Original Article

46, XY gonadal dysgenesis: new SRY point mutation in two siblings with paternal germ line mosaicism


Familial recurrence risks are poorly understood in cases of de novo mutations. In the event of parental germ line mosaicism, recurrence risks can be higher than generally appreciated, with implications for genetic counseling and clinical practice. In the course of treating a female with pubertal delay and hypergonadotropic hypogonadism, we identified a new missense mutation in the SRY gene, leading to somatic feminization of this karyotypically normal XY individual. We tested a younger sister despite a normal onset of puberty, who also possessed an XY karyotype and the same SRY mutation. Imaging studies in the sister revealed an ovarian tumor, which was removed. DNA from the father's blood possessed the wild type SRY sequence, and paternity testing was consistent with the given family structure. A brother was 46, XY with a wild type SRY sequence strongly suggesting paternal Y-chromosome germ line mosaicism for the mutation. In disorders of sexual development (DSDs), early diagnosis is critical for optimal psychological development of the affected patients. In this case, preventive karyotypic screening allowed early diagnosis of a gonadal tumor in the sibling prior to the age of normal puberty. Our results suggest that cytological or molecular diagnosis should be applied for siblings of an affected DSD individual.

Conflict of interest

The authors have no conflict of interest of any nature.

46, XY complete gonadal dysgenesis (CGD) is characterized by a 46, XY karyotype, normal female external genitalia, completely undeveloped (‘streak’) gonads, no sperm production, and presence of normal Müllerian structures. Although many different genes are required for both male and female sex determination, the Y chromosome plays a primary role through the Y-linked gene SRY. This gene is critical for testis
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determination, and its absence results in the development of a female gonad in chromosomally male embryos (1). Human SRY is a 204 residue nuclear protein comprising three domains: a central HMG-box domain, which is highly conserved among mammalian species (approximately 80 residues in length), surrounded by N- and C-terminal domains (2). Mutations of the SRY gene [henceforth referred to as SRY (−)] are the cause of 46, XY CGD in approximately 10–15% of 46, XY disorders of sex development (DSD) (3). Mutations in the SRY gene include point, frameshift and deletion mutations, with the majority located within the HMG-box affecting DNA binding. The majority of the mutations described to date are singletons although 15 familial cases have been described which show vertical transmission and/or recurrence in siblings.

We report a novel point mutation within the SRY HMG-box domain shared by two sisters, which markedly lowers DNA binding activity. Only one sister was symptomatic at the time of molecular diagnosis; and we tested the second female sibling as a preventive measure despite signs of normal female puberty. Importantly, the younger sister with 46, XY CGD had developed a mixed ovarian tumor whereas her older sister showed no evidence of neoplasia at the time of gonadectomy. Another sister was 46, XX and phenotypically normal. Their father and a 46, XY phenotypically normal brother do not carry the mutation in leukocyte DNA, suggesting paternal germline mosaicism. We review the literature on familial SRY mutations and the implications of germline mosaicism for recurrence rate calculations and genetic counseling.

Materials and methods

Subjects

Patient 1

The proband, a 16-year-old healthy adolescent, was referred because of pubertal delay with hypergonadotrophic hypogonadism (FSH 74 IU/l, n = 12–30, ovulatory phase; LH 17 IU/l, n = 40–200, ovulatory phase). The patient had begun estrogen replacement therapy 12 months before her first visit to our clinic. Family history revealed a 14-year-old prepubertal sister and a twin brother and sister aged 11 years, all in good health (Fig. 1a). Her unrelated, Caucasian parents had no endocrine disorder or history of delayed puberty but her mother had received clomiphene citrate before the twin pregnancy. On physical examination, the patient’s weight was 75 kg (90th–97th), her height was 170 cm (90th). She had adipomastia, but Tanner stage 2 breasts and pubic hair. Her external genitalia were that of a normal female. Imaging revealed a bone age delay of 3 years and a bone mineral density revealed osteopenia (Z score = −2.4). Anti-ovarian antibodies were negative. The karyotype was 46, XY and a FISH analysis confirmed the homogenous presence of the SRY locus in all cells examined. The patient underwent laparoscopic removal of streak gonads; pelvic exploration confirmed the presence of Müllerian structures. Following completion of pubertal induction, the patient was cycled with an oral contraceptive and Vitamin D and calcium was introduced.

