Identification of novel SRY mutations and SF1 (NR5A1) changes in patients with pure gonadal dysgenesis and 46,XY karyotype

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Submitted on October 18, 2010; resubmitted on January 12, 2011; accepted on January 12, 2011

ABSTRACT: Primary amenorrhea due to 46,XY disorders of sexual development (DSD) is complex with the involvement of several genes. Karyotyping of such patients is important as they may develop dysgerminoma and molecular analysis is important to identify the underlying mechanism and explore the cascade of events occurring during sexual development. The present study was undertaken for the genetic analysis in seven patients from five families presenting with primary amenorrhea and diagnosed with pure gonadal dysgenesis. Karyotyping was done and the patients were screened for underlying changes in SRY, desert hedgehog (DHH), DAX1 (NR0B1) and SF1 (NR5A1) genes, mutations in which are implicated in DSD. All the patients had 46,XY karyotype and two novel SRY mutations were found. In Family 1 (Patient S1.1) a missense mutation c.294G>A was seen, which results in a stop codon at the corresponding amino acid (Trp98X) and in Family 2 (Patients S2.1, S2.2 and S2.3), a missense mutation c.334G>A (Glu112Leu) was identified in all affected sisters. Both mutations were seen to occur in the conserved high mobility group box of SRY gene. One heterozygous change c.427G>A resulting in Glu143Lys in DHH gene in one patient and two heterozygous changes in the intronic region of SF1 (NR5A1) gene (c.244+80G>A+ c.1068–20C>T) in another patient were noted. One individual did not show changes in any of the genes analyzed. These results reiterate the importance of SRY and others, such as SF1 (NR5A1) and DHH, that are involved in the cascade of events leading to sex determination and also their role in sex reversal.

Key words: Swyer syndrome / pure gonadal dysgenesis / SRY / DHH / DAX1 (NR0B1) / SF1 (NR5A1) / mutation

Introduction

Sex determination in humans is a complex process involving interplay of several genes, and malfunctioning of any one of them leads to disorders of sex development (DSD). DSD is a term that characterizes incomplete or disordered genital or gonadal development leading to discordance between genetic, gonadal and phenotypic sex (Hughes et al., 2006; Nabhan and Lee, 2007). Gonadal development is bipotential during the first 7–8 weeks of mammalian embryogenesis and differentiation occurs subsequently to give rise to testes or ovaries in individuals with a 46,XY or 46,XX karyotype, respectively. DSD is a heterogeneous disorder that occurs as either pure gonadal dysgenesis (PGD) or partial gonadal dysgenesis with a frequency of 1 in 3000 births (Camerino et al., 2006). PGD is characterized by fully developed female-type external genitalia and normal Müllerian structures. Testes are not seen and only streak gonads are present. Partial gonadal dysgenesis is distinguished by genital ambiguity and dysgenetic and/or streak gonads. Several genes encode for proteins required for gonad development, including SRY, SF1 (NR5A1), DAX1 (NR0B1), desert hedgehog (DHH), WT1, WNT4 and SOX9, mutations in which can lead to gonadal disorders of sex development.

The SRY gene (MIM#480000) located on chromosome Yp11.3 plays a pivotal role in testis determination. SRY consists of two open reading frames. Its key sequence involves a high mobility group (HMG) box encompassing codons 13–82, which shares characteristics with other DNA-binding sequences. Occurrence of the majority of mutations in the HMG domain proves the role of SRY in testes differentiation by DNA bending (Giese et al., 1994) and juxtaposing...
more than one testicular determining gene and, hence, facilitating transcription or interaction between certain gene products. Perturbations of SRY are seen in 10–15% of XY gonad dysgenesis cases (Berta et al., 1990) associated with both pure (Cameron and Sinclair, 1997; Margaret et al., 1998; Assumpcao et al., 2002) and partial gonadal dysgenesis (Domenice et al., 1998). Loss of function SRY mutations are shown to result in the female phenotype in 10–15% of cases (Cameron and Sinclair, 1997; Margaret et al., 1998; Assumpcao et al., 2002). Most of the reported mutations are de novo but cases documenting inheritance of mutations from the unaffected father have also been reported (Isidor et al., 2009).

Apart from SRY, DSD also result from mutations in genes that are involved in the gonadal development such as SF1 (NR5A1), DAX1 (NR0B1), DHH, WTI, WNT4 and SOX9.

