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ORIGINAL ARTICLE

Cytogenic and molecular analyses of 46,XX male syndrome with clinical comparison to other groups with testicular azoospermia of genetic origin

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KEYWORDS
46,XX male syndrome;
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Background/Purpose: XX male is a rare sex chromosomal disorder in infertile men. The purpose of this study was to distinguish the clinical and genetic features of the 46,XX male syndrome from other more frequent, testicular-origin azoospermic causes of male infertility.

Methods: To study 46,XX male syndrome, we compared clinical and endocrinological parameters to other groups with testicular-origin azoospermia, and to an age-matched group of healthy males and females as normal control. Fluorescent *in situ* hybridization for detection and localization of the sex-determining region of the Y gene (*SRY*), array-based comparative genomic hybridization screening, and real-time qualitative polymerase chain reaction of *FGF9*, *WT1*, *NR5A1*, and *SPRY2* genes were performed in this genetic investigation.

Results: Our three patients with 46,XX male syndrome had a much higher follicular-stimulating hormone level, lower body height, lower testosterone level, and ambiguous external genitalia. One of the three patients with 46,XX male syndrome was *SRY*-negative. A further genetic study, including a comparative genomic hybridization array and real-time polymerase chain reaction, showed a gain of *FGF9* copy numbers only in the *SRY*-negative 46,XX male. The genetic copy number of the *FGF9* gene was duplicated in that case compared to the normal female control and was significantly lower than that of the normal male control. No such genomic gain was observed in the case of the two *SRY*-positive 46,XX males.

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Conclusion: Similar to clinical manifestations of 46,XX male syndrome, genetic evidence in this study suggests that *FGF9* may contribute to sex reversal, but additional confirmation with more cases is still needed.

Introduction

Chromosomal abnormalities are possible causes of male subfertility and infertility. The overall incidence of chromosome abnormalities in infertile men was estimated by several studies to be around 5.8%,[1] and 0.38% in newborns delivered after successful intracytoplasmic sperm injection procedures.[2] Microdeletion of genes in the AZF area of the Y chromosome is also a well-known etiology of azoospermia and severe oligoasthenospermia in patients with male infertility,[3] and the overall frequency of microdeletions was estimated in our series to be 12.2% in azoospermic men and 3.4% in oligozospermic men.[4,5] Y-chromosome abnormalities can be either numerical abnormalities or a variety of structural aberrations. The total absence of the Y chromosome may be seen as a molecularly visible defect by a polymerase chain reaction (PCR) diagnosis and can be proven by a cytogenic analysis of the chromosome karyotype.[6]

During the screening of the 555 infertile male patients, we found 47 cases of a Y chromosome gene deletion. Among these patients, there were three cases for which we could detect no genetic expression of the Y chromosome in the PCR analysis. The cytogenetic analysis of the chromosome revealed a 46,XX male karyotype. Furthermore, detection and localization of the sex-determining region of the Y gene (*SRY*) can have a variable result.[7–9] Two of the three cases were found to have the SRY gene according to fluorescent *in situ* hybridization (FISH), while the other case was a 46,XX male who was SRY-negative. In addition to the cytogenic and molecular analyses of 46,XX male syndrome, this study also made clinical and endocrinological comparisons with other groups with testicular azoospermia such as idiopathic hypogonadotropic hypogonadism (IHH) syndrome and Klinefelter's syndrome. The purpose of this study was to distinguish the clinical and genetic features of the 46,XX male syndrome from other, more frequently occurring genetic disorders. The information can be a good reference for counseling and treatment of male infertility.

Materials and methods

Participants

In total, 47 patients screened from 555 infertile males were found to have a microdeletion of the Y chromosome, and three patients were found to have no Y chromosome according to a PCR analysis. Among these 47 patients, 34 individuals were azoospermic and 13 were severely oligozoospermic or asthenospermic.

In order to compare the clinical and endocrinological features with those three cases with 46,XX male syndrome, 13 cases of IHH and 31 cases of Klinefelter's syndrome were also selected from our 555 patients with male infertility (by a *post hoc* approach).

