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NOTES

Fertility preservation in a family with a novel NR5A1 mutation

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Abstract. The common phenotype of nuclear receptor superfamily 5, group A, member 1 (NR5A1) gene mutations in 46,XY is gonadal dysgenesis without adrenal deficiency. Though the phenotype of gonadal dysgenesis is variable, ranging from complete female to normal male genitalia, an asymptomatic 46,XY male is rare. Preserved fertility has so far been described in only three affected 46,XY males with different mutations, but no functional analysis of these mutations has been performed. Here, we report on male siblings with hypospadias and their asymptomatic father in whom we identified a heterozygous NR5A1 mutation of c.910G>A, p.E304K. Western blotting and subcellular localization revealed no significant difference between the wild type (WT) and E304K. Electrophoretic mobility shift assay experiments showed that E304K abrogated DNA-binding ability. E304K reduced transactivation and had no dominant negative effect. In conclusion, we report on a novel hypomorphic NR5A1 mutation, which may be associated with the phenotype of the family.

Key words: NR5A1 gene, Fertility, Hypospadias

NUCLEAR RECEPTOR SUPERFAMILY 5, GROUP A, MEMBER 1 (NR5A1) gene encodes nuclear receptor that regulates the transcription of genes involved in reproduction, steroidogenesis, and sexual differentiation. It is expressed in the adrenal glands, gonads, pituitary gland and ventromedial hypothalamic nucleus [1-3].

The common phenotype of heterozygous NR5A1 mutations in human is considered to be gonadal dysgenesis without adrenal deficiency. In 46,XY individuals, phenotype of external genitalia ranges from complete female-type to normal male-type. However, an asymptomatic and fertile 46,XY male with heterozygous NR5A1 mutations is rare.

To date preserved fertility in 46,XY male who carrying NR5A1 mutations have been reported in only three individuals [4-6]. Two of the three males were asymptomatic and one presented hypospadias. One of the asymptomatic males carried the mutation in a mosaic pattern. All their sons and grandsons who inherited the mutations in heterozygous state had various degrees of 46,XY disorder of sex development (DSD). In these literatures, functional analyses of the mutations have not been performed.

Here we report on a pair of male siblings with isolated severe hypospadias and their father carrying a novel heterozygous mutation of c.910G>A, p.E304K in NR5A1 gene. Their asymptomatic father, preserved fertility, also carried the p.E304K. Functional analyses revealed that this novel mutation retained partial activity, which may result in asymptomatic 46,XY male phenotype with preserved fertility.

Patients

Patient 1 was a 3-year-old boy, who presented with penoscrotal hypospadias. He was referred to our hospital for endocrinological assessment (Fig. 1A). He was the first child of healthy nonconsanguineous Japanese parents and was born through spontaneous vaginal delivery at 39 weeks of gestational age, following a natural and uneventful pregnancy. His birth weight and height were 3060 g (0.26 SD) and 49 cm (0.07 SD),
respectively. He was diagnosed with penoscrotal hypospadias at birth. He had no other manifestations including adrenal deficiency. He underwent surgical treatment at the age of 3 years, when his penile length reached 45 mm. Testicular volume was both 1 mL; the testes were not completely descended in the scrotal region, but could easily descend manually. Karyotype was 46,XY.

Endocrinological evaluation is summarized in Table 1. Basal gonadotropin and testosterone levels, GnRH stimulating test values and human chorionic gonadotropin (HCG) stimulation test values were normal.

Presently, Patient 1 is a 16-year-old teenager with spontaneous pubertal development. Adrenal function was considered normal, based on the dehydroepiandrosterone-sulfate (DHEA-S) level. Current height is 168.8 cm (−0.12 SD), testicular volume is both 15 mL, and pubic hair is at Tanner stage IV. Basal gonadotropin and testosterone levels were normal but anti-müllerian hormone (AMH) was low for his Tanner stage.

Patient 2 was a 3-month-old boy, who is the younger brother of Patient 1 (Fig. 1A). He presented with peno-scrotal hypospadias and was referred to our hospital for endocrinological assessment. He was also born by spontaneous vaginal delivery at 39 weeks of gestational age, following a natural and uneventful pregnancy. Birth weight and height were 2730 g (−1.07 SD) and 47.0 cm (−0.97 SD), respectively. Testicular volume was both 1 mL; the testes were completely descended. He had bifid scrotum. Penile length was 37 mm. No other manifestations including adrenal deficiency were presented. Karyotype was 46,XY.

Endocrinological evaluation is summarized in Table 1. Basal gonadotropin and testosterone levels were normal. 

