Wide Spectrum of NR5A1-Related Phenotypes in 46,XY and 46,XX Individuals

Sorahia Domenice[1], Aline Zamboni Machado[1], Frederico Moraes Ferreira[2], Bruno Ferraz-de-Souza[1], Antonio Marcondes Lerario[1], Lin Lin[3], Mirian Yumie Nishi[1], Nathalia Lisboa Gomes[1], Thatiana Evelin da Silva[1], Rosana Barbosa Silva[1], Rafaela Vieira Correa[4], Luciana Ribeiro Montenegro[1], Amanda Narciso[1], Elaine Maria Frade Costa[1], John C Achermann[3], and Berenice Bilharinho Mendonca*[1]

Steroidogenic factor 1 (NR5A1, SF-1, Ad4BP) is a transcriptional regulator of genes involved in adrenal and gonadal development and function. Mutations in NR5A1 have been among the most frequently identified genetic causes of gonadal development disorders and are associated with a wide phenotypic spectrum. In 46,XY individuals, NR5A1-related phenotypes may range from disorders of sex development (DSD) to oligo/azoospermia, and in 46,XX individuals, from 46,XX ovotesticular and testicular DSD to primary ovarian insufficiency (POI). The most common 46,XY phenotype is atypical or female external genitalia with clitoromegaly, palpable gonads, and absence of Müllerian derivatives. Notably, an undervirilized external genitalia is frequently seen at birth, while spontaneous virilization may occur later, at puberty. In 46,XX individuals, NR5A1 mutations are a rare genetic cause of POI, manifesting as primary or secondary amenorrhea, infertility, hypoestrogenism, and elevated gonadotropin levels. Mothers and sisters of 46,XY DSD patients carrying heterozygous NR5A1 mutations may develop POI, and therefore require appropriate counseling. Moreover, the recurrent heterozygous p.Arg92Trp NR5A1 mutation is associated with variable degrees of testis development in 46,XX patients. A clear genotype-phenotype correlation is not seen in patients bearing NR5A1 mutations, suggesting that genetic modifiers, such as pathogenic variants in other testis/ovarian-determining genes, may contribute to the phenotypic expression. Here, we review the published literature on NR5A1-related disease, and discuss our findings at a single tertiary center in Brazil, including ten novel NR5A1 mutations identified in 46,XY DSD patients. The ever-expanding phenotypic range associated with NR5A1 variants in XY and XX individuals confirms its pivotal role in reproductive biology, and should alert clinicians to the possibility of NR5A1 defects in a variety of phenotypes presenting with gonadal dysfunction.

Introduction

NR5A1 (nuclear receptor subfamily 5 group A, member 1), previously known as SF1 (Steroidogenic Factor-1) and Ad4BP (Adrenal 4-Binding Protein), was first cloned by Parker and colleagues in 1992, in an attempt to identify a protein that could activate the promoters of steroid hydroxylase enzymes (Lala et al., 1992).

NR5A1 is expressed in steroidogenic tissues (Honda et al., 1993; Ikeda et al., 1993; Morohashi et al., 1994; Parker and Schimmer, 1997; Ramayya et al., 1997; Morohashi,

1999), pituitary gonadotrophs (Barnhart and Mellon, 1994; Ingraham et al., 1994; Ngan et al., 1999), and neurons located at the dorsomedial portion of the ventromedial hypothalamus (VMH) (Ramayya et al., 1997; Morohashi et al., 1999). In developing embryos, *Nr5a1* is expressed in the urogenital ridge, representing the first marker of gonadal and adrenal differentiation (Ikeda et al., 1994; Morohashi et al., 1995, 1999; Ramayya et al., 1997; Hanley et al., 1999, 2001). Throughout human development, NR5A1 is expressed in the steroid-secreting adrenal cortex, in Leydig and Sertoli cells, as well as in granulosa and theca cells.

NR5A1 stimulates the expression of several genes required for the development and maintenance of the male differentiation cascade. It regulates the expression of *LHCGR* and the steroidogenic enzymes *STAR, CYP11A1*, and *CYP17A1* in Leydig cells, required for testosterone biosynthesis. NR5A1 also increases the expression of insulin-like polypeptide 3 (INSL3), which regulates testicular descent and is a survival factor for male germ cells in adults (Zimmermann et al., 1998; Tremblay and Robert, 2005). Anti-Mullerian hormone (AMH) and its receptor, AMHR2, essential factors for male reproductive tract development, are also regulated by NR5A1. In Sertoli cells, NR5A1 regulates the expression of the testis-determining genes *SRY* and *SOX9* (Jeyasuria et al., 2004).

Mutations in *NR5A1* are emerging as a frequent genetic cause of human 46,XY disorders of sex development (DSD), having been identified throughout the globe. In South America, familial and sporadic DSD patients bearing NR5A1 defects have been described in Brazil and Argentina (Lourenço et al., 2009; Ciaccio et al., 2012; Gabriel Ribeiro de Andrade et al., 2014; Fabbri et al., 2016). Here, we review the phenotype associated with ten novel and one previously described *NR5A1* allelic variants identified in a Brazilian cohort of 46,XY and 46,XX DSD patients followed at a single tertiary center. Our findings are discussed in light of previously published reports, and several aspects of the phenotypic spectrum associated with NR5A1 mutations in humans are reviewed.

Patients

This is a retrospective study approved by the Ethics Committee of Hospital das Clinicas, University of Sao Paulo Medical School. Written informed consents were obtained from all patients or their parents/guardians.

The full cohort of DSD patients followed at the Developmental Endocrinology Unit at Hospital das Clinicas consisted of 83 patients with 46,XY DSD (48 patients with 46,XY gonadal dysgenesis and 35 patients with 46,XY DSD of unknown cause) and 90 patients with 46,XX DSD (70 patients with primary ovarian failure (44 patients had primary amenorrhea and 26 patients had secondary amenorrhea) and 20 patients with 46,XX SRY negative disorders

of ovary development (16 with 46,XX ovotesticular DSD (OTDSD) and 4 with 46,XX testicular DSD (TDSD)).

Molecular Analysis

Mutational analysis of *NR5A1* (Ensembl transcript ESNT00000373588) was performed by Sanger sequencing and by targeted massively parallel sequencing (TMPS), as detailed in Supporting Information.

Identified allelic variants were analyzed according to the joint American College of Medical Genetics and Genomics Association for Molecular Pathology Guidelines (Richards et al., 2015) (see Supporting Information)

IN SILICO ANALYSIS

Alignment of wild-type and mutated *NR5A1* sequences was performed using MUSCLE (Edgar, 2004) and the NCBI's Reference Sequence (RefSeq) database. To evaluate the impact of NR5A1 nonsynonymous mutations at the tertiary protein structure, several structural models were built for wild-type and mutated molecules in the YASARA suite (Krieger et al., 2004), using as templates the crystal structures of the human liver receptor homologue 1 (LRH-1, NR5A2) DNA-binding domain (DBD) in complex with the *hCYP7A1* promoter (PDB accession code 2A66), and the human NR5A1 ligand-binding domain (LBD) in complex with di-pamitoyl-3-SN-phosphatidylethanolamine (PDB accession code 1ZDT). Stereo chemical quality of generated structural models was accessed with MOLPROBITY (Davis et al., 2007). Two molecular mechanics simulations were carried in explicit solvent for each model. Trajectories were calculated with the YAMBER3 force field. Ten thousand simulation steps of 2.5 fs were recorded for each wild type and its corresponding mutated molecules. The final confirmation of the lowest energy models was confirmed for each pair of simulations. The volumes of the cavities of the models were calculated using KVFINDER (Oliveira et al., 2014). Molecular models were drawn using PyMOL (www.pymol.org).