An important gene involved in early gonadal development is SF1 (Steroidogenic factor 1) (MIM#184757), located at 9q33, a member of the nuclear hormone receptor family, also known as NR5A1 and is expressed in the developing urogenital ridge, hypothalamus, anterior pituitary and the adrenal glands (Parker et al., 2002; Ferraz-de-Souza et al., 2006). It encodes a nuclear transcription factor regulating the expression of a number of genes that participate in sexual development. SF1 knockout mice fail to develop adrenal glands and gonads and die at birth (Lala et al., 1992). In humans, heterozygous SF1 mutations in XY individuals lead to adrenal and gonadal failure (Köhler and Achermann, 2010), cryptorchidism (Wada et al., 2005), microgenis (Wada et al., 2006) and male infertility (Bashamboo et al., 2010).

DAX1 (OMIM#300473, dosage sensitive sex reversal adrenal hypoplasia congenital critical region of the X chromosome gene I), located on chromosome Xp21.3–p21.2, is a member of the nuclear receptor superfamily (NR0B1) and encodes a 470-amino-acid nuclear receptor. It has a triple repeat motif in the DNA-binding domain at the N-terminus and a C-terminus ligand-binding domain that resembles several nuclear receptors. It is considered to play a role in a global negative transcriptional regulator of steroid hormone production by repressing the expression of multiple genes involved in the steroidogenic pathway (Swain et al., 1996). DAX1 mutations are associated with X-linked primary adrenal insufficiency and hypogonadotropic hypogonadism (Achermann et al., 1999). It antagonizes the action of SRY resulting in a sex-reversed phenotype (Swain et al., 1998; Domenice et al., 2004).

Another gene involved is the DHH (MIM#233420) located on 12q13, which is a member of the hedgehog family of signaling proteins that encodes a 396-amino-acid protein. DHH is expressed in the Sertoli cells and is a positive regulator of the differentiation of steroid-producing Leydig cells in the fetal testis. Studies using murine homolog (Dhh) have demonstrated that the differentiation of peritubular myoid cells and the consequent formation of testis cords are regulated by Dhh (Clark et al., 2000; Pierucci-Alves et al., 2001), substantiating its role in gonadal differentiation. Mutations in DHH have been reported in cases of partial and PGD with 46,XY karyotype (Canto et al., 2004).

WTI (MIM#67102), located on chromosome 11p13, is essential for the development of the urogenital tract, where it regulates expression of SRY, and later it plays a pivotal role, together with SF1, in the production of Anti-Müllerian hormone in Sertoli cells (Nachtigal et al., 1998; Hossain and Saunders, 2001). Heterozygous missense and splice site WTI mutations are associated with Denys–Drash syndrome (MIM#194080) and Frasier syndrome (MIM#136680).

WNT4 (MIM#603490), located on chromosome 1p36.23–p35, belongs to a family encoding cystein-rich glycoproteins, which act as extracellular signaling proteins. Female patients carrying heterozygous WNT4 mutation present with Mullerian duct abnormalities along with clinical and biological evidence of hyperandrogenism (Philibert et al., 2008). WNT4 increases the expression of DAX1 in Sertoli and Leydig cells and duplication of WNT4 in a chromosomal male has been associated with XY gonadal dysgenesis (Jordan et al., 2001).

The transcription factor SOX9 (MIM#142920) located at 17q23 is necessary for cartilage formation and testis differentiation, mutations of which lead to campomelic dysplasia, in which about two-thirds of 46,XY individuals also have a partial or complete form of XY gonadal dysgenesis (Wagner et al., 1994). Recently, compound heterozygosity for two missense mutations in the polycomb gene chromobob homolog 2 (CBX2) (MIM#602770) at 17q25 has been documented as being causative for XY gonadal dysgenesis (Biaison-Laubet et al., 2009).

Despite the knowledge of the role of these genes in the cascade of events during sexual development, the cause of gonadal dysgenesis is still unclear and needs thorough investigation.

In view of the importance of the SRY, SF1 (NR5A1), DAX1 (NR0B1) and DHH genes in normal gonadal development, the present study was undertaken with an aim to systematically identify the underlying changes in these genes in seven patients from five families who were clinically diagnosed with PGD and a 46,XY karyotype.

Materials and Methods

The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Committee. Informed consent was obtained from all the patients and their family members before being enrolled for the study. Clinical examinations and laboratory investigations were carried out, detailed family history was collected and pedigree charts were drawn (Fig. 1).

