CASE REPORT

A novel mutation in the accessory DNA-binding domain of human steroidogenic factor 1 causes XY gonadal dysgenesis without adrenal insufficiency

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Abstract

Objective: Steroidogenic factor 1 (SF1), officially designated NR5A1, is a nuclear receptor that plays key roles in endocrine development and function. Previous reports of human SF1 mutations revealed a spectrum of phenotypes affecting adrenal function and/or gonadal development and sex differentiation. We present the clinical phenotype and functional effects of a novel SF1 mutation.

Patient: The patient is a 22-year-old 46, XY Japanese patient who presented with dysgenetic testes, atrophic vasa deferentia and epididymides, lack of Müllerian structures, and clitoromegaly. Endocrine studies revealed normal adrenal function.

Results: Analysis of the SF1 gene revealed compound heterozygosity for a previously described p.G146A polymorphism and a novel missense mutation (p.R84C) in the accessory DNA-binding domain. The father carried the p.G146A polymorphism and the mother had the p.R84C mutation; both were clinically and reproductively normal. Functional studies demonstrated that the p.R84C SF1 had normal nuclear localization but decreased DNA-binding affinity and transcriptional activity compared with wild-type SF1; it did not exhibit any dominant negative activity.

Conclusions: These results describe the human phenotype that results from compound heterozygosity of the p.G146A polymorphism and a novel p.R84C mutation of SF1, thereby extending the spectrum of human SF1 mutations that impair testis development and sex differentiation in a sex-limited manner while preserving normal adrenal function.

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Introduction

The nuclear receptor steroidogenic factor 1 (SF1, officially designated NR5A1) initially was identified as a regulator of the steroidogenic cell-specific expression of the cytochrome P450 steroid hydroxylases (1, 2). Subsequent studies have shown that SF1 is a key regulator of endocrine function within the hypothalamic–pituitary–steroidogenic organ axis (3). Knockout mice lacking SF1 have adrenal and gonadal agenesis, XY sex reversal, structural abnormalities of the ventromedial hypothalamic nucleus, and altered gonadotropin expression by pituitary gonadotropes (4). Structurally, SF1 consists of a zinc finger DNA-binding domain, ligand-binding domain, and activation function-2 domain characteristic of nuclear receptors. It also contains a novel accessory DNA-binding domain – sometimes designated the C-terminal extension – which confers binding site stability and specificity by

interacting with nucleotides 5' of the nuclear receptor half-site (5, 6). The C-terminal extension lies just downstream of the canonical zinc finger DNA-binding domain and is further divided into the T box, which interacts with the DNA backbone, and the A box, which interacts with the minor groove of DNA (6, 7).

To date, 11 published SF1 mutations have been described in human patients. Although the first three cases (8–10) presented with adrenal insufficiency, the others had normal adrenal function (11–15). Moreover, these individuals displayed varying degrees of gonadal dysfunction, with apparently normal ovarian function in a genotypically female patient (10). Additional SF1 mutations have been described in abstract form; most of these were heterozygous, causing gonadal dysgenesis without adrenal insufficiency (16–19). In addition to the known mutations of SF1, a polymorphism (p.G146A) has been described that slightly reduces transactivation activity (20). Here, we report a patient

with compound heterozygosity for the p.G146A polymorphism and a novel SF1 mutation (p.R84C) in the accessory DNA-binding domain, that results in mild gonadal dysgenesis but no adrenal insufficiency.

Patient and methods

Patient

This Japanese patient was born at term to non-consanguineous parents. No abnormalities in the external genitalia were apparent at birth, and the patient was reared as a female. At 11 months of age, she was referred to the Urology Division with clitoromegaly. Genital examination revealed slight clitoromegaly and posterior labial fusion, with presumed gonads that were palpable bilaterally in the inguinal region. The patient's karyotype was 46, XY. At 17 months, two small gonads located in the inguinal regions were removed. Grossly, atrophic deferent ducts and epididymides were noted. Microscopic examination of gonads (Fig. 1) revealed seminiferous tubules containing immature Sertoli cells and decreased germ cells. Interstitial Leydig cells were prominent. Genitography revealed a blind vaginal pouch and urogenital sinus. No Müllerian derivatives were found at laparotomy, and feminizing genitoplasty was performed at 3 years of age.

The patient was referred for puberty induction at 12.5 years of age, and an endocrine evaluation was performed. Serum testosterone and estradiol levels were undetectable, and basal gonadotropin levels were elevated, with a predominance of follicle-stimulating hormone over luteinizing hormone (FSH, 76.0 IU/l; LH, 19.5 IU/l). Estrogen therapy begun at that time induced normal breast development.

