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Functional Characterization of Two Mutations Located in the Ligand Binding Domain in the SF1

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Abstract

Purpose: Since SF1 gene mutations located in the ligand binding domain are associated with a wide phenotypic spectrum in 46,XY subjects, the functional and structural characterization of these variations is of great interest. The aim of this study is to evaluate the clinical phenotype, hormonal pattern and molecular studies (genetic, functional data and protein structural analysis) in two non-related 46,XY disorder of sex development (DSD) index patients.

Methods: Clinical characteristics, genomic DNA sequencing analysis, protein prediction software study and protein structure analysis, and functional characterization of the mutations was carried out.

Results: Both index DSD patients showed a similar phenotype, however several affected members of Family 1 showed variable phenotypes. While in Family 1 a previously reported heterozygous missense point mutation (p.Arg313His) was found, in Family 2 a novel heterozygous missense point mutation (p.Ser303Arg) was detected. Both mutations were predicted to be as *"probably damaging"*. The transcriptional activity of SF1 mutants p.Arg313His and p.Ser303Arg, studied using two different promoters in two cell lines, exhibited significant reductions of transactivation activity. Structural analysis showed differences between both mutants, such as changes in the flexibility of the receptor backbone and in the tertiary structure around the ligand and in the AF-2 domain.

Conclusions: One of these ligand binding domain mutations in SF1 showed phenotypic heterogeneity among family members, while both variations showed similarities in prepubertal phenotype, as well as in damage prediction and experimental decreases in transcriptional activity, but marked differences in structural consequence predictions. Finally the present study reinforces the concept of the wide variability in the clinical phenotype in affected 46,XY DSD patients.

Keywords: Steroidogenic factor-1; SF1; *NR5A1* gene; LBD mutations; 46,XY DSD

Introduction

Steroidogenic factor-1 (SF1/AD4BP/FTZF1) plays a key role in the regulation of adrenal and reproductive organs differentiation. The SF1 gene (*NR5A1*) is an autosomal gene located on chromosome 9q33 and it is the member 1 of the nuclear receptor subfamily 5, group A [1]. It expands over 30 kb of genomic DNA and it is comprised of one non-coding exon (I) followed by six coding exons (II to VII).

The protein consists of 461 amino acids that include a DNA-binding domain (DBD) containing two zinc fingers, a hinge region containing a first functional activation domain (AF-1), a ligand-binding domain (LBD) of 12 helices (H1-H12) that includes a second functional activation domain (AF-2), and an accessory region [2]. It is expressed in undifferentiated gonads even before SRY and it is necessary for testis determination and differentiation. SF1 protein is highly expressed in steroidogenic tissues, such as gonads, adrenals, and placenta, and regulates almost all the enzymes related to this process. It is also expressed in the ventromedial hypothalamic nucleus and pituitary gonadotropes with relevant physiological roles in the central nervous system [3].

Homozygous null mice (*Nr5a1*−/−) have adrenal and gonadal agenesis, persistent Müllerian structures in XY karyotype, partial hypogonadotropic hypogonadism, and other features such as hyposplenism, abnormalities of the ventromedial hypothalamus, and late-onset obesity [4,5].

In humans, mutations in *NR5A1* were reported to cause both 46,XY and 46,XX gonadal dysgenesis with or without adrenal failure [6-8]. Variable loss of SF1 function is associated with a wide phenotypic spectrum indicating that SF1 dosage is critical. In the approximately 81 patients so far described with impairment to the protein function mutations in the *NR5A1* gene the clinical phenotype is largely variable including, female genitalia, mild clitoromegaly, isolated hypospadias, anorchia and even normal male genitalia with infertility [6,9]. In 46,XX affected female patients, mutations in *NR5A1* are associated with different phenotypes including primary and secondary amenorrhea, premature menopause and decreased ovarian reserve, but preserved fertility [7,8,10].

We are reporting the clinical phenotype, hormonal pattern and molecular studies (genetic, functional data and protein structural analysis) in two non-related 46,XY, DSD index patients and their affected family members. A novel heterozygous *NR5A1* gene mutation, c.909G>A (p.Ser303Arg), and a previously described [8,10] heterozygous *NR5A1* gene mutation, c.938G>A (p.Arg313His), both located in exon 5, in the

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highly conserved helix 5 (H5) of the ligand binding domain of the protein (LBD), are presented. Functional studies and protein structure analyses shed light into the reasons sustaining the pathogenicity of the mutations.

Subjects and Methods

Clinical material

Two families with an index case presenting with a 46,XY DSD were evaluated. Four generations of Family 1 and two generations of Family 2 are depicted in the family tree of Figure 1.

The clinical and biochemical findings in the affected members of the first family kindred have been previously reported [10]. Family 2 has not been previously reported.

This study was approved by the Ethics Committee of the Garrahan Pediatric Hospital. Written informed consent for the study was obtained from all adult patients or patients´ parents or tutors.

