

CLINICAL STUDY

Testosterone production during puberty in two 46,XY patients with disorders of sex development and novel NR5A1 (SF-1) mutations

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Abstract

Background: Steroidogenic factor 1 (SF-1, NR5A1) is a key transcriptional regulator of many genes involved in the hypothalamic–pituitary–gonadal axis and mutations in NR5A1 can result in 46,XY disorders of sex development (DSD). Patients with this condition typically present with ambiguous genitalia, partial gonadal dysgenesis, and absent/rudimentary Müllerian structures. In these cases, testosterone is usually low in early infancy, indicating significantly impaired androgen synthesis. Further, Sertoli cell dysfunction is seen (low inhibin B, anti-Müllerian hormone). However, gonadal function at puberty in patients with NR5A1 mutations is unknown.

Subjects and methods: Clinical assessment, endocrine evaluation, and genetic analysis were performed in one female and one male with 46,XY DSD who showed spontaneous virilization during puberty. The female patient presented at adolescence with clitoral hypertrophy, whereas the male patient presented at birth with severe hypospadias and entered puberty spontaneously. Molecular analysis of NR5A1 was performed followed by *in vitro* functional analysis of the two novel mutations detected.

Results: Testosterone levels were normal during puberty in both patients. Analysis of NR5A1 revealed two novel heterozygous missense mutations in the ligand-binding domain of SF-1 (patient 1: p.L376F; patient 2: p.G328V). The mutant proteins showed reduced transactivation of the CYP11A promoter *in vitro*.

Conclusion: Patients with 46,XY DSD and NR5A1 mutations can produce sufficient testosterone for spontaneous virilization during puberty. Phenotypic females (46,XY) with NR5A1 mutations can present with clitoromegaly at puberty, a phenotype similar to other partial defects of androgen synthesis or action. Testosterone production in 46,XY males with NR5A1 mutations can be sufficient for virilization at puberty. As progressive gonadal dysgenesis is likely, gonadal function should be monitored in adolescence and adulthood, and early sperm cryopreservation considered in male patients if possible.

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Introduction

Steroidogenic factor 1 (SF-1, NR5A1), a member of the nuclear receptor superfamily, is a key transcriptional regulator of many genes involved in the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes (e.g. inhibin α , DAX1 (NROB1), StAR (STAR), CYP11A (CYP11A1), CYP17A1, HSD3B2, CYP19A1, MIS (AMH), oxytocin, and SOX9) (1). SF-1 is highly expressed in steroidogenic tissues from very early stages of development, including the developing gonad and adrenal, and continues to be expressed in these tissues into adult life (2). SF-1 is also expressed in the ventromedial hypothalamic nucleus and pituitary gonadotropes (3, 4, 5). Consistent with this central

role in reproductive and adrenal development, XY *Sf-1* knockout mice (*Sf-1*^{-/-}) lack adrenal glands and show male to female 'sex reversal' due to complete gonadal dysgenesis (6). Furthermore, male gonad-specific *Sf-1*^{-/-} mice display hypoplastic testes and internal genitalia, undescended testes, and infertility (7). SF-1 interacts *in vitro* with several other major factors playing a role in gonadal determination and differentiation (e.g. SRY, GATA4, and SOX9) (8, 9, 10).

To date, about 50 different NR5A1 mutations have been reported in humans with disorders of sex development (DSD). NR5A1 mutations were first described in two 46,XY patients with complete gonadal dysgenesis and primary adrenal failure (11, 12) and in one 46,XX female with primary adrenal failure (13).

More recently, however, many more heterozygous *NR5A1* mutations have been identified in patients with 46,XY DSD without adrenal insufficiency. The majority of these 46,XY DSD patients without adrenal failure have a phenotype of ambiguous genitalia at birth, partial gonadal dysgenesis, and absent or rudimentary Müllerian structures (14, 15, 16). The frequency of *NR5A1* mutations in patients with this phenotype has been estimated to be about 15% (17, 18). Furthermore, *NR5A1* mutations have also been identified in 46,XY patients with hypospadias and cryptorchidism, which is likely to be caused by a milder form of partial gonadal dysgenesis (19, 20), as well as in association with vanishing testis syndrome, micropenis, or infertility (21, 22, 23). In addition, SF-1 plays a role in ovarian development and function and mutations have been reported in women (46,XX) with primary ovarian insufficiency and premature ovarian failure (24). *NR5A1* mutations rarely cause isolated adrenal insufficiency (25). Therefore, it is currently thought that disruption of SF-1 has a more significant effect on human gonadal development and function compared with human adrenal development and function (25).