Adrenal function was evaluated at 22 years of age after identification of a mutation in the *NR5A1* gene.

Figure 1 Gonadal histology reveals seminiferous tubules occupied by immature Sertoli cells and few germ cells (400× magnification; hematoxylin and eosin stained).

Physical examination revealed a normotensive, phenotypic female with a height of 163.3 cm, a weight of 61.3 kg, and a body mass index of 23. Breasts were Tanner stage 5, and pubic hair was Tanner stage 4. The serum cortisol level increased normally from 223.5 to 775.3 nmol/l after acute adrenocorticotropin (1–24) stimulation without any abnormal accumulation of adrenal steroid precursors. Further history revealed that no adrenal crisis had occurred even during surgery.

The younger sister of the proband's maternal grandmother had a son with hypospadias. The proband's mother had a total of three children including a healthy daughter (46, XX) and a son (46, XY) with hypospadias. She experienced menarche at 14 years, menopause at 46 years, and never had adrenal crisis. At the time of this study, the mother was 50 years old and had a serum LH level of 26.7 IU/l, FSH level of 37.3 IU/l, and estradiol level of <36.7 pmol/l. The proband's father was a normal healthy male aged 52 years at the time of this study. Both parents appear to have normal fertility and reproductive function.

Mutational analysis

With informed consent under a protocol approved by the Institutional Review Board of Kobe Children's Hospital, leukocyte DNA was extracted using standard methods, and sequencing of the *SF1* gene was performed on PCR-amplified target regions. Oligonucleotide primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html).

Plasmids

pcDNA.hSF1 (pcDNA3.1/Zeo(+), Invitrogen) containing human SF1 cDNA was used to generate expression vectors carrying either the p.R84C mutation or the previously described p.G146A polymorphism (20, 21). The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce a c.C250T mutation (designated p.R84C SF1) or a c.G437C mutation (designated p.G146A SF1). Similarly, pcDNA.hSF1 HA tag was used as template to generate the p.R84C mutant SF1 N-terminal hemagglutinin (HA)-tagged fusion protein. All mutations were confirmed by DNA sequencing. The human 17 α -hydroxylase (CYP17) promoter plasmid includes 1.1 kb CYP17 5'-flanking region inserted into pGL3 Basic (Promega) (22).

Immunofluorescence

To determine subcellular localization of the mutated SF1 protein, transfection analyses in COS-7 cells were performed. Cells were plated at a density of 50 000 cells per well on Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY, USA). After 24 h, the cells were

transiently transfected with empty vector or HA-tagged WT or p.R84C SF1 expression vector using FuGENE 6 Transfection Reagent (Roche). Forty-eight hours after transfection, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunofluorescence analysis was performed using anti-HA primary antibody (HA.11, CRP Inc., Berkeley, CA, USA) at a 1:1000 dilution and FITC-conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:200 dilution. Expression of HA-tagged SF1 was visualized with an Optiphot microscope (Nikon, Melville, NY, USA).

Gel mobility shift assays

Complementary synthetic oligonucleotides (5'-GGCCA-CAGATTCTCCAAGGCTGAT-3'; 5'-GGCATCAGCCTTG-GAGAATCTGT-3') containing the mouse 21-hydroxylase (Cyp21)-140 SF1-responsive element were annealed and end-labeled with [³²P]dCTP using Klenow fragment. *In vitro* translated wild-type and p.R84C SF1 were produced with the TNT coupled reticulocyte lysate system (Promega). Comparable amounts of wild-type and p.R84C SF1 proteins in the *in vitro* translation reactions were confirmed by immunoblot analysis with an anti-SF1 antibody (data not shown). Gel mobility shift assays were performed with 2 µg poly (dI·dC/-dI·dC) as non-specific competitor and the indicated amounts of reticulocyte lysate (23).

Cell culture and luciferase assays

Analyses of transcriptional activity were carried out in both steroidogenic and non-steroidogenic cell lines. Y1 mouse adrenocortical tumor cells were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (FBS). Monkey kidney COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. For luciferase assays, cells were plated on 96-well plates at a density of 5000 cells per well. Transient transfections of Y1 and COS-7 cells were performed using FuGENE 6 Transfection Reagent (Roche). Cells were cotransfected with constant concentrations of the CYP17 promoter plasmid (10 ng), and expression plasmids driving expression of wild-type SF1 (5 ng), p.G146A SF1 (5 ng), or increasing concentrations of p.R84C SF1 (5–50 ng). For analysis of dominant negative activity, cells were cotransfected with the promoter plasmid (10 ng) and wild-type SF1 expression vector (5 ng) in the absence or presence of increasing concentrations of p.R84C SF1 expression vector (5–50 ng). Empty vector was used to equalize the total DNA concentration in each transfection. Cells were lysed 48 h after transfection and luciferase assays were performed. Data analyzed by Student's *t*-test are presented as means ± s.d. from at least three experiments performed in triplicate.