Mutation analysis of the *NR5A1* gene

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. The coding exons (exons 2-7) and flanking intronic regions of the *NR5A1* gene were amplified by PCR using specific primers gently provided by Dr. J. C. Achermann, University College London, UK. The PCR products were purified (Qia Quick PCR purification kit, Qiagen, Buenos Aires, Argentina) and sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Buenos Aires, Argentina) on an ABI PRISM 3130 Genetic Analyzer capillary DNA Sequencer (Applied Biosystems, Buenos Aires, Argentina). The primers used for sequencing were the same as those used for PCR. The nucleotide sequences obtained were compared with the NCBI entry of *NR5A1* gene: NG_008176.1.

In silico protein analysis

The sequence homology-based tool SIFT (Sorting Intolerant from Tolerant; http://sift.jcvi.org/), version 2.0.6 and the structure-based tool PolyPhen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/) were used to predict the pathogenicity of the missense variants p.Arg313His and p.Ser303Arg using default settings. The original sequence of the protein was obtained from the Ensembl and Uniprot/Swiss-Prot databases.

Site-directed mutagenesis

For promoter activity experiments, an expression vector containing human wild type SF1 cDNA (p-SF1wt) was constructed in the pcDNA3 vector (Invitrogen, Buenos Aires, Argentina). The SF1 cDNA was PCR amplified using specific primers, SF1for_pcDNA3 (5′TGGTGTGAGGGGGTTTCTG3′) and SF1rev_pcDNA3 (5′GAGAGGAGGAAGGGATGACC3′) carrying *Eco*RI and *Xho*1 restriction enzyme sites, from RT-PCR of H295R cell line (human adrenocortical carcinoma cell line). The 1921-bp PCR product was 2% agarose gel-purified using the Zymoclean Gel DNA Recovery Kit (ZYMO RESEARCH, Buenos Aires, Argentina). The fragment was digested with *Eco*RI/*Xho*1 and cloned into the *Eco*RI and *Xho*1 sites of the pcDNA3 vector. The accuracy of the construct was confirmed by sequencing. *NR5A1* expression vectors containing the p.Arg313His and p.Ser303Arg variants were generated by PCR-based site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene, Buenos Aires, Argentina) with specific primers, using p-SF1wt as a template (p-R313H and p-S303R). The entire sequence of all mutant plasmids was confirmed by direct sequencing prior to functional studies.

In vitro functional studies of *NR5A1* mutations

All transient gene expression studies to assess *NR5A1*/SF1 function were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen. Buenos Aires, Argentina) according to the manufacturer's protocol. Each expression vector (p-SF1wt, p-R313H or p-S303R) was co-transfected into SMAT1 cell line (murine immature Sertoli cells) or into Y1 cell line (murine adrenocortical tumor cells) with reporter plasmid (PGL2, Promega, Buenos Aires, Argentina) containing hAMH promoter kindly provided by Dr. Rodolfo Rey (Centro de Investigaciones Endocrinológicas (CEDIE), Hospital de Niños R. Gutiérrez, Buenos Aires, Argentina), p-*P*hAMH or with reporter plasmid (PGL3, Promega, Buenos Aires, Argentina) containing the h3BHSD2 promoter, p-*P*h3BHSD2. Both promoters have response elements for SF1. The cells were lysed 48 hrs after transfection and assayed for luciferase activity (Dual Luciferase Reporter Assay System; Promega, Buenos Aires, Argentina). Co-transfection of CMV Renilla luciferase was used as a marker of transfection efficiency. Results are shown as the mean ± SEM of three independent experiments, each performed in triplicate and represents the ratio of luciferase activity