DNA isolation and PCR analysis of microdeletions of the Y chromosome

DNA was extracted from peripheral blood leukocytes of patients, and genomic DNA was isolated using a Genomic DNA Isolation Kit (PUREGENE, Madison, WI, USA) following the manufacturer's protocol. The concentration of the isolated genomic DNA was determined by a spectrophotometric analysis at 260 nm. All DNA samples were processed for Yq microdeletions using a PCR. The STS and genes used were as follows: AZFa: sY84 and sY86; AZFb: sY127 and sY134; and AZFc: sY254 and sY255. *SRY* and *ZFY* were included as internal controls.[10]

Cytogenetic analysis

Whole blood was collected, and plasma was prepared with heparin. Peripheral blood lymphocytes were cultured in two 25T flasks using RPMI media supplemented with 10% fetal calf serum. Metaphase chromosomes were analyzed by a standard Wright G-banding technique. Karyotyping was performed in 50 metaphases by a conventional method.[11]

FISH for SRY detection and localization

The *SRY* probe, LSI SRY/CEPX, was obtained from Vysis (Abbott Molecular Diagnostics, Hoofddorp, the Netherlands). At room temperature, 7 μl of LSI hybridization buffer, 1 μl of the LSI DNA probe, and 2 μl of purified H_2O were mixed, centrifuged for 1–3 seconds, vortexed, then recentrifuged, heated for 5 minutes in a 73°C water bath, and placed on a slide warmer set to 45–50°C. Hybridization areas were marked with a diamond-tipped scribe on a slide. The slide was immersed in a 73 ± 1°C denaturing bath (70% formamide/2× SSC) for 5 minutes. If the metaphase chromosome morphology was problematic, a denaturing temperature of 70–73°C possibly provided better results. The slide was dehydrated for 1 minute in 70% ethanol (EtOH), 1 minute in 85% EtOH, and 1 minute in 100% EtOH. The slide was dried and placed on a 45–50°C slide warmer for 2 minutes. Ten microliters of probe mix was applied to the slide. The coverslip was immediately applied when the probe was placed on the slide, and was sealed with diluted rubber cement. The slide was placed in a prewarmed humidified box, and hybridization was allowed to proceed overnight for 12–16 h in a 37°C incubator. One wash tank with 0.4× SSC/0.3% NP-40 was prepared, and the slide was placed in a 73 ± 1°C water bath for at least

30 minutes. A second tank of 2× SSC/0.1% NP-40 was prepared at room temperature. The rubber cement seal and coverslip were removed, and the slide was immediately placed into a wash tank (0.4× SSC/0.3% NP-40), which was agitated for 1−3 seconds. This was repeated for a maximum of four slides, and then all slides were left in a Coplin jar for 2 minutes. The coverslips from several slides were not removed before placing the slides in the wash bath. The timing of incubation began when the last slide was added to the wash bath. Slides were washed in 2× SSC/0.1% NP-40 at room temperature for 5−60 seconds, with agitation for 1−3 seconds as a slide was placed in the bath. The slide was allowed to air-dry in darkness. Ten microliters of DAPI II counterstain was applied to the target area of the slide, and a coverslip was added. The slide was viewed using a suitable filter set, and then stored at −20°C in the dark.[12]

Array-based comparative genomic hybridization analysis using Affymetrix Genome Wide Human SNP Array 6.0

Experiments were performed according to standard protocols for the Affymetrix Genome Wide Human SNP Array 6.0 (Affymetrix Human SNP Assay 6.0 User's Guide for Automated Target Preparation; Affymetrix, Santa Clara, CA, USA). Quality control, genotype calling, probe-level normalization, and raw microarray images were analyzed using Affymetrix® Genotyping Console™ Software vers. 3.0.1. Reference set 44 used related Han Chinese samples obtained from the HapMap project. Genetic gains (with a copy number of $\geq$3) and losses (with a copy number of $\leq$1) were defined according to the working criteria of the Genotyping Console™ Software. To avoid false-positive copy number changes due to random noise in the signal intensity at each single-nucleotide polymorphism (SNP), we set a minimum physical length of at least 10 consecutive SNPs for putative genetic alterations. The physical positions of all SNPs on the arrays were mapped according to the Human Genome Sequence (http://www.ncbi.nlm.nih.gov/genome/guide/human/; Build 36). To consider existing structural variations of interest, we included public copy number variation (CNV) data (http://projects.tcag.ca/variation/) in our analysis.