In most published cases of 46,XY DSD due to *NR5A1* mutations, testosterone has been low indicating significantly impaired androgen synthesis. Sertoli cell hypofunction has been suggested because of low levels of inhibin B and anti-Müllerian hormone (AMH) and elevated FSH. However, normal testosterone levels at birth have been reported in one patient with 46,XY DSD and low normal testosterone levels during puberty have been described in another patient (23, 26). We report two novel *NR5A1* mutations in two patients with 46,XY DSD who demonstrate normal male testosterone production during puberty.

Subjects

Written informed consent for genetic analysis was obtained according to institutional ethical guidelines from the parents of patient 1 and from patient 2.

Patient 1

Patient 1 is a female who presented at puberty with clitoral hypertrophy. She was born to nonconsanguineous German Caucasian parents. The clinical presentation, endocrine and laparoscopic findings, and gonadal histology are summarized in Table 1. Psychological evaluation of the patient showed female gender identity. At 14 6/12 years, bilateral gonadectomy was performed with the patient's consent and estrogen substitution was initiated. Mutations of 5- α reductase type 2 (*SRD5A2*), 17- β hydroxysteroid dehydrogenase type 3 (*HSD17B3*), and androgen receptor (*AR*) genes were excluded. Basal cortisol was normal at puberty.

Patient 2

Patient 2 is a 28-year-old male who first presented at birth with severe penoscrotal hypospadias, hypoplastic phallus, and small inguinal testes. He was born to nonconsanguineous German Caucasian parents. In early infancy, a two-step surgical correction of hypospadias was carried out. The clinical presentation, testicular histology, results of laparoscopy, pubertal development, hormones, and semen analysis of the patient are summarized in Table 1. Mutations of the *SRD5A2*, *AR*, and LH/choriogonadotropin receptor (*LHCGR*) genes were excluded. Testosterone substitution was started at 28 years of age. Basal and stimulated cortisol (Synacthen test) levels were normal in adulthood.

Methods

Molecular analysis of *NR5A1* gene

Molecular analysis of exons 2–7 of the gene encoding SF-1 (*NR5A1*) was performed as described previously (18). DNA mutation numbering is based on GenBank reference DNA sequence NM_004959.4, with the A of the ATG initiation codon designated +1 (www.hgvs.org/mutnomen).

Functional characterization

In vitro functional activities of the *NR5A1* mutations on the murine *Cyp11a* promoter was assessed by transient gene expression assays in human embryonic kidney TSA-201 cells as described previously (17).

Results

Molecular genetic analysis and functional characterization

Analysis of the *NR5A1* gene revealed a novel heterozygous missense mutation c.1126C>T (p.L376F, g.127253372) in exon 6 in patient 1 and a novel heterozygous missense mutation c.983G>T (p.G328V, g.127255316) in exon 5 in patient 2. Both mutations are located in the ligand-binding domain of SF-1 (Fig. 1). The p.L376F alteration is a *de novo* mutation as neither parents of patient 1 harbored this change. Only the mother of patient 2 was available for genetic analysis and she did not harbor the mutation. The mutated amino acids, leucine at position 376 and glycine at position 328, are highly conserved in SF-1 orthologs (Fig. 1). The c.1126C>T and c.983G>T variants are unlikely to be neutral nonpathological polymorphisms as they are not present in the database of single-nucleotide variants (dbSNP132) or the 1000 Genomes data (www.1000genomes.org/data).

Table 1 Clinical presentation, endocrine data, and other investigations of patients. Conversion to SI units: testosterone ng/ml \times 3.47 for nmol/l; inhibin B pg/ml \times 1.0 for pmol/l. Normal values for testosterone in males with Tanner stages 2–3, 0.62–2.26 ng/ml; Tanner stages 4–5, 1.68–6.0 ng/ml; normal testosterone peak after stimulation with HCG 5000 IU/m² at 0 h 0.20–4.0 ng/ml and at 72 h 3.0–10.0 ng/ml; basal FSH in males with Tanner stage 1, <1–1.3 IU/l; Tanner stages 2–3, <1–4.0 IU/l; Tanner stages 4–5, 1.4–5.1 IU/l; basal LH in males with Tanner stage 1, <1–1.5 IU/l; Tanner stages 2–3, 1–4.1 IU/l; Tanner stages 4–5, 3.4–7.5 IU/l; inhibin B, 74–470 pg/ml.

