BIOLOGIJA. 2007. Vol. 53. No. 3. P. 16–24

Sex reversal mutation analysis in a family with pure gonadal dysgenesis of the XY female type (Swyer syndrome)

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Gonadal dysgenesis with XY male-to-female sex reversal has been attributed to mutations and gene dosage differences in at least seven genes. We present a family of three sisters with a pure gonadal dysgenesis (Swyer syndrome) with an 46, XY karyotype. The sisters have a common X-chromosomal haplotype in Xp21.3-p11.3, the region of the X-chromosomal Swyer syndrome which includes *NR0B1.* We excluded mutations in *SRY* and flanking sequences, in *NR0B1*and its promoter, in *DMRT1*, *WT1, SOX9, and NR5A1*. Also the X-chromosomal ATRX region and the *SOX3* gene were excluded by segregation analysis with polymorphic markers. We excluded a duplication of *NR0B1* and a deletion of *DMRT1* by gene copy dosage measurement. Since the pedigree suggests an X-chromosomal mode of inheritance, we suggest that there is either another yet unknown gene in the common interval on Xp which may cause Swyer syndrome or, more likely, that a gene in this interval is aberrantly regulated during gonad differentiation. Our data do not formally exclude another autosomal gene.

Key words: sex determination, Swyer syndrome, gonadal dysgenesis, sex reversal

INTRODUCTION

Male-to-female sex reversal giving rise to XY females with ambiguous genitalia, hypoplastic ovaries, or complete gonadal dysgenesis is the result of failure of testis determination or differentiation due to mutations or dosage alterations of genes from the testis determination pathway. The term "46,XY gonadal dysgenesis" describes various conditions of aberrant gonadal differentiation. Affected subjects with normal Müllerian structures and female genitalia and pure gonadal dysgenesis are subsumed under Swyer syndrome (OMIM: 306100 [1]). Patients with pure XY gonadal dysgenesis lack testicular development, are of normal stature in contrast to Turner syndrome (45,X) patients, and they do not show any additional symptoms. Usually, they are not diagnosed before puberty. In puberty, girls with pure gonadal dysgenesis neither develop secondary sexual characteristics nor do they menstruate. However, if seminiferous tubules or their remnants can be detected by histological examination of the gonad, a regression from an already differentiated gonad has to be considered (embryonic testicular regression sequence [2]). Swyer syndrome is a syndrome with genetic heterogeneity, which is caused only in about 20% of the cases by mutations of *SRY* (Sex-determining region on the Y chromosome), the initiator of male sex determination. It is the key determinant for testis formation from the undifferentiated bipotent gonad [3, 4]. *SRY* point mutations or deletions may lead to 46,XY pure gonadal dysgenesis [5–7]. Usually, mutations arise *de novo*. However, familial cases of Swyer syndrome with *SRY* mutations have also been reported, where the father showed somatic and germ-cell mosaicism for

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the *SRY* alleles [8–10] or at least gonadal mosaic had been suggested [11]. A sequence variant in the 5' non-HMG part of the coding sequence in a sex-reversed individual was also found in somatic cells and sperm of the normal father and a healthy brother [12], indicating incomplete penetrance.

For male-to-female sex reversal with gonadal dysgenesis a few candidate genes have been reported: *SRY, WT1*, *NR0B1*, *SOX9*, *NR5A1*, *ATRX*, *DMRT1, DHH*, *LHX9, EMX2, WNT-4*, *FGF9*, *GATA4* and *FOG2* [13–35]. Their temporal and spatial expression pattern and their mutual interaction have led to an arrangement of some of them in a hypothetical pathway of sex determination and differentiation [36, 37]. The interaction of these genes and their contribution to sex determination is poorly understood [38] and is studied intensively on the molecular level. All these candidate genes and their role in sex determination have been delineated from cases of XY sex reversal by reverse genetics and from animal models. Mutations in some of the genes of the mammalian sex determination pathway may lead to XY sex reversal even in the presence of an intact *SRY* gene. A duplication of a defined 160 kbp region (DSS region = dosage-sensitive sex reversal) on the short arm of the human X chromosome causes gonadal dysgenesis with XY sex reversal [39]. Within this region, the gene *NR0B1* (also known as *DAX1*) has been mapped. Hitherto, no mutations in *NR0B1* were reported that resulted in sex reversal.

The Wilms tumor suppressor gene *WT1* plays a key role in genitourinary development and subsequent normal function. Wilms tumor is caused by mutations or heterozygous deletions of *WT1*. Isolated gonadal dysgenesis without kidney cancer due to mutations in *WT1* has not been described yet [40].

DMRT1, a gene which is expressed exclusively in testis [24], has been reported to be another candidate gene for XY sex-reversal. Deletions in the subtelomeric region of chromosome 9p were detected in patients with gonadal dysgenesis and an intact *SRY* gene [41]. The critical interval for the gene that causes gonadal dysgenesis has been mapped very close to marker D9S1779 on chromosome 9p24.3 [27].

Mutations in *SOX9* or chromosomal rearrangements in the neighboring chromosomal region on the long arm of chromosome 17 cause campomelic dysplasia (CD) and may result in XY sex-reversal with dysgenetic gonads [18, 19]. Only about 75% of karyotypically male CD patients also have sex-reversal. CD and sex-reversal result from haploinsufficiency of *SOX9*.

Three out of these genes, namely *SRY*, *DMRT1*, and *NR0B1* have been analysed in a family with 46,XY gonadal dysgenesis. No mutation was found, suggesting another gene locus that might contribute to Swyer syndrome when mutated [42]. An analysis of families with idiopathic Swyer syndrome revealed another candidate gene region in Xp11.21-Xp11.23 [43].

We describe three sisters with an XY karyotype and pure gonadal dysgenesis. We examined *SRY*, not only in the highly conserved HMG box domain, but also its promoter and downstream sequences. Segregation analyses of markers in distal 9p, proximal 11p, and of the pericentric region of the X chromosome pointed to common parental haplotypes in the affected girls. Common haplotypes prompted a sequence analysis of *DMRT1, WT1,* and *NR0B1* on genomic DNA of the sibs. Candidate genes *SOX9* and *NR5A1* were sequenced directly without preceding segregation analysis.