Real-time qualitative PCR of the FGF9, WT1, NR5A1, and SPRY2 genes

Multiples of changes in the *FGF9*, *WT1*, *NR5A1*, and *SPRY2* gene copy numbers were determined by a quantitative PCR using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Molecular Systems, Branchburg, NJ, USA) and LightCycler Instrument 1.5 (Roche Molecular Systems Branchburg, NJ, USA). Quantification was performed by comparing the target locus to the reference *ATP2B4* gene. Relative quantities of copy numbers of the *FGF9*, *WT1*, *NR5A1*, and *SPRY2* genes were normalized using Promega Human Genomic DNA: Female (Promega, Madison, WI, USA) as a calibrator. Cycling conditions were as follows: preincubation at 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 10 seconds. The specific oligonucleotide primer pairs were selected from the Universal Probe Library (Roche Molecular Systems), and the specificity of each primer pair was verified. The mean threshold cycle number (Ct) for each gene in each sample was obtained from triplicate experiments. The primers were *WT1*, 5′-aggaggatagcacggaagaa-3′ and 5′-cagttccccaaccactcatt-3′; *NR5A1*, 5′-gtccctcctcctcctaaa-3′ and 5′-aatgaatgaatgggcaaagg-3′; *FGF9*, 5′-aaaagccgag tcctctgatg-3′ and 5′-cccaggtggtcacttaacaaa-3′; *SPRY2*, 5′-agatcagagccatccgaaac-3′ and 5′-ctctcgtgtttgtgctgagtg-3′; and *ATP$_2$B$_4$*, 5′-ccacgaacaccactcctg-3′ and 5′-accctagtcc-caaacttagaagcc-3′.

Statistical analysis

Statistical analysis was performed using SAS vers. 8 (SAS Institute, Cary, NC, USA). A *Z*-test was used to test the two proportions between both the AZFb- and AZFc-deletion group and the AZFc-only-deletion group. A *t*-test was used to test differences among the body height (BH), follicle-stimulating hormone (FSH), and testosterone concentration of patients and normal controls. Significance was accepted at $p < 0.05$.

Results

We performed a cytogenic analysis of the 47 patients with a PCR molecular diagnosis of Y chromosome microdeletions, including three patients whose entire Y chromosome could not be identified. In these three patients with the complete absence of the Y chromosome on PCR screening, evaluation of the G-banded chromosome preparation from phytohemagglutinin-stimulated lymphocytes revealed a 46,XX karyotype. Cytogenetic studies confirmed a nonmosaic karyotype in all metaphases analyzed. The presence of the *SRY* gene was confirmed by PCR. Seven of the 11 patients (63.6%) with AZFb and AZFc area deletions were found to have variable Y chromosome abnormalities, whereas only five of 31 patients (16.1%) with AZFc area deletions were found to have chromosomal abnormalities. The Y chromosome had higher rates of cytogenic anomalies when the deletion area was found to be larger in the PCR analysis ($p = 0.0008$) (Table 1).

Clinically, there were no remarkable variabilities in physical characteristics; the genitals were normal among the different groups with microdeletions of the Y chromosome. However, the general feature of patients with 46,XX male syndrome was quite unusual, even though they were completely masculinized. Their average body height (160.3 cm) was significantly less than that of the aged-matched 46,XY healthy male controls of a normal population (171.8 cm, $p = 0.048$). They were also significantly shorter than the group of males with AZFb and AZFc deletions (172.6 cm, $p = 0.02$), Kleinefelter's syndrome males (175.9 cm, $p = 0.01$), and males with IHH (Table 2).

The three cases of 46,XX male syndrome also had prominent clinical features of atrophic testicles and ambiguous external genitalia. One had cryptorchism, and another had hypospadia (Table 3). Their endocrinological picture reflected remarkable testicular atrophy. Elevation of the FSH level of 46,XX males was even more prominent than that of patients with Kleinefelter's syndrome

Table 1 Variable chromosomal abnormalities in the molecular and cytogenetic screening of the 47 patients with Y chromosome gene deletion (including 3 patients with total absence of Y chromosome).