Patient 2

This 14-year-old sister of the proband was seen for screening, following the diagnosis of her sister. On physical examination, her weight was 70 kg (90th–97th), her height was 170 cm (90th). She had adipomastia, but Tanner stage 2 breasts and pubic hair. Her external genitalia were that of a normal female. She was also found to have hypergonadotropic hypogonadism (FSH: 66 IU/l, n = 12–30; LH: 13 IU/l, n = 40–200; Estradiol: <7; 3 pmol/l despite breast budding, and a normal female level of testosterone (0.64 nmol/l) and DHEAS (3.35 μmol/l) as well as normal thyroid function test (TSH: 2.10 mU/l). The karyotype was also 46, XY without mosaicism. Imaging studies revealed the presence of a right adnexal mass of 46 × 42 × 29 mm, and tumor markers were positive (βHCG: 14 IU/l, n = <5; afp: 1.4 μg/l, n < 10). Pathological examination of the surgically removed...
Mutation in SRY in two feminized siblings

Fig. 2. Investigation of patient II-2: (a) Hypointense mass of 46 × 42 × 29 mm on cross section T1-weighted magnetic resonance imaging (arrow); (b) macroscopic picture of the right removed gonad. An arrow indicates the fallopian tube; (c) histopathologic section, H&E staining, magnification ×25. Region 1. Seminiferous tubules with hyaline contents corresponding to a gonadoblastoma (magnification ×630) (arrow 1). Region 2. High cellularity and anarchic architecture corresponding to a dysgerminoma (magnification ×630) (arrow 2).

gonads revealed a mixed ovarian tumor showing multiple islands of dysgerminoma and less numerous foci of gonadoblastoma surrounded by Sertoli cells; the left adnex showed no evidence of tumor and consisted of a highly fibrous ovarian structure with no demonstrable follicles (Fig. 2).

Methods

SRY mutation analysis with Hpy188I

Initial mutation analysis of the SRY coding region was performed by Gendia (Antwerp, Belgium), testing DNA from the proband’s peripheral blood leukocytes. Following a preliminary report of a point mutation in the SRY HMG box, mutation status was confirmed by restriction fragment length polymorphism (RFLP) analysis performed at Saint-Justine Hospital; family members were likewise screened by RFLP at Sainte-Justine Hospital. Polymerase chain reaction (PCR) amplification of a fragment of SRY exon 1 was performed with primers, PCR conditions and minor modifications to those described in the study of Semerci et al. (4). Platinum Taq DNA polymerase (Invitrogen Corporation, Burlington, ON, Canada) was used with the following cycling parameters: initial denaturation at 94°C for 5 min followed by 30 cycles of: denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s. The PCR products (270 bp) was then subjected to digestion with Hpy188I in recommended buffer (New England Biolabs Inc, Pickering, ON, Canada) and the fragments analyzed on 7% polyacrylamide mini-gel. The normal allele (A allele) leads to 4 fragments of 132, 88, 45 and 4 bp whereas the mutation (C allele) destroys 1 restriction site and produces 3 fragments of respectively 177, 88 and 4 bp.

Markers used for paternity testing

Microsatellite and single nucleotide polymorphism (SNP) markers used for paternity testing, along with their chromosomal location and reference for PCR amplification, can be found in Table S1, Supporting Information.

Plasmid construction

The pcDNA3.1(+) plasmid vector (Invitrogen) containing a BamHI and EcoRI cloning site was used for in vitro SRY synthesis. The SRY DNA insert was generated using both wild type and mutant DNA from peripheral blood leukocytes; the forward primer sequence (5’-GCGGATCCgcacctttcaattttgtcgc-3’) contained a BamHI restriction site and the SRY -39 to -20 site. The reverse primer sequence (5’-GCGAATTCTACAGCTTTGTCCAGTGTCG-3’) contained an EcoRI restriction site and the SRY TAG site.

The PCR amplification was performed in a 50-μL reaction volume containing PrimeSTAR Buffer (10 mM Tris–HCl (pH 8.2), 20 mM NaCl, 0.02 mM EDTA,
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0.2 mM DTT, 1 mM MgCl₂, 200 μM each of dNTP (dATP, dCTP, dGTP, dTTP), 15 pmol of oligonucleotide primers, 65 ng of DNA, and 1.25 IU Taq DNA Polymerase (TaKaRa:R010A). PCR thermal cycling conditions were: a 2-min predenaturation period at 94°C and 35 cycles of the following: 98°C for 10 s, 60°C for 5 s, and 72°C for 60 s. The SRY cDNA sequence was 663 nt and both the wild type (pcDNA3.1(+) -wt) and mutant vectors (pcDNA3.1(+) -E89A) were verified by sequencing.