MATERIALS AND METHODS

Subjects

Informed consent was obtained from all patients in this study. The family came to our attention due to primary amenorrhea of the eldest daughter of a non-consanguineous couple. This woman, II.1, was clinically examined at the age of 18. With a body height of 165 cm (61st percentile) and a weight of 53 kg (35th percentile), she was within normal range. She had scanty axial and pubic hair. Mammary glands were underdeveloped with sparsely pigmented nipples. By means of ultrasonography a hypoplastic uterus of 2.5 × 1.5 × 3 cm was found. The right gonad measured 3.5 × 2.0 × 1.5 cm, whereas the smaller left gonad measured only 2.0 × 1.5 × 1.0 cm. Tubae were long with the infantile stage of development. Hormone treatment was supplied to promote menstruation and improve the secondary sexual characteristics.

Her younger sister, II.2, was examined when she was 15. At this age her height was 160 cm (39th percentile) and her body weight was 52.8 kg (53rd percentile). Endocrinological investigations showed only elevated levels for FSH (118.26 mU/ml vs. normal: 3–22 mU/ml) and prolactin (709.16 µU/ml vs. 20–500 µU/ml), whereas the other values were in a normal range: LH (41.65 vs. normal: 0.7–78.9 mU/ml), testosterone (3.51 nmol/l vs. normal <3.5 nmol/l in females), and estradiol (83.96 pmol/l vs. normal 10–180 pmol/l). Clinical examination showed a low anterior and posterior hairline. Secondary pubic hair was of male characteristic along the abdominal midline. Breast development had started at the age of 13–13.5 years. She had widely spaced small nipples. Labia minora were small and only in the frontal part normally developed. Ultrasound examination revealed a retroverted uterus infantilis (size of corpus: 2 × 1.2 × 2 cm; size of cervix: 1.7 × 0.9 cm) and an underdeveloped mucosa. Gonadectomy was carried out for both elder sisters by laparotomy. Gonads were compact and white-colored. The size of the left gonad was 3 × 2 × 1 cm, the right gonad measured 4 × 2 × 1.5 cm. On re-examination at 18 years, her height was 163 cm and her weight was 64 kg. The retroverted uterus measured 7.5 × 3.2 × 4.8 cm. She received hormone therapy and has normal breast development and normal menstruation cycles from that time on.

The youngest sister was clinically examined at the age of 7 years. Her height was 120 cm (37th percentile) and her weight was 30 kg (93rd percentile). Her physical and female sexual development was typical of her age.

Histological examination of gonads of subjects II.1 and II.2 showed only ovarian tissues in each gonad. We found hypoplastic ovarian stroma with degenerated primary and secondary follicles. The poorly differentiated gonads of the patients showed no testicular structures like seminiferous tubules which might have been a hint to an embryonic testicular regression syndrome rather than to pure gonadal dysgenesis. Testicular feminization, another XY male-to-female sex reversal syndrome, was excluded in the family because the sisters had testosterone levels of 3.5 nmol/l, whereas girls with androgen insensitivity syndrome usually have elevated levels like males in the range of 9.4–27 nmol/l. Moreover, our patients had no inguinal testes and no testicular structures in their gonads.

METHODS

Analysis of *SRY*

Genomic DNA isolated from peripheral blood lymphocytes of the 46,XY girls (II.2 and II.3) and their parents was prepared according to standard protocols [44]. DNA from fixed, HE-stained gonadal sections (subject II.1) was extracted with the QIAamp Tissue kit (Qiagen, Hilden, Germany).

The presence of the coding region of *SRY* was proven after PCR amplification with primers *SRY*A-f and *SRY*A-r (all primer sequences and amplification conditions are available on request) flanking the coding region. In order to find the mutations that might be responsible for XY male-to-female sex reversal, PCR-amplified products were sequenced with the nested primers XES10 and XES11 [45] from both ends. The primers' 5' ends were Cy5-labeled and the sequencing reaction was performed with a Thermo Sequenase fluorescent labeled primer Cycle Sequencing kit with 7-deaza-Dgtp (GE Healthcare, Freiburg, Germany). The sequence was analysed on an ALFexpress fluorescence sequencer. Sequence data were compared to a published reference sequence (GenBank accession number: gi:292513) by means of a standard BLAST [46] alignment. Promoter and 5' UTR of *SRY* were amplified by PCR, cloned and sequenced. In order to exclude a terminal deletion of telomeric Yp sequences that might be responsible for male-to-female sex reversal [47], a 0.4 kb fragment distal to *SRY* was PCR-amplified with primers SRYF and SRYR.

Sequence analyses on further genes (*SOX9*, *NR5A1*, *DMRT1*, *NR0B1*, *WT1*) were performed on PCR-amplified DNA of exonic parts of the sequence, including about 50 bp on each side of intronic sequence in order to discover splice site mutations. Sequences were analysed either on an ALFexpress or an Applied Biosystems 3100 capillary sequencer.

Segregation analyses for *DMRT1*, *WT1*, and X-chromosomal genes (*NR0B1*, *ATRX*, *SOX3*)

For segregation analyses, primer sequences were taken from the GenomeDataBase (GDB; http://www.gdb.org). The marker sequences were amplified via PCR according to standard PCR protocols (95 °C / 30 s; 52–55 °C / 30 s; 72 °C / 30 s; 30–35 cycles; 1.5–2.5 mM $MgCl_2$). PCR products were separated on denaturing 6% acrylamide gels, alkali-blotted onto a nylon membrane and hybridized with a γ-^{32}P-ATP terminally labeled $(CA)_{11}$ oligonucleotide probe. Allele sizes of markers from the pedigree were entered in Cyrillic Pedigree Analysis software (Cyrillic Software, Reading, UK).