Group	Microdeletion of Y chromosome by PCR molecular diagnosis	*N*	Cytogenic analysis of abnormal chromosome karyotype	*n*	Sum of patients with abnormal karyotype for each group ($n/N \times 100$) %
1	Cannot detect the entire AZF region of Y chromosome	3	46,XX	3	3 (100)
2	Both AZFb and AZFc deletion	11	46,X,+mar	2	7 (63.6)[a]
			46,X,del (Y) (q11.22)	2	
			mos 45,X/46,XY	1	
			mos 46,X,idic(Y)(q11.22)/45,X	1	
			47,XY,+mar	1	
3	Only AZFb deletion	2	—	0	0 (0)
4	Only AZFc deletion	31	46,X,del (Y) (q11.22)	1	5 (16.1)
			46,XY,inv(15)	1	
			46,XY,inv(9qh)	1	
			46,XY,9qh+	1	
			46,X,Yqh−	1	
	Total	47		15	15 (31.9)

[a] *Z*-test was used to test the two proportions between group 2 and group 4 ($p = 0.0008$). The significance level was set at $p < 0.05$.

($p = 0.07$). A lower serum testosterone level could not be differentiated between groups with 46,XX male Kleinefelter's syndrome and IHH in a state of untreated hypogonadism (Table 2).

Cytogenetic studies confirmed a nonmosaic 46,XX karyotype in the three cases. Two of them were identified to possess the *SRY* gene by FISH; one case had no signal for the *SRY* gene (Fig. 1). For the case of the 46,XX male that was *SRY*-negative, we performed a further array-based comparative genomic hybridization (array-CGH) analysis and validated the CNVs. Duplication of the *FGF9* and *SPRY2* genes was detected (Fig. 2). Real-time PCR analysis with multiple controls confirmed significant differences in these two genes in the *SRY*-negative 46,XX male (Fig. 3).

Discussion

46,XX male syndrome represents a rare, poorly characterized form of male hypogonadism in the literature. By 1996, only 150 patients with classical XX male syndrome had been reported[13]; however, more than 100 cases were described in the next 10 years (1996−2006).[14] Some of the newly found patients were in the neonatal stage[15] with genital ambiguities and were detected by a chromosomal screening analysis. As in this study, increasing numbers of cases were detected in groups with male infertility, especially among patients diagnosed with testicular azoospermia.[16] This means that 46,XX males are very often phenotypically male. Their social life and daily life are normal as a man until they discover the problem of infertility. Although this genetically belongs to a complicated mechanism of female-to-male sex reversal in the pregonadal stage, a 46,XX male or 46,XX sex reversal was reasonably renamed a 46,XX testicular disorder[17] of sex development based on clinical manifestations.

Usually, we perform a PCR analysis to detect microdeletions of the Y chromosome, and a cytogenetic analysis of the chromosome karyotype for all patients with azoospermia or severe oligozoospermia of testicular origin. 46,XX male syndrome patients are detected as a group with the greatest severity of defects or the entire loss of the Y chromosome. Among the three groups with variable microdeletions of the Y chromosome (46,XX, AZFb + AZFc deletion, and only the AZFc deletion), a higher prevalence of an abnormal chromosome karyotype was found in patients with a wider microdeletion of the Y chromosome

Table 2 BH, hormonal comparison among the groups of 46,XX males, microdeletion of Y chromosome with AZFb and/or AZFc,47,XXY, IHH and healthy individuals.

	(1) 46,XX male ($n = 3$)	(2) Normal male	(3) Normal female	(4) AZFb and/or AZFc ($n = 30$)	(5) 47,XXY ($n = 31$)	(6) IHH ($n = 8$)
BH (cm)	160.3 ± 4.5	171.8[a]	158.9	172.6 ± 6[a]	175.9 ± 6.1[a,b]	170.3 ± 10.1
FSH (mIU/mL)	54.8 ± 8.9	1.7−11	3.9−10	14.6 ± 7.6[c]	39.5 ± 10.3[d]	1.7 ± 1.3[c]
Testosterone (ng/mL)	1.88 ± 0.39[e]	2.5−8.5	0.06−0.86	3.79 ± 1.73[e]	1.68 ± 1.53[e]	2.8 ± 4.2

The level of significance of the *t*-test was set at $p \leq 0.05$.
[a] BH: (1) versus (2), (4), (5), $p \leq 0.05$.
[b] BH: (2) versus (5), $p \leq 0.05$.
[c] FSH: (1) versus (4), (6), $p \leq 0.05$.
[d] FSH: (4) versus (5), $p \leq 0.05$.
[e] Testosterone: (2) versus (1), (4), (5), $p \leq 0.05$.

Table 3 *SRY* and other related component determination, localization, and clinical relevance of three patients with the 46,XX male syndrome.