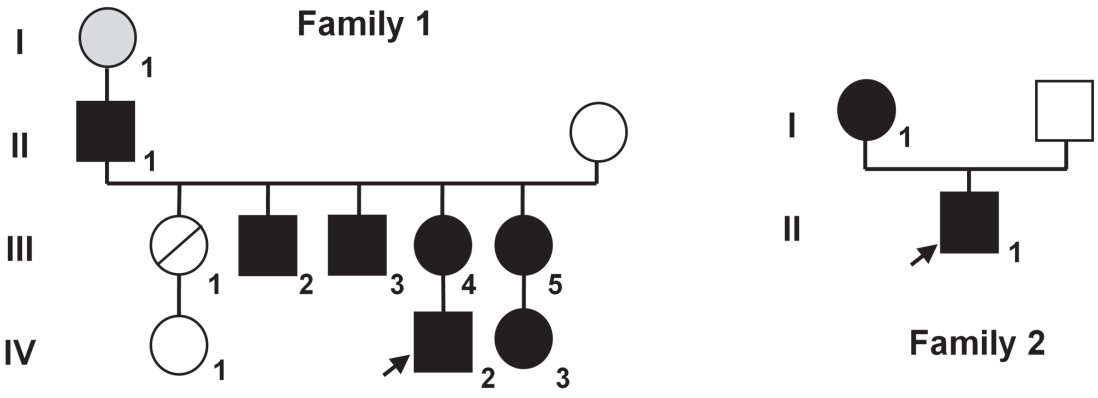

Figure 1: Family trees of both kindreds. Squares represent males and circles represent females. Black squares represent affected 46, XY subjects who were raised as boys, and black circles represent affected 46, XX subjects. Index case in each family is indicated by an arrow. Family 1 the circle with a slash through it represents a deceased female who was not studied. Subject I-1 (gray circle) experienced menopause at the age of 30 years and was inferred to be affected (not available for study).

of each SF1 and mutants compare to the empty vector. All data were standardized for Renilla activity. Statistical significance was examined using ANOVA test. Values of $p<0.05$ were considered significant.

In silico structural analysis of the wt protein and variants

Protein complexes in solution were prepared using as scaffold the X-ray crystallographic structure 1ZDT (chain B) of human SF1 wt LBD bound to a co-regulator peptide (NCoA-2, KENALLRYLLDKD) and a phospholipid ligand (di-palmitoyl-3-SN-phosphatidylethanolamine, PEF). Ser303Arg and Arg313His point mutations were introduced *in silico* with Discovery Studio Visualizer 3.5 [11]. Protonation states of the ionizable residues at pH 7.4 were defined by visual inspection after addition of H atoms missing in the 2.10 Å resolution crystal structure. Each structure was solvated by a truncated octahedral box of TIP3P water extended up to 12 Å around each complex, adding K^+ ions to electroneutrality (7, 6, or 8 counterions respectively for wt, p.Ser303Arg and p.Arg313His). A standard classical minimization protocol (2500 steps relaxing solvent plus ions while maintaining the complex restrained with 500 kcal/mol.Å, followed by 20000 steps of unrestrained optimization of the whole system) was applied under periodical boundary conditions with the *sander* module of the AMBER12 suite [12], assigning the *ff03* force field to both SF1 and the co-regulator peptide, and the GAFF parameters to the phospholipid ligand. RESP atomic charges were also obtained with Gaussian09 rev. A.02 [13]. Long-range electrostatics was treated using the *Particle-mesh Ewald* (PME) approach [14], and a 10 Å cut-off was used for direct space interactions. Molecular dynamics simulations (MD) were run in explicit water for each complex at 300 K under the same conditions, using a 2 fs integration step and applying restrictions to H atoms with SHAKE algorithm [15]. After 100 ps equilibration in a NVT ensemble using a Langevin thermostate, 5 ns of NPT simulations were produced with *pmemd* from the AMBER12 suite of programs [12]. MD trajectory post-processing was conducted with the *ptraj* module of AmberTools 12 [12] and representative structures corresponding to the most populated cluster (among five clusters generated with the averaged-linkage algorithm) were obtained for each complex.

Results

Clinical features

The index case Family 1 [10], patient IV-2 was born with ambiguous genitalia (2 cm length phallus, labioscrotal folds, severe hypospadias, and small inguinal gonads). Hormonal determinations revealed elevated serum FSH and low AMH levels, and normal steroidogenic response to hCG stimulation (Table 1). Male sex was assigned. Laparoscopic examination and bilateral orchidopexy was performed at 10 months of age. Müllerian structures were observed and a biopsy of the right gonad revealed signs of testicular dysgenesis, absence of Leydig cells and atypical heterochromatic germ cells. No lesions of carcinoma in situ were observed. Three other 46,XY individuals in the family (subjects II-1, III-2 and III-3) presented with severe hypospadias at birth, all of them developed spontaneous male puberty, and one (subject II-1) has fathered 5 children. Among the family members one 46,XX individual (subject I-1) developed early menopause (this subject was not available for the biochemical and genetic evaluation) and other three 46,XX family members (subjects III-4, III-5 and IV-3) presented high FSH and low AMH levels.

The index case of Family 2 (patient II-1) was the first child of non-consanguineous parents. The baby was born with ambiguous genitalia and was initially assigned the female sex at birth. The patient was referred for further evaluation at three months of age. On physical examination, the baby presented a 2.5 cm length phallus with well developed corporal tissue, complete labioscrotal fusion and scrotal hypospadias; both inguinal gonads were palpable. Hormonal determinations revealed elevated serum FSH and low AMH levels, as well as normal steroidogenic response to hCG stimulation (Table 1). Sex was re-assigned to male. Müllerian structures were present on ultrasound and confirmed by laparoscopy. A right gonad biopsy at 10 months of age revealed mild testicular dysgenesis, presence of Leydig cells, and few germ cells. His mother reported difficulties getting pregnant and presented low AMH and high basal FSH serum levels. His father referred normal sexual development and fertility. All affected individuals studied presented normal adrenal function.