IN VITRO FUNCTIONAL STUDIES

Transient gene expression assays were performed using human embryonic kidney TSA-201 cells to analyze transcriptional activation of murine *Cyp11a1* and rat *Cyp19* promoters by wild-type or mutant NR5A1 (Lin et al., 2007) (see Supporting Information).

Results

We identified 11 pathogenic *NR5A1* variants, including 10 novel variants in 46,XY DSD patients and one previously reported variant in a 46,XX testicular DSD patient.

CLINICAL AND HORMONAL CHARACTERISTICS OF 46,XY DSD PATIENTS BEARING PATHOGENIC *NR5A1* VARIANTS

Nine of ten 46,XY DSD patients presented with atypical genitalia at birth and one had female external genitalia. Nine patients were assigned as female at birth, one of

TABLE 1. *Clinical Phenotype of Ten 46,XY DSD Patients and One 46,XX Testicular DSD Patient in Whom NR5A1 Mutations Were Identified*

Patient	Karyotype	Age at first evaluation (years)	External genitalia	Mullerian structures	Age at surgery	Sex of rearing	Family
1	46,XY	0.5	Atypical Phallus 3 cm, perineal hypospadias, palpable testes	A	Genitoplasty at 1.6 years	Male	NA
2[a]	46,XY	0.7	Atypical Clitoromegaly, palpable gonads	A	Gonadectomy at 9 m[a]; Genitoplasty at 2 years	Female	NA
3	46,XY	4.9	Atypical Single perineal opening, palpable gonads	A	Gonadectomy and Genitoplasty at 4.9 years	Female	M – WT, F – NA
4[a]	46,XY	12	Atypical Clitoromegaly (2.0 cm),single perineal opening, palpable gonads	A	Gonadectomy at 3 years	Female	NA
5	46,XY	12.3	Atypical Clitoromegaly (4.0 cm), two perineal openings, no palpable gonads. Virilization at puberty	A	Gonadectomy and Genitoplasty at 12.5 years	Female	Mother is a carrier of the mutation and presented with POI
6	46,XY	13	Atypical Clitoromegaly (3cm), labial fusion, no palpable gonads	P	Gonadectomy and Genitoplasty at 15.5 years	Female	Mother is a carrier of the mutation, F – NA
7[a]	46,XY	16.7	Atypical Clitoromegaly, left gonad palpable	P	Gonadectomy at 14 years; Genitoplasty at 15 years	Female	NA
8	46,XY	21	Female genitalia	P	Not performed	Female	NA
9[a]	46,XY	26	Atypical Phallus 6.5 cm, perineal hypospadias, palpable testes	A	Genitoplasty at 26 years	Female to Male (9 years)	NA
10[a]	46,XY	39	Atypical, clitoromegaly (3cm), two perineal opening	A	Gonadectomy at 14 years; Genitoplasty at 39 years	Female	NA
11[a]	46,XX	59	Atypical, no palpable gonads	A	Genitoplasty in childhood	Male	NA

[a]Surgery performed previously to the evaluation in our center.

P, present; A, absent; F, father; M, mother; NA, not available; years, years of age; POI, primary ovarian insufficiency.

whom reassigned to male at 9 years of age (patient 9, Table 1); only one patient was assigned as male at birth (patient 1, Table 1). The initial assessment at our specialist unit ranged from 0.75 to 39 years of age. Only three patients were seen in childhood, and the remaining after puberty; four of these patients had previously undergone bilateral gonadectomy. The patient with normal-appearing female external genitalia was referred at 21 years of age due to primary amenorrhea (patient 8, Table 1).

Basal hormonal evaluation was available for seven patients. Gonadotropin levels were available in three

prepubertal and in four postpubertal patients. All postpubertal patients had elevated gonadotropin levels, mainly FSH. Among four postpubertal patients, basal testosterone levels were normal for age in three (360, 155, and 414 ng/dl, respectively) and low in the single patient with normal female external genitalia (patient 8). A stimulation test with hCG was performed in four patients (two prepubertal and two pubertal) and in all of them testosterone levels increased adequately (Table 2).

Seven patients underwent bilateral gonadectomy; age at gonadectomy ranged from 9 months to 15.5 years. The two patients who were raised as males had inguinal testes and underwent bilateral orchiopexy. Gonadectomy was not performed in one patient who discontinued follow-up care. In three patients, a uterus was identified by pelvic ultrasound or MRI (Table 1).

None of the patients had symptoms or signs of adrenal insufficiency, and normal basal adrenal function was documented in six patients.

A family history of DSD was presented in three cases. Patient 6 had two paternal cousins with atypical genitalia. Patient 7 reported two second-degree cousins with primary amenorrhea. Patient 5's mother, who also carried the NR5A1 variant, developed ovarian insufficiency at 39 years.

CLINICAL AND HORMONAL CHARACTERISTICS OF THE 46,XX DSD PATIENT BEARING A PATHOGENIC NR5A1 VARIANT

Although mutations in NR5A1 have been reported in a small proportion of women with primary ovarian insufficiency (POI), NR5A1 mutations were not identified in 70 patients with POI in our cohort. However, the previously described p.Arg92Trp NR5A1 mutation was found in heterozygous state in one 46,XX patient with testicular DSD. This patient (Patient 11, Table 1) was first seen in our Unit when he was 59 years old. He was born with atypical genitalia and had undergone masculinizing genitoplasty during childhood. Spontaneous secondary sexual characteristics such as phallic enlargement were absent and testosterone replacement was started at 15 years of age. Bilateral mastectomy was performed at 18 years of age.

TABLE 2. *Hormonal Profiles of Brazilian 46,XY DSD and 46,XX Testicular DSD Patients in Whom NR5A1 Mutations Were Identified*

Patient	Age (years)	FSH (U/l)	LH (U/l)	Testosterone (ng/dl)	
				Basal	After hCG
1	0.5	NA	NA	11	458
	13	6	5	NA	508
2[a]	0.7	2	4	NA	12
3	4.9	5	<0.6	14	169
4[a]	12	NA	NA	NA	NA
5	12.3	77	13	414	NA
6[a]	13	NA	NA	NA	277
7	13	69	24	155	295
8	21	60	16	14	NA
11[a]	26	21	14	360	499
9[a]	39	NA	NA	NA	NA
10[a]	59	45	28	178	NA

