Identical *NR5A1* Missense Mutations in Two Unrelated 46,XX Individuals with Testicular Tissues

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Abbreviations: DSD, disorders of sex development; ExAC, the Exome Aggregation Consortium; FOXL2, Forkhead box L2; HGVB, the Human Genetic Variation Browser; NR0B1, nuclear receptor subfamily 0 group B member 1; NR5A1, nuclear receptor subfamily 5, group A, member 1; SOX9, SRY-box 9; TESCO, testis enhancer sequence core element.

Abstract

Context: The association between monogenic mutations and 46,XX testicular/ovotesticular disorders of sex development (DSD) remains rather speculative. Although mutations in *NR5A1* are known to cause 46,XY gonadal dysgenesis and 46,XX ovarian insufficiency, such mutations have not been implicated in the testicular development of 46,XX gonads.

Case Description: Patients 1 and 2 were unrelated 46,XX DSD patients who manifested genital abnormalities at birth. Clinical examinations confirmed the presence of ovotesticular and/or testicular tissues in the gonads and the absence of uterus and vagina. Molecular analysis of 28 genes involved in gonadal development identified a heterozygous p.R92W mutation of *NR5A1* in both patients. This mutation was absent from the clinically normal mothers of patients 1 and 2 and has not been detected in the general population. *In silico* analysis suggested that p.R92W is probably pathogenic and likely causes conformational changes at the DNA-binding site. *In vitro* assays demonstrated that the mutant protein is resistant to the NR0B1 (nuclear receptor subfamily 0 group B member 1)-induced suppression on *SOX9* TESCO (testis enhancer sequence core element) activity. While patient 1 carried additional rare polymorphisms in *FOXL2* and *WWOX*, patient 2 had no other mutations in tested genes.

Conclusions: This study provides the first indication that specific mutations in *NR5A1* may underlie testicular development in 46,XX individuals.

46,XX testicular/ovotesticular disorders of sex development (DSD) are rare conditions in which the developmental process of 46,XX gonads is switched toward testicular formation. 46,XX testicular DSD usually results from translocations of *SRY*-containing DNA fragments from Y chromosomes to X chromosomes (1), while a small percentage of cases is attributed to chromosomal rearrangements that affect *cis*-regulatory regions of *SOX9* or *SOX3* (2). Likewise, 46,XX ovotesticular DSD frequently arises from chromosomal mosaicism or chimerism (3). To date, monogenic mutations have not been associated with 46,XX testicular/ovotesticular DSD, except for those in *RSPO1* and *WNT4*, which were identified in a few patients with syndromic DSD.

Nuclear receptor subfamily 5 group A member 1 (NR5A1, also known as SF1/Ad4BP) is a transcription factor that regulates gonadal and adrenal development. In mice and possibly in humans as well, NR5A1 and SRY-box-9 (SOX9) synergistically activate the testis enhancer sequence core element (TESCO) of *Sox9*/*SOX9* (4). The cooperative transactivation of TESCO by NR5A1 and SOX9 is antagonized by the nuclear receptor subfamily 0 group B member 1 (NR0B1, also known as DAX1) (5). More than 40 loss-of-function mutations of *NR5A1* have been identified in patients with gonadal dysgenesis and/or adrenal insufficiency (6). *NR5A1* abnormality represents one of the major causes of gonadal dysgenesis in genetic males and accounts for a small fraction of primary ovarian insufficiency in genetic females (6). Nevertheless, *NR5A1* mutations have not been implicated in 46,XX testicular/ovotesticular DSD. Here, we identified an *NR5A1* missense mutation in two unrelated 46,XX individuals with testicular tissues.

Subjects and Methods

Case reports

Detailed clinical information of patients 1 and 2 is shown in Table 1 and in the Supplemental Information. These patients are unrelated Japanese individuals with a 46,XX karyotype.

Patient 1 was raised as a female. At birth, this patient manifested ambiguous external

genitalia of Prader stage 3–4. Laparoscopy and cystoscopy confirmed the absence of vagina and uterus. The patient's blood testosterone level was within the reference range of age-matched males, while gonadotropin levels were elevated. At 1 year of age, she underwent gonadectomy. The right and left gonads were found to be immature testis-like tissues and SOX9- and Forkhead box L2 (FOXL2)-positive ovotestis, respectively.