Since deletions in distal 9p (*DMRT1*) may result in XY sex reversal, we used markers from the 9p24–p22 interval to look for hemizygosity in this chromosomal region. Markers used were from tel→cen: D9S1779, D9S129, D9S288, D9S1813, D9S132, D9S286, and D9S157. The common paternal haplotype in this region prompted a sequence analysis of the *DMRT1* gene. All five exons of *DMRT1* were amplified by PCR [25] and sequenced on the genomic level.

For the segregation analysis of markers from the *WT1* region, we used the microsatellite markers D11S4154, PAX6, D11S935, and D11S4203. The markers span the region 46.62 Mbp to 48.91 Mbp (from the telomere) on chromosome 11. *WT1* exons 1–10 together with flanking sequences were PCR-amplified and sequenced.

Inasmuch as the pedigree is compatible with an X-chromosomal mode of inheritance and because X-chromosomal inheritance had been suggested for Swyer syndrome, we performed a segregation analysis with 27 polymorphic microsatellite markers (Fig. 1) of the X chromosome. The markers cover the region between Xp22.3 and Xq28 in intervals of 200 Kb to 20.8 Mb. Large intervals were chosen for a coarse coverage of the entire X chromosome, and small intervals were chosen for the fine localization of crossovers. The common X-chromosomal interval that was inherited from the mother to all daughters may include a candidate gene from this region. The most prominent candidate in our view was the *NR0B1* gene. Both exons of *NR0B1* together with flanking sequences were PCR-amplified and sequenced on the genomic level.

Quantitative Southern blot analyses for detection of dosage differences of *NR0B1* and of *DMRT1*

Southern blots have been prepared applying 10 µg genomic DNA of subject II.2, her mother, and six control persons with karyotypes 46, XX or 46,XY to the gel. DNA was cut with restriction enzyme *Eco*RI or *Hin*dIII. First, blot filters were hybridized with a 389 bp *NR0B1* probe that was created by PCR amplification with primers 1Aa [48], and then filters were re-hybridized with an autosomal probe pC63 (locus D17S21) of chromosome 17. This autosomal probe served as an internal standard to compensate for gel loading differences or different DNA amounts in the samples. Quantitation of signal levels was performed with a Molecular Dynamics Phosphorimager (GE Healthcare, Freiburg, Germany). The copy number of *NR0B1* was calculated and set in relation to the pC63 signals by means of the "Filter" software [49].

For the measurement of dosage levels in 9p we used a 280 bp PCR-generated probe of *DMRT1* for hybridization on *Hin*dIII-digested DNA of subjects II.3, II.2, mother I.2, and four controls. The probe was amplified from genomic DNA with primers DMT1MH1-f and DMT1MH1-r.

RESULTS AND DISCUSSION

By definition, Swyer syndrome is an XY pure gonadal dysgenesis associated with mutations in *SRY* or linked to the chromosome region Xp22.11–p21.2. For an X-chromosomal localization, no candidate gene has been published yet, although at least three genes (DSS-region, *ATRX*, *SOX3*) have been proposed to be involved in sex reversal [17, 22, 48, 50]. Moreover, mutations in several autosomal genes may also lead to XY sex reversal. The goal of our study was to identify a candidate gene or a candidate region for XY male-to-female sex reversal in a family of non-consanguineous parents with three Swyer-syndrome daughters. Genes from the pathway of mammalian sex determination were studied whether their alleles cosegregate with the phenotype and were sequenced in order to find mutations. Thus, we sequenced *SRY* (with promoter), *NR0B1* (with promoter), *DMRT1*, *WT1*, *SOX9*, and *NR5A1,* and we measured *NR0B1* and *DMRT1* gene dosages, and finally we analysed *ATRX* and *SOX3* by segregation analysis in order to find whether mutant alleles might have caused XY sex reversal.

The reported increased FSH values are a symptom of dysgenetic ovaries. Estrogen levels in our subjects were within a normal range, indicating estrogen production from follicles of a more or less intact ovary. Therefore, some basic steps of ovary development had been passed successfully during embryogenesis. It has been shown recently that gene targeting of *FOG2* or *GATA4* results in sex-reversed XY gonads with a different morphology than control XX gonads [30]. Thus, it seems not adequate to suggest an involvement of these genes upstream of *SRY* in the XY sex reversal in the three sisters.

In males, *SRY* triggers the differentiation of the bipotent gonad into a testis and initiates the differentiation of Sertoli cells from the supporting cells precursors. This step failed in the sisters studied, since we could not detect any testicular structures. Mutations in downstream genes that are usually activated by *SRY* (either directly or mediated by other downstream gene products) may abolish gonad differentiation even in the presence of an intact *SRY*. Upstream and downstream of SRY, there are several genes which may interfere with gonadal differentiation. Activation of SRY transcription and changes in its transcript levels during early gonadogenesis are maintained by upstream genes like *NR5A1* (SF1), *WT1*, *GATA4* and *FOG2*. Mutations in these genes may reduce the amount of *SRY* transcripts and thus also lead to sex reversal, despite an intact *SRY*.

Most cases of pure gonadal dysgenesis with XY sex reversal (Swyer syndrome) are caused by *de novo* mutations in *SRY*. In our study, the *SRY* ORF and flanking sequences were PCR-amplified and sequenced. In DNA of individuals II.2 and II.3, a *SRY* PCR product was obtained, whereas DNA of II.1 could not be amplified due to the poor quality of DNA isolated from histological samples. We found no *SRY* mutations in the ORF of subjects II.2 and II.3 (reference sequence gi:292513). Pure or partial gonadal dysgenesis was been attributed to deletions immediately 5' and 3' to *SRY* [47, 51]. Consequently, we sequenced another 1.1 kb (g.-1100_g.+1) of 5' sequence comprising the 5' UTR (g.-148_g.-1) and the proximal parts of the promoter including the functional promoter of SRY [52, 53]. Again, we did not find any mutation (reference gi:10801470). Loss of sequences 3' of *SRY* which would point to a Ypter deletion that might be associated with sex reversal [47] was excluded by PCR of a 400 bp DNA fragment 1151 bp downstream of the *SRY* Stop codon. It was present in the patients' DNA, in DNA of a male control, but not in a female control excluding a 3' deletion.

