analysis, this would be expected to be associated with the presence of identical alleles for informative proximal DNA markers and differences in informative distal DNA markers due to crossing over in first meiosis. In the current case, multiple DNA markers covering both centromeric and distal chromosomal regions were evaluated but only one set of maternally derived alleles was detected in both twins. Hence, fertilization of the second polar body could have occurred only in association with extreme suppression of recombination in meiosis I and is, therefore, very unlikely.

Whatever, the preceding events, a chimeric embryo developed and at some point in early gestation a division or twinning event occurred, resulting in two separate embryos. Asymmetry of this division event could explain the difference in the ratio of XX:XY cells and difference in the paternal genetic contributions.

The twins show different paternal and identical maternal contributions in skin fibroblasts. Profiles were identical in peripheral lymphocytes with three alleles detected for three markers demonstrating chimerism in peripheral lymphocytes. Blood chimerism is a well-documented phenomenon in dizygous twins although the mechanism by which this arises is not clear (Van Dijk et al. 1996). Unfortunately, no placental pathology was performed and so the chorionicity is not known.

Several hundred cases of TH have been reported in the medical literature. Of 282 published cases reviewed by Krob et al. (1994), 20% had either chromosomal mosaicism or chimerism involving a Y chromosome, most commonly 46,XX/46,XY. A 46,XX/46,XY karyotype may result from mosaicism (the presence of cell populations of more than one genotype within an individual and arising from a single zygote), or chimerism (the presence of genetically distinct populations of cells within an individual derived from more than one zygote) (Benirschke 1970). Multiple DNA markers may distinguish between mosaicism and chimerism and indicate the underlying mechanism. Mosaicism may arise through postzygotic non-dysjunction in a 46,XY conceptus or by multiple non-dysjunction events in an embryo with a pre-existing chromosome abnormality, such as 47,XXY (Niu et al. 2002). Previous case reports have implicated fusion of two embryos to form a tetragametic chimera (Uehara et al. 1995; Strain et al. 1998), fertilization of an ovum and a large polar body by two different sperm (Dewald et al. 1980; Repas-Humpe et al. 1999), and fertilization of an ovum that has undergone immediate cleavage prior to fertilization by two different sperm, as the mechanism underlying chimerism (Strain et al. 1995; Giltay et al. 1998). Although fertilization of a parthenogenetically activated ovum by two sperm has previously been implicated in TH (Giltay et al. 1998), the current case is unique in its association with a spontaneously conceived, viable twin pregnancy.

Conclusions

We report a case of 46,XX/46,XY twins determined through molecular genetic studies to possess a single maternal genetic contribution and two paternal genetic contributions. As a result, the twins are more genetically alike than dizygotic twins and less alike than monozygotic twins. This probably represents a rare event, but demonstrates that not all twins conform to the traditionally accepted classification of dizygotic and monozygotic. Investigation of the twins was prompted because one of the twins was a true hermaphrodite while the co-twin was a phenotypically normal male. This observation suggests the existence of other similar twins that have not yet been, and may never be, identified.

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