Patient 2 was raised as a male. At birth, he manifested male-type external genitalia with hypospadias and bifid scrotum. Gonadal biopsy and laparoscopy at 6 months of age confirmed the presence of testicular tissues and spermatic cord. Magnetic resonance imaging indicated the absence of vagina and uterus. His gonadotropin and testosterone levels were almost comparable to those of unaffected boys. At 9 years and 7 months of age, the patient had descended testes of 1 mL in volume.

Molecular analyses

This study was approved by the Institutional Review Board Committee and performed after obtaining informed consent. Detailed methods are described in the Supplemental Information.

Genomic DNA samples were obtained from patients 1 and 2. Twenty-eight genes known or predicted to regulate gonadal development were analyzed by next-generation or Sanger sequencing. We also analyzed copy-number alterations in the genome.

The population frequency of identified substitutions was analyzed using the Exome Aggregation Consortium (ExAC) Browser, dbSNP, and the Human Genetic Variation Browser (HGVB). The functional consequences of these mutations were predicted by Polyphen-2 and MutationTaster, and the three-dimensional structure of mutated NR5A1 was predicted by PyMOL. When possible, DNA samples of the parents were also analyzed.

***In vitro* functional assays**

Detailed methods are available in Supplemental Information. We performed luciferase reporter assays for wildtype NR5A1 and p.R92W. Briefly, Chinese hamster ovary-K1 cells were

transiently transfected with expression vectors for *NR5A1*, *SOX9*, and *NR0B1*, a reporter vector containing *SOX9* TESCO, and an internal control vector. Relative luciferase activity was measured 48 hours after transfection by the dual luciferase method.

Results

Molecular analyses

The same heterozygous substitution (c.274C>T, p.R92W) in the A-box motif of the NR5A1 DNA-binding domain was detected in patients 1 and 2 (Fig. 1A and B). This substitution was not found in databases. This substitution was scored "probably damaging" and "disease causing" by Polyphen-2 and MutationTaster, respectively. Protein modeling predicted that the mutation induces conformational changes at the DNA-binding site (Fig. 1C).

Patient 1 carried additional substitutions in *FOXL2* (c.1045C>G, p.R349G) and *WWOX* (c.550C>T, p.L184F), the allele frequencies of which in the ExAC Browser are 32/111,232 and 6/120,756, respectively (Fig. S1). Both substitutions were scored "probably damaging" and "disease causing". Patient 2 had no additional mutations in tested genes. Copy-number alterations were not detected.

In patient 1, the substitutions in *NR5A1* and *FOXL2* were inherited from the father, while the *WWOX* substitution was transmitted from the mother. In patient 2, the *NR5A1* substitution was absent from the mother, indicating paternal or *de novo* origin.

In vitro functional assays

Both wildtype NR5A1 and the p.R92W mutant activated *SOX9* TESCO in cooperation with SOX9. While NR0B1 repressed the TESCO activation by wildtype NR5A1 in a dose dependent manner, it did not affected transactivating activity of the p.R92W mutant (Fig. 1D).

Discussion

We detected identical *NR5A1* mutations in two unrelated 46,XX individuals with testicular tissues. The p.R92W mutation affected the functionally important A-box motif of the DNA-binding domain (7), and has not been identified in the general population. *In silico* analysis suggested that p.R92W is "probably damaging" and causes protein conformational changes. These data indicate that p.R92W is a pathogenic mutation, rather than a benign polymorphism. Testicular formation in patients 1 and 2 can be explained by assuming that p.R92W is a gain-of-function mutation that triggers *SOX9* overexpression, which is known to induce the testicular development of immature 46,XX gonads (2). Indeed, *in vitro* assays demonstrated that p.R92W is resistant to the NR0B1-induced suppression on *SOX9* TESCO activity. The absence of p.R92W in the patients' mothers is consistent with the possible association between this mutation and 46,XX DSD. It is noteworthy that a homozygous mutation in the same codon, p.R92Q, has been identified in an individual with 46,XY gonadal dysgenesis and adrenal insufficiency (8), suggesting the functional importance of the arginine residue at the 92nd position. Differences in the clinical manifestation of p.R92Q and p.R92W possibly reflect differences in the transactivating activity of these mutants. Indeed, *in silico* analysis predicted a structural difference between the mutants (Fig. 1C). Nevertheless, we cannot exclude the possibility that DSD in our patients developed independently of p.R92W. Further studies are necessary to clarify the phenotypic consequences of this mutation.

