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NOTE

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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),

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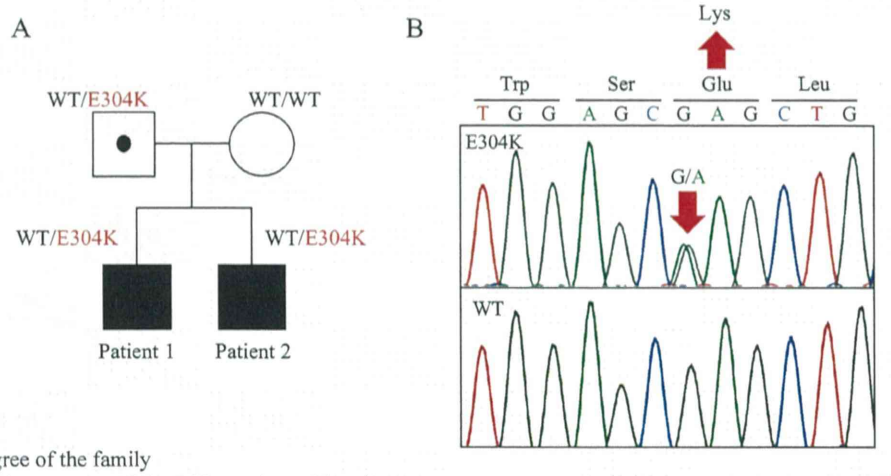

Fig. 1 A Pedigree of the family
B Partial sequences of the PCR products. The chromatogram represents heterozygous substitution of glutamic acid (GAG) in place of lysine (AAG) at codon 304. The red down-pointing arrow indicates the mutated nucleotide.

Table 1

Patient		**1**	**2**
Age		**3 years old**	**4 months old**
Karyotype		46,XY	
GnRH test Basal/Maximum	LH (mIU/mL)	0.5 / 3.82 (60 min)	1.45 / 9.04 (30 min)
	FSH (mIU/mL)	0.92 / 5.13 (90 min)	2.07 / 5.48 (90 min)
HCG test Basal/Maximum	T (ng/mL)	<0.1 / 2.8	1.0 / 7.8
	DHT (ng/mL)	N/A / 0.54	0.52 / 1.57
Age		**16 years old**	**13 years old**
LH (mIU/mL)		7.12	2.59
FSH (mIU/mL)		5.04	4.30
T (ng/mL)		3.94	3.81
AMH (pmol/L)		11.3	5.88

HCG, human chorionic gonadotropin; T, testosterone; DHT, dihydrotestosterone; AMH, anti-müllerian hormone; N/A, not available

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 penoscrotal 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 and 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 for his age. He underwent surgical treatment at 1

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*, *FGF8*, *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 ß2-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) for 1 h. After washing, the cells were incubated with Alexa Fluor Secondary Antibodies (1:1000; Cell Signaling Technology) in the blocking buffer for 1 h. Cells were imaged using the IX-71 fluorescence microscope (Olympus, Tokyo, Japan).

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 $MgCl_2$, 2.5% glycerol, 0.05% NP-40, and 1μg of poly (dI-dC) for 20 min.

For competition experiments, an excess (200×) of unlabeled competitor oligonucleotides were included in the binding reactions. The protein–DNA complexes