Only about 20% of cases with gonadal dysgenesis are due to *SRY* mutations which may explain the failure to find a mutation in the DNA of the three XY sisters in this report. One study on 49 females with XY sex-reversal [54] had shown mutations about 2 kbp 5' of the translation start. One of these mutations turned out to be a polymorphism and another mutation was present not only in the proband's DNA but also in her father's *SRY*. Although a mutation had been found in the father's *SRY*, he nevertheless was fertile and had fathered a daughter (XY). These weak genotype-phenotype relations prompted us to refrain from a mutation search far outside of the coding region, although it might be indicative of an incomplete penetrance of *SRY* mutations. Reports on familial cases of sex-reversed persons with gonadal dysgenesis are rather rare [9, 45, 55, 56]. Within the group of familial cases there is a 3-generation family with two males (one of them fertile) and 3 XY females [56]. All of them with an XY karyotype have an identical *SRY* mutation g.177G > C which leads to a conserved amino acid exchange p.V60L. Therefore, this identical mutation with a different phenotype might be indicative of incomplete penetrance. This uncertainty and the low percentage of sex-reversed persons with *SRY* mutations call for the study of other loci where mutations may cause gonadal dysgenesis with XY sex reversal.

DMRT1 and deletion mapping in the distal part of chromosome 9p

Deletions of the distal part of chromosome 9p have been reported as another cause for XY sex reversal. *DMRT1* maps to 9p24.3 and is transcribed exclusively in the testes. Its expression in the testes is regulated by GATA4 [57]. In mice and chicken it is expressed in the genital ridge [58, 59]. DM-domain genes are supposed to play a conserved role in male-specific development – not only in mammals, but also in other vertebrates and in the invertebrates *Drosophila melanogaster* (*dsx*) and *Caenorhabditis elegans* (*mab-3*). A comprehensive overview of *DMRT1* genetics has been recently given by Ferguson-Smith [60]. Gene targeting of the murine *Dmrt1* caused severe defects in the adult testis [61]. In contrast to humans, where (heterozygous) deletions of 9pter lead to sex reversal (e. g., see [62]), both *Dmrt1* copies of the mouse gene have to be deleted to cause sex reversal, suggesting a recessive mode of inheritance. In our study, we determined the gene copy number by duplicate quantitative Southern Blots with a *DMRT1* probe. We found no changes of the gene copy number after phosphor-image analysis. For II.2, II.3, and their mother we measured a number of two gene copies (Table 1). This result is congruent with the value of 100% that was used arbitrarily for the control persons. This indicates that *DMRT1* gene copies exist in duplicate and no deletion had occurred.

In order to exclude deletions in the vicinity of the *DMRT1*, *DMRT2* and *DMRT3* loci, seven markers from 9p24–p22, where *DMRT1* maps very close to D9S1779 [26, 27], were

Table 1. ***DMRT1*** **gene copy numbers as determined by quantitative Southern Blot**

Subject	Gene dosage % from 2 measurements	Relative amount of *DMRT1* DNA	Deduced number of gene copies
♀ control	104.9/97.6	2.02	2
♀ control	93.1/83.4	1.77	2
♂ control	124.1/91	2.15	2
♂ control	96.9/112.3	2.09	2
II.3	83.3/92.7	1.76	2
II.2	90.4/105.9	1.96	2
I.2 (mother)	112.8/128.2	2.41	2