Notably, patient 1 carried additional rare "probably damaging" polymorphisms in *FOXL2* and *WWOX*. While maternal inheritance of the *WWOX* polymorphism argues against its association to 46,XX DSD, the paternally transmitted *FOXL2* substitution may have affected gonadal development. Indeed, FOXL2 is known to interact with NR5A1 (9), and multiple mutations in *FOXL2* have been reported to increase the risk of 46,XX testicular DSD (10). However, the clinical significance of *FOXL2* substitutions seems limited, if any, because nucleotide changes in the *FOXL2* coding region were not found in patient 2, who had a more severe phenotype than patient 1.

In summary, this study provides the first indication that specific mutations in *NR5A1* may underlie testicular development in 46,XX individuals. This notion needs to be validated in future studies.

Acknowledgements

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Figure legends

Fig. 1.

(A) The *NR5A1* mutation identified in patients 1 and 2. The mutated nucleotide is indicated by an arrow. This mutation was scored "probably damaging" by Polyphen-2.

(B) Position of p.R92W. Black and white boxes indicate the coding and non-coding exons of *NR5A1*, respectively. This mutation affected the A-box motif (blue box) in the DNA-binding domain (DBD). HD, hinge domain; LBD, ligand binding domain.

(C) Three-dimensional protein modeling of p.R92W and a known R92Q mutation. NR5A1 and target DNA are illustrated in pink and blue, respectively. Amino acids at the 92nd codon are highlighted in red. The p.R92W and p.R92Q mutations probably induce conformational changes at the DNA-binding site.

(D) Representative results of luciferase assays using the *SOX9* TESCO reporter. Expression vectors for *NR5A1* and *SOX9* were transfected into CHO-K1 cells, along with the reporter vecotor and various doses of *NR0B1* expression vector (6.25, 25, 100, 400 ng/well). Results are expressed as mean ± standard deviation. WT, wildtype NR5A1; MUT, the p.R92W mutant.

Table 1. Clinical features of patients 1 and 2.

	Patient 1				Patient 2		
Physical findings at birth							
External genitalia	ambiguous external genitalia (Prader stage 3-4)				male-type genitalia with hypospadias and bifid scrotum		
Gonad	not palpable				palpable		
Histology	testis-like (right), ovotestis (left)				testis		
Uterus	absent				absent		
Hormonal findings							
Age at exam[a]	days 6 and 13[b]		1 month		1-5 months[c]		14.8 years
	basal	stimulated	basal	stimulated	basal	stimulated	basal
LH (mIU/mL)[d]	9.1 [0.3-1.9 (M), 0.2-0.7 (F)]	…	…	…	0.5 [0.1-0.4 (M, F)]	6.0 [0.4-6.0 (M), 1.6-4.8 (F)]	14.0 [0.5-3.1 (M), 1.3-33.4 (F)]
FSH (mIU/mL)[d]	13.6 [0.8-3.0 (M), 1.8-8.6 (F)]	…	…	…	2.7 [0.6-3.0 (M), 2.1-6.1 (F)]	9.4 [6.3-15.6 (M), 14.5-21.9 (F)	32.0 [0.9-6.7 (M), 6.3-8.1 (F)]
Testosterone (ng/dL)[e]	…	…	164 [115-404 (M), 0-15 (F)]	566 [>200 (M)]	124 [115-404 (M), 0-15 (F)]	424 [>200 (M)]	140 [119-349 (M), 21-67 (F)]
AMH (ng/mL)	12.6 [55.6 ± 21.3 (M), 1.0 ± 0.9 (F)]	…	…	…	…	…	…
E_2 (pg/mL)[f]	21 [96 ± 44 (M), 31 ± 32 (F)]	…	…	…	10 [46 ± 46 (M), 33 ± 37 (F)]	10 [No reference data]	…
ACTH (pg/mL)	47 [24 ± 11 (M), 26 ± 12 (F)]	…	…	…	…	…	21 [23 ± 6 (M), 22 ± 17 (F)]
Cortisol (µg/dL)[g]	13.4 [12.4 ± 5.3 (M), 12.8 ± 7.1 (F)]	46.7 [38.2 ± 4.4 (M), 10.0 ± 8.1 (F)]	…	…	17.2 [12.4 ± 5.3 (M), 12.8 ± 7.1 (F)]	29.3 [38.2 ± 4.4 (M), 40.0 ± 8.1 (F)]	7.7 [9.5 ± 2.9 (M), 10.1 ± 2.8 (F)]
17-OHP (ng/mL)[g]	…	…	…	…	1.7 [<20.0 (M, F)]	3.9 [<16.6 (M, F)]	…