![Fig. 1 A Pedigree of the family B Partial sequences of the PCR products. The chromatogram represents heterozygous substitution of glutamic acid (GAG) in place of lysine (AAG) at codon 304. The red down-pointing arrow indicates the mutated nucleotide.](image)

Table 1 Endocrinological evaluation

<table>
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<tr>
<th>Patient</th>
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<tr>
<td>Age</td>
<td>3 years old</td>
<td>4 months old</td>
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<tr>
<td>Karyotype</td>
<td>46,XY</td>
<td></td>
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<tr>
<td>GnRH test Basal/Maximum</td>
<td>LH (mIU/mL)</td>
<td>0.5 / 3.82 (60 min)</td>
</tr>
<tr>
<td></td>
<td>FSH (mIU/mL)</td>
<td>0.92 / 5.13 (90 min)</td>
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<tr>
<td>HCG test Basal/Maximum</td>
<td>T (ng/mL)</td>
<td>&lt;0.1 / 2.8</td>
</tr>
<tr>
<td></td>
<td>DHT (ng/mL)</td>
<td>N/A / 0.54</td>
</tr>
<tr>
<td>Age</td>
<td>16 years old</td>
<td>13 years old</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
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<td>2.59</td>
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<tr>
<td>FSH (mIU/mL)</td>
<td>5.04</td>
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</tr>
<tr>
<td>T (ng/mL)</td>
<td>3.94</td>
<td>3.81</td>
</tr>
<tr>
<td>AMH (pmol/L)</td>
<td>11.3</td>
<td>5.88</td>
</tr>
</tbody>
</table>

HCG, human chorionic gonadotropin; T, testosterone; DHT, dihydrotestosterone; AMH, anti-müllerian hormone; N/A, not available.
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year of age for penoscrotal hypospadias.

Presently, he is a 13-year-old teenager with spontaneous pubertal development. Current height is 155.6 cm (−0.41 SD). Testicular volume of the both sides are 12 and 15 mL, respectively, and his pubic hair is at Tanner stage III–IV. Basal gonadotropin and testosterone levels were normal but AMH level was low for his Tanner stage, as well as his brother.

The father of patients 1 and 2 is a 46-year-old healthy Japanese man, who self-reported normal external genitalia. He had two children by a healthy nonconsanguineous Japanese woman without infertility treatment. At the time of birth of his children, he was aged 30 and 33 years, respectively. He refused hormonal evaluation, and no endocrinological assessments were performed for him.

Mutational Analysis

Genomic DNA in the patients’ peripheral leukocytes and the saliva was extracted by using standard techniques. Genomic DNA samples were analyzed for mutations in 25 known causative/candidate/susceptible genes for non-syndromic hypospadias, i.e., AR, ATF3, BMP4, BMP7, BNC2, CTGF, CYP1A1, CYR61, DGKK, EGF, ESR1, ESR2, FGFR2, GSTM1, GSTT1, HOXA4, HOXB6, HSD3B2, HSD17B3, MAMLD1, MID1, NR5A1, SRD5A2, and WT1. Mutations were screened by the Haloplex method (Agilent Technologies, Palo Alto, CA) on a Miseq next-generation sequencer (Illumina, San Diego, CA, USA) (Kon et al. in submission). A NR5A1 mutation indicated by the screening analysis was confirmed by Sanger sequencing.

To determine the presence of somatic mosaicism of the father, we subcloned PCR products of the DNA from the two different tissues; saliva and nails, took 100 colonies of each, screened for the mutation by PCR-Direct sequence, and calculated the ratio of mutant to wild-type alleles.

Crystal Structure Modeling

The crystal structure of the NR5A1 ligand-binding domain (LBD) (Protein Data Bank ID 1YPO; http://www.rcsb.org/pdb/) was used as a reference wild-type structure for modeling the structure of E304K NR5A1 by using the PyMol Molecular Graphics System (http://www.pymol.org).

Functional Studies

Construction of expression vectors

To generate wild-type (WT) NR5A1 expression vectors, NR5A1-coding region was amplified from human ovary library cDNA and cloned into pCMV-myc (Clontec, Palo Alto, CA). Then the E304K mutation was introduced by site-directed mutagenesis by using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan).

Western blotting

Empty, WT, and mutant type (MT) vectors (2 μg) were transfected into COS1 cells in a plate with a diameter of 35 mm. Cells were harvested 48 h after transfection, and their whole cell proteins were extracted using the M-PER Mammalian Protein Extraction Reagent (Thermo). Western blotting was conventionally performed with mouse anti-myc monoclonal antibody (Invitrogen) and rabbit anti β2-microglobulin monoclonal antibody (Abcam) used as an internal control.

Subcellular localization analysis

WT and MT vectors (2.5 µg) were transfected in COS1 cells in a dish with a diameter of 15 mm. They were fixed in 4% paraformaldehyde, permeabilized with 0.2% TritonX-100, and blocked using 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 24 h after transfection. The cells were then incubated with a mouse anti-myc monoclonal antibody (Invitrogen) and rabbit anti β2-microglobulin monoclonal antibody (Abcam) used as an internal control.