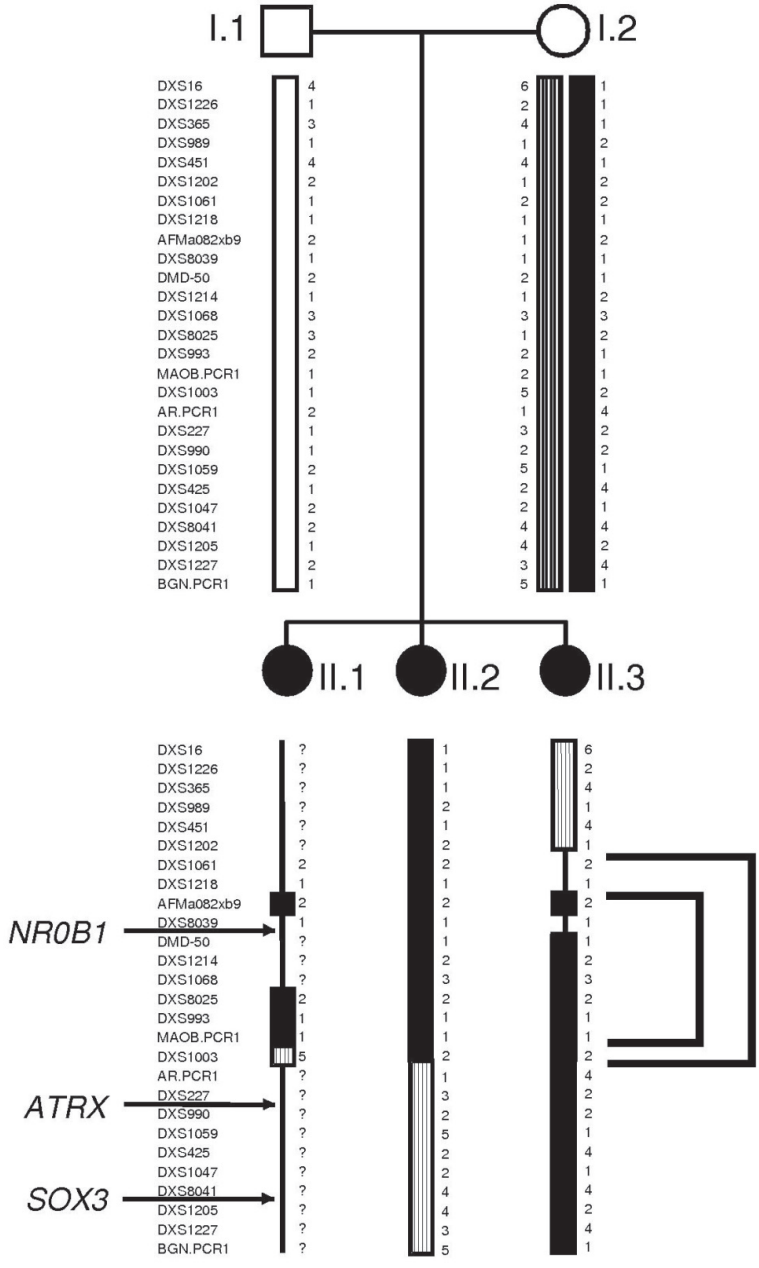

Fig. 1. Segregation analysis with 27 markers of the X chromosome
The 46,XY sex-reversed girls had inherited a common maternal haplotype from DXS1202 to DXS1003 (exclusive; outer bracket). The markers DXS1218, DXS8039, and DXS1061 (distal Xp) are not informative and, therefore, a recombination event may have occurred proximal to DXS1061, limiting the interval from AFMa082xb9 to MAOB.PCR1 (inclusive; inner bracket).
"?" in the marker analysis of histological sections of II.1 means "not determined". In the DNA of II.1 only the markers were that contributed to the definition of the interval boundaries analysed, because the DNA extracted from HE-stained sections was of poor quality and did not amplify for all markers.

proofed for heterozygosity. All but one microsatellite markers in the family were informative. We found heterozygosity for five markers (D9S1779, D9S129, D9S288, D9S1813, and D9S157) in all sisters (Fig. 2). The non-informative marker D9S132 was homozygous in II.1 and II.2, but heterozygous in II.3. Marker D9S286 was homozygous in II.3 and heterozygous in II.2. The results with all markers excluded hemizygosity, and thus excluded a deletion of a gene(s) in this region. It may be noteworthy that all girls had inherited the same paternal interval (haplotype), at least from the distal marker D9S1779 to D9S1813. This common haplotype prompted a sequence analysis of *DMRT1*. We sequenced all five exons including splice junctions of *DMRT1* on genomic DNA. We did not find any mutation in the transcribed sequence of the sisters. At the 3' end of intron 4 we found a G > C transversion (g.IVS4-4G > C) in the consensus splice acceptor sequence which is known from the SNP database (http://www.ncbi.nlm.nih.gov/SNP/) under accession number rs279895. With the exclusion of any mutation in the transcribed part of *DMRT1*, we exclude this gene as a candidate for familial XY sex reversal in the sisters.

With the exception of one nucleotide substitution in an XY female [25] in a recent study with 87 XY sex-reversed individuals, no other sequence variants have been identified in *DMRT1* cDNA. With regard to the ethnic background of this patient, a naturally occurring polymorphism in this population could not be ruled out. Thus, it is not clear whether mutations of *DMRT1* can cause sex reversal at all or whether the sex reversal is caused by haploinsufficiency or decreased levels of *DMRT1* expression.

WT1

With four markers D11S4154, PAX6, D11S935, and D11S4203 in a 2.29 Mbp interval we have established the haplotypes of the family members. All girls had inherited the same maternal but different paternal haplotypes (not shown), thus excluding pater-

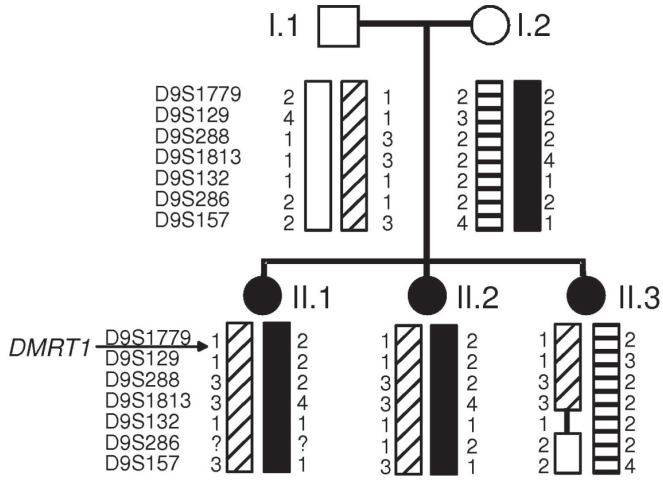

Fig. 2. Segregation analysis with markers of chromosome 9p
All girls had inherited the same paternal alleles from D9S1779 to D9S1813 or D9S132 (not informative). No deletion of *DMRT1* was found, which maps between D9S1779 and D9S129.
"?" indicates a PCR failure from DNA of a histological section.

nal *WT1* mutations that might have caused the male-to-female sex reversal. A maternally inherited mutation may not be expected to cause symptoms in the mother, unless the phenotype is influenced by variable expressivity or incomplete penetrance. Although isolated gonadal dysgenesis without kidney cancer has not been reported yet for *WT1* mutations, we decided to search for *WT1* mutations in the DNA of the sisters by sequencing, because the broad phenotypic spectrum of *WT1* mutations and functional aspects of the different WT1 isoforms asked for a *WT1* study in more detail. In order to find or exclude a maternally inherited *WT1* mutation, we have sequenced the 10 PCR-amplified exons with splice sites in one daughter and her mother. In the 5' UTR we detected a T/G heterozygosity c.-7T/G, which is known as a SNP rs2234582. In summary, we exclude *WT1* as a candidate for familial Swyer syndrome in this family because we did not find any mutation in the *WT1* transcript.