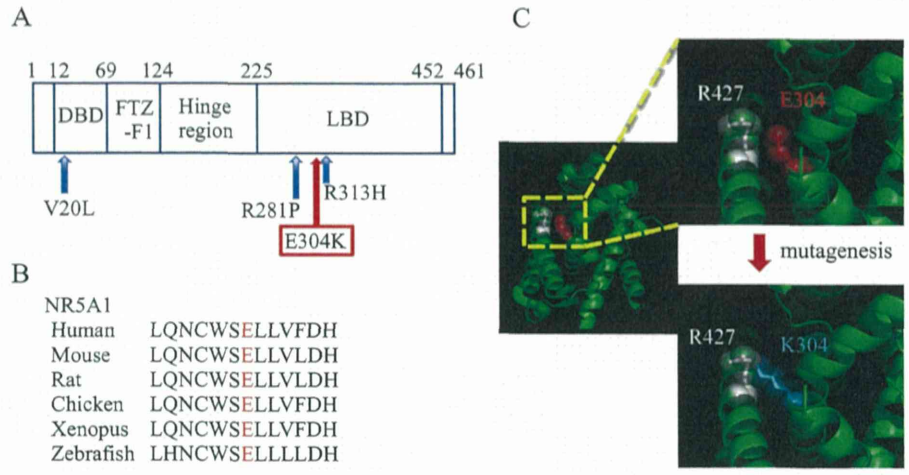

Fig. 2 A Schematic diagram of the functional domains of NR5A1. Arrows indicate the mutations in patients with preserved fertility (details in the main text). Red arrow indicates the mutation of this case.
B Homology study revealed that the glutamic acid residue at codon 304 is highly conserved throughout the species.
C A modeled structure of E304K in comparison with that of WT (upper panels). Crystal structure modeling showing E304K NR5A1 was predicted to make an abnormal binding to R427.

were subjected to gel electrophoresis and transferred onto a nylon membrane. The biotin-labeled probe was detected using the Lightshift Chemiluminescent EMSA Kit (Pierce).

Transcription analysis

Transactivation assay was performed in COS1 cells by using a dual-luciferase reporter assay system (Promega, Madison, Wisc, USA). Luciferase reporter vector was constructed by inserting the *CYP11A1* promoter sequence (−110 to +49) into a pGL4.24 [luc2P/minP] vector (Promega) [7]. The reporter vector (0.3 μg) was transfected to COS1 cells with empty (0.2 μg), WT (0.2 μg), MT (0.2 μg), WT (0.1 μg) plus empty (0.1 μg), MT (0.1 μg) plus empty (0.1 μg) or WT (0.1 μg) plus MT vector (0.1 μg) and PRL-CMV vector (2.5 ng) used as an internal control for the transfection. Empty vector was used to equalize the total DNA concentration in each transfection. Luciferase assays were performed 48 h after the transfection. All assays were performed in triplicate and repeated thrice. The statistical significance was determined by *t* test.

Ethics

Written informed consent was obtained from the patients and their parents. The study protocol was approved by the Institutional Ethical Review Board of the Tokyo Metropolitan Children's Medical Center and National Research Institute for Child Health and Development.

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).