Chronological age corresponding to the hormonal evaluation.
Conversion factors to SI units: T, ng/dl to nmol/l, multiply by 0.0347
[a]Surgery performed previously to the evaluation in our Center
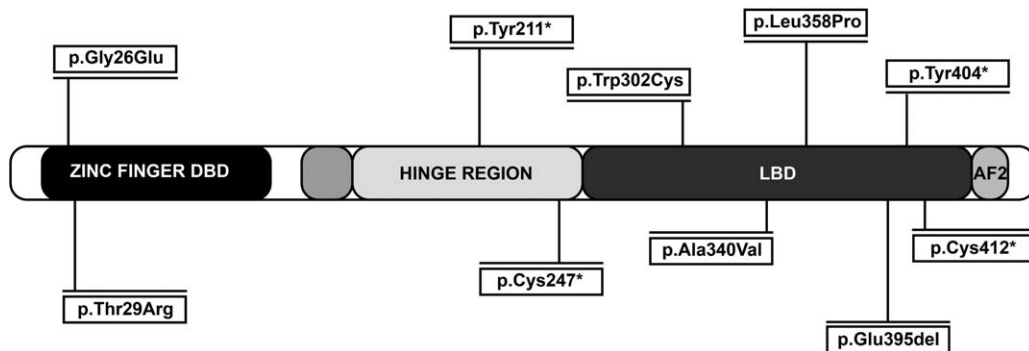NA, not available.

FIGURE 1. *The ten novel mutations identified in 46,XY DSD patients and their localization on the NR5A1 protein. DBD, DNA-binding domain; LBD, ligand-binding domain; AF2, activation function domain.*

TABLE 3. *Molecular Characterization of NR5A1 Defects Identified in Heterozygous State in Ten 46,XY and in One 46,XX Testicular DSD Patients*

Patient	Mutation (DNA)	Mutation (protein)	Domain	Functional impact	Bind energy
8	c.77G>A	p.Gly26Glu	DBD (1st zinc finger)	In silico prediction: damaging	↓
9	c.86C>G	p.Thr29Arg	DBD	In silico prediction:damaging	NA
5	c.663C>G	p.Tyr211*	Hinge	Truncated protein	NA
2	c.741C>A	p.Cys247*	Hinge	Truncated protein	NA
7	c.906G>C +c.437G>C	p.Trp302Cys +p.Gly146Ala	LBD Hinge	In silico prediction: damaging	↑
1	c. 1019C>T	p.Ala340Val	LBD	In silico prediction: damaging	NA
3	c.1073T>C	p.Leu358Pro	LBD	Impaired transactivation[fs a]	↑
6	c.1183_1185del	p.Glu395del	LBD	In silico prediction: damaging	↓
10	c.1212C>G +c.437G>C	p.Tyr404* +p.Gly146Ala	LBD Hinge	Truncated protein	↑
11	c.1236C>A	p.Cys412*	LBD	Truncated protein	NA
4	c.274C>T	p.Arg92Trp	DBD (A box)	Ref	NA

The alignment of the rows is according to mutation location in the NR5A1protein. Binding energy to the "ligand di-pamitoyl-3-SN-phosphatidylethanolamine of the mutated complex compared to the non-mutated complex.

Functional studies of mutated NR5A1 activity shown a reduction in the transcriptional activity of Cyp11a1 and Cyp19 promoters.

↓, reduced bind energy; ↑, increased bind energy; DBD, DNA-binding domain; LBD, ligand-binding domain; NA, not available.

He did not have symptoms or signs of adrenal insufficiency, and basal adrenal profiling was normal. Peripheral blood leukocyte DNA was PCR-analyzed for Y-specific genes such as *SRY, TSPY, AMGY, DYZ3, DYS280*, and *DYS1*, all of which were not amplified and therefore considered absent. Pelvic MRI performed at 59 years of age revealed small bilateral testes in the inguinal regions; a uterus was not identified.

MOLECULAR CHARACTERISTICS OF NOVEL NR5A1 DEFECTS

Ten novel heterozygous *NR5A1* mutations were identified in 46,XY DSD patients (Fig. 1), including five nonsynonymous variants (p.Gly26Glu, p.Thr29Arg, p.Trp302Cys, p.Ala340Val, p.Leu358Pro), four stop-gain variants (p.Tyr211*, p.Cys247*, p.Tyr404*, p.Cys412*), and one frameshift variant (p.Glu395-del) (Table 3, Fig. 1). In three cases DNA samples were obtained from patients' mothers; two of them carried *NR5A1* mutations (Table 3).

The previously described common nonsynonymous p.Gly146Ala variant (rs1110061) was identified in two patients in association with p.Trp302Cys and p.Tyr404* *NR5A1* mutations, respectively.

IN SILICO STUDIES OF THE NR5A1 VARIANTS IDENTIFIED IN 46,XY PATIENTS

Primary sequence analyses showed that all NR5A1 mutated amino acids are extremely conserved among non-redundant homologous and relative sequences. Regarding potential and binding energies studies, native and mutated energy distributions of the p.Gly26Glu, p.Trp302Cys, p.Leu358Pro, p.Tyr404*, and p.Glu395del variants were compared. The LBD domain mutations p.Trp302Cys and p.Tyr404* both led to a higher energy state and therefore to a less stable molecule in comparison with the wild-type protein (Fig. 2).

The p.Gly26Glu variant, located in the DBD, establishes a polar contact not found in the original molecule, increasing its affinity for DNA binding and making the NR5A1 dissociation from the DNA helix difficult, thus potentially impairing transcriptional regulation. Interestingly, the p.Gly26Glu variant seems to stabilize the DBD, leading to a smaller energy compared to that of the native domain (Fig. 2). As a consequence, an opposite behavior was observed for binding energies. While all LBD mutated molecules studied increased the bind energy to the ligand di-pamitoyl-3-SN-phosphatidylethanolamine, p.Gly26Glu provided smaller binding energy to the DNA helix. Potential and binding energy means, variations, and overall conformations between mutated and native molecules are shown in Table 4.

Figure 3 presents the lowest energy molecular models of all mutated domains superposed to their respective native versions. Replacement of Gly by Glu at position 26 creates an additional contact not present in the original
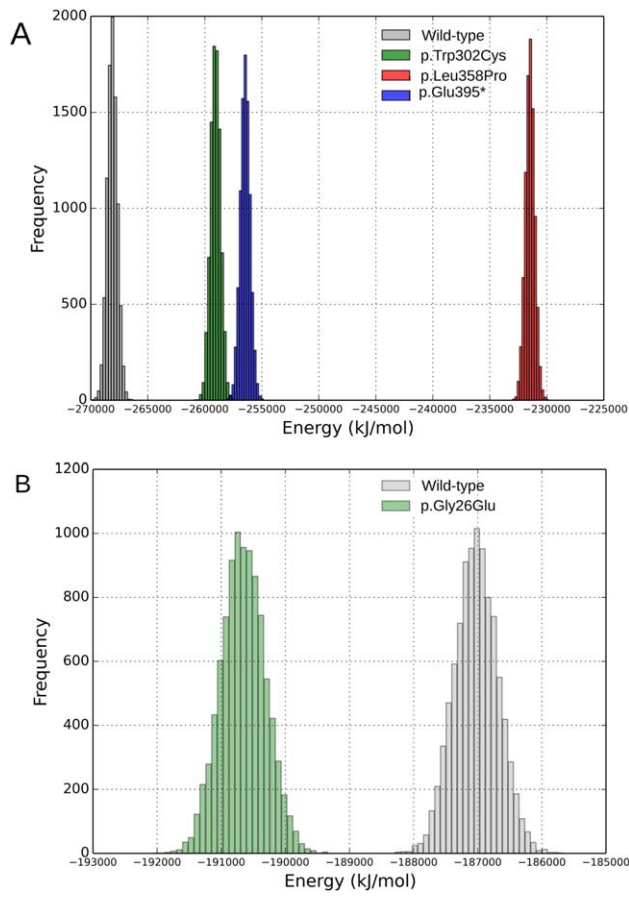
TABLE 4. *Variation of the Potential and Binding Energies Means Overall 9.000 Conformations Between Mutated and Native Molecules*