X chromosomal genes

Because the pedigree of our family suggests an X-chromosomal inheritance, we looked for cosegregation of X-chromosomal markers with the disorder. The alleles of 27 markers in an interval from Xp22.3 to Xq28 have been determined in the DNA of the sisters and their parents (Fig. 1). Individual II.2 has one crossover between markers DXS1003 and AR.PCR1. As far as markers could be analysed from the DNA of histological sections of sister II.1, a different recombination of the X chromosome was detected between markers MAOB.PCR1 and DXS1003. In individual II.3, a crossover must have occurred between markers DXS1202 and AFMa082xb9. It might be possible that the genuine crossovers were different, because the allelic phase of maternal X chromosomes is unknown. However, interpretation of results is independent of this consideration. Taking together the marker data from all sisters, a possible region for a candidate gene of XY sex reversal can be defined by a common haplotype ranging from DXS1202 to DXS1003 in proximal Xp (exclusive, spanning about 19.4 Mbp) or from AFMa082xb9 (Xp21.3) to MAOB.PCR1 (inclusive, approx. 13.65 Mbp; inner bracket in Fig. 1). This interval is flanking, but not overlapping, a recently described interval in Xp11.21–Xp11.23 for an idiopathic form of male-to-female sex reversal [43].

Mutations in *ATRX* (also known as *XH2* X-linked helicase 2; alternative symbol *XNP* = X-Linked Nuclear Protein; [63]), a DNA helicase which maps to the ATRX (X-chromosomal α-thalassemia and mental retardation; OMIM: 301040) interval, may lead to sex reversal with severe mental retardation. The segregation analysis excludes the ATRX region in Xq13.3 [22]. We also excluded *SOX3*, another putative sex-determining factor, albeit the involvement of *SOX3* in testis determination is discussed rather controversially [50, 64]. Supposing X-chromosomal inheritance for XY male-to-female sex reversal in this family, the common X-chromosomal haplotype of the sisters might point to a candidate gene for sex reversal in the 13.65 Mbp region that we found. The DSS region maps to this interval. Duplication of the DSS region in Xp21 leads to XY sex reversal in persons with an intact *SRY* gene. The DSS region includes the *NR0B1* gene. It was found that mice with transgenic overexpression of *Nr0b1* show delayed testis development, but a complete sex reversal occurs only in mouse strains with a "weak" *Sry* allele [65]. That is why only a precisely scheduled spatial and temporal expression of *SRY* and *NR0B1* leads to the induction of a male gonad from the undifferentiated gonad during early embryogenesis. Since this kind of sex reversal is dosage-dependent, we performed quantitative Southern blots in the Xp21 region with a cloned *NR0B1* probe. Blots of the patients were hybridized in order to determine the number of *NR0B1* gene copies. Table 2 summarizes the results of this analysis. The DNA of II.2 had only a single copy of the *NR0B1* gene, whereas her mother I.2 had two copies as expected from the XX karyotype. This result excludes a duplication of the DSS interval as a cause of Swyer syndrome. In order to rule out *NR0B1* mutations causing Swyer syndrome in our patients, we sequenced both exons and flanking sequences of *NR0B1* on PCR-amplified genomic DNA. We sequenced the 1168 bp translated sequence of exon 1 together with 200 bp of the promoter (g.-200_-1) and parts of intron 1 (g.IVS1+1_+150 and g.IVS1 −120_−1) and exon 2 (245 bp) together with 250 bp downstream of it. In exon 1 we found a sequence variant (g.10C > G) at position 1589 (gi:1163076) and another change (g.114T > C) at position 1693 of the reference sequence which is known as SNP rs6150. Both sequence variants were found in the *NR0B1* sequence of individual II.2, homozygous in the DNA of the mother, (hemizygous) in the DNA of the healthy father, and in a male control. Another published sequence (gi:5016089) shows the same sequence at position g.10 as ours and, hence, this nucleotide substitution might represent rather a common

Table 2. ***NR0B1*** **gene copy numbers as determined by quantitative Southern Blot**

Subject	Gene dosage % from 2 measurements	Relative amount of *NR0B1* DNA	Deduced number of gene copies
♀ control	95.3/82.7	1.78	2
♀ control	94.5/78.6	1.73	2
♂ control	58.1/46.0	1.04	1
♂ control	50.3/51.0	1.01	1
♂ control	48.8/48.2	0.97	1
♂ control	50.4/60.3	1.11	1
II.2	56.1/53.2	1.09	1
I.2 (mother)	112.6/101.8	2.14	2

polymorphism than a mutation. We have carefully checked for a sequence polymorphism by screening 100 X chromosomes of individuals with the same ethnic background. All these chromosomes have the same sequence as the three 46,XY sisters, and hence they may represent the wildtype sequence. The database entry with a different sequence might have originated from an individual of a different ethnic background. These findings also exclude the gain of function mutations in *NR0B1* that might lead to the XY sex reversal.

Sequence analysis of *SOX9*

SOX9, another member of the SOX genes group, is possibly a direct *SRY* target and a key module in sex differentiation. Haploinsufficiency of it leads to the autosomal dominant semilethal skeletal malformation syndrome – Campomelic dysplasia with or without male-to-female XY sex reversal [18, 19]. A duplication of the *SOX9* region leads to the opposite, i. e. 46,XX female-to-male sex reversal [66]. Since no case with pure XY gonadal dysgenesis and *SOX9* mutations has been reported, we have sequenced all three *SOX9* exons in the sex-reversed girls. We found no sequence variant in comparison with the reference gi:51511734.

Sequence analysis of *NR5A1* (SF1)

NR5A1 is a 7 exons gene on chromosome 9q33. SF1 regulates enzymes of adrenal corticosteroid synthesis. Its expression in gonadal tissue starts before the onset of *SRY* transcription and is confined mainly to the Sertoli cells, later SF1 localizes to the Leydig cells [67]. Its synergistic activity with NR0B1 and WT1 during male sexual development [68] prompted us to search for mutations in this gene, too. We found one SNP (rs915034) in the 3' UTR with the variant c.1468C > T (gi: 24432033), the T allele being very frequent (81.8%) in the European population. No further sequence variants have been found. This finding excludes *NR5A1* as a candidate for male-to-female sex reversal in the study family.

CONCLUSIONS

We found no Swyer-syndrome causing mutations or sequence variants in our patients, with the exception of some DNA polymorphisms in splice junctions, non-coding and coding sequences of some genes of sex determination. A detailed analysis of six genes excludes the coding sequence of candidate genes *SRY* (with promoter), *DMRT1*, *WT1*, *NR5A1*, *SOX9*, and *NR0B1* (with promoter) in the family. We excluded *ATRX* and *SOX3* by segregation analysis.