Mutational analysis

The *NR5A1* gene molecular study revealed the mutation c.938G>A (p.Arg313His) in heterozygous state in the first family and a novel mutation, c.909G>A (p.Ser303Arg) in heterozygous state in the second family. Both mutations are located in exon 5 in the highly conserved H5 of the LBD. In order to determine if these alterations are present with a high frequency in the general population (Single Nucleotide Polymorphism Database), we looked for these variations in the NCBI databases and ensembl genome browser and we did not find them, suggesting that these two variations would not be common polymorphisms. In addition, we search for the mutations in 100 healthy subjects (200 alleles) by DNA sequencing and no allele carrying this mutation was detected.

NR5A1 gene mutation prediction model

To assess the potential deleterious effect of the amino acid changes, we used two software programs, SIFT and PolyPhen-2. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences. Arginine residue in position 313 and serine residue in position 303 are highly conserved between species. These mutations were evaluated to the option *"affects protein function"* with a highly deleterious tolerance index score of 0.00 and 0.02 respectively. The same assessment was performed with PolyPhen-2. This software predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Both mutations were predicted to be as *"probably damaging"* with a score of 1.000 and 0.999 respectively.

Structural analysis

The two variants containing point mutations p.Ser303Arg and p.Arg313His were compared to human SF1 wt. Both amino acid changes are located in the H5 helix of the LBD. In particular, p.Ser303Arg is located in the vicinity of the area of interaction with the co-regulator peptide (mainly delimited by H12, H3, and H4) and the p.Arg313His mutation is part of a salt bridge H2…H5 compacting the LBD structure of the receptor and stabilizing its active form [2,16].

From a comparison of the dynamical behavior of the protein backbone at the three SF1 complexes in solution emerges that whereas p.Ser303Arg displays a similar structural evolution than that of SF1 wt, for the p.Arg313His variant it takes longer (near 4 nanoseconds (ns)) to stabilize the 3D structure, which globally differs from the other two variants due to the loss of the salt bridge interaction (Figure 2A).

Since it has been reported that restrictions in the flexibility of *NR5A1* receptors knock down their activation [17,18], we have compared this issue between the three variants using the root mean square fluctuations of the protein by residue (RMSF) calculated at the stabilized part of each 5 ns simulation (Figure 2B). As it can be immediately appreciated, whereas the p.Ser303Arg mutation turns the protein significantly more flexible throughout all its structure as compared to the wt (particularly in the H2-H3 loop and in the H12 helix), the p.Arg313His mutation turns, in contrast, the receptor more rigid in several regions.

The overall comparison of the tridimensional arrangement of alpha-helices and loops of the LBD across the three variants (Figures 2C and

Subject	CA (years)	LH(IU/l) LHRH		FSH(IU/l) LHRH		T(ng/ml)		E2 (pg/ml)	AMH (pmol/l)	Inhibin B (pg/ml)	Cortisol (ug/dl)	ACTH (pg/ml)
		basal	peak	basal	peak	basal	hCG					
Family 1 (*) II-1 46,XY	57	6.0		**32.4** (1-14)		2.87			**<7.1** (22-38)	**<30** (30-200)	14.5	38.6
III-2 46,XY	31	2.3		4.6		2.97			**<2.9** (22-38)	**60.8** (50-450)	12.1	25
III-3 46,XY	29	4.4		13.1		3.09			**20** (22-38)	**54.7** (75-375)	10.1	38.9
III-4 46,XX	27	12.1		**47.5** (1.1-9.6)		0.56		**<9**	**<0.3** (0-75)		8.2	22
III-5 46,XX	25	11.7		**25.1**(1.1-9.6)		0.31		28.3	**5.9** (0-75)		5.6	15.5
IV-2 46,XY	0.03	**9.6** (2.5 ± 1.7)	**24.1**	**13.3** (2.4 ± 1.7)	**24.5**	1.04	3.95	<9	**58** (76-381)	ND	16.6 [1]	57.3 [1]
	1.8	<0.1		2.6		<0.05			**<2.9** (360-638)	**31.9** (80-325)		
IV-3 46,XX	3.8	<0.1		**12.4**(2.8±1.9)		<0.05		<9	**<0.3** (0-74)		14.1	15.7
Family 2 I-1 46,XX	23	12.3		**17.8** (1.1-9.6)		ND		30.6	**<1.3** (0-75)		8.9	
II-1 46,XY	0.3	3.75		**13.6** (2.4±1.7)		0.72	1.36		**133** (251-679)	**39.9** (80-325)	11.7	20

Table 1: Serum hormone levels in the affected individuals available for the study.
Boldface numbers indicate values outside the reference range; normal values are shown in parentheses. SI conversion factors: testosterone (nanomoles per
liter), 3.47; estradiol (picomoles per liter), 3.671; cortisol (nanomoles per liter), 27.59.
CA= Chronological age. ND=Not determined
[1]at the age of 0.83 years
(*) Ciaccio et al. [10]

2D) highlights that both point mutations induce changes in the tertiary structure of the receptor around the ligand (H2-H3/H6-H7 loops, beta hairpin, and H6/H7 helices) and in the AF-2 domain (H4, H11, and H12), resulting the latter in a different positioning of the co-activator peptide.

