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DATA REPORT

A novel C-terminal truncating *NR5A1* mutation in dizygotic twins

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Nuclear receptor subfamily 5, group A, member 1 (NR5A1) is a nuclear receptor involved in gonadal and adrenal development. We identified a novel C-terminally truncating *NR5A1* mutation, p.Leu423Trpfs*7, in dizygotic twins with 46,XY disorders of sex development. Our results highlight the functional importance of C-terminal region of NR5A1 and indicate that *NR5A1* mutations can be associated with intrafamilial phenotypic variations, progressive testicular dysfunction, hypogonadotropic hypogonadism, and borderline adrenal dysfunction.

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Nuclear receptor subfamily 5, group A, member 1 (NR5A1; alias SF-1 and Ad4BP) is a nuclear receptor involved in the development of gonads and adrenal glands.[1,2] NR5A1 consists of 461 amino acids and contains DNA binding and ligand-binding domains.[1,2] The ligand-binding domain is located in the carboxyl (C)-terminal region of the protein and harbors the activation function-2 (AF-2) domain, which mediates the interaction between NR5A1 and its cofactors.[1] Although NR5A1 is known as an orphan receptor, recent studies have suggested that phosphatidylinositol (3,4,5)-trisphosphate (PIP3) may be a ligand of NR5A1.[3] Blind *et al.*[3] showed that three loop structures in the ligand-binding domain, which are designated as L2–3, L6–7, and L11–12, are essential for the binding of NR5A1 to PIP3.

To date, more than 40 heterozygous mutations in *NR5A1* (NM_004959.4) have been identified in patients with 46,XY disorders of sex development (DSD).[2] A small percentage of mutation-positive patients exhibited adrenal insufficiency, indicating that during development, testes are more vulnerable to NR5A1 dysfunction than adrenal glands.[1,2] Previously reported *NR5A1* mutations include five nucleotide alterations in the C-terminal region, namely, p.Val424del, p.Arg427Alafs*139, p.Leu437Gln, p.Leu437Thrfs*57, and p.Glu445*.[4–8] Of these, p.Arg427Alafs*139, p.Leu437Thrfs*57, and p.Glu445* remove the L11–12 loop structure and the AF-2 domain, whereas p.Val424del and p.Leu437Gln affect amino acids adjacent to L11–12 (Supplementary Figure S1). *In vitro* analysis confirmed that p.Val424del and p.Leu437Gln had low-transactivating activities.[4,6] Five individuals carrying these C-terminal mutations invariably manifested 46,XY DSD without adrenal insufficiency (Supplementary Figure S1). These findings indicate that the C-terminal region of NR5A1 contains a component essential for protein function in the developing testes. However, considering the limited number of previous reports, further studies are necessary to determine the phenotypic consequences of mutations in the C-terminal region of NR5A1.

Here, we report a hitherto unreported C-terminal truncating mutation in *NR5A1* identified in dizygotic male twins. This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. Written informed consent was obtained from the patients' parents. The twins (patients 1 and 2) were born to nonconsanguineous Japanese parents at 38 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, both patients manifested hypomasculinized male-type external genitalia characterized by a micropenis, hypospadias, a bifid scrotum, and undescended testes (Table 1). The patients were otherwise healthy and had no skin pigmentation. They had 46,XY karyotype. Ultrasonography detected bilateral testes in the upper part of scrotum but no Müllerian derivatives. Blood hormone examinations at birth revealed normal testosterone values in patients 1 and 2. However, examinations at two months of age showed compromised testosterone production in both patients; testosterone responses to human chorionic gonadotropin stimulation were blunted in patient 1 and completely absent in patient 2 (Table 1). In addition, patient 2 manifested low-gonadotropin levels at two months of age. Elevated blood levels of adrenocorticotropic hormone in patient 2 and blunted cortisol responses to adrenocorticotropic hormone stimulation in patient 1 suggested subnormal glucocorticoid production. Thus, patients 1 and 2 were diagnosed as having testicular dysfunction and borderline adrenal dysfunction.

We performed molecular analysis of *NR5A1* in patients 1 and 2 and their parents. Genomic DNA samples were extracted from peripheral leukocytes. The entire coding regions of *NR5A1* were PCR-amplified and sequenced. The primer sequences are available upon request. Through this analysis we identified a hitherto unreported heterozygous frameshift mutation (c.1267delC, p.Leu423Trpfs*7) in patients 1 and 2 (Figure 1a,b). The mutation was located within the last exon of *NR5A1* and satisfied the requirement to escape nonsense-mediated mRNA decay.[9] Hence, this mutation was predicted to encode a

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Table 1. Clinical findings of the patients