Patients

All the seven patients presented with the chief complaint of primary amenorrhea and were subjected to clinical and genetic evaluations. Physical examinations revealed well-developed secondary sexual characters with normal breast development; pelvic ultrasound showed small infantile uterus and hormone levels were noted to be irregular in all the seven patients. A total of 5 ml of peripheral blood samples were collected in heparin from all the patients and their unaffected relatives for cytogenetic and molecular investigations. Karyotyping was done using conventional g bands by trypsin using giemsa banding on cultured blood lymphocytes.

Mutation analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. The SRY, DHH, DAX1 (NR0B1) and SF1 (NR5A1) genes were amplified using custom-synthesized oligonucleotide primers. PCR amplification of the entire coding region of SRY gene was done using the primers as described previously (Rajender et al., 2006) using 70–100 ng DNA, 1.5 mM MgCl₂, 0.25 mM of each dNTP, 10 pM of each primer and 1.0 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA) in a 25-μl volume mixture in a thermocycler [ABI 9700 Applied Biosystems (ABI), Foster City, CA]. A positive (normal male 46,XY) and a
negative (normal female 46,XX) control were included with the amplification of SRY gene.

The entire coding region of DHH gene was amplified using four primer pairs as described previously (Umehara et al., 2000), to identify coding region and splice-site changes. Amplification was done using 10 ng DNA, 1.5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 units of Taq polymerase (Invitrogen) in a total reaction volume of 25 μl.

PCR amplification of the entire coding region and splice sites of DAX1 (NR0B1) gene was done using primer pairs and conditions as described previously (Achermann et al., 1999). Amplification was done using 10 ng DNA, 1.5 mM MgCl₂, 0.25 mM of each dNTP, 10 pM of each primer and 0.5 units of Taq polymerase (Invitrogen) in a 25-μl reaction volume.

The entire coding region of SF1 (NR5A1) gene and the flanking region of all the exons were amplified using primer pairs as described previously (Lourenc¸o et al., 2009). The 25-μl reaction for PCR consisted of 1X Pyrostart™ Fast PCR Master Mix (2X, Fermentas, Life Sciences), 10 pM of each primer and 40–80 ng of DNA template.

Sequencing
All the amplified products were subjected to gel purification using QIAamp gel extraction kits (Qiagen, GmBH, Hilden) and the purified PCR products were sequenced bidirectionally using BigDye Terminator Mix version 3.1 (ABI) and were analyzed on an ABI-3100 Genetic Analyzer (ABI).

Nucleotide sequences were compared with the published cDNA sequences of SRY (GenBank accession number ENSG00000184895), SF1 (NRS1) (GenBank accession number ENSG00000169297) and DHH gene (GenBank accession number ENSG00000139549).

Polyphen 2 and SIFT analysis
In silico analysis using Polyphen 2 and SIFT (Sorting Intolerant From Tolerant) tools was carried out for the novel mutations, to look for the pathogenicity of the identified changes. The SIFT tool (http://blocks.fhcrc.org/sift/SIFT.html) generates multiple sequence alignments of a gene over different species and assesses the degree of conservation of the substituted positions over the course of evolution. It gives a value as a score, and a score whose value of <0.05 is considered potentially damaging.

Results

Cytogenetic analysis
All the affected patients showed the male karyotype (46,XY) on chromosome analysis using conventional cytogenetics. Biochemical parameters showed irregular levels of LH, FSH and testosterone (Table I).

Mutation analysis
SRY gene: Two novel pathogenic mutations were identified in SRY gene.
Family 1: In patient S1.1, a novel mutation c.294G>A was identified, which generated a premature stop codon at amino acid position 98 (Trp98X).
Family 2: Three affected patients (S2.1, S2.2 and S2.3) showed the mutation c.334G>A resulting in amino acid substitution Glu112Leu. The father and the brother were not available for any examination. In silico analysis revealed that the amino acid at position 112 is conserved among orthologs with a score being 0.05 and the substitution was not tolerated.

DHH gene: Family 3: Sequencing of DHH gene revealed a heterozygous mutation C.427G>A resulting in amino acid substitution Glu143Lys in an individual (Patient S3.1). SIFT and Polyphen2 analysis revealed that the amino acid position was conserved among orthologs during evolution.

SF1 (NR5A1) gene: Sequence analysis of SF1 (NR5A1) gene did not reveal any changes in the coding region. One patient revealed two heterozygous changes in two different introns. The heterozygous changes c.244+80G>A and c.1068–20C>T were seen in introns 3 and 4, respectively in the individual from Family 5 (S5.1). No other changes were identified in this patient in the other genes that were screened.

