The novel p.Cys65Tyr in NR5A1 gene in three siblings with 46,XY disorders of sex development

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**Background:** Disorders of sex development (DSD) is the term used for congenital conditions in which development of chromosomal, gonadal, or phenotypic sex is atypical. There are several genes that participate in both sex determination and differentiation processes. Nuclear receptor subfamily 5, group A, member 1 gene (*NR5A1*) encoding steroidogenic factor 1 (SF1), a transcription factor, is involved in gonadal development and regulates adrenal steroidogenesis. Mutations in the *NR5A1* gene may lead to different 46,XX or 46,XY DSD phenotypes with or without adrenal failure. We report a Brazilian family with a novel *NR5A1* mutation causing ambiguous genitalia in 46,XY affected individuals and signs of premature ovarian failure in their mother. **Case presentation:** Three siblings presenting 46,XY DSD with ambiguous genitalia and normal testosterone production and their parents were included in the study. Molecular analyses were carried out for *AR*, *SRD5A2* and *NR5A1* genes. *AR* and *SRD5A2* gene sequencing did not reveal any mutation. However, *NR5A2* sequence analysis indicated that all three siblings were heterozygous for p.Cys65Tyr mutation which was inherited from their mother. **Conclusion:** The p.Cys65Tyr mutation located within the second zinc finger of DNA binding domain was considered deleterious upon analysis with predictive algorithms. The identification of heterozygous individuals with this novel mutation may bring additional knowledge on structural modifications that may influence *NR5A1* DNA-binding ability, and may also contribute to genotype-phenotype correlations in DSD since hormone dosages in the three sibs with 46,XY DSD and in their mother suggest late-onset adrenal and ovarian abnormalities, respectively.

**Keywords:** disorders of sex development, *NR5A1* mutation, premature ovarian failure
**Background**

Steroidogenic factor 1 (SF1), denominated as nuclear receptor subfamily 5 group A member 1 (NR5A1 [OMIM +184757]), is a protein that regulates several steps of adrenal and gonadal development [1,2]. It is encoded by NR5A1 gene, which is an autosomal gene mapped to 30 kb within 9q33. NR5A1 gene sequence includes one non-translated exon (exon 1), six coding exons (exon 2-7) and six introns [3,4]. The SF1 protein contains 461 amino acid divided into a two zinc-finger DNA-binding domain (DBD), a ligand-binding domain (LBD), two functional activation domains (AF-1 and AF-2), an accessory region, and a hinge region [5]. SF1 protein is extremely conserved among species, presenting 95% overall amino acid homology between human and mouse [6].

NR5A1 is expressed in the developing urogenital ridge, steroidogenic tissues (as gonads, adrenals, and placenta), hypothalamus and anterior pituitary [7-9]. In general, it activates the expression of AMH in Sertoli cells leading to the regression of Müllerian structures [1,2,9]; in Leydig cells it activates the expression of several enzymes involved in steroidogenesis, resulting in virilization of external genitalia and testicular descent [1,2,9]; and, in ovaries, NR5A1 is expressed in the granulosa and theca cells where it regulates genes required for ovarian steroidogenesis and follicle growth maturation [8,9]. Genes such as: CYPs, 3β-HSD, StAR, SOX9, DAX1, and others are among the gene targets for NR5A1 regulation [5]. As an essential transcription regulator for adrenal and gonadal development NR5A1 is very important in the sex differentiation process, although it also plays important physiological roles in the central nervous system [10]. Therefore, mutations in NR5A1 may lead to Disorders of Sex Development (DSD) defined as incomplete or disordered gonadal or genital development, causing divergences between genetic sex, gonadal sex and phenotypic sex [11,12].

p.Gly35Glu and p.Arg92Gln were the first two mutations described in human NR5A1. They had been identified in patients with primary adrenal insufficiency, complete gonadal dysgenesis and Müllerian duct persistence [13,14]. After those, over 50 mutations have been reported in a large number of 46,XY DSD individuals with apparently normal adrenal function, in 46,XX individuals with premature ovarian failure and normal female phenotype and also with
male infertility [15-19]. In addition, several reports demonstrated that NR5A1 variations might also be associated with hypospadias, anorchia, and in some cases of adrenal tumors and endometriosis [20,21].

The findings in the literature indicate a complex expressivity of phenotype, penetrance and modes of inheritance for NR5A1 mutations. Therefore, it is difficult to establish a direct phenotype-genotype correlation [22].