A closer inspection of the hydrogen-bond network surrounding each mutation promptly shows that whereas the interaction with Lys434 is the most affected for p.Ser303Arg (in comparison to SF1 wt), Arg313 ability of establishing hydrogen bonds with Glu237 and Asp309 in wt is completely lost when replaced by His313 in p.Arg313His (Table 2).

Finally, a closer look in the comparison of the interactions with the ligand at the LBD across variants shows that as the p.Ser303Arg mutation affects SF1 interaction with the phospholipid mainly through changes in Lys440 positioning and the opening of the channel's entrance defined by H2-H3 and H6-H7 loops (this resulting in PEF's polar head more exposed to the solvent), p.Arg313His mutation does not produce significant changes on the ligand-receptor recognition (Figures 2E and 2F).

Functional studies of NR5A1 mutation activities

To examine the transcriptional activity of the SF1 mutants (p-R313H and p-S303R) and the wild type SF1 (p-SF1wt), we performed transient co-transfection assays with different SF1 target gene promoters (p-*P*hAMH and p-*P*h3BHSD2) in steroidogenic cell lines (SMAT1 and Y1).

In both steroidogenic cell lines, p-SF1wt significantly ($p<0.05$) increased reporter gene expression driven by the human AMH or 3BHSD2 promoters. Transfection with each of the two missense mutations p-R313H or p-S303R showed impaired transactivation activity compared with p-SF1wt (Figure 3).

In details, in the SMAT1 cell line the co-transfection with p-*P*hAMH and p-SF1wt significantly increased the luciferase activity (3.00 ± 0.09 AU, mean ± SEM, $p<0.05$ ANOVA) while mutants p-R313H and p-S303R

Protein Residue:atom pairs	Distance (Å)	Angle (°)
WT		
Ser303:O···Lys434:NH3	3.268	144.7
Ser303:OH···Glu304:O	2.969	146.7
Ser303:C=O···Val307:NH	2.830	175.9
S303R		
Arg303:NE···Glu304:O	4.372	106.4
Arg303:NE···Val307:NH	3.026	174.3
WT		
Arg313:NE···Glu237:OE2	3.153	168.2
Arg313:NH2···Glu237:OE1	2.616	166.8
Arg313:NH2···Asp309:OD2	3.158	154.4
Arg313:NH1···Asp309:OD1	2.689	156.1
Arg313:NH2···Asp309:OD1	2.864	155.6
R313H		
Interactions completely lost		

Table 2: Structural analysis of the hydrogen bond interactions around each point mutation

significantly decreased luciferase activity compared to p-SF1wt (1.32 AU ± 0.05 and 1.09 AU ± 0.01 respectively, mean ± SEM, $p<0.05$ ANOVA) (Figure 3A). Similar results were obtained in the SMAT1 cell line with the co-transfection with p-*P*h3BHSD2; a significant increased luciferase activity in the presence of p-SF1wt (6.92 AU ± 0.25, mean ± SEM, $p<0.05$ ANOVA) while when assaying the effect of the mutants p-R313H and p-S303R a significant reduction of transactivation