Mutation/domain	ΔE (kJ/mol)	ΔE_{bind} (kJ/mol)
p.Gly26Glu/DBD	3633	−1746
p.Trp302Cys/LBD	9053	36
p.Leu358Pro/LBD	36,639	36
p.Glu395 del/LBD	11,656	59

All samples distributions passed Shapiro-Wilk normality test, being means compared with Welsh's t-test.

All resultant *p* values were less than 2.2×10^{-16}.

FIGURE 2. (**A**) *Energy distributions of the native NR5A1 LBD domain and mutated molecules. A higher energy is associated with a less stable molecule compared to the native due loss of amino acid contacts.* (**B**) *Energy distributions of the native NR5A1 DBD domain and its p.Gly26Glu mutated molecule. A smaller energy is associated to a more stable molecule, compared to the native due additional amino acids contacts.*

DBD domain. The negatively charged Glu26 Oε2 forms a salting bridge with the Arg79 Nε, which makes the β-hairpin 21-20 less flexible and closer to the ligand DNA. In the native LBD domain, the Try302imidazolic ring contributes to form a hydrophobic environment of the active site bottom cleft required to accommodate fatty ligands. Removal of such a bulky amino acid at that position induces a whole structural reorganization so that the central cavity of the protein shrinks, decreasing the occluded volume from 885 Å3 to 724 Å3. In turn, the p.Leu358Pro mutation is located in a hydrophobic core surrounded by four α-helices. In the original molecule, Leu358 establishes several contacts with a network of hydrophobic residues. Although proline is a non-polar amino acid, its side chain is too small compared to leucine and cannot effectively reach the surrounded hydrophobic residues. Therefore, many hydrophobic contacts were lost, which may explain

the potential energy increasing. This change may be responsible for the reduction of transcriptional activity observed in the functional studies described below.

The variants p.Tyr211* and p.Cys247* are located in hinge region of NR5A1 that is important for stabilizing the LBD and interaction with other proteins that control NR5A1 transcriptional activity.

Finally, the Glu385 deletion is located within LDB helix-9, which is in the opposite direction of the ligand cleft. Together with the N-terminal helix-1, these two external helices tightly embrace the entire domain. In addition, the removal of the Glu385 slightly rotates the helix-9 axial axis; weakening several lateral contacts and making the helix unroll half a turn. All these modifications are predicted to be transmitted throughout the whole domain decreasing its affinity to the ligand.

The results of the molecular simulation analyses strongly support the hypothesis that all these novel mutations potentially interfere with the activity of the NR5A1 protein. Its structural stability and its ability to bind the di-palmitoyl-3-SN-phosphatidylethanolamine and probably other fat can be affected by the presence of the mutations. The function of such fat acid has not been cleared; however, it certainly provides additional contacts and stability to the whole molecule to perform its activities. Altogether, the simulation results strengthen the hypothesis that the studied mutations are very likely deleterious.

IN VITRO FUNCTIONAL STUDIES OF THE P.LEU358PRO NR5A1 VARIANT

In vitro studies showed a markedly impaired transcriptional activity of the p.Leu358Pro mutant NR5A1, with a reduction of more than 70% and 80% in the transactivation of the *Cyp11a1* and *Cyp19* promoters, respectively (Fig. 4).

Discussion

NR5A1 MUTATIONS IN 46,XY INDIVIDUALS

Since the first 46,XY DSD patient with the p.Gly35Glu *NR5A1* mutation described by Achermann et al. (1999), the spectrum of phenotypes associated with *NR5A1* mutations has greatly expanded. This initial patient bearing the
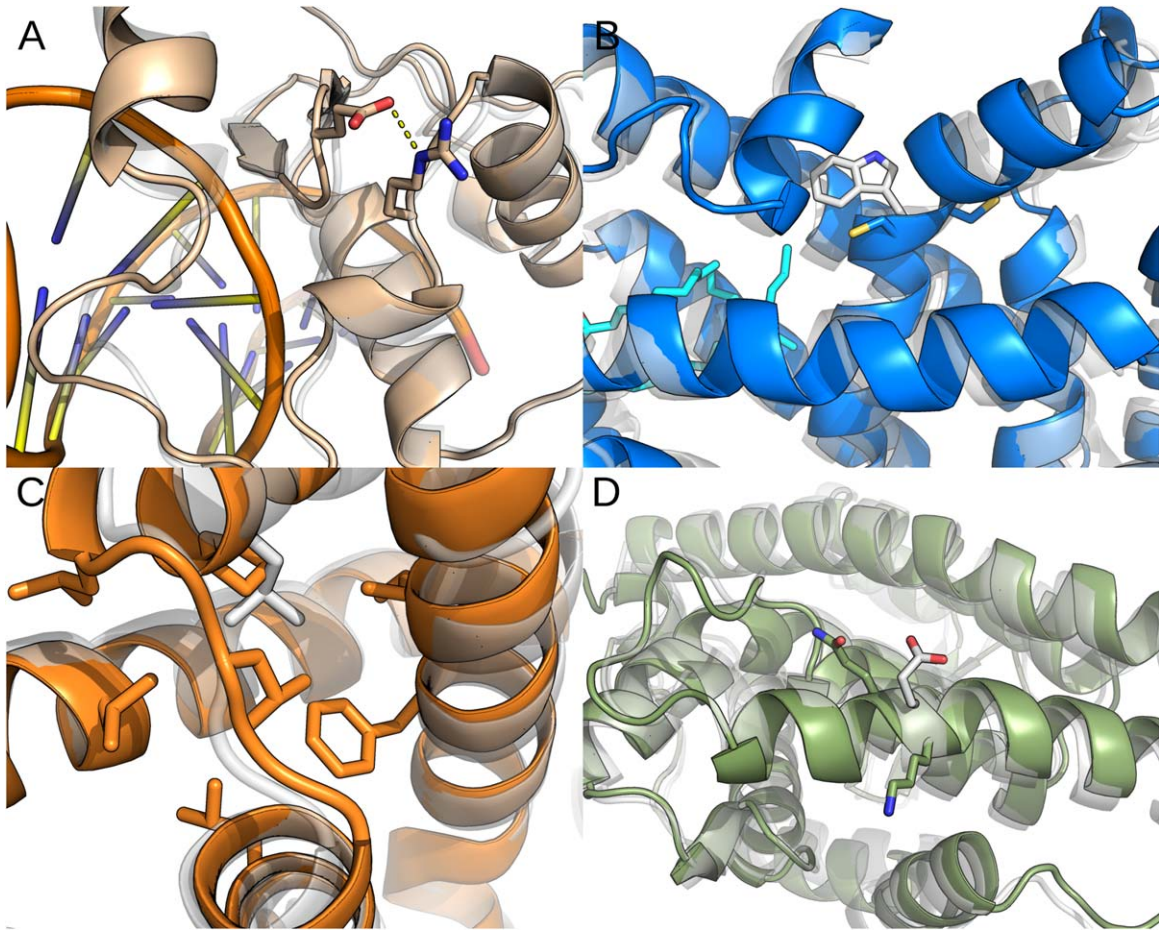
FIGURE 3. *Superimposed Models: Superimposition of the native (white) and mutated smallest energy NR5A1 models (colored): (**A**) DBD domain showing Glu26 and Arg79 salt bridge; (**B**) LBD domain showing Try302Cys; (**C**) LBD hydrophobic network with Leu358Pro in the center; and (**D**) LBD helix-9 rotated and unrolled half a turn with the Glu385 deletion.*