In this report, we describe the novel NR5A1 gene mutation c.195G>A identified in three siblings with 46,XY DSD. The siblings were born of non-consanguineous parents, and had been brought to medical care due to genital ambiguity. Molecular analyses showed that the putative p.Cys65Tyr missense was inherited from the mother who presented with signs of premature ovarian failure.

**Case Presentation**

**Case report**

The study was undertaken under an institutionally approved ethic protocol and informed consent was obtained from all subjects and relatives.

Three affected siblings with 46,XY DSD had been evaluated (Fig. 1A). The index case, now aged 14, was born at term to healthy non-consanguineous parents, after an uneventful pregnancy. He was first seen with genital ambiguity, at the age of 2 months when he still had a female sex assignment. Physical examination revealed a 2-cm phallus, a single perineal opening, and the gonads were palpable in the labioscrotal folds. There were high levels of FSH but normal levels of LH, and a normal testosterone response to hCG test. The karyotype was 46,XY and pelvic ultrasound showed absence of mullerian derivatives. Female to male sex reassignment had been carried out at the age of 1 year and 4 months, when he underwent hypospadias repair. Spontaneous puberty began at the age of 11 years, with sustained high levels of FSH, initially normal LH levels which became elevated with time, and normal levels of testosterone (Table 1). He is now at Tanner stage G4P5 without sex hormone replacement.
His height is near the target. Recent evaluation of the adrenal function revealed slightly elevated ACTH with normal basal cortisol levels (Table 1).

The second sib, now aged 6 years, was born at term after an uneventful pregnancy, with a 2-cm phallus, penoscrotal hypospadias and palpable gonads in the labioscrotal folds. His karyotype was 46,XY. Hormone investigation was performed with the age of 2 month, with high levels of LH and normal levels of FSH and testosterone (Table 1). He had a male sex assignment and hypospadias repair. Recent evaluation of the adrenal function revealed slightly elevated ACTH with normal cortisol levels (Table 1).

The third sib, now aged 5 years, was born at term after an uneventful pregnancy with a 1.3-cm phallus, a single perineal urogenital opening, and both gonads were palpable in the inguinal region. He had a normal 46,XY karyotype and, at the age of 2 months, normal levels of FSH, LH, testosterone and dihydrotestosterone (Table 1). He was assigned as male and underwent further hypospadias repair and orchidopexy. Recent evaluation of the adrenal function revealed slightly elevated ACTH with normal cortisol levels (Table 1).

The 32-year-old mother was subject to a thorough hormone evaluation after a NR5A1 mutation was detected (see below). Hormonal results revealed high FSH, normal to high LH and normal to low estradiol levels with normal adrenal function (Table 1). She also referred irregular menses as a clinical symptom suggesting premature ovarian failure.

Methods

Genomic DNAs from patients and parents were purified from peripheral leukocytes by proteinase K lysis, phenol/chloroform extraction, and ethanol precipitation, according to standard techniques. Sequencing of both AR (androgen receptor) and SRD5A2 (5α-reductase) genes had been performed as described elsewhere [23,24]. The NR5A1 exons and 5’ and 3’ untranslated flanking regions were amplified by polymerase chain reaction (PCR) using specific primers designed based on the normal gene sequence (ENSG00000136931, www.ensembl.org). Independent PCR fragments were purified in 1% agarose gel electrophoresis with the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA), and both sense and antisense
strands were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Grand Island, NY, USA) with the same primers used in PCR reactions. The Chromas Lite 2.0 (Technelysium Pty Ltd) and CLC Sequence Viewer v.6.8.1 free software (CLC bio) were used to analyze and compare sequences with the reference NR5A1 sequence. Structural analyses were performed using PDB ID: 2FF0 – chain A as template. The native and mutant models were constructed by SWISS MODEL web-served program. Internal contacts were evaluated by STING Millenium (http://www.cbi.cnptia.embrapa.br) and visualized by PyMol®.

Results

DNA sequence analyses of AR and SRD5A2 genes did not show any mutation. However, NR5A1 gene sequencing revealed a novel transition G>A within exon 3 in the three heterozygous siblings and also in their mother (Fig. 1B). The nucleotide change c.195G>A is predicted to cause the substitution of a cysteine by a tyrosine at the amino acid residue 65 (p.Cys65Tyr).