DAX1 gene: Two previously reported polymorphisms (rs2269345 and rs6150) were seen in the DAX1 gene in two patients (Patients S1.1, S3.1). No other pathogenic changes were noted in this gene in

Figure 1 Pedigree of the patients with PGD. Filled boxes represent affected individuals. Open boxes represent unaffected individuals. Arrowhead indicates the proband.
**Table I** Details of clinical features, hormone levels and mutation status of the individuals diagnosed with PGD.

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Physical examination</th>
<th>Endocrine studies</th>
<th>Ultrasonography reports</th>
<th>Gene with underlying mutation</th>
<th>cDNA position of pathogenic change identified</th>
<th>Amino acid position of pathogenic change identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1.1</td>
<td>18/F</td>
<td>Breast: Tanner stage III</td>
<td>LH 20.4 FSH 275.7 Testo 2.10</td>
<td>Small infantile uterus with small ovaries</td>
<td>SRY c.294G-&gt;A</td>
<td>Trp98X</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S2.1</td>
<td>22/F</td>
<td>Breast: Tanner stage IV</td>
<td>LH 6.35 FSH 22.70 Testo 1.75</td>
<td>Small uterus and ovaries</td>
<td>SRY c.334G-&gt;A</td>
<td>Glu112Leu</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S2.2</td>
<td>26/F</td>
<td>Breast: Tanner stage IV</td>
<td>LH ND FSH ND Testo ND</td>
<td>Hypogonadism with small uterus and ovaries</td>
<td>SRY c.334G-&gt;A</td>
<td>Glu112Leu</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S2.3</td>
<td>16/F</td>
<td>Breast: Tanner stage III</td>
<td>LH ND FSH ND Testo ND</td>
<td>Hypogonadism with small uterus and ovaries</td>
<td>SRY c.334G-&gt;A</td>
<td>Glu112Leu</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>S3.1</td>
<td>14/F</td>
<td>Axillary &amp; pubic hair: Tanner stage IV</td>
<td>LH 14.19 FSH 11.19 Testo 5.74</td>
<td>Uterine agenesis with small uterus and two ovoid structures seen in the superficial inguinal region</td>
<td>DHH c.427G-&gt;A</td>
<td>Glu142Lys</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>S4.1</td>
<td>18/F</td>
<td>Breast: Tanner stage II</td>
<td>LH 23.7 FSH 50.1 Testo 1.2</td>
<td>Small uterus and ovaries</td>
<td>No change in any of the genes tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>S5.1</td>
<td>24/F</td>
<td>Breast: Tanner stage III</td>
<td>LH 14.90 FSH 56.7 Testo 0.83</td>
<td>Rudimentary uterus and small sized ovaries</td>
<td>NR5AI c.244+80G-&gt;A +c.1068—20C&gt;T (changes in introns 3 and 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference range for hormones

<table>
<thead>
<tr>
<th>FSH (mIU/ml)</th>
<th>LH (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular phase</td>
<td>3.03–8.08</td>
</tr>
<tr>
<td>Mid-cycle peak</td>
<td>2.55–16.69</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>1.38–5.47</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>26.72–133.41</td>
</tr>
<tr>
<td>Males</td>
<td>0.95–11.95</td>
</tr>
</tbody>
</table>

F, female; LH, luteinizing hormone; FSH, follicle stimulating hormone; Testo, testosterone; ND, not done; NA, details not available.
any of the affected patients. The individual from Family 4 (S4.1) did not show any sequence changes in the genes analyzed.

All these sequence changes were seen to occur only in the patients and not in any of the 50 healthy and fertile controls who were also studied.

Discussion

Several studies have shown the importance of SRY in testicular development. Mutations in SRY are reported in ~10–15% of 46,XY females with pure or partial gonadal dysgenesis (Hersmus et al., 2009). It has been reported that the mutations in the open reading frame of SRY in XY female patients leads to dysgenesis of gonads (Cameron and Sinclair, 1997; Margarit et al., 1998; Assumpcao et al., 2002). In the present study, we identified two novel mutations in SRY gene both leading to PGD. One mutation occurred de novo while the other mutation was identified in a familial case with three affected individuals. Both mutations were identified at highly conserved positions within the HMG box.