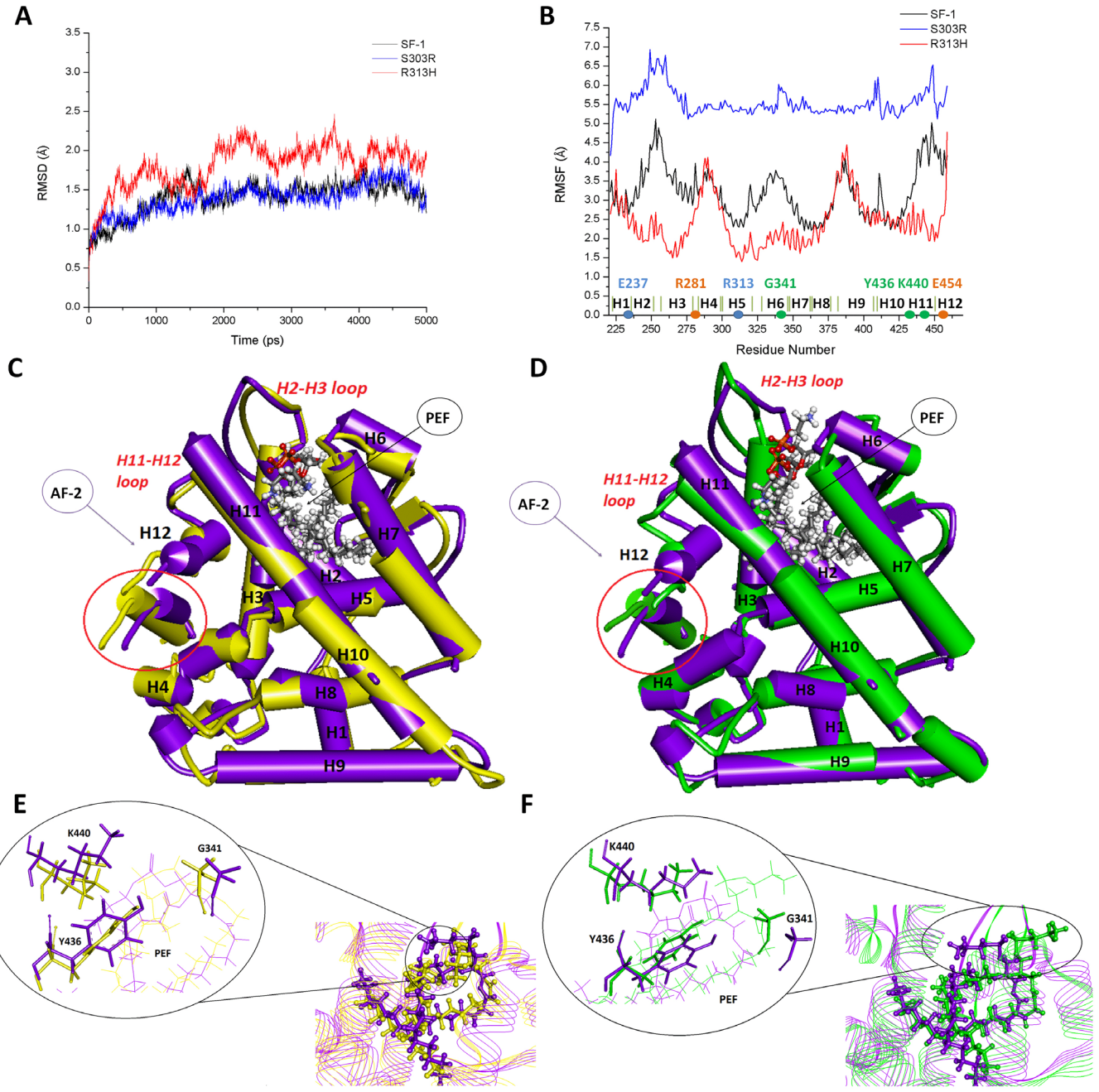

Figure 2: Structural analysis of SF1 mutations. Panel A - Structural evolution of Cα RMSD for each SF1 variant along the simulation taking the initial structure as reference. Panel B - Flexibility by protein residue calculated as RMS fluctuations from the time-averaged Cα positions along the stabilized part of each simulation. Panels C and D - Overlay by pairs of protein variants, evidencing the main differences on α-helices, β-hairpin, and loops 3D disposition in the LBD. In purple the SF1 wt protein, in yellow the R313H mutant, and in green the S303R mutant. AF-2 is the ligand-dependent activation function and PEF a phospholipid ligand. Circled in red is the coactivator peptide. Panels E and F – Detail of the region around the ligand, compared by pair of protein variants. In purple the SF1 wt protein, in yellow the R313H mutant, and in green the S303R mutant.

capacity was observed compared to p-SF1wt (1.91 AU ± 0.07 and 2.00 AU ± 0.08 respectively, mean ± SEM, $p<0.05$ ANOVA) (Figure 3B).

In the Y1 cell line, similar responses as in the SMAT1 cell line were observed with p-*P*h3BHSD2 and p-SF1wt (9.27 AU ± 0.54, mean ± SEM, $p<0.05$ ANOVA) and mutants p-R313H and p-S303R (4.55 AU ± 0.32 and 4.08 AU ± 0.17 respectively, mean ± SEM, $p<0.05$ vs. SF1wt, ANOVA) (Figure 3C). Transactivating studies of both mutants with the p-*P*hAMH in the Y1 cell line were not possible as the p-SF1wt did not increase luciferase activity.

Discussion

We are reporting the molecular characterization of two missense mutations (p.Arg313His and p.Ser303Arg) in the LBD of the SF1 gene, in two non-related 46,XY DSD patients, both in heterozygous state.