heterozygous p.Gly35Glu mutation presented with adrenal failure and gonadal dysgenesis with persistent Mullerian derivatives (Woo et al., 2015), a phenotype that closely resembled the phenotype observed in murine *Nr5a1* knockout models (Luo et al., 1994). Gonadal dysgenesis and adrenal insufficiency were also present in the second reported NR5A1-related 46,XY DSD, in a patient bearing the homozygous p.Arg92Gln mutation (Achermann et al., 2002).

The expected phenotype associated with *NR5A1* mutations was shifted when a heterozygous 8-bp microdeletion was found in a 46,XY DSD patient who presented with clitoromegaly, absence of uterus and gonads but normal adrenal function (Correa et al., 2004). After this first report, the study of several cohorts of individuals with 46,XY DSD has shown that adrenal insufficiency is a rare finding in patients with NR5A1 defects (Lin et al., 2006; Guran et al., 2016).

Reported heterozygous *NR5A1* mutations support the model that partial NR5A1 dysfunction can result in several degrees of impaired Leydig cell function and androgen biosynthesis, leading to predominantly abnormal gonadal phenotypes, which can range from complete testicular dysgenesis with Mullerian structures, through mild clitoromegaly or atypical genitalia without Mullerian derivatives, to proximal hypospadias associated with undescended testis (Köhler et al., 2009), or even micropenis with absent gonads (Philibert et al., 2007).

Currently, *NR5A1* mutations represent one of the most frequent defects associated with 46,XY gonadal dysgenesis, accounting for up to 20% of cases (Suntharalingham et al., 2015).

More than 80 different *NR5A1* variants, distributed across the full length of the protein, have been described and the majority are nonsynonymous mutations (Pedace et al., 2014; Tantawy et al., 2014; Woo et al., 2015; Fabbri et al., 2016). Most of these mutations are located in the DBD and are in a heterozygous state or compound heterozygous state with the p.Gly146Ala (rs1110061) variant,
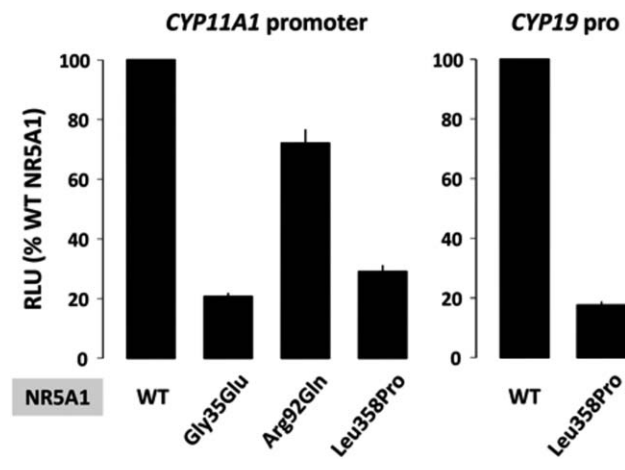
FIGURE 4. *Transcriptional activation of target gene promoters by wild-type (WT) and mutant NR5A1 in human embryonic kidney TSA-201 cells. In vitro activation of CYP11A1 and CYP19 gene promoters by the novel NR5A1 mutant p.Leu358Pro was reduced, in comparison to WT NR5A1 (29% and 18% of WT, respectively). Notably, the degree of transcriptional activity impairment at the CYP11A1 promoter by p.Leu358Pro NR5A1 was comparable to that of the well-known NR5A1 mutant p.Gly35Glu (Lin et al., 2007) and more severe than p.Arg92Gln (Achermann et al., 2002). Results represent the mean (SEM) for three assays, each performed in triplicate, and shown relative to wild-type transactivation. RLU, relative luciferase units.*

with the exception of two mild mutations described in homozygous state (Achermann et al., 2002; Soardi et al., 2010). These findings reinforce the concept that NR5A1 dosage is critical to normal gonadal development. However, a clear correlation between the location of a mutation, its in vitro functional performance, and the associated phenotype is not observed. Indeed, family members bearing the same *NR5A1* mutation may present with variable phenotypes (Warman et al., 2011).

There are some hypotheses to explain the phenotypic variation associated with a similar *NR5A1* mutation. First, interallelic association with the known p.Gly146Ala polymorphism (rs1110061) may further reduce NR5A1 activity and contribute to more severe phenotypes (WuQiang et al., 2003; Hasegawa et al., 2004; Wada et al., 2006; Reuter et al., 2007; Köhler et al., 2008; Lourenço et al., 2009; Bashamboo et al., 2010a; Paris et al., 2011; Camats et al., 2012). However, two patients in our cohort also bore the p.Gly146Ala variant besides the pathogenic NR5A1 variants, p.Trp302Cys and p.Tyr404*, and their phenotype was not strikingly distinct or more severe.

The contribution of other genetic modifiers has also been suggested to explain phenotypic variability. Exome sequencing analyses of DSD patients have identified pathogenic variants or variants of uncertain significance in several genes involved in sexual development (Bashamboo et al., 2010b; Bashamboo et al., 2014). In a 46,XY patient

with atypical external genitalia, palpable inguinal gonads, absent uterus in pelvic ultrasonography, and poor testosterone response to hCG stimulation, Mazen and colleagues (2016) identified, by exome sequencing, the previously described p.Arg313Cys *NR5A1* mutation in compound heterozygous state with a p.Gln237Arg *MAP3K1* variant. This *NR5A1* mutation was previously reported in association with mild hypospadias (Allali et al., 2011), and a possible digenic inheritance was proposed to explain the phenotypic heterogeneity (Mazen et al., 2016).

Progressive androgen production and virilization in adolescence has been observed in several XY patients with *NR5A1* mutations, in contrast to the severe undervirilized external genitalia found in most patients (Cools et al., 2012; Gabriel Ribeiro de Andrade et al., 2014; Tantawy et al., 2014; Fabbri et al., 2016). The almost normal testosterone levels after hCG stimulation or at pubertal age suggest that NR5A1 action may be less implicated in pubertal steroidogenesis than during fetal life. NR5A1 is expressed in the both fetal and adult Leydig cells, but differences in its actions at both stages are not completely known. The study of a hypomorphic mouse model of *Nr5a1* indicated differential impairment of fetal and adult Leydig cell development, whereby *Nr5a1* may regulate the differentiation of fetal Leydig cells, whereas in the adult it may regulate progenitor cell formation and/or survival (Karpova et al., 2015). Different actions of NR5A1 in fetal and postnatal Leydig cells populations might contribute to the switch from birth to pubertal phenotypes.