In vitro translation
The TNT® T7 Quick Coupled Transcription/Translation Systems (Promega:L1170) was used to synthesize 35S-methionine-labeled SRY in a reaction mix containing [35S]-methionine (Perkin Elmer, Waltham, MA; NEG709A), TNT Quick Master Mix and 1.0 μg of the expression vector in a final reaction volume of 50 μl. The mixture was incubated at 30°C for 90 min, and the labeled wild type and mutant SRY proteins were analyzed by 12% SDS-PAGE; autoradiography showed a single, 30 kD band as expected for both proteins.

Preparation of oligonucleotide probe
The SRY-binding sequence upstream of SOX9 (AAC AAT) (5) was incorporated into a 32P-labeled oligonucleotide probe as previously described by Shahid et al. (6). The oligonucleotides used were: F: 5′-GGGT TAACGT AACAAAT GAATCTGGTAGTA-3′, R: 5′-GT ACTACCAGATTC ATTTGT ACGTTAACC-3′.

Electrophoretic mobility shift assay
The ability of the SRY wt and mutant proteins to bind to DNA was assessed in a binding reaction containing increasing amount of wild type or mutant proteins in the binding buffer described in the study by van de Wetering et al. (7). After a pre-incubation of 20 min on ice of the proteins in binding buffer, the probe was added and the incubation resumed for 60 min on ice. The samples were then electrophoresed through a non-denaturing 12% polyacrylamide gel run in 0.5× TBE buffer at room temperature. The gel image was analyzed using the Fuji phosphoimager FLA-7000.

Computational analysis
Amino acid alignments of SRY protein from different species were performed with Polyphen (http://genetics.bwh.harvard.edu/pph/) (Table S2) (8). A picture of the nuclear magnetic resonance (NMR) structure of the wild-type HMG-Box domain of SRY protein complexed with DNA (pdb accession number 1J46) was designed with the PyMOL Molecular Graphic System (http://www.pymol.org/) (Fig. 3b).

Fig. 3. Novel SRY mutation. (a) Schematic of the SRY protein showing the location of the DNA binding domain (HMG box) in relation to the identified missense mutation, which causes a glutamic acid (E) to alanine (A) change in amino acid 89. (b) Nuclear magnetic resonance structure of the wild-type HMG-Box domain of the SRY protein complexed with DNA (pdb accession number 1J46). The side chain of the involved amino acid is depicted (Glu89) (arrow). The picture was designed with the PyMOL Molecular Graphic System (http://www.pymol.org/). (c) Synthesize SRY protein in vitro. An autoradiograph of in vitro-translated 35S-labeled SRY-wt, SRY-E89A proteins analyzed by 12% SDS-PAGE, showing a single 30-kDa band. Luc, luciferase (negative control). (d) Electrophoretic Mobility Shift Assay. As explained in Materials and Methods, increasing concentrations of wt or recombinant SRY protein were added to a 32P-labeled oligonucleotide probe encompassing the SRY binding region of SOX9. Lane 1 shows a luciferase control. The binding capacity of the wt protein surpasses that of the mutant protein at all concentrations tested.
Results

Chromosome analysis (performed in a peripheral hospital and based on examination of 20 metaphases) from peripheral cultured blood lymphocytes revealed a 46, XY karyotype in the proband, a phenotypic female (see pedigree, Fig. 1a). Sequence analysis of the SRY gene in the proband showed a single point mutation c.266A>C (p.Glu89Ala) which was confirmed by an RFLP assay designed to distinguish the wild type and mutant alleles at this position in the gene. This missense mutation is located in the DNA binding domain of the protein, and the substitution of an uncharged amino acid for one with a positive charge suggests that protein structure in this region is perturbed; Polyphen analysis supported this prediction and also showed that Glu89 in SRY HMG-Box domain is highly conserved across species (Table S2) (8) (Fig 3a,b). The variant has not been reported among unaffected males nor in the known SRY mutation databases (Patrick Willems, Gendia, personal communication), nor in the Human Genome Mutation Database (HGMD).