Age (years)	Clinical presentation	Testosterone (ng/ml)	FSH (IU/l)	LH (IU/l)	Other investigations
Patient 1 14	B1, P4, A1 Clitoromegaly	2.1	35.9	2.3	Karyotype: 46,XY Laparoscopy: abdominal testes, no Müllerian structures Testicular histology: bilateral testicular tissue, no spermatogenesis Inhibin B: <10 pg/ml Steroid profile: normal
Patient 2 At birth	Severe penoscrotal hypospadias Small inguinal testes Penile length 1 cm	NA	NA	NA	Karyotype: 46,XY
3.5	Penile length 2.5 cm	NA	0.6	0.9	Laparoscopy: vaginal rest Testicular histology: reduction of spermatogonia, increased intertubular tissue, thickening of the tunica albuginea
13.5	G2, P2, A1 Gynecomastia B2 (3 cm \varnothing) Penile length 4.7 cm Right testis 5 ml, left testis 4 ml	3.9 \rightarrow 6.5 (after HCG 5000 IU/m ²)	25.9	3.4	–
16	G4, P5, A3 Gynecomastia B4–5 (9 cm \varnothing) Penile length 7.1 cm Right testis 13 ml, left testis 15 ml	2.6	35.4	7.0	–
19	Penile length 6 cm Right testis 6 ml, left testis 6 ml	3.3	37.9	10.9	Surgery for gynecomastia
28	Right testis 6 ml, left testis 6 ml	2.6	58.7	14.0	Inhibin B: undetectable Spermiogram: azoospermia

NA, not available; HCG, human chorionic gonadotrophin.

Functional analysis of the mutants showed reduced activities to 12% (p.L376F) and 8% (p.G328V) of wild-type activity on the *Cyp11a* promoter (Fig. 2). No functional activity of the mutants on the *AMH*, *TESCO*, and *INSL3* promoter was investigated as the two patients did not show either persistent uterine development as a sign of disturbed AMH secretion or severe gonadal dysgenesis as a result of impaired testes determination.

Discussion

We report two novel *NR5A1* mutations (p.G328V and p.L376F) in two 46,XY DSD patients with normal male testosterone production at puberty. To date, knowledge about gonadal function at puberty in patients with 46,XY DSD and *NR5A1* defects is very scarce, as most

cases identified have undergone gonadectomy in early infancy. Only one 46,XY DSD patient with low normal testosterone production during puberty has been reported (26).

The first patient we describe (patient 1) is a 46,XY female who presented at puberty with clitoral hypertrophy, bilateral dysgenetic abdominal testes, no uterus, and a testosterone level within the normal male range for mid to late puberty. The phenotype of this patient resembles the pubertal presentation of partial 5 α -reductase type 2 deficiency, 17 β -HSD type 3 deficiency, or partial androgen receptor or LH receptor defects, all of which can also present with virilization at puberty. However, the hormone profile (elevated FSH, low inhibin B) is consistent with partial gonadal dysgenesis, which is not present in androgen synthesis defects. We hypothesize that maturation of other compensatory steroidogenic enzymes or cofactors

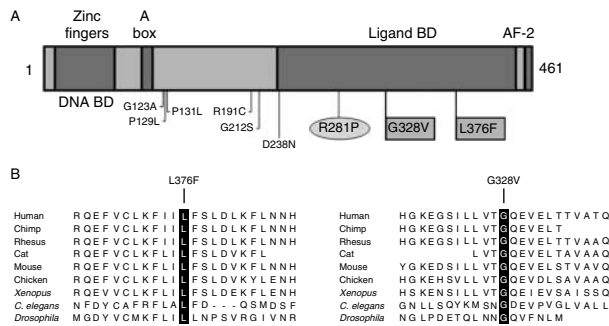


Figure 1 (A) Structure of the gene encoding SF-1 (*NR5A1*) showing different mutations: the two novel mutations reported in this study (p.L376F and p.G328V); mutations identified in patients with male infertility (p.G123A, p.P129L, p.P131L, p.R191C, p.G212S, and p.D238N) (23); mutation of a 46,XY DSD patient with hypospadias and low-normal testosterone production (p.R281P) (26). (B) The mutated leucine at position 376 and glycine at position 328 are conserved in SF-1 orthologs.

during puberty in patients with *NR5A1* mutations can result in significant testosterone production in some cases.