In contrast, fetal Sertoli cell function seems to be preserved in the most patients with heterozygous *NR5A1* mutations, based on the common observation of absent Müllerian derivatives and primitive seminiferous tubules on histology. In our cohort, three of 10 patients had Müllerian derivatives although in one of them the uterine volume remained infantile after three years of estrogen replacement.

The reviewed data of seventy-two 46,XY DSD patients with *NR5A1* mutations reported in the literature, for whom information on presence or absence of Müllerian derivatives was available, suggested that Müllerian derivatives are present in about 24% of the cases (Pedace et al., 2014; Tantawy et al., 2014; Woo et al., 2015; Fabbri et al., 2016). However, persistently elevated FSH levels after puberty found in all patients studied suggest an impairment of Sertoli cells function in post pubertal age (Pedace et al., 2014).

Male infertility has been also related to the presence of *NR5A1* defects (Bashamboo et al., 2010a; Ferlin et al., 2015). Patients with moderate/severe oligospermia or azoospermia and *NR5A1* mutations may have normal testosterone and normal low or low inhibin B levels, but they are at potential risk of deterioration of testicular hormonal secretion with age and may need counseling regarding

preservation of sperm and regular monitoring of endocrine function (Bashamboo et al., 2010a).

The presence of ovotestis was described in a 46,XY girl, with the 9q33.3-q34.1 deletion encompassing *NR5A1* and *LMX1B* genes causing genitopatellar syndrome associated with female external genitalia and clitoromegaly (Schlaubitz et al., 2007).

NR5A1 MUTATIONS IN 46,XX SUBJECTS

The first reported 46,XX patient with a *NR5A1* mutation (p.Arg255Leu) presented with isolated adrenal insufficiency, but a follow-up description of ovarian function at postpubertal age is lacking (Biason-Lauber and Schoenle, 2000). Subsequently, it was shown that *NR5A1* mutations are also a genetic cause of POI, with phenotypes ranging from primary to secondary amenorrhea, associated with infertility, hypoestrogenism, and elevated gonadotropin levels (Lourenço et al., 2009; Camats et al., 2012) Sisters and mothers of 46,XY DSD patients carrying heterozygous *NR5A1* mutations may also develop premature ovarian failure (Lourenço et al., 2009; Camats et al., 2012; Fabbri et al., 2016).

Several genes required for ovarian steroidogenesis, and follicle growth and maturation are regulated by *NR5A1*, explaining why mutant *NR5A1* may lead to progressive loss of ovarian function and female reproductive capacity (Lourenço et al., 2009; Camats et al., 2012; Fabbri et al., 2016). Women bearing *NR5A1* defects should receive appropriate counseling and fertility guidance, enabling potential oocyte cryopreservation, due to the risk of developing ovarian insufficiency later in life.

In contrast to its prominent pathogenic role in 46,XY DSD, NR5A1 defects are a rare cause of sporadic POI of unknown etiology in women (1.4–1.6%). (Suntharalingham et al., 2015) Sixteen different *NR5A1* variants distributed across the full length of the protein have been described in patients with premature ovarian failure, and the majority of them are non-synonymous mutations (Suntharalingham et al., 2015)

The spectrum of disorders associated with *NR5A1* variants in 46,XX individuals was further expanded by the demonstration of the specific recurrent heterozygous p.Arg92Trp NR5A1 variant, in association with variable degrees of testis development in 46,XX patients from unrelated families (Baetens et al., 2016; Bashamboo et al., 2016; Igarashi et al., 2017). The p.Arg92Trp *NR5A1* mutation was described in ten *SRY* negative 46,XX DSD patients, six with testicular DSD and four with ovotesticular DSD. This mutation was found in two familial cases: the first one comprising two 46,XX ovotesticular DSD sisters and the second one involving one 46,XX testicular DSD patient and his 46,XY sister with partial gonadal dysgenesis. In these two families a maternal inheritance was identified. Atypical genitalia was the most frequent presentation (six patients), but a phenotypic spectrum ranging from female external genitalia with clitoromegaly (one patient), to male external genitalia with micropenis (two patients) or

penoscrotal hypospadias (one patient) was observed. Three of eight patients had a uterus, and two had a hemi-uterus. Gonads were palpable in seven patients and the other three 46,XX ovotesticular patients had bilateral abdominal ovotestis.

The p.Arg92Gln NR5A1 mutation, located at the same residue, was previously described in homozygous state in a 46,XY phenotypic female with adrenal insufficiency and in homozygous state in a 46,XX girl with early-onset primary adrenal insufficiency. This variant was also present, in heterozygous state, in her phenotypically normal sister, mother, and father. (Achermann et al., 2002; Guran et al., 2016) Differences in the transactivating activity of the p.Arg92Gln and the p.Arg92Trp mutants may be responsible for the significant phenotypic differences observed (Igarashi et al., 2017) Nevertheless, the contribution of modifying allelic variants in other genes involved in gonadal development may not be completely excluded.

In vitro assays demonstrated that the p.Arg92Trp mutant lost its binding capacity to a consensus NR5A1 response element and reduced activation of some minimal promoters (*Amh, Cyp11a1*) (Bashamboo et al., 2016). This mutated protein was also less sensitive to NR0B1-induced suppression on the *SOX9* TESCO element, leading to testis formation (Igarashi et al., 2017). These findings suggest a dysregulation of normal female gonadal development by p.Arg92Trp NR5A1, tipping the balance toward testis development in 46,XX individuals.

Conclusion

In a single-center Brazilian cohort, we have identified 10 novel *NR5A1* mutations in 46,XY DSD patients and the previously described p.Arg92Trp mutation in one 46,XX testicular DSD patient. Additionally, no mutations were found in 70 patients with POI. Our findings reinforce those of previous reports and contribute to expanding our understanding of NR5A1-related phenotypes in humans.

NR5A1 mutations are associated with a wide spectrum of disorders of gonadal development, ranging from DSD to oligo/azoospermia in 46,XY individuals and 46,XX ovotesticular and testicular phenotypes to primary ovarian failure in 46,XX individuals. The most frequent phenotype in 46,XY patients is atypical or female external genitalia with clitoromegaly, palpable gonads, and absence of Müllerian derivatives. Postnatally, Leydig cells function seems to be preserved in several patients with *NR5A1* mutation, while Sertoli and germ cells are more profoundly affected. The severely undervirilized external genitalia frequently seen at birth, contrasts with spontaneous virilization at puberty in several patients. Phenotypic variability observed in patients with similar NR5A1 defects may be related to the association with genetic modifiers or pathogenic variants in other testis/ovarian-determining genes.

References

http://mutationassessor.org/r3/MutationAssessor_white_paper.pdf.

Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL. 1999. A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. Nat Genet. 22(2):125–126.