The binding activity of SRY lies in the HMG domain (Hersmus et al., 2009), which is composed of three α-helices forming an L-shaped structure. The glutamic acid-to-leucine change at position 112 introduces a small non-polar residue for an acidic residue. The substitution may not alter the structure of the protein but can significantly alter its binding properties and therefore glutamic acid residue at position 112 is conserved among the orthologs throughout the course of evolution. In silico analysis showed that substitution of glutamic acid with leucine is not tolerated and is therefore considered pathogenic. This mutation occurs in the third α helix of the HMG domain and thus might disrupt the binding activity of the HMG box, consequently affecting the transcription of other testis-determining genes.

All the affected individuals of Family 2 had the Glu112Leu mutation. The presence of this change in other family members, especially the father, could not be studied, as they were not available for analysis.

The second mutation Trp98X leads to the generation of a premature stop codon and results in a truncated protein. Tryptophan at position 98 lies in the second α helix of the HMG box and the truncated protein so formed may not be able to bind to the DNA, resulting in the loss of function mutation.

The activation of DHH transcription occurs immediately after the initiation of SRY expression and is one of the first indications of male-specific development (Bitgood and McMahon, 1995). The phenotypic presentation of patients with DHH gene mutations may be pure or mixed gonadal dysgenesis. Most of the cases with PGD have been reported with homozygous mutations, while a heterozygous exon 3 mutation (1086delG) was seen in a patient with mixed gonadal dysgenesis (Canto et al., 2009). In the present study, a heterozygous mutation c.427G>A was identified in one patient with PGD. The corresponding amino acid (glutamic acid at position 143) is evolutionarily conserved and thus might be important for the functioning of the protein. Based on previous studies, it is known that DHH is a key gene in mammalian gonadal differentiation (Bitgood and McMahon, 1995; Clark et al., 2000; Canto et al., 2009). The absence of nucleotide changes in this patient in any of the other genes screened in the present study indicates that DHH mutation may be pathogenic even in the heterozygous state, although the presence of mutations in other genes involved in sexual development cannot be ruled out.

Lourenço et al. (2009) described novel SF1 (NR5A1) mutations associated with 46,XY DSD and ovarian insufficiency. All of them were heterozygous, except one identified in two Brazilian sibs who were homozygous for a Asp293Asn mutation: one with 46,XY DSD and the other with 46,XX primary ovarian insufficiency. Recently SF1 (NR5A1) mutations have been shown to cause male infertility and the authors believe that the mutations may be associated with altered sex hormone levels and mild abnormalities in cellular structure of the testes (Bashamboo et al., 2010).

In the present study, we did not find any SF1 (NR5A1) coding region changes but a compound heterozygous change (c.244+80G>A and c.1068–20C>T) was identified in two consecutive introns. The effect of these changes was not assessed by functional studies but it is believed that the changes may affect pre-mRNA splicing, leading to altered protein structure. This may in turn affect its crucial role of effective action of male sex hormone on testicular tissue and thus can lead to the phenotype as seen in the patient.

We also identified two synonymous polymorphisms c.114C>T (rs6150) and c.498G>A (rs2269345) in DAX1 gene in two patients. Both are located within the first exon, and neither changes the predicted amino acid. Although these DNA changes have not been examined experimentally for their effect on mRNA or protein expression, repeated observations by different groups that these nucleotide changes do not co-segregate with the disease phenotype confirms that these are true polymorphisms (Phelan and McCabe, 2001).

One individual from Family 4 (S4.1) did not show sequence changes in any of the genes analyzed. This individual may have an underlying molecular defect in the other genes like WT1, WNT4 or SOX9, mutations in which have also been implicated in gonadal dysgenesis (de Santa Barbara et al., 2000).

In conclusion, we report novel mutations in the SRY gene in two families, a heterozygous change in DHH gene in one patient and compound heterozygous changes in the introns of SF1 (NR5A1) in one patient, all of whom were phenotypic females with 46,XY karyotype. Primary amenorrhoea due to 46,XY DSD is complex with several factors involved in the sex determination pathway, including SF1 (NR5A1), DAX1 (NR0B1), DHH, WT1, WNT4 and SOX9. These genes should be systematically analyzed in individuals with primary amenorrhoea due to 46,XY DSD, as early diagnosis of this condition is essential due to the high risk of developing dysgerminoma by such patients.

Authors’ roles

P.P., S.B. and A.S. carried out the cytogenetic and molecular analysis. P.P. drafted the manuscript. A.K. and R.K. were instrumental in patient recruitment and detailed clinical workup of the patients and their family members. A.S. planned and supervised the experiments, analyzed the results and wrote the paper.

Acknowledgements

The authors thank the patients and their family members for their participation in the study.