	Patient 1		*Patient 2*	
Physical findings at birth				
Birth weight (g)	2,614		2,574	
Birth length (cm)	45.7		45.7	
Penile length (mm)	11 (−3.6 s.d.)		6 (−4.6 s.d.)	
Hypospadias	Urethral opening at the proximal penile shaft		Urethral opening at the proximal penile shaft	
Scrotum	Bifid scrotum		Bifid scrotum	
Gonads	Bilateral testes in the upper part of scrotum		Bilateral testes in the upper part of scrotum	
Müllerian structures	Absent		Absent	
Endocrinological findings				
At birth				
LH (mIU/ml)	≦0.10		≦0.10	
FSH (mIU/ml)	0.10		0.11	
Testosterone (ng/ml)	*1.84* (0.66 ± 0.42)		1.06 (0.66 ± 0.42)	
ACTH (pg/ml)	*69.1* (7.2–63.3)		*308* (7.2–63.3)	
Cortisol (μg/dl)	14.0 (6.2–19.4)		19.0 (6.2–19.4)	
Two months after birth	Basal	Peak	Basal	Peak
LH (mIU/ml)[a]	5.53 (1.20–7.90)	14.70	≦**0.10** (1.20–7.90)	2.01
FSH (mIU/ml)[a]	*8.41* (0.80–5.70)	17.13	3.44 (0.80–5.70)	15.89
Testosterone (ng/ml)[b]	1.37 (0.84–4.78)	3.75 (3.67–13.77)	≦**0.03** (0.84–4.78)	≦**0.03** (3.67–13.77)
Cortisol (μg/dl)[c]	14.3 (6.2–19.4)	**17.7** (≧20.0)	6.94 (6.2–19.4)	23.6 (≧20.0)

Abbreviations: ACTH, adrenocorticotropic hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone. The conversion factor to the SI unit: LH 1.0 (IU/l), FSH 1.0 (IU/l), testosterone 3.47 (nmol/l), ACTH 0.22 (pmol/l) and cortisol 27.6 (nmol/l). Reference values in normal boys are shown in parentheses. Hormone values below the reference ranges are boldfaced and those above the reference ranges are italicized.
[a]LH releasing hormone stimulation test (100 μg/m^2 bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 min).
[b]Human chorionic gonadotropin stimulation test (3,000 IU/m^2 i.m. for 3 consecutive days; blood sampling on days 1 and 7).
[c]ACTH stimulation test (250 μg/m^2 i.v.; blood sampling at 0, 30, and 60 min).

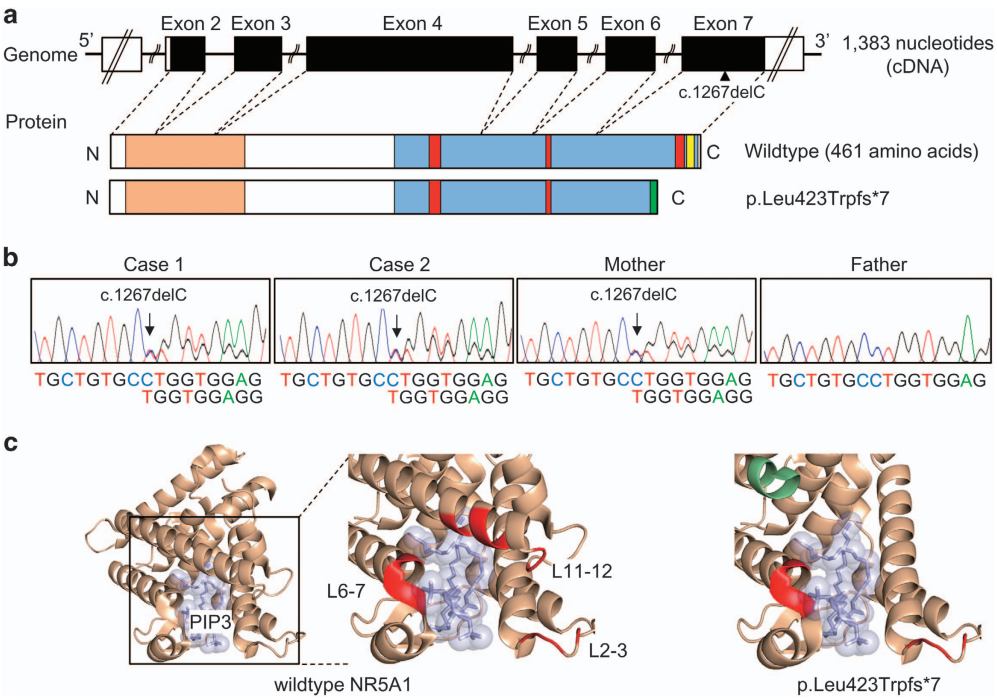

Figure 1. The *NR5A1* mutation identified in this study. (**a**) Schematic representation of wildtype and mutant *NR5A1*/NR5A1. The black and white boxes in the upper panel indicate the coding and non-coding regions, respectively. The orange and blue boxes in the lower panel indicate the DNA binding and ligand-binding domains, respectively. The activation function-2 (AF-2) domain and three loop structures (L2–3, L6–7, and L11–12) are indicated by yellow and red boxes, respectively. The p.Leu423Trpfs*7 mutation deletes L11–12 and the AF-2 domain and adds six aberrant amino acids (green box). (**b**) Chromatograms of the c.1267delC mutation. Arrows indicate the mutated nucleotides. (**c**) Three-dimensional models of NR5A1 and its putative ligand PIP3. Residues in three loop structures (L2–3, L6–7, and L11–12) and H11 mediating the binding between NR5A1 and PIP3 are shown in red. Aberrant amino acids in the mutant protein are shown in green. NR5A1, nuclear receptor subfamily 5, group A, member 1; PIP3, phosphatidylinositol (3,4,5)-trisphosphate.