The clinical and biochemical phenotypes of these two 46,XY index patients were similar. Hormonal determinations showed normal basal and peak testosterone, high serum FSH and low serum AMH levels for the child's age. Evidence of testicular dysgenesis and the presence of Müllerian

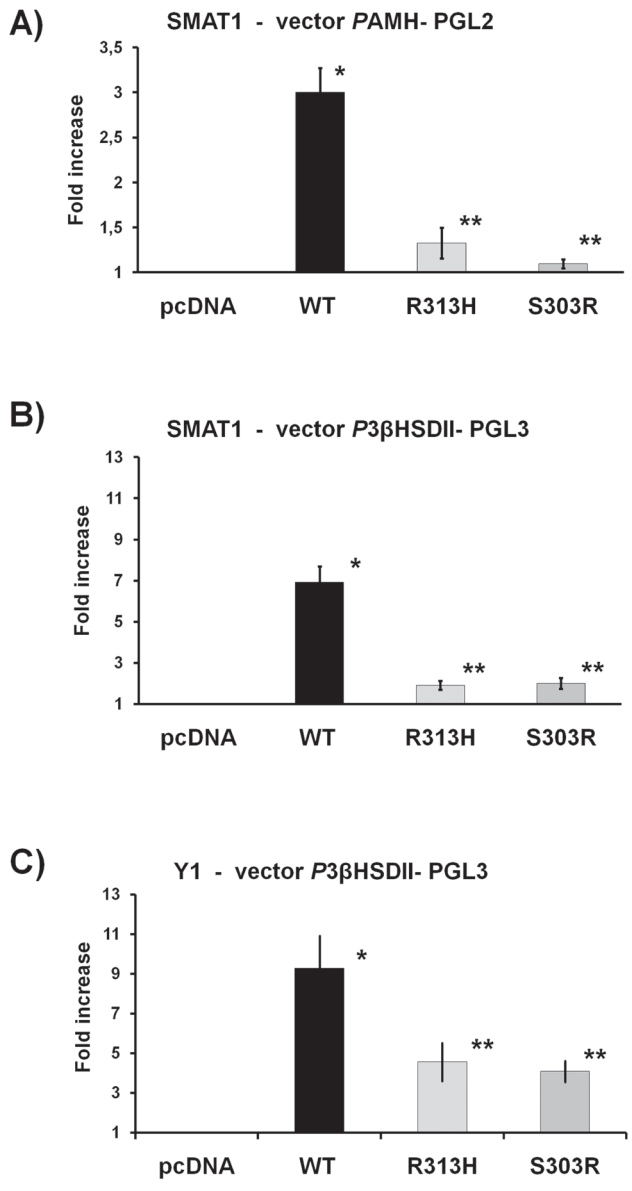

Figure 3: Functional analysis in SMAT1 and Y1 of the R313H and S303R mutations. The transcriptional activity of wild-type SF1 (p-SF1wt) and mutants p.Arg313His and p.Ser303Arg (p-R313H and p-S303R respectively) were studied using *P*h3BHSD2 and *P*hAMH promoters in SMAT1 cell line and *P*h3BHSD2 promoter in Y1 cell line. Both mutants in both cell line and with both promoters exhibited a reduction of transactivation activity. The results are expressed as fold increase of luciferase activity compared with empty vector (mean ± SD). All values represent the means ± SEM of three separate transfection experiments, each performed in triplicate. * vs. empty vector; ** vs. SF1, p<0.05, ANOVA.

structures suggested a failure of embryonic fetal Sertoli cells to secrete AMH during the sensitive prenatal period, which is consistent with the low levels of AMH detected during infancy. The baby of the second family, evaluated at the age of minipuberty, also presented low serum inhibin B levels along with elevated FSH. As it was proposed in an aromatase deficient boy, inhibin B might be the major contributor in the regulation of serum FSH secretion in normal infant males [19]. Both patients were initially assigned the female sex at birth, but after a careful evaluation, male sex re-assignation was recommended to, and accepted by the parents, on the basis of adequate basal T secretion for age, including normal response to hCG stimulation, and potential for successful intercourse in adulthood. This decision was also supported by the follow-up of other 46,XY DSD members of the first family who developed spontaneous male puberty, one with preserved fertility [10] and lack of reports of development of testicular tumors in 46,XY *NR5A1* dysgenetic testes.

There was a striking variability among the affected relatives in the first family that range from severe ambiguous genitalia to normal male external genitalia and preserved fertility, as it was previously reported by us [10]. A wide phenotypic spectrum has been described in patients with heterozygous *NR5A1* mutations including when the mutation is located in the LBD (Table 3) [5,6,9,20-32]. Phenotype variability in *NR5A1* mutations makes genotype-phenotype correlations very difficult. In 46,XX individuals carrying heterozygous *NR5A1* mutations, variable gonadal phenotypes have also been described [7,8,10,20,33-36]. In this report, four 46,XX affected women presented high serum FSH levels with low levels of AMH. The presence of regular menses in the three young-adult women studied and successful maternity in two, reflect a compensated ovarian dysfunction. These women may go through a stage of decreased ovarian reserve before developing clinical signs or symptoms of ovarian failure, as it has been reported [20].

Adrenal function was normal in both index patients and their relatives carrying mutations in *NR5A1*. To date, adrenal insufficiency was reported in only three patients harboring *NR5A1* mutations [37-39].