To determine the DNA binding characteristics of the E89A variant, we performed an electrophoretic mobility shift assay using in vitro synthesized wild type or mutant protein, and an oligonucleotide probe containing the SOX9 promoter SRY binding sites. As seen in Fig. 3d, the mutant protein displayed markedly decreased binding activity at all protein concentrations employed. These data suggest that the E89A variant has a pathogenic effect on protein function and is not simply a rare neutral polymorphism.

By the RFLP assay, neither of the proband’s parents carried the SRY mutation, based on analysis of blood leukocyte-derived DNA (Fig. 1b). The father showed no unusual phenotype characteristics with regard to primary and secondary sexual traits, suggesting that the father might be a germ line mosaic for the mutation. Correct paternity was confirmed by genotyping eight highly polymorphic microsatellites and three SNPs on six different chromosomes; informative loci are shown in the Table S1. All markers were consistent with the given family structure.

Based on the current standard of practice, there would normally be no special reason to perform any genetic tests on the proband’s siblings (one brother, two sisters). However, we chose to verify the karyotype (examination of 30 metaphases) of all three siblings, and unexpectedly one sister was also 46, XY despite a clinical examination that suggested breast budding compatible with a Tanner stage B2-3. By our RFLP assay, we determined that the proband’s nominal sister was also carrying the SRY mutation, while the brother carried a normal SRY gene sequence (Fig. 1b, lanes 7). The youngest sister had a normal 46, XX karyotype. On further examination, the 46, XY SRY− sister was found to have a right side ovary tumor showing multiple islands of dysgerminoma and less numerous foci of gonadoblastoma surrounded by Sertoli cells (Fig. 2). The left ovary was not tumorous. Bilateral ovaries were removed surgically from both 46, XY sisters, who are now receiving hormone therapy to allow normal secondary sexual development.

Discussion

We have identified a novel point mutation p.Glu89Ala within the HMG box of the SRY gene shared by two 46, XY phenotypically female ‘sisters’. Functional studies support that this variant is a pathogenic mutation and not a neutral polymorphism, as the mutation decreases the SRY DNA-binding capacity in a well validated in vitro assay. Their father is wild type for SRY sequence as is a 46, XY phenotypically normal brother. The brother’s (dizygotic) twin sister has a normal 46, XX karyotype.

Previously reported familial cases of 46, XY CGD as a result of SRY mutation are summarized in Table 1. There are 16 different mutations within 16 such families, but only one family had evidence of paternal germ cell mosaicism as in our family. However in that study correct paternity was not confirmed explicitly (9).

Most aspects of phenotype of the two 46, XY sisters described here are typical of patients with 46, XY CGD. However, the younger sister exhibited a seemingly normal onset of puberty, which is atypical in patients with SRY mutations. In retrospect, although the low estradiol levels compatible with breast budding (Tanner B2) are currently unmeasurable by our clinically available assay (lower limit 73 pmol/l), we suspect that the ovarian tumor may have secreted sufficient estrogens to initiate puberty. Notably, as seen in Table 1, five of the 46, XY SRYmut females exhibiting breast development at diagnosis (and not yet on estrogen therapy), four out of five were found to have an ovarian tumor as in our younger patient. Villanueva et al. (10) reported the case of a 17-year-old girl with 46, XY CGD and a breast development corresponding to Tanner stage V. She was found to have bilateral gonadoblastoma and right dysgerminoma. The estrogen levels in that patient, although persistently in the range of follicular phase concentrations, were higher than those of postmenopausal women and apparently were high enough to ensure breast development but not menarche. As her estrogen and androgen levels dropped markedly postoperatively, her dysgenetic gonads were obviously the source of sex steroids. Barakat et al. reported a 16-year-old girl with 46, XY CGD and spontaneous breast development that occurred at 13 years followed by menarche at 14 years. She was referred for secondary amenorrhea and clitoromegaly 2 years later, and was found to have bilateral gonadoblastoma. The authors showed that both gonads produced estradiol in culture (11). The low estradiol levels in our patient’s younger sibling despite her spontaneous breast development could also be explained by conversion of dehydroepiandrosterone to estrogen in mammary tissue as suggested by Cantu et al. (12), although the Tanner B3 breasts and the absence of the finding in the older affected sister argue against this.