AMH, anti-Müllerian hormone; E_2, estradiol; 17-OHP, 17-hydroxyprogesterone.

Conversion factors to the SI unit: LH, 1.0 (IU/L); FSH, 1.0 (IU/L); testosterone, 0.0347 (nmol/L); AMH, 7.14 (pmol/L); E_2, 3.671 (pmol/L); ACTH, 0.22 (pmol/L); cortisol, 27.59 (nmol/L) and 17-OHP, 3.03 (nmol/L).

[a] Reference ranges for age-matched male (M) and female (F) children are shown in brackets.

[b] LH, FSH, AMH and E_2 were measured at day 6, while ACTH and cortisol were measured at day 13.

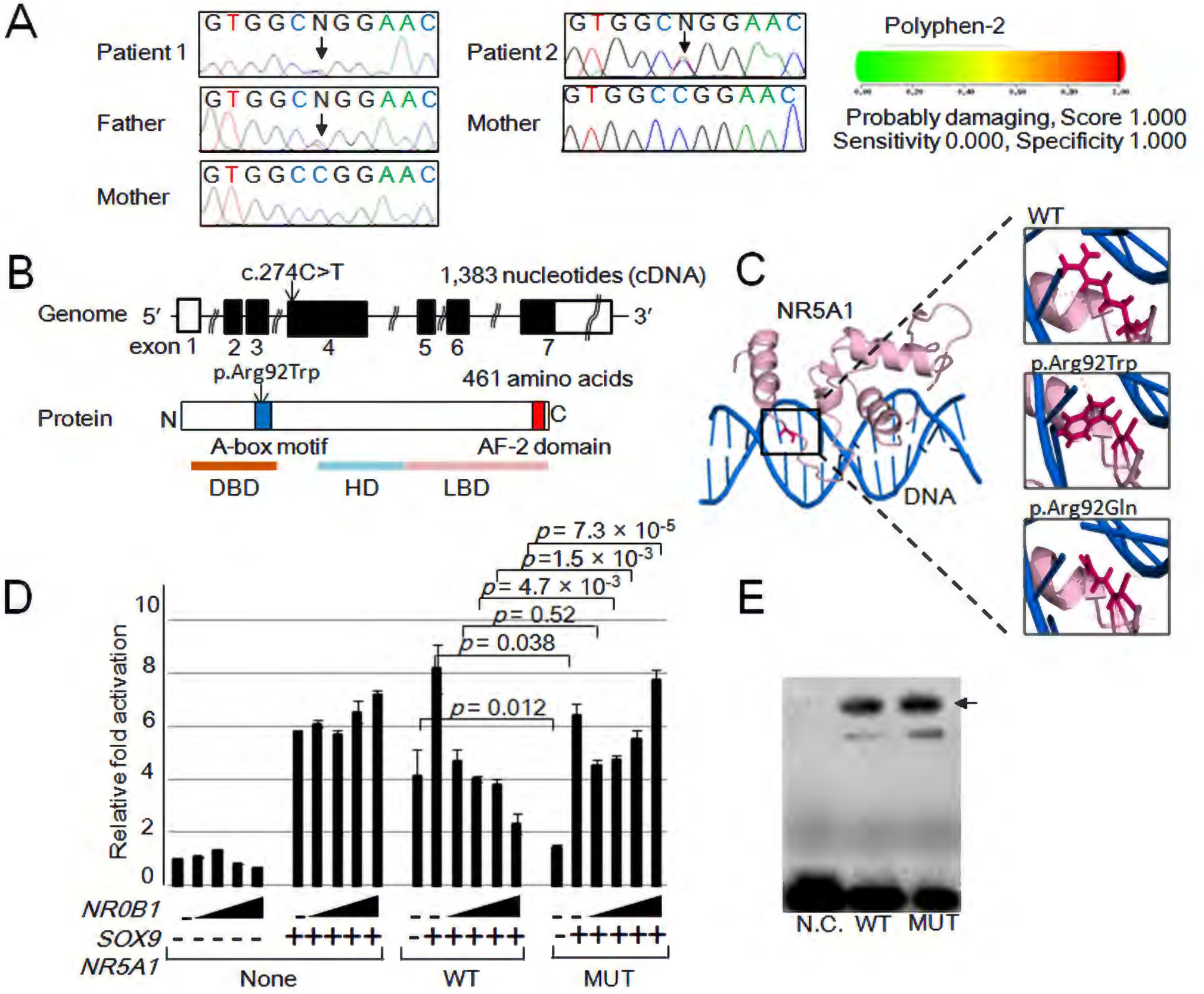
[c] LH and FSH were measured at 5 months of age, testosterone and E_2 were measured at 2 months of age, and cortisol and 17-OHP were measured at 1 month of age.