Results

Mutational analysis

Direct sequencing of SF1 in DNA from the patient identified a heterozygous c.C250T mutation in exon 4 (Fig. 2), which resulted in an arginine (CGC)-to-cysteine (TGC) substitution at codon 84 (p.R84C). The patient's mother was heterozygous for the mutated and wild-type alleles. Since the mutation eliminates a BssH II site, the heterozygous states of the patient and her mother were confirmed by restriction enzyme analysis (Fig. 2). This mutation was absent in 55 normal controls (110 alleles). The patient and her father were heterozygous for a previously described p.G146A polymorphism in exon 4 of the SF1 gene. DNA could not be obtained from the patient's sister and brother. Although both parents are heterozygous for variant SF1 alleles, they have apparently normal fertility and reproductive function.

Functional characterization of the mutation

To explore the molecular basis for the endocrine disorder in our patient, we examined the effect of the p.R84C missense mutation and the p.G146A polymorphism on SF1 function. We first performed gel

Figure 2 Sequence analysis of SF1 mutation. (A) DNA sequence analysis revealed a heterozygous mutation in exon 4 of SF1 causing an arginine-to-cysteine substitution at codon 84 (p.R84C). (B) As the mutation eliminates a BssH II site, restriction enzyme analysis was used to confirm the heterozygous state of the patient and her mother.

mobility shift assays with *in vitro* translated wild-type SF1 or p.R84C SF1 and a probe from the mouse-140 Cyp21 SF1-responsive element. Both proteins bound the probe to form a shifted complex, although the apparent affinity of the p.R84C SF1 for DNA was consistently lower than that of wild-type SF1 (Fig. 3A). The decreased affinity of the p.R84C SF1 is likely because the mutation resides in the accessory DNA-binding region, which stabilizes DNA binding of SF1 and related receptors by interacting with nucleotides 5' of the classic recognition half-site (5, 24). These data indicate that the p.R84C mutation decreases but does not totally abolish its ability to bind to its cognate response element.

To examine the effect of the p.R84C mutation on expression and subcellular localization of SF1, we transiently transfected wild-type or p.R84C SF1 expression vectors carrying a hemagglutinin epitope tag into COS-7 cells and then examined location and intensity of immunofluorescent staining. The wild-type and p.R84C SF1 proteins were expressed at comparable levels

and localized exclusively to the nucleus (Fig. 3B). Thus, although the p.R84C mutation is located immediately adjacent to the proposed nuclear localization sequence of SF1 (bases 89–101, (25)), it does not alter its trafficking or stability. Previous studies have confirmed the proper nuclear localization of p.G146A SF1 (20).

To assess the transcriptional activity of the wild-type, p.R84C and p.G146A SF1 proteins, we performed transient transfection assays. In both Y1 adrenocortical and COS-7 monkey kidney cells, cotransfection with 5 ng wild-type SF1 significantly ($P < 0.01$) stimulated reporter gene expression driven by the human (CYP17) promoter. In contrast, the p.R84C mutation required larger amounts (15 ng, $P < 0.05$; 50 ng, $P < 0.01$) of transfected p.R84C SF1 to significantly stimulate CYP 17 promoter activity (Fig. 4A). This finding suggests that the mutation in the accessory DNA-binding domain decreases but does not abolish SF1 transcriptional activity. Contrary to previous reports, we did not see any decreased transactivation activity of the p.G146A SF1 polymorphism in our transfection studies (Fig. 4A). When wild-type and p.R84C SF1 expression plasmids were cotransfected (Fig. 4B), the mutated SF1 did not inhibit transcriptional activity of wild-type protein, suggesting that it does not act as a dominant negative inhibitor. Rather, the p.R84C SF1 mutation augmented the transcriptional activity of wild-type SF1 in a dose-dependent manner, consistent with its partial function. In a preliminary experiment, similar results were obtained when p.G146A SF1 and p.R84C SF1 were cotransfected (data not shown).