Only the approach to test cosegregation of the disorder with genetic markers near candidate genes may allow identifying the involvement of genes from the pathway of mammalian sex determination in sex reversal in our patients. An X-chromosomal mode of inheritance seems most likely for the girls in our study, although we cannot exclude formally any other autosomal gene. The known X-linked genes for sex-reversal were excluded either by segregation analysis or by sequence analysis of the coding region and the promoter. A similar study without marker analysis has been performed in a large pedigree with 46,XY gonadal dysgenesis, where autosomal dominant inheritance had been suggested [69]. By means of genome-wide linkage analysis, another group suggested a sex-determining locus on chromosome 5q11.2 [70]. Cosegregation of the X-chromosomal candidate gene region with XY sex reversal in our family does not necessarily implicate a mutation in a coding sequence. It may be a hint also to changes in the regulation of gene activity. However, even an intact gene on the DNA sequence analysis level may contribute to sex reversal by an attenuated or increased gene activity. Therefore, we have sequenced parts of the *NR0B1* promoter in our patients, but could not find any mutation. However, we do not know anything about transcript levels of *NR0B1* during the decisive phase of sex determination. A small imbalance in the concerted action of SF1, NR0B1 and N-CoR [71] may lead to altered MIS (Müllerian inhibiting substance; Anti-Müllerian hormone) levels. Regulation by varying CpG methylation of the *NR0B1* promoter during early development or upregulation by *WNT4* [72] may increase *NR0B1* expression and thus lead to sex reversal. However, these effects, which may be tissue-specific and specific for developmental stages, cannot be studied in the DNA of lymphocytes of adult individuals. The interaction of the genes *SRY*, *SOX9*, *DMRT1*, *NR0B1*, *WT1*, *SF1*, *GATA4* and *FOG2* is still poorly understood, and only animal models or *in vitro* studies may help to further elucidate the molecular interactions of the partners of sex determination.

Abbreviations

MIM – Mendelian Inheritance In Man; ORF – open reading frame; UTR – untranslated region; NCBI – National Center for Biotechnology Information; DSS – dosage-sensitive sex reversal.

Received 16 April 2007
Accepted 8 June 2007

References

1. McKusick VA Mendelian Inheritance in Man. Catalogs of Human Genes and Genetic Disorders. Baltimore: John Hopkins University Press, 1998; 12.
2. Marcantonio SM, Fechner PY, Migeon CJ et al. Am J Med Genet 1994; 49: 1–5.
3. Sinclair AH, Berta P, Palmer MS et al. Nature 1990; 346: 240–4.
4. Koopman P, Münsterberg A, Capel B et al. Nature 1990; 348: 450–2.
5. Zeng YT, Ren ZR, Zhang ML et al. J Med Genet 1993; 30(8): 655–7.
6. Battiloro E, Angeletti B, Tozzi MC et al. Hum Genet 1997; 100(5–6): 585–7.
7. Guidozzi F, Ball J, Spurdle A Obstet Gynecol 1994; 49(2): 138–46.
8. Bilbao JR, Loridan L, Castaño L Hum Genet 1996; 97: 537–39.
9. Schmitt-Ney M, Thiele H, Kaltwaβer P et al. Am J Hum Genet 1995; 56: 862–9.
10. Hines RS, Tho SP, Zhang YY et al. Fertil Steril 1997; 67(4): 675–9.
11. Affara NA, Chalmers IJ, Ferguson-Smith MA Hum Mol Genet 1993; 2: 785–9.
12. Domenice S, Yumie NM, Correia Billerbeck AE et al. Hum Genet 1998; 102(2): 213–5.
13. Berta P, Hawkins RJ, Sinclair AH et al. Nature 1990; 348: 448–50.
14. Jäger RJ, Anvret M, Hall K et al. Nature 1990; 348: 452–4.
15. Pelletier J, Bruening W, Kashtan CE et al. Cell 1991; 67: 437–47.
16. Pelletier J, Bruening W, Li FP et al. Nature 1991; 353(6343): 431–4.
17. Muscatelli F, Strom TM, Walker AP et al. Nature 1994; 372: 672–6.
18. Foster JW, Dominguez-Steglich MA, Guioli S et al. Nature 1994; 372: 525–30.
19. Wagner T, Wirth J, Meyer J et al. Cell 1994; 79: 1111–20.
20. Luo X, Ikeda Y, Parker KL Cell 1994; 77: 481–90.
21. Achermann JC, Ito M, Ito M et al. Nat Genet 1999; 22(2): 125–6.
22. Ion A, Telvi L, Chaussain JL et al. Am J Hum Genet 1996; 58(6): 1185–91.
23. Gibbons RJ, Picketts DJ, Villard L et al. Cell 1995; 80(6): 837–45.
24. Raymond CS, Shamu CE, Shen MM et al. Nature 1998; 391(6668): 691–5.
25. Raymond CS, Parker ED, Kettlewell JR et al. Hum Mol Genet 1999; 8(6): 989–96.
26. Flejter WL, Fergestad J, Gorski J et al. Am J Hum Genet 1998; 63(3): 794–802.
27. Calvari V, Bertini V, De Grandi A et al. Genomics 2000; 65(3): 203–12.
28. Veitia R, Nunes M, Brauner R et al. Genomics 1997; 41(2): 271–4.
29. Umehara F, Tate G, Itoh K et al. Am J Hum Genet 2000; 67(5): 1302–5.
30. Tevosian SG, Albrecht KH, Crispino JD et al. Development 2002; 129(19): 4627–34.
31. Ottolenghi C, Moreira-Filho C, Mendonca BB et al. J Clin Endocrinol Metab 2001; 86(6): 2465–9.
32. Ogata T, Muroya K, Sasagawa I et al. Kidney Int 2000; 58(6): 2281–90.
33. Birk OS, Casiano DE, Wassif CA et al. Nature 2000; 403(6772): 909–13.
34. Vainio S, Heikkila M, Kispert A et al. Nature 1999; 397(6718): 405–9.
35. Colvin JS, Green RP, Schmahl J et al. Cell 2001; 104(6): 875–89.
36. Parker KL, Schimmer BP, Schedl A Cell Mol Life Sci 1999; 55(6–7): 831–8.
37. Schafer AJ, Goodfellow PN BioEssays 1996; 18(12): 955–63.
38. Veitia RA, Salas-Cortes L, Ottolenghi C et al. Mol Cell Endocrinol 2001; 179(1–2): 3–16.
39. Bardoni B, Zanaria E, Guioli S et al. Nat Genet 1994; 7: 497–501.
40. Nordenskjöld A, Fricke G, Anvret M Hum Genet 1995; 96: 102–4.
41. Veitia RA, Nunes M, Quintana-Murci L et al. Am J Hum Genet 1998; 63(3): 901–5.
42. Kempe A, Engels H, Schubert R et al. Gynecol Endocrinol 2002; 16(2): 107–11.
43. Rajender S, Thangaraj K, Gupta NJ et al. J Clin Endocrinol Metab 2006; 91(10): 4028–36.
44. Sambrook J, Russell DW Molecular Cloning. A Laboratory Manual. 2001; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45. Hawkins JR, Taylor A, Goodfellow PN et al. Am J Hum Genet 1992; 51: 979–84.
46. Altschul SF, Gish W, Miller W et al. J Mol Biol 1990; 215: 403–10.
47. McElreavey K, Vilain E, Barbaux S et al. Proc Natl Acad Sci USA 1996; 93(16): 8590–4.
48. Zanaria E, Muscatelli F, Bardoni B et al. Nature 1994; 372: 635–41.
49. Geerkens C, Vetter U, Just W et al. Hum Genet 1995; 96(1): 44–52.
50. Bergstrom DE, Young M, Albrecht KH et al. Genesis 2000; 28(3–4): 111–24.
51. McElreavey K, Vilain E, Abbas N et al. Proc Natl Acad Sci USA 1992; 89: 11016–20.
52. Su H, Lau YFC Am J Hum Genet 1993; 52: 24–38.
53. Hossain A, Saunders GF J Biol Chem 2001; 276(20): 16817–23.
54. Kwok C, Tyler-Smith C, Mendonca BB et al. J Med Genet 1996; 33: 465–8.
55. Jaeger RJ, Harley VR, Pfeiffer RA et al. Hum Genet 1992; 90: 350–5.
56. Vilain E, McElreavey K, Jaubert F et al. Am J Hum Genet 1992; 50: 1008–11.
57. Lei N, Heckert LL Mol Cell Biol 2004; 24(1): 377–88.
58. Nanda I, Shan Z, Schartl M et al. Nat Genet 1999; 21(3): 258–9.