Tumor risk in patients with 46, XY CGD has been estimated at 30%, but may be as high as 50–70%
Table 1. Familial cases of 46, XY females with SRY mutations

<table>
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<th>References</th>
<th>Case number</th>
<th>Mutation</th>
<th>Type</th>
<th>SRY domain</th>
<th>In vitro analysis</th>
<th>Age at Dx</th>
<th>Tanner</th>
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<td>Screening</td>
<td>Gb/ Dg R</td>
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B, breast development; bilat, bilateral; DG, dysgerminoma; Dx, diagnosis; E2, estrogen, Gb, gonadoblastoma; L, left; Lk, leukocytes; NA, not available; No, no tumor found; P, pubic hair; R, right; unil, unilateral.

<sup>1</sup> Father with testicular dysgenesis syndrome (cryptorchidism, seminoma in this testis diagnosed at 39 years, severe hypospadias, oligoasthenozoospermia).

<sup>2</sup> 46, XY mutation positive in normal brother and uncle.

<sup>3</sup> 46, XY mutation positive in normal fertile brother.

<sup>4</sup> Mosaicism in father’s sperm.

<sup>5</sup> 46, XY mutation positive in normal paternal uncle.

<sup>6</sup> 46, XY mutation positive in two brothers with ambiguous genitalia diagnosed at 8 and 10 days, and in three normal brothers. 46, XY mutation positive in a paternal aunt with ambiguity.

<sup>7</sup> DNA from periarticular inflammatory tissue of the deceased father.
in the third decade of life, and 80% at 40 years. Patients may present with either gonadoblastoma or the more invasive dysgerminoma (13). The presence of at least a part of the Y chromosome is necessary for development of a gonadoblastoma in a dysgenetic gonad. This so-called gonadoblastoma locus (GBY) on Yp11.1 contains a potential oncogene named testis specific protein Y-encoded (TSPY) involved in cell cycle regulation (14–16). The TSPY gene is expressed in both fetal and adult testes, and it has been hypothesized that it regulates the normal proliferation of both embryonic prespermatagonia/gonocytes and adult spermatogonial cells (15,17).

The finding of germ cell mosaicism in two of the 16 familial examples of SRY mutations has significant implications for genetic counseling. There are multiple genetic diseases (>60) in which confined germline mosaicism has been observed (18). Although recurrence risk calculations are problematic, Duchenne muscular dystrophy has one of the highest risks of transmission by leukocyte mutation negative females, estimated at between 14% and 20%. There are two possibilities for such a high recurrence. One is that the mutation occurs very early before the formation of the germ cells and is therefore present in both somatic and germinal cells, although this might be expected to cause a mutation in 100% of the germ cells, rather than mosaicism. Alternatively, a mutation may occur following germline-soma divergence but early in the germinal lineage. The examination of several different tissues (buccal, smear, muscle, or hair follicle) may discriminate between these alternatives, although in the case of 46, XY CGD, molecular analyses of single paternal sperm would be the ideal test to determine probably recurrence risk (19). However, sperm are subject to clonal expansions, in some cases with a suggestion of deleterious mutations actually providing a competitive advantage in the testis, thus it is probably impossible to provide families with an accurate recurrence risk generally (20–22).

In conclusion, our identification of a novel point mutation in the SRY gene shared by two 46, XY CGD sisters revealed probable paternal germline mosaicism. Functional analysis indicates that this mutation decreases the DNA-binding activity of the SRY protein. More importantly, this family illustrates that upon identification of the proband, even sisters with pubertal onset should be screened cytologically and/or for SRY mutations, because even in the case of germ cell mosaicism a high recurrence risk is possible. In fact, pubertal onset in a mutation-bearing patient may herald the presence of a gonadal tumor. It is important to note that preventive screening of siblings, in families with one sporadic 46, XY feminized case, is not necessarily the current standard of practice. Our decision to screen both seemingly unaffected sisters, leading to the identification in one of a mutation requiring hormonal treatment, and a tumor requiring surgical excision, had major impact to improve the feminized sister’s long-term quality of life.

Supporting Information
The following Supporting information is available for this article: Table S1. Paternity confirmation.
Table S2. Amino acid alignments across species in the regions flanking E89 (red box) performed with Polyphen (http://genetics.bwh.harvard.edu/pph/).
Additional Supporting information may be found in the online version of this article.
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References


Mutation in SRY in two feminized siblings