Electrophoretic Mobility Shift Assay (EMSA)

The sequences from nucleotides −52 to −31 of human CYP11A1 contain the putative wild type NR5A1 binding site, which were biotin-labeled double-stranded oligonucleotide and used as a probe in the EMSA experiments [7]. Ten micrograms of the extracted nuclear protein was incubated at room temperature in a 20-µL binding reaction mixture containing 20-fmol probe, 50 mM KCl, 5 mM MgCl2, 2.5% glycerol, 0.05% NP-40, and 1µg of poly (dl-dC) for 20 min. For competition experiments, an excess (200×) of unlabeled competitor oligonucleotides were included in the binding reactions. The protein–DNA complexes
Results

Mutational analysis

We identified a novel heterozygous c.910G>A (p. E304K) missense mutation in NR5A1 gene in patients 1 and 2. We performed Sanger sequencing on PCR products from genomic DNA to confirm the variants (Fig. 1B). Glu304 was located in the LBD region (Fig. 2A), which is highly conserved among NR5A1 proteins (Fig. 2B). Familial analysis revealed that their asymptomatic father carried the same heterozygous mutation, while the mother had no mutation.

The subcloning analysis revealed the ratio of mutant to wild-type alleles was 47/53 in the saliva, and 46/54 in the nail. This suggests that the father carries the mutation in heterozygous state, not in mosaic in nature.

Crystal Structural Modeling of the LBD region of NR5A1

Glu304 has no connection with Arg427. The glutamic acid to lysine substitution was predicted to make an abnormal residue-residue binding to Arg427 (Fig. 2C).
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Although several bands were detected for E304K protein, they were also identified in wild type and were not competed by the cold competitors. They were considered to be non-specific bands.

Transactivation analyses
In COS1 cells, WT NR5A1 stimulated the transcription of the CYP11A1 reporter in a dose-dependent manner. E304K NR5A1 decreased transcription activity to 40%, compared to WT (P < 0.01). Dominant negative effect was not observed from the co-transfected pattern of WT and E304K NR5A1 (Fig. 3D).

Discussion
Functional analyses revealed that nuclear localization and protein expression of E304K were not disrupted but DNA binding and transcriptional activity were impaired. Since the location of E304K is in the LBD, it is com-

**Fig. 3** Functional Analyses

A Western blotting: WT and E304K NR5A1 were detected as same sized bands.

B Subcellular localization analyses: WT and E304K NR5A1 were localized to the nucleus.

C EMSA experiments: WT and E304K NR5A1 showed specific binding to DNA, which were displaced by an excess amount of cold competitors. In contrast to the WT NR5A1, E304K NR5A1 proteins showed decreased DNA binding affinity.

D Transactivation assays: WT NR5A1 stimulated the transcription of the CYP11A1 reporter in a dose-dependent manner. E304K NR5A1 exhibited reduced transactivation and had no dominant negative effect.
compatible to maintain the nuclear localization and translation of the protein. According to the crystal structural modeling of the LBD region of NR5A1, E304K was predicted to make an abnormal binding of Lys304 to Arg427. This may affect the structure of ligand binding pocket. Glu304 and Arg427 are residues of Helix5 and Helix10 of the LBD, respectively. Helix5 and Helix10 are both component of the ligand-binding pocket [8]. The structural conversion of the ligand-binding pocket may affect the DNA binding. This could be one of the mechanisms underlying the effect of LBD mutation (E304K) on DNA binding. Two previously published reports described the DNA-binding affinity of the mutations in the LBD region [9-10]. In these reports, R255L lost the affinity, while L437Q retained it. Ours is the third report examining the DNA-binding affinity of mutations in the LBD region.

The severity of underandrogenization resulting from 46,XY gonadal dysgenesis is variable, ranging from complete female to normal male genitalia, even in familial cases. Similar to the previously reported familial cases involving 46,XY fertile male, the phenotype of the external genitalia also differed between the two sons and their father in this case. One of the reported fertile 46,XY males carried the heterozygous NR5A1 mutation in mosaic pattern. This is compatible that he was asymptomatic and fertile, and his son inherited the mutation in heterozygous state to be symptomatic. From the subcloning analysis, the father studied in this report was suggested to carry the mutation in heterozygous state, not in the mosaic in nature.

Little is known about the prognosis of gonadal functions such as pubertal development and reproductive functions of 46,XY males with mild undervirilization of the external genitalia due to heterozygous NR5A1 mutations. The two sons and their father in this family had spontaneous pubertal development. The asymptomatic father was obviously fertile. But two sons showed low AMH levels at the latest evaluation. Fertility of the two sons must be confirmed.

Recently, NR5A1 mutations were identified in men with idiopathic spermatogenic failure [11]. In some of these patients, spermatogenesis appeared to be gradually decline with age. Bashamboo et al. [11] hypothesized a progressive loss of gonadal function over time, so that fecundity may be achieved in early adulthood before the development of spermatogenic failure. In this case, the father had his children at the age of 30 and 33 years. At present, we have not evaluated the reproductive function of the father.

In conclusion, we report on two siblings with hypospadias and their healthy father, indicating preserved fertility, who all have a novel hypomorphic NR5A1 mutation.

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Disclosure

The authors have nothing to declare.

References