PID	Age	*SRY*	Localization	Testis and external genital organs	Virilization	Sperm sample	FSH (mIU/mL)	LH (mIU/mL)	Testosterone (ng/mL)	Prolactin (ng/mL)
M1	33	+	X chromosome	Left :1.0 cm Right: <0.5 cm	+	Azoospermia	46.58	17.63	2.03	27.05
M2	34	+	Not investigated	Left: cryptochirm Right :0.8 cm (migrating)	+	Azoospermia	54.33	19.57	2.17	8.15
M3	52	−	−	Left: <0.5 cm, glandular hypospadia Right : <0.5cm	+	Azoospermia	64.31	20.16	1.44	16.08

screened by a PCR molecular diagnosis (Table 1). In the cytogenic analysis, 12 patients had structural anomalies of the Y chromosome. Patients with the Y chromosome structural anomaly in combination with variable Y chromosome microdeletions had a statistically significant ($p < 0.05$) higher prevalence of azoospermia (10/12, 83%) compared to those with no structural anomalies (20/32, 62.5%). However, patients with loss of the entire Y chromosome (46,XX male syndrome) had completely different clinical features compared to patients with structural anomalies that occurred in only certain fragments of the Y chromosome. This indicates that through the pathogenesis of sex reversal, phenotypic changes of 46,XX male syndrome go beyond loss of function of the Y chromosome.

All three patients with 46,XX male syndrome had total azoospermia with atrophic testes and highly elevated FSH and lower testosterone levels. In groups with severe microdeletions in the variable AZFb or AZFb + AZFc areas of the Y chromosome, only 30.7% (4/13) had atrophic testes,

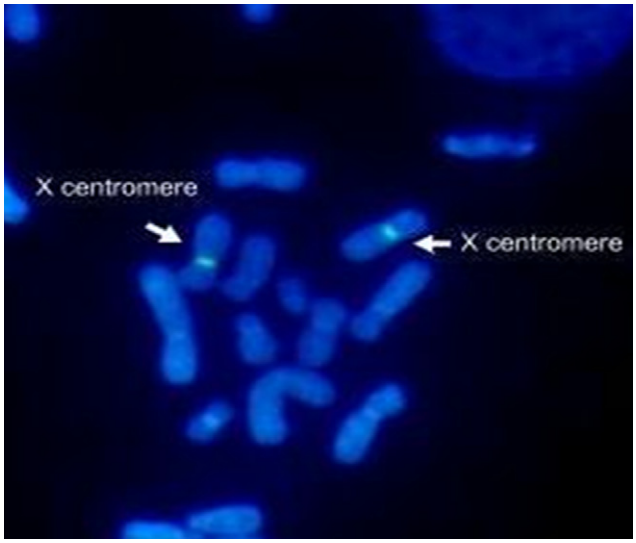

Figure 1 Fluorescent *in situ* hybridization (FISH) with the LSI *SRY* (orange)/CEP X (green) probes. No orange signal corresponding to the *SRY* gene was detected at all chromosomes but two green signals corresponding to the centromeric region of both chromosome X.