Patient 2, a 46,XY male born with ambiguous genitalia, had sufficient testosterone synthesis at puberty for spontaneous virilization and male pubertal development to occur. However, toward the end of puberty, he developed progressive gonadal failure affecting both Leydig and Sertoli cells, with evidence of deteriorating function through adulthood. At 28 years, his testosterone was very low, inhibin B was undetectable, and azoospermia was present. The delayed orchidopexy may have contributed to the azoospermia but is unlikely to have been causative. Previously, we have reported *NR5A1* mutations in about 10% of patients with a similar phenotype of complex hypospadias at birth (severe hypospadias and/or hypoplastic phallus and cryptorchidism) (19, 20). However, only one other patient with complex hypospadias at birth and testosterone production during puberty has been reported; Philibert *et al.* (26) described a 46,XY boy with hypospadias and micropenis at birth who was found to harbor a heterozygous *NR5A1* mutation (p.R281P) in the ligand-binding domain of the protein. He entered puberty spontaneously with a low-normal rise of testosterone but had elevated FSH and decreased inhibin B concentrations, reflecting Leydig and Sertoli cell hypofunction.

Both patients reported in our study showed various degrees of underandrogenization at birth revealing that Leydig cell function had already been disturbed during embryonic and fetal development. In contrast, embryonic/fetal Sertoli cell function was sufficient for adequate AMH production and subsequent Müllerian duct regression in patient 1. In patient 2, partially impaired AMH production is suggested by the presence of a small Müllerian rest. Neither of the patients we describe showed any signs or symptoms of adrenal

insufficiency as is the case with the majority of patients with heterozygous *NR5A1* mutations published previously. Although detailed tests of adrenal function are not available for patient 1, we would currently recommend more comprehensive adrenal testing (e.g. cortisol response to Synacthen, and basal ACTH) and an ongoing awareness of the potential risk of adrenal failure in these patients. Further studies are required to systematically address the risk of adrenal dysfunction in such individuals in the long term.

In a recent study on male infertility, *NR5A1* mutations were described in seven out of 199 (3.5%) males with moderate/severe oligospermia or azoospermia (23). All mutations were missense mutations in the hinge region or proximal ligand-binding domain of the protein. Notably, normal testosterone and low normal/undetectable inhibin B levels were found in two patients under 30 years of age while the older patients had low to undetectable testosterone and inhibin B levels. These observations suggest that infertile males with *NR5A1* mutations are at risk of deterioration of gonadal function with age (23).

Our study and the studies of Philibert *et al.* and Bashamboo *et al.* show that patients with *NR5A1* mutations can produce significant levels of testosterone in adolescence and can enter puberty spontaneously. However, progressive gonadal dysgenesis resulting in oligospermia or azoospermia is potentially likely in individuals where spermatogenesis has occurred. Therefore, we strongly recommend regular monitoring of gonadal function in adolescence and adulthood in

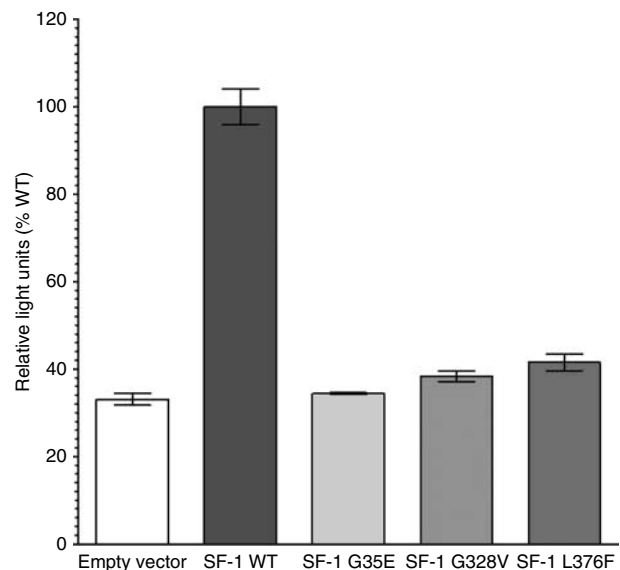


Figure 2 Effect of the SF-1 mutants on transcriptional activity of the minimal promoter of *Cyp11a* in TSA-201 cells. A previous loss of function change in SF-1/*NR5A1* (p.G35E) was used as a control for loss of activity. All values represent the means of three separate transfection experiments (\pm S.E.M.).

males with *NR5A1* mutations. If sperms are detected, early cryopreservation could be undertaken with appropriate genetic counseling in an attempt to preserve future chances of fertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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