Achermann JC, Ozisik G, Ito M, et al. 2002. Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose-dependent manner. J Clin Endocrinol Metab 87(4):1829–1833.

Adzhubei IA, Schmidt S, Peshkin L, et al. 2010. A method and server for predicting damaging missense mutations. Nat Methods 7(4):248–249.

Allali S, Muller JB, Brauner R, et al. 2011. Mutation analysis of NR5A1 encoding steroidogenic factor 1 in 77 patients with 46,XY disorders of sex development (DSD) including hypospadias. PLoS One 6(10):e24117.

Auton A, Brooks LD, Durbin RM, et al. 2015. A global reference for human genetic variation. Nature 526(7571):68–74.

Baetens D, Stoop H, Peelman F, et al. 2016. NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development. Genet Med doi:10.1038/gim.2016.118 [Epub ahead of print].

Barnhart KM, Mellon PL. 1994. The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. Mol Endocrinol 8(7):878–885.

Bashamboo A, Brauner R, Bignon-Topalovic J, et al. 2014. Mutations in the FOG2/ZFPM2 gene are associated with anomalies of human testis determination. Hum Mol Genet 23(14):3657–3665.

Bashamboo A, Donohoue PA, Vilain E, et al. 2016. A recurrent p.Arg92Trp variant in steroidogenic factor-1 (NR5A1) can act as a molecular switch in human sex development. Hum Mol Genet 25(16):3446–3453.

Bashamboo A, Ferraz-de-Souza B, Lourenço D, et al. 2010a. Human male infertility associated with mutations in NR5A1 encoding steroidogenic factor 1. Am J Hum Genet 87(4):505–512.

Bashamboo A, Ledig S, Wieacker P, et al. 2010b. New technologies for the identification of novel genetic markers of disorders of sex development (DSD). Sex Dev 4(4–5):213–224.

Biason-Lauber A, Schoenle EJ. 2000. Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. Am J Hum Genet 67(6):1563–1568.

Camats N, Pandey AV, Fernández-Cancio M, et al. 2012. Ten novel mutations in the NR5A1 gene cause disordered sex development in 46,XY and ovarian insufficiency in 46,XX individuals. J Clin Endocrinol Metab 97(7):E1294–1306.

Chun S, Fay JC. 2009. Identification of deleterious mutations within three human genomes. Genome Res 19(9):1553–1561.

Ciaccio M, Costanzo M, Guercio G, et al. 2012. Preserved fertility in a patient with a 46,XY disorder of sex development due to a new heterozygous mutation in the NR5A1/SF-1 gene: evidence of 46,XY and 46,XX gonadal dysgenesis phenotype variability in multiple members of an affected kindred. Horm Res Paediatr 78(2):119–126.

Cools M, Hoebeke P, Wolffenbuttel KP, et al. 2012. Pubertal androgenization and gonadal histology in two 46,XY adolescents with NR5A1 mutations and predominantly female phenotype at birth. Eur J Endocrinol 166(2):341–349.

Correa RV, Domenice S, Bingham NC, Billerbeck AE, Rainey WE, Parker KL, Mendonca BB. 2004. A microdeletion in the ligand binding domain of human steroidogenic factor 1 causes XY sex reversal without adrenal insufficiency. J Clin Endocrinol Metab. 89(4):1767–1772.

Davis IW, Leaver-Fay A, Chen VB, et al. 2007. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res 35(Web Server issue):W375–W383.

Davydov EV, Goode DL, Sirota M, et al. 2010. Identifying a high fraction of the human genome to be under selective constraint using GERP++. PLoS Comput Biol 6(12):e1001025.

Dong C, Wei P, Jian X, et al. 2015. Comparison and integration of deleteriousness prediction methods for nonsynonymous SNVs in whole exome sequencing studies. Hum Mol Genet 24(8):2125–2137.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5):1792–1797.

Fabbri HC, Ribeiro de Andrade JG, Maciel-Guerra AT, et al. 2016. NR5A1 loss-of-function mutations lead to 46,XY partial gonadal dysgenesis phenotype: report of three novel mutations. Sex Dev 10(4):191–199.

Ferlin A, Rocca MS, Vinanzi C, et al. 2015. Mutational screening of NR5A1 gene encoding steroidogenic factor 1 in cryptorchidism and male factor infertility and functional analysis of seven undescribed mutations. Fertil Steril 104(1):163–169.e161.

Gabriel Ribeiro de Andrade J, Marques-de-Faria AP, Fabbri HC, et al. 2014. Long-term follow-up of patients with 46,XY partial gonadal dysgenesis reared as males. Int J Endocrinol 2014:480724.

Garber M, Guttman M, Clamp M, et al. 2009. Identifying novel constrained elements by exploiting biased substitution patterns. Bioinformatics 25(12):i54–i62.

Gnirke A, Melnikov A, Maguire J, et al. 2009. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nat Biotechnol 27(2):182–189.

Guran T, Buonocore F, Saka N, et al. 2016. Rare causes of primary adrenal insufficiency: genetic and clinical characterization of a large nationwide cohort. J Clin Endocrinol Metab 101(1):284–292.

Hanley NA, Ball SG, Clement-Jones M, et al. 1999. Expression of steroidogenic factor 1 and Wilms' tumour 1 during early human gonadal development and sex determination. Mech Dev 87(1–2): 175–180.

Hanley NA, Rainey WE, Wilson DI, et al. 2001. Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15(1): 57–68.

Hasegawa T, Fukami M, Sato N, et al. 2004. Testicular dysgenesis without adrenal insufficiency in a 46,XY patient with a heterozygous inactive mutation of steroidogenic factor-1. J Clin Endocrinol Metab 89(12):5930–5935.

Honda S, Morohashi K, Nomura M, et al. 1993. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. J Biol Chem 268(10):7494–7502.

Igarashi M, Takasawa K, Hakoda A, et al. 2017. Identical NR5A1 missense mutations in two unrelated 46,XX individuals with testicular tissues. Hum Mutat 38(1):39–42.

Ikeda Y, Lala DS, Luo X, et al. 1993. Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. Mol Endocrinol 7(7):852–860.

Ikeda Y, Shen WH, Ingraham HA, Parker KL. 1994. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Mol Endocrinol 8(5):654–662.

Ingraham HA, Lala DS, Ikeda Y, et al. 1994. The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. Genes Dev 8(19):2302–2312.

Jeyasuria P, Ikeda Y, Jamin SP, et al. 2004. Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. Mol Endocrinol 18(7):1610–1619.

Karpova T, Ravichandiran K, Insisienmay L, et al. 2015. Steroidogenic factor 1 differentially regulates fetal and adult leydig cell development in male mice. Biol Reprod 93(4):83.

Kircher M, Witten DM, Jain P, et al. 2014. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 46(3):310–315.

Krieger E, Darden T, Nabuurs SB, et al. 2004. Making optimal use of empirical energy functions: force-field parameterization in crystal space. Proteins 57(4):678–683.

Köhler B, Lin L, Ferraz-de-Souza B, et al. 2008. Five novel mutations in steroidogenic factor 1 (SF1, NR5A1) in 46,XY patients with severe underandrogenization but without adrenal insufficiency. Hum Mutat 29(1):59–64.