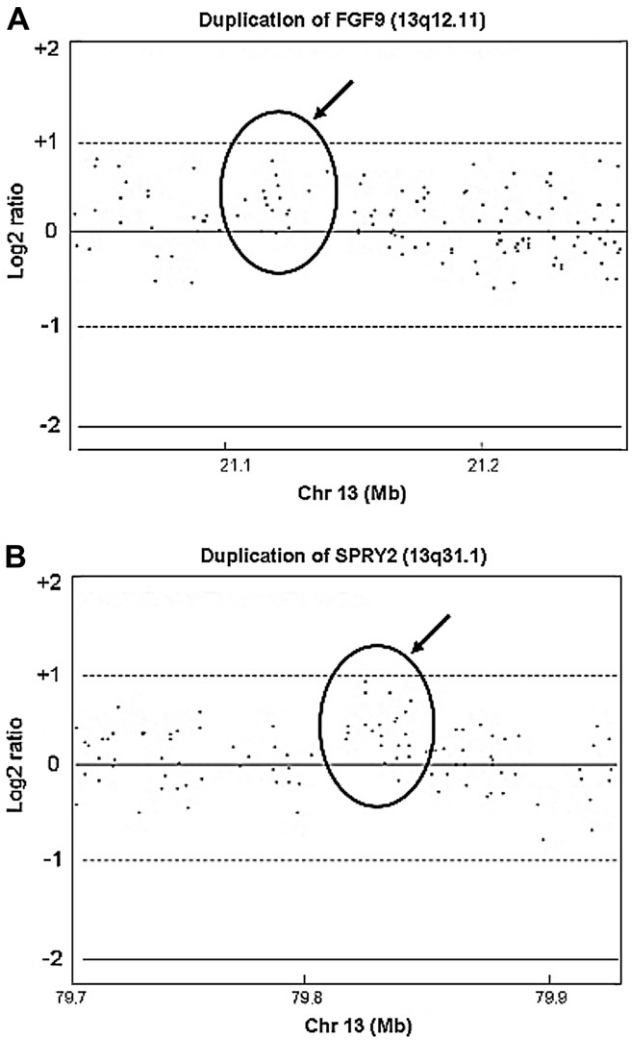

Figure 2 Array-based comparative genomic hybridization (array-CGH) profiles are shown for an *SRY*-negative 46,XX male patient. The Y-axis marks the intensity ratios plotted on a $\log_2$ scale. The X-axis shows the chromosome coordinate in megabases (Mb). (A) *FGF9* gene. The duplicated 13q12.11 region (21.143874—21.174184 Mb) is marked with a circle. (B) *SPRY2* gene. The duplicated 13q31.1 region (79.807500—79.813700 Mb) is marked with a circle.

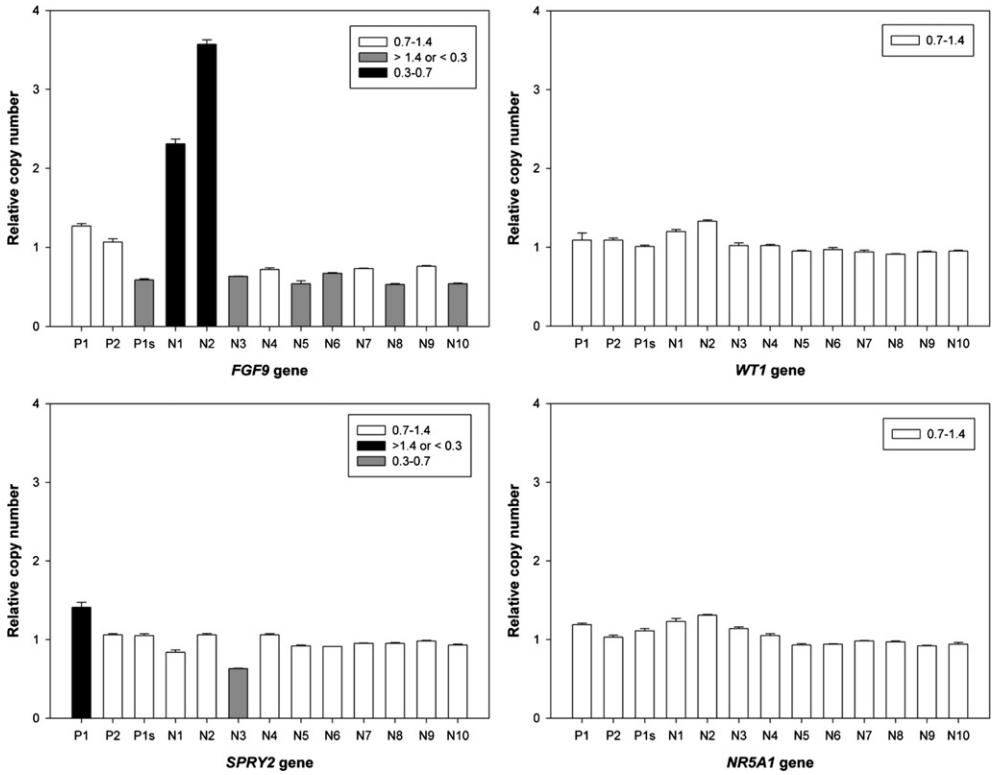

Figure 3 The real-time quantitative PCR (qPCR) results for four sex-determining related genes (*FGF9*, *SPRY2*, *WT1*, *NR5A1*). P1, 46,XX,SRY(−); P2, 46,XX,SRY(+); P1s, the sister of the P1. The fold change in gene copy number for the target gene relative to the endogenous reference gene (*ATP2B4*) was compared for the patients (P1—P2) and the control DNA (P1s, N1—N10) as described in the Materials and methods section. The normalized fold changes were interpreted as follows: no change (0.7—1.4, white bar), homozygous loss (<0.3, black bar), over-representation (>1.4, black bar), and ambiguous (0.3—0.7, gray bar).