59. Raymond CS, Kettlewell JR, Hirsch B et al. Dev Biol 1999; 215(2): 208–20.
60. Ferguson-Smith M Sex Dev 2007; 1: 2–11.
61. Raymond CS, Murphy MW, O'Sullivan MG et al. Genes Dev 2000; 14(20): 2587–95.
62. Muroya K, Okuyama T, Goishi K et al. J Clin Endocrinol Metab 2000; 85(9): 3094–100.
63. Villard L, Lacombe D, Fontes M Eur J Hum Genet 1996; 4(6): 316–20.
64. Lim HN, Berkovitz GD, Hughes IA et al. Hum Genet 2000; 107(6): 650–2.
65. Swain A, Narvaez V, Burgoyne PS et al. Nature 1998; 391(6669): 761–7.
66. Huang B, Wang S, Ning Y et al. Am J Med Genet 1999; 87(4): 349–53.
67. Hanley NA, Ball SG, Clement-Jones M et al. Mech Dev 1999; 87(1–2): 175–80.
68. Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL et al. Cell 1998; 93(3): 445–54.
69. Le Caignec C, Baron S, McElreavey K et al. Am J Med Genet 2003; 116(1): 37–43.
70. Jawaheer D, Juo SH, Le Caignec C et al. Clin Genet 2003; 63(6): 530–5.
71. Crawford PA, Dorn C, Sadovsky Y et al. Mol Cell Biol 1998; 18(5): 2949–56.
72. Jordan BK, Mohammed M, Ching ST et al. Am J Hum Genet 2001; 68(5): 1102–9.

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LYTIES REVERSIJOS MUTACIJŲ ANALIZĖ ŠEIMOJE SU MOTERIŠKO TIPO GRYNĄJA GONADŲ DISGENEZE (SWYER SINDROMU)

Santrauka

Manoma, kad XY individuose moterišką fenotipą gali nulemti bent septynių genų mutacijos ir skirtingos genų dozės. Mes tyrėme šeimą, kurioje yra trys XY seserys su grynąja gonadų disgeneze (Swyer sindromu). Visos seserys turėjo bendrą X chromosomos haplotipą Xp21,3–p11,3 intervale, apimančiame ir *NR0B1*. Buvo ekskliuduotos mutacijos ir flanginės sekos *SRY, NR0B1* gene ir jo promotoriuje, taip pat *DMRT1, NT1, SOX9, NR5AA*. Su polimorfiniais žymenimis analizuodami šeiminę segregaciją ekskliudavome X chromosomos mutacijas *ATRX* rajone ir *SOX3* gene. Matuojant genų dozes, buvo ekskliuduota *NR0B1* duplikacija ir *DMRT1* delecija. Paveldėjimo pobūdis šeimoje atitinka sukibusį su X chromosoma tipą, tad galima manyti esant dar vieną (kol kas nežinomą) geną minėtame Xp intervale, sukeliantį Swyer sindromą. Labiau tikėtina, kad šiame intervale esantis genas neteisingai reguliuojamas, kai diferencijuojasi gonados. Gauti duomenys taip pat neatmeta formalios galimybės, kad egzistuoja dar kitas autosominis genas.