C-terminally truncated protein lacking L11–12 and the AF-2 domain (Figure 1a). Crystal structure analysis using the PyMol Molecular Graphics System (http://www.pymol.org) suggested that the mutation altered the protein structure of the PIP3 binding site (Figure 1c). The mutation was shared by the phenotypically normal mother of the twins. Next, we performed microsatellite analysis of 13 loci on various chromosomes for the patients and their parents. The results indicated that patients 1 and 2 are dizygotic twins (Supplementary Table S1).

The aforementioned results indicate that loss of the 38 amino acids at the C-terminal end of NR5A1 weakens the transactivating activity in the fetal testis. Our findings, in conjunction with data of previously reported for five C-terminal mutations,[4–8] highlight the functional importance of the C-terminal region of NR5A1 (Supplementary Figure S1). It appears that L11–12 and the AF-2 domain in this region are critical for NR5A1 function because L11–12 has been implicated in PIP3 binding and the AF-2 domain likely mediates the interaction between NR5A1 and other proteins.[3] In this regard, the apparently normal phenotype of the mother of patients 1 and 2 is consistent with previous findings that heterozygous *NR5A1* mutations usually permit fertility in females, although such mutations can cause decreased ovarian reserve and early menopause.[2]

Phenotypic analysis of patients 1 and 2 provided several notable findings. First, patient 2 had more severe micropenis and testosterone deficiency than patient 1. These findings are consistent with prior observations that identical *NR5A1* mutations can be associated with broad phenotypic variations.[8,10,11] Indeed, C-terminal mutations of *NR5A1* have been shown to cause 46,XY DSD of various clinical severities without any genotype–phenotype correlations (Supplementary Figure S1).[4–8] Our case provides an additional example of the intrafamilial phenotypic variation of *NR5A1* abnormalities. Since patients 1 and 2 are dizygotic twins, some genetic factors specific to patient 2 may have enhanced testicular dysfunction in this individual. Second, hormonal evaluation of patient 2 at two months of age showed complete testosterone deficiency, although this individual had partially masculinized external genitalia indicative of moderate androgen exposure at around eight to twelve weeks gestation. Such discrepancy between genital appearance and hormone data were also observed in other male patients with *NR5A1* mutations[5,6,11–14] These data imply that *NR5A1* mutations contribute to progressive testicular dysfunction around birth. Consistent with this, an *NR5A1* mutation was identified in a patient with testicular regression syndrome, an extremely rare condition in which 46,XY patients manifest male-type external genitalia in the absence of gonads.[10] Additionally, progressive deterioration of testosterone deficiency was reported in a boy carrying a *NR5A1* mutation.[15] Third, patient 2 manifested low-gonadotropin levels, indicative of hypogonadotropic hypogonadism. In this regard, p.Leu437Gln, one of the five previously reported C-terminal mutations, caused partial hypogonadotropic hypogonadism in addition to testicular dysfunction.[6] Gonadotropin deficiency was also reported in pituitary-specific *Nr5a1* knockout mice.[16] Thus, hypogonadotropic hypogonadism might have contributed to poor testosterone production in patient 2, although we should follow up with this individual until puberty to clarify whether he has gonadotropin deficiency. Finally, hormonal examinations of patients 1 and 2 indicated mildly compromised glucocorticoid production. Thus far, obvious adrenal insufficiency has been documented only in a small fraction of patients with 46,XY DSD due to *NR5A1* heterozygous mutations.[2] Notably, none of the five previously reported patients with C-terminal mutations manifested adrenal insufficiency.[4–8] Our data suggest that mild glucocorticoid deficiency can be hidden in patients with various types of *NR5A1* mutations.

In conclusion, we identified a hitherto unreported *NR5A1* frameshift mutation in a dizygotic twin pair. Our results highlight the functional importance of the C-terminal region of NR5A1. Furthermore, the results of this study indicate that *NR5A1* mutations, including those in the C-terminal region, can be associated with intrafamilial phenotypic variations, progressive testicular dysfunction, hypogonadotropic hypogonadism, and borderline adrenal dysfunction.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.955.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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Supplementary Information for this article can be found on the Human Genome Variation website (http://www.nature.com/hgv).