and 84.6% (11/13) were azoospermic. Generally, they had normal testosterone levels, and FSH levels were dependent on the width of the microdeletion; however, on average, they were normal or borderline and significantly lower than those of patients with 46,XX male syndrome. This clearly shows that deletion of the AZF area of the Y chromosome in 46,XX male syndrome is the cause of the defects in spermatogenesis. The X chromosome inactivation mechanism can disrupt normal *SRY* expression.[18] The *SRY* gene might not be sufficient to induce adequate function of Sertoli's cells in XX (*SRY*+) mice,[19] and a complete Sertoli's cell-only syndrome and hyperplasia of Leydig cells were found in other studies of 46,XX males with testicular biopsies.[20] In our case of the 46,XX male who was *SRY*(−), we found that the genetic copy number of *FGF9* was duplicated, compared to the normal female control. However, it was significantly lower than that of the normal male control. The function of the *FGF9* gene was recently confirmed to be correlated with postnatal testicular development. Complete testicular atrophy of 46,XX males should theoretically occur through some pathogenesis of the sex-reversal mechanism.

Compared to the age-matched group of healthy males in the normal population, our three patients with 46,XX male syndrome had a higher FSH level, lower body height, lower testosterone level, and ambiguous external genitalia. The body height of 46,XX males was also significantly lower than that of the other male groups with chromosomal anomalies. Patients with 47,XXY are much taller than normal males and 46,XX males can be supported by the existence of a Y-linked growth gene.[21,22] 46,XX males were even shorter than patients with IHH, and their body height was close to that of normal females, which might indicate that hypogonadism contributes less to growth. The Y-linked growth gene cannot be switched to 46,XX males in the early sex-reversal process, which might cause them to exhibit a female stature.

Previous studies showed that patients with classical 46,XX male syndrome have normal-for-age testosterone levels during puberty but frequently exhibit hypergonadotropic hypogonadism in adulthood.[23] The average serum testosterone level of our three 46,XX male patients (1.88 ± 0.39 ng/mL) was lower than normal values (2.5—8.5 ng/mL), but not significantly lower than that of patients with Klinefelter's syndrome or patients with hypogonadotropic hypogonadism. All three patients acted as healthy adult males in society with no symptoms of male hypogonadism. One of them had bilateral mal-descended testicles, and one had hypospadia, but none had gynecomastia. This means that the lower levels of testosterone that affected our three patients with 46,XX male syndrome was limited, variable, and independent of their serum testosterone levels (Table 3).[24] The clinical and endocrinological profiles of our three cases with 46,XX males may also be evidence to support the genetic function of sex reversal in the embryological stage.

One of our three patients with 46,XX male syndrome was *SRY*-negative. Even though the hormone profile of this *SRY*-negative 46,XX male was more prominent as a picture of hypertrophic hypogonadism than that of the other two *SRY*-

positive 46,XX males (Table 3), we could find no significant difference in clinical manifestations as described in a previous report.[25] Furthermore, our epigenetic study showed a gain in *FGF9* copy numbers in the *SRY*-negative 46,XX male. No such genomic gain was observed in the case of the *SRY*-positive 46,XX male.[26,27] Furthermore, the copy number of the *SPRY2* gene in our *SRY*-negative 46,XX male patient was higher than that of the *SRY*-positive 46,XX male, as well as higher than those of the tested normal males and females. In the literature, the *SPRY2* gene is involved in male sex organogenesis by controlling *FGF9* gene-induced mesonephric cell migration to the developing testis.[28] The higher amount of *SPRY2* in this *SRY*-negative 46,XX male may have some meaning combined with the higher amount of *FGF9*. The higher amount of *FGF9* interferes with the expression of *WNT4* in the embryo, thereby retarding ovarian development of 46,XX males, even in the absence of the *SRY* gene.[28,29] Additional screening of 46,XX males is needed to elucidate the role of *FGF9* and other genetic mechanisms of sex reversal.

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