Figure 3 DNA binding and nuclear localization of wild-type and mutated SF1. (A) WT and p.R84C SF1 proteins were produced by coupled *in vitro* transcription/translation and used in EMSAs with an oligonucleotide probe comprising the Cyp21-140 SF1-responsive element as described in Patient and methods. WT SF1 (4 μ l lysate) formed a shifted complex at the indicated position, while the amount of shifted complex formed by the p.R84C SF1 protein (1, 4, and 8 μ l lysate) was decreased. The (–) lane contained lysate programmed only with empty expression vector. (B) Nuclear localization of WT and p.R84C SF1. COS-7 cells were transiently transfected with WT or p.R84C expression plasmids, and SF1 was detected by immunohistochemistry as described in Patient and methods.

Discussion

Compound heterozygosity for a novel SF1 mutation, p.R84C, and a previously described SF1 polymorphism, p.G146A, was identified in a 46, XY sex-reversed patient with mildly dysgenetic testes and normal adrenocortical function. Upon histological analysis, the testes were found to be relatively intact, perhaps because gonadectomy was performed as early as 17 months of age. Müllerian regression had occurred in our patient, suggesting that the prenatal Sertoli cell function was conserved.

Functional analysis of the p.R84C SF1 mutation revealed decreased DNA binding and attenuated transactivation activity. The p.R84C mutation is the third known mutation located in the accessory DNA-binding region of human SF1. Achermann and colleagues reported a p.R92Q mutation that, like the p.R84C mutation, partially impaired DNA binding and transactivation activity with no dominant negative activity (9). A second mutation, p.G91S, results in a protein with decreased transactivation activity and DNA binding, and mild dominant negative activity present only at high ratios of mutated to wild-type expression plasmids (14). These findings are consistent with earlier mutational studies of

Figure 4 Effect of the SF1 mutation on transcriptional activity. Transient transfection assays with the WT, p.R84C, and p.G146A SF1 expression plasmids and the human CYP17-luciferase reporter plasmid were performed in the indicated cells lines as described in Patient and methods. The fold inductions over reporter alone (—) with the wild-type (WT), p.G146A SF1, and p.R84C SF1 plasmids are indicated. Numbers below graphs specify nanograms of transfected DNA. (A) p.R84C SF1 shows significantly attenuated transcriptional activity. (B) p.R84C SF1 does not exhibit dominant negative activity in transient transfection assays. Data are presented as means $\pm$ s.d. from at least three experiments performed in triplicate. * $P \leq 0.05$; ** $P \leq 0.01$.

the accessory DNA-binding region of Ftz-F1, the *Drosophila* SF1 homolog, in which mutations of R581 and R589 – the counterparts of R84 and R92 of human SF1 – partially impaired DNA binding and transcriptional activation. Combined mutation of G587 and G588 – the counterparts of G90 and G91 – showed a marked decrease in DNA-binding affinity (5). Of the three known mutations in the accessory DNA-binding domain, p.R92Q has the highest conserved transcriptional activity, while p.R84C appears to have the lowest (14). Notably, the phenotypic consequences of the three mutations do not correlate precisely with their relative levels of impaired transcriptional activity *in vitro*. Whereas the patients carrying either the p.R84C or p.G91S mutation had a sex-limited autosomal dominant disorder that resulted in normal adrenal function and small dysgenetic testes (14), the p.R92Q mutation caused a more severe phenotype with adrenal insufficiency and impaired regression of Müllerian structures that were inherited in an autosomal recessive manner (9).

Normal reproductive function in the mothers carrying either the p.R92Q (9), p.M78I, or p.G91S (14) mutations, among others (18), and the apparently

normal ovaries in the patient described by Biason-Laubier & Schoenle (10) have led to the proposal that ovarian development requires only one functional SF1 allele. Consistent with this model, the patient's mother had apparently normal sex differentiation and fertility despite carrying the p.R84C mutation.

The p.G146A polymorphism, which is located in the hinge region of SF1 and mildly diminishes transactivation activity (20), has been proposed as a susceptibility factor for adrenal disease, cryptorchidism, severe micropenis, and type 2 diabetes (20, 26–28). In our experiments, the p.G146A SF1 mutation did not impair transcriptional activation. The studies differed in the SF1-responsive promoters and cell lines; thus, the apparent discrepancy may merely reflect different experimental conditions. The compound heterozygosity of the patient for the p.G146A mutation and the p.R84C mutation may have perturbed SF1 function sufficiently to allow the phenotypic expression of the p.R84C mutation in a heterozygous state. Further analyses of human subjects carrying the p.G146A polymorphism in combination with other mutated alleles will be needed to define the precise genetics involved.

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