Residue 65 in the NR5A1 protein corresponds to a highly conserved cysteine in mammal corresponding proteins (Fig. 2A). It is located at the second zinc finger of DNA binding domain, as illustrated by Little et al. [25] (Fig. 2B,C). Structural analyses demonstrated that C65 in the native protein, besides binding directly to zinc atom, it makes a hydrogen bond with R69 and hydrophobic interaction with C68 (Fig. 2D). The mutant Y65 maintains both interactions. However, a new hydrophobic interaction by 3.42 Ångstrons is established with C55 (Fig. 2E).

Three predictive methods to evaluate the effect of the amino acid substitution were used: PolyPhen (Polymorphism Phenotyping) that gives scores ranging from 0 (neutral) to a positive (damaging) number; SIFT (Sorting Intolerant From Tolerant) whose scores range from 0 (damaging) to 1 (neutral); and Aling GV-GD that classifies the amino acid change into classes ranging from C0 to C65, where C0 is considered tolerant and C65 deleterious [26,27]. The p.Cys65Tyr mutation resulted in PolyPhen score of 1.0, SIFT score of 0 and Aling GV-GD put
it into class C65 indicating a protein damage, probably leading to patients’ phenotypes. In order to discard the possibility of the nucleotide variation being a frequent polymorphism, 86 healthy controls (172 alleles) were analyzed and c.195G>A was not identified in any allele.

Discussion

We present here the follow-up of patients with 46,XY DSD in a Brazilian family. The three sibs described here presented different hormone profiles at minipuberty: isolated elevation of FSH (sib 1), isolated elevation of LH (sib 2); and normal FSH, LH and testosterone levels (sib 3) (Table 1). Initially, AR and SRD5A2 gene sequence analyses were performed due to the 46,XY karyotype, genital ambiguity and normal testosterone production. Nevertheless, mutations in those genes had not been identified and the patients remained idiopathic, as the etiologic diagnosis was not defined, even though the severity of genital ambiguity and the familial recurrence clearly indicated a genetic origin. After long-term follow-up, the etiology of 46,XY DSD in these cases had been clarified due to the identification of p.Cys65Tyr mutation on the NR5A1 gene which was investigated based on the recent knowledge on variable phenotypic expression of NR5A1 mutations, and on the possibility of the mutation inheritance from a fertile mother, which mimicked an X-linked recessive pattern.

Several mutations have already been described in NR5A1 in different cases of 46XY DSD [15,17,28,29], however a mutation in the C65 residue has been here described for the first time. The nucleotide change c.195G>A results in the p.Cys65Tyr is predicted as damaging by in silico tools. It is well known that cysteine influences the overall three-dimensional structure of proteins. Its sulfur group reacts quite readily with other sulfur groups, forming disulfide bonds that play important role in the folding and stability of proteins. Cysteine residues also play a valuable role in crosslinking proteins, which increases protein rigidity and also confers proteolytic resistance [30]. Conversely, tyrosine is an aromatic and partially hydrophobic amino acid [30]. The aromatic side chain is usually involved in stacking interactions with other aromatic side chains [30]. Tyrosine can also be involved in phosphorylation within intracellular protein [30]. In the structural analyses, a new hydrophobic interaction with C55 was observed.
for Y65. In addition, this novel mutation is located within the second zinc finger of DNA binding domain and C65 itself binds directly to the zinc atom [25], therefore the change to tyrosine may influence NR5A1 DNA-binding ability by destabilizing zinc-finger conformation. Although further functional analyses will be necessary for the formal demonstration of a deleterious effect for p.Cys65Tyr mutation, both predictive and structural analyses indicate that it might correlate with the DSD phenotype in the three heterozygous siblings. Their fertile 32-year-old mother who is also heterozygous for the mutation referred irregular menses, after molecular diagnosis. Upon endocrine investigation, levels for gonadotropins and estradiol suggest premature ovarian failure. Taking together, those data indicate that p.Cys65Tyr mutations may also compromise the ovarian functional maintenance similar to other NR5A1 mutations described in the literature [15-17].

Considering the slightly elevated ACTH levels in all three patients with 46,XY DSD it may be inferred that p.Cys65Tyr mutation might probably present a late-onset effect upon adrenal function, justifying a long term follow-up on such patients.

In conclusion, based on recent knowledge concerning the phenotypic expression of NR5A1 mutations, the analysis of this gene becomes an important tool for diagnosing patients with DSD including the cases with normal testosterone secretion.

Consents
Written informed consent was obtained from each member of the family for publication of this Case Report and any accompanying images. A copy of the written consent is available for review by the Series Editor of this journal.