Köhler B, Lin L, Mazen I, et al. 2009. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1,

NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 161(2):237–242.

Lala DS, Rice DA, Parker KL. 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. Mol Endocrinol 6(8):1249–1258.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25(14):1754–1760.

Lin L, Gu WX, Ozisik G, et al. 2006. Analysis of DAX1 (NR0B1) and steroidogenic factor-1 (NR5A1) in children and adults with primary adrenal failure: ten years' experience. J Clin Endocrinol Metab 91(8):3048–3054.

Lin L, Philibert P, Ferraz-de-Souza B, et al. 2007. Heterozygous missense mutations in steroidogenic factor 1 (SF1/Ad4BP, NR5A1) are associated with 46,XY disorders of sex development with normal adrenal function. J Clin Endocrinol Metab 92(3):991–999.

Lourenço D, Brauner R, Lin L, et al. 2009. Mutations in NR5A1 associated with ovarian insufficiency. N Engl J Med 360(12): 1200–1210.

Luo X, Ikeda Y, Parker KL. 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77(4):481–490.

Mazen I, Abdel-Hamid M, Mekkawy M, et al. 2016. Identification of NR5A1 mutations and possible digenic inheritance in 46,XY gonadal dysgenesis. Sex Dev 10(3):147–151.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16(3):1215.

Morohashi K. 1999. Gonadal and extragonadal functions of Ad4BP/SF-1: developmental aspects. Trends Endocrinol Metab 10(5):169–173.

Morohashi K, Hatano O, Nomura M, et al. 1995. Function and distribution of a steroidogenic cell-specific transcription factor, Ad4BP. J Steroid Biochem Mol Biol 53(1–6):81–88.

Morohashi K, Iida H, Nomura M, et al1994. Functional difference between Ad4BP and ELP, and their distributions in steroidogenic tissues. Mol Endocrinol 8(5):643–653.

Morohashi K, Tsuboi-Asai H, Matsushita S, et al. 1999. Structural and functional abnormalities in the spleen of an mFtz-F1 gene-disrupted mouse. Blood 93(5):1586–1594.

Ng PC, Henikoff S. 2006. Predicting the effects of amino acid substitutions on protein function. Annu Rev Genomics Hum Genet 7: 61–80.

Ngan ES, Cheng PK, Leung PC, Chow BK. 1999. Steroidogenic factor-1 interacts with a gonadotrope-specific element within the first exon of the human gonadotropin-releasing hormone

receptor gene to mediate gonadotrope-specific expression. Endocrinology 140(6):2452–2462.

Oliveira SH, Ferraz FA, Honorato RV, et al. 2014. KVFinder: steered identification of protein cavities as a PyMOL plugin. BMC Bioinformatics 15:197.

Paris F, De Ferran K, Bhangoo A, et al. 2011. Isolated 'idiopathic' micropenis: hidden genetic defects? Int J Androl 34(6 Pt 2): e518–e525.

Parker KL, Schimmer BP. 1997. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 18(3):361–377.

Pedace L, Laino L, Preziosi N, et al. 2014. Longitudinal hormonal evaluation in a patient with disorder of sexual development, 46,XY karyotype and one NR5A1 mutation. Am J Med Genet A 164A(11):2938–2946.

Philibert P, Zenaty D, Lin L, et al. 2007. Mutational analysis of steroidogenic factor 1 (NR5a1) in 24 boys with bilateral anorchia: a French collaborative study. Hum Reprod 22(12):3255–3261.

Ramayya MS, Zhou J, Kino T, et al. 1997. Steroidogenic factor 1 messenger ribonucleic acid expression in steroidogenic and non-steroidogenic human tissues: northern blot and in situ hybridization studies. J Clin Endocrinol Metab 82(6):1799–1806.

Reuter AL, Goji K, Bingham NC, et al. 2007. A novel mutation in the accessory DNA-binding domain of human steroidogenic factor 1 causes XY gonadal dysgenesis without adrenal insufficiency. Eur J Endocrinol 157(2):233–238.

Richards S, Aziz N, Bale S, et al. 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17(5):405–424.

Schlaubitz S, Yatsenko SA, Smith LD, et al. 2007. Ovotestes and XY sex reversal in a female with an interstitial 9q33.3-q34.1 deletion encompassing NR5A1 and LMX1B causing features of Genitopatellar syndrome. Am J Med Genet A 143A(10):1071–1081.

Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. 2010. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods 7(8):575–576.

Shihab HA, Gough J, Cooper DN, et al. 2013. Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. Hum Mutat 34(1):57–65.

Siepel A, Pollard KS, Haussler D. 2006. New methods for detecting lineage-specific selection. In: Apostolico A, Guerra C, Istrail S,

et al., editors. Research in computational molecular biology: Proceedings of the 10th annual international conference, RECOMB 2006, Venice, Italy, April 2-5, 2006. Berlin: Springer. pp. 190–205.

Soardi FC, Coeli FB, Maciel-Guerra AT, et al. 2010. Complete XY gonadal dysgenesis due to p.D293N homozygous mutation in the NR5A1 gene: a case study. J Appl Genet 51(2):223–224.

Song W, Gardner SA, Hovhannisyan H, et al. 2016. Exploring the landscape of pathogenic genetic variation in the ExAC population database: insights of relevance to variant classification. Genet Med 18(8):850–854.

Suntharalingham JP, Buonocore F, Duncan AJ, Achermann JC. 2015. DAX-1 (NR0B1) and steroidogenic factor-1 (SF-1, NR5A1) in human disease. Best Pract Res Clin Endocrinol Metab 29(4): 607–619.

Tantawy S, Mazen I, Soliman H, et al. 2014. Analysis of the gene coding for steroidogenic factor 1 (SF1, NR5A1) in a cohort of 50 Egyptian patients with 46,XY disorders of sex development. Eur J Endocrinol 170(5):759–767.

Tremblay JJ, Robert NM. 2005. Role of nuclear receptors in INSL3 gene transcription in Leydig cells. Ann N Y Acad Sci 1061: 183–189.

Wada Y, Okada M, Fukami M, et al. 2006. Association of cryptorchidism with Gly146Ala polymorphism in the gene for steroidogenic factor-1. Fertil Steril 85(3):787–790.

Wang K, Li M, Hakonarson H. 2010. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38(16):e164.

Warman DM, Costanzo M, Marino R, et al. 2011. Three new SF-1 (NR5A1) gene mutations in two unrelated families with multiple affected members: within-family variability in 46,XY subjects and low ovarian reserve in fertile 46,XX subjects. Horm Res Paediatr 75(1):70–77.

Woo KH, Cheon B, Kim JH, et al. 2015. Novel heterozygous mutations of NR5A1 and their functional characteristics in patients with 46,XY disorders of sex development without adrenal insufficiency. Horm Res Paediatr 84(2):116–123.

WuQiang F, Yanase T, Wei L, et al. 2003. Functional characterization of a new human Ad4BP/SF-1 variation, G146A. Biochem Biophys Res Commun 311(4):987–994.

Zimmermann S, Schwarzler A, Buth S, et al. 1998. Transcription of the Leydig insulin-like gene is mediated by steroidogenic factor-1. Mol Endocrinol 12(5):706–713.