The amino acid substitution of both missense mutations (p.Arg313His and p.Ser303Arg) takes place in a highly conserved site among species observed by *in silico* analysis.

The structural analysis showed that, since it turns to be a less rigid protein, interactions are expected to be more labile for p.Ser303Arg mutant. On the other hand, the loss of flexibility induced by p.Arg313His mutation occurs in regions which are involved in several interactions of relevance for structural packing (H2-E238 and H5-R313) and co-activator recruiting at the activation function domain AF-2 (H3-R281 and H12-E454). The H11 C-term region where the phospholipid polar head binds at the entrance of the ligand channel is also highly affected. In agreement with this, crystallographic studies on the SF1 ligand binding domain revealed different phospholipids bound in its hormone binding pocket [2,16-18,40-42].

In vitro assays used to assess the functional impact of these two mutations, on h3BHSD2 and hAMH promoters and in two different steroidogenic cell lines (SMAT1 and Y1), showed impaired transactivation activity. On this line, besides the known interaction with phospholipids, its LBD might interact with some unknown co-activators to trigger adrenal and gonadal development [43]. Even though the *in vitro* studies in some reports of *NR5A1* gene mutations located in the LBD, confirm the deleterious effect (Table 3), the clinical phenotype variability observed among the affected patients reported remains poorly understood.

Even though, SF1 is known to be engaged in the interaction with numerous co-activators acting over the promoter region of several steroidogenic enzymes and factors involved in reproduction, steroidogenesis and sexual differentiation [1,6,39], the molecular mechanisms of heterozygous mutations remain to be elucidated.

The majority of the patients previously described, including the present report, carried heterozygous *NR5A1* gene mutations in the LBD, supporting the concept of dose dependence of SF1 action (Table 3).

In summary, we are reporting, in two non-related 46,XY DSD patients, the clinical phenotype, hormonal studies, molecular characterization, and protein structural analysis of one missense mutation of the *NR5A1*

Mutation	Sex Assign.	Genital Phenotype	Puberty	Fertility	In silico prediction**	Functional charaterization	Reference
p.Glu237Lys	Male	Normal Male	Spontaneous	Azoospermia	Damaging	Not done	[21]
p.Asp238Asn	Male	Normal Male	Spontaneous	Severe failure	Not done	Impaired transcription	[22]
p.Trp279Arg	Male	Micropenis, Perineal hypospadias	Not reported	Not reported	Damaging	Not done	[23]
p.Arg281Pro	Male	Micropenis, Perineal hypospadias	Spontaneous	High sFSH Asymptomatic carrier father	Damaging	Impaired transcription	[24]
p.Ser303Arg*	Male	Perineal hypospadias, EMS 8/12	Prepuberty	Prepuberty	Damaging	Impaired transcription	This report
p.Glu304Lys*	Male	Perineal hypospadias	Prepuberty	Asymptomatic carrier father	Not done	Impaired transcription	[25]
p.Arg313Cys	Male	Glandular hypospadias	Not reported	Not reported	Not done	Impaired transcription	[23]
p.Arg313His	Male	Perineal hypospadias	Not reported	Asymptomatic carrier father	Not done	Not done	[26]
p.Arg313His*	Male	Perineal hypospadias	Spontaneous	Preserved	Damaging	Impaired transcription	[10] and this report
p.Gly328Val	Male	Perineal hypospadias	Spontaneous. Hypergon. hypogonadism	Azoospermia	Not done	Impaired transcription	[27]
p.Val355Met*	Male	Micropenis, bilateral anorquia	Induced	Not reported	Not done	Impaired transcription	[28]
p.Cys370Trp	Male	DSD (EMS 3/12)	Not reported	Not reported	Damaging	Not done	[29]
p.Leu376Phe	Female	DSD (female) Neonatal Clitoromegaly	Spontaneous (clitoromegaly)	Pubertal gonadectomy	Not done	Impaired transcription	[27]
p.Asp380Tyr	Female	DSD (female)	Not reported	Adolescent	Damaging	Not done	[30]
p.Leu437Gln	Male	Perineal hypospadias	Induced	Not reported	Not done	Impaired transcription	[31]

Table 3: Missense mutations in the SF-1 ligand binding domain of 46,XY patients

*_In silico_ structural analysis performed;

**Analyzed using SIFT and/or PolyPhen-2 tools.

sFSH: serum FSH.

EMS: External Masculinization Score (minimum: 1, maximum: 12) [32]

gene (p.Arg313His) previously described without any functional study performed, and a novel missense mutation (p.Ser303Arg), both in heterozygous state, located in the LBD. *In vitro* and *in silico* experiments argued for their functional impact and also provided insight into the structure-function relationship of the SF1 protein. Finally the present study reinforces the concept of the wide variability in the clinical phenotype in affected 46,XY DSD patients.

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