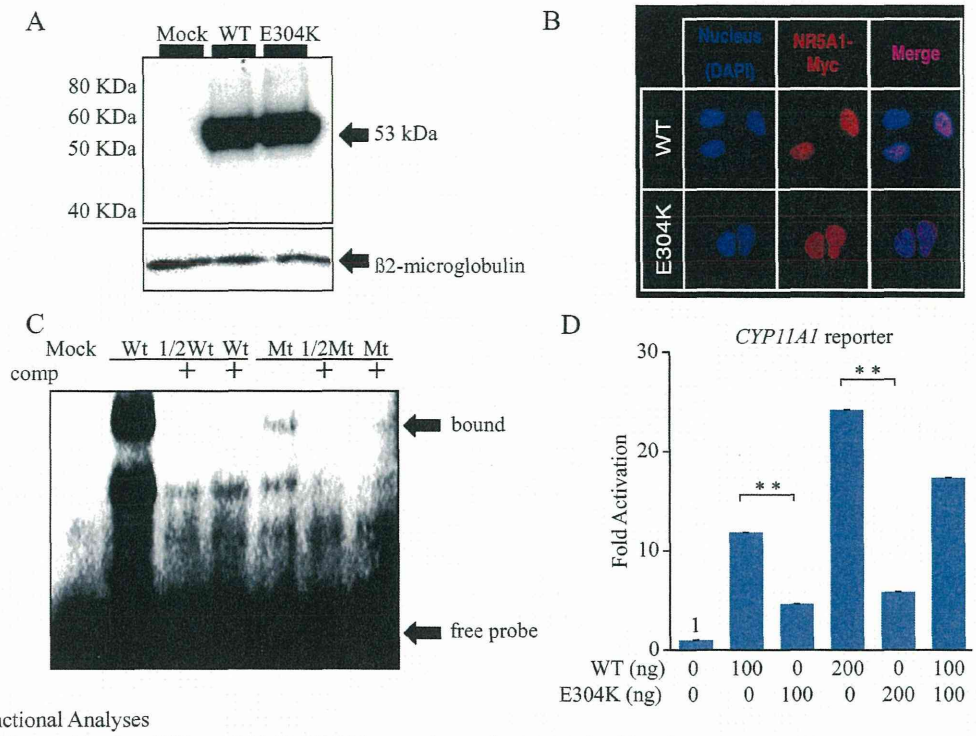

Fig. 3 Functional Analyses
A Western blotting: WT and E304K NR5A1 were detected as same sized bands.
B Subcellular localization analyses: WT and E304K NR5A1 were localized to the nucleus.
C EMSA experiments: WT and E304K NR5A1 showed specific binding to DNA, which were displaced by an excess amount of 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.

Functional Analyses

Western blotting

WT and E304K NR5A1 proteins were expressed in the same size of 53 kDa and same amounts (Fig. 3A). It suggests that translation of E304K is equivalent to WT.

Subcellular localization analyses

WT and E304K NR5A1 proteins localized exclusively in the nucleus (Fig. 3B). Expression and localization pattern are equivalent between WT and E304K, indicating that nuclear targeting were not affected by this mutation.

EMSA

WT NR5A1 proteins specifically bound to the DNA (shown in black arrow with 'bound'); this binding was offered competition by excess (200×) amount of cold competitors. In contrast to WT NR5A1, E304K NR5A1 showed obviously decreased affinity of DNA binding (Fig. 3C).

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 *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-

patible 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.

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CLINICAL REPORT

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Compound Heterozygous Deletions in Pseudoautosomal Region 1 in an Infant With Mild Manifestations of Langer Mesomelic Dysplasia

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Haploinsufficiency of *SHOX* on the short arm pseudoautosomal region (PAR1) leads to Leri–Weill dyschondrosteosis (LWD), and nullizygosity of *SHOX* results in Langer mesomelic dysplasia (LMD). Molecular defects of LWD/LMD include various microdeletions in PAR1 that involve exons and/or the putative upstream or downstream enhancer regions of *SHOX*, as well as several intragenic mutations. Here, we report on a Japanese male infant with mild manifestations of LMD and hitherto unreported microdeletions in PAR1. Clinical analysis revealed mesomelic short stature with various radiological findings indicative of LMD. Molecular analyses identified compound heterozygous deletions, that is, a maternally inherited ~46 kb deletion involving the upstream region and exons 1–5 of *SHOX*, and a paternally inherited ~500 kb deletion started from a position ~300 kb downstream from *SHOX*. In silico analysis revealed that the downstream deletion did not affect the known putative enhancer regions of *SHOX*, although it encompassed several non-coding elements which were well conserved among various species with *SHOX* orthologs. These results provide the possibility of the presence of a novel enhancer for *SHOX* in the genomic region ~300 to ~800 kb downstream of the start codon.

Key words: *SHOX*; langer mesomelic dysplasia; deletion; enhancer

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INTRODUCTION

SHOX on the short arm pseudoautosomal region (PAR1) is a transcription factor gene exclusively expressed in the developing limbs and pharyngeal arches [Rao et al., 1997; Clement-Jones et al., 2000]. Haploinsufficiency of *SHOX* leads to idiopathic short stature and Leri–Weill dyschondrosteosis (LWD; OMIM #127300), and nullizygosity of *SHOX* results in Langer mesomelic dysplasia (LMD; OMIM #249700) [Rao et al., 1997; Belin et al., 1998; Shears et al., 1998; Zinn et al., 2002]. Previous studies in patients with LWD and LMD have identified several copy-number abnormalities in PAR1 that involved coding exons and/or the putative upstream or downstream enhancer regions, together with multiple point

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