Competing interests
The authors declare that they have no competing interests.
Authors’ contributions

HCF carried out the molecular genetic studies with NR5A1 gene, participated in the NR5A1 sequence alignment and drafted the manuscript. JGRA carried out the clinical genetic studies and contributed with writing clinical description of the cases for the manuscript draft. FCS contributed with the structural analysis of normal and mutant proteins. FLC conducted the molecular genetic studies with SRD5A2 gene and sequence alignment investigation. RJP was responsible for the molecular genetic studies with AR gene and sequence alignment comparison. ATM-G participated in the design of the study and was responsible for the clinical genetic evaluation of the patients. GG-J participated in the design of the study and was responsible for the endocrine evaluation of the patients. MP-de-M conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

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References


Legend of the Figures

**Figure 1.** A) Pedigree of the family. The three siblings and the mother carry the mutation c.195G>A. B) Electropherogram showing part of NR5A1 exon 3 sequence where the c.195G>A heterozygous transition leading to p.Cys65Tyr mutation occurred.

**Figure 2.** A) Multiple alignment of NR5A1 protein family using ClustalW: the conserved residue C65 is shown in red. B) Scheme of the two zinc fingers from the DNA-binding domain (DBD). Red circle denotes the C65 residue (adapted from Little *et al.* [25]). C) Structural complex of NR5A1 bound to DNA showing the C65 residue ligated to the zinc atom at the zinc-finger binding site within the DNA binding domain. D) Structural model of the native protein showing internal contacts. The C65 interacts by hydrogen bond with R69 and hydrophobic interaction with C68. E) Mutant protein internal contacts. The Y65 establish new hydrophobic contact with C55.
Table 1: Hormonal values for the three patients.

<table>
<thead>
<tr>
<th>Patient, Age (yr)</th>
<th>Clinical Presentation</th>
<th>Testosterone (nmol/L)</th>
<th>FSH (IU/L)</th>
<th>LH (IU/L)</th>
<th>Estradiol (nmol/L)</th>
<th>Cortisol (nmol/L)</th>
<th>ACTH (pmol/L)</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.75</td>
<td>micropenis and perineal hypospadia</td>
<td>1.04 (pre hCG)</td>
<td>11.90</td>
<td>2.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Started spontaneous puberty</td>
<td>6.59 (after hCG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td></td>
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<tr>
<td>13</td>
<td>Tanner IV</td>
<td>12.87</td>
<td>27.25</td>
<td>11.96</td>
<td>-</td>
<td>-</td>
<td>376.46</td>
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<tr>
<td>14</td>
<td>Tanner IV-V</td>
<td>15.89</td>
<td>23.51</td>
<td>10.95</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td><strong>Patient 2</strong></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>0.17</td>
<td>micropenis and penoscrotal hypospadia</td>
<td>7.63</td>
<td>4.85</td>
<td>10.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6</td>
<td>Tanner I</td>
<td>0.66</td>
<td>1.15</td>
<td>&lt;0.10</td>
<td>-</td>
<td>-</td>
<td>576.01</td>
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<tr>
<td><strong>Patient 3</strong></td>
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<tr>
<td>0.17</td>
<td>micropenis, perineal urogenital opening and bilateral cryptorchidism</td>
<td>0.66 (pre hCG)</td>
<td>4.62</td>
<td>0.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Tanner I</td>
<td>0.66 (after hCG)</td>
<td>6.94</td>
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<tr>
<td><strong>Mother</strong></td>
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</tr>
<tr>
<td>32</td>
<td>Irregular menses</td>
<td>-</td>
<td>48.88</td>
<td>19.83</td>
<td>109.11</td>
<td>218.31</td>
<td>9.66</td>
</tr>
</tbody>
</table>

Normal Range

Newborn: 2.43-13.88
Children: <5.89
Puberty: Tanner I - <0.69
Tanner II - <14.92
Tanner III – 2.25- 27.07
Tanner IV – 6.47-26.37
Tanner V – 6.59-30.54
Male 18-49 yrs: 8.64-29.01

Male 18-49 yrs: 8.64-29.01

Female (menopause): 25.80-7.70
Children: 0.20-3.80
Tanner: 2.25- 27.07
Tanner IV – 6.47-26.37
Tanner V – 6.59-30.54
Male 18-49 yrs: 8.64-29.01

Female (menopause): 25.80-7.70
Children: 0.20-3.80
Tanner: 2.25- 27.07
Tanner IV – 6.47-26.37
Tanner V – 6.59-30.54
Male 18-49 yrs: 8.64-29.01
Figure 1

Figure 2