[d] Gonadotropin releasing hormone stimulation test (100 µg/m^2, max. 100 µg bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 minutes).

[e] Human chorionic gonadotropin stimulation test (750 U, i.m. for 3 consecutive days; blood sampling on days 1 and 4).

[f] Human menopausal gonadotropin stimulation test (100 U, i.m. for 3 consecutive days; blood sampling on days 0 and 4).

[g] Human ACTH stimulation test (250 µg/m^2, i.v.; blood sampling at 30 and 60 minutes).


A
Patient 1
Father
Mother
Patient 2
Mother
G T G G C N G G A A C
G T G G C C G G A A C
Polyphen-2
Probably damaging, Score 1.000
Sensitivity 0.000, Specificity 1.000
B
c.274C>T
1,383 nucleotides (cDNA)
Genome 5′
3′
exon 1 2 3 4 5 6 7
p.Arg92Trp
461 amino acids
Protein N C
A-box motif
AF-2 domain
DBD HD LBD
C
NR5A1
DNA
WT
p.Arg92Trp
p.Arg92Gln
D
p= 7.3 × 10-5
p=1.5 × 10-3
p= 4.7 × 10-3
p= 0.52
p= 0.038
p= 0.012
Relative fold activation
0 2 4 6 8 10
NR0B1
SOX9
NR5A1
None WT MUT
E
N.C. WT MUT

SUPPORTING INFORMATION

Subjects and Methods

Subjects

Patients 1 and 2 were unrelated patients identified by genital abnormalities at birth. These patients manifested no additional clinical features. Patient 1 was conceived by *in vitro* fertilization. Both patients were born at 41 weeks gestation as the sole child of non-consanguineous Japanese parents, and had no family history of disorders of sex development (DSD). G-banding analysis confirmed a 46,XX karyotype. PCR for *SRY* yielded no amplification products.

Ethical approval

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent from the participants or their parents.

Sequence analysis

Genomic DNA samples were obtained from patients 1 and 2, as well as from the parents of patient 1 and the mother of patient 2. Exome sequencing was performed using the All Exome v5 Kit (Agilent Technologies, Palo Alto, CA, USA) and the HiSeq 1500 sequencer (Illumina, San Diego, CA, USA). Sequencing data were analyzed by BWA 0. 6. 2 (http://bio-bwa.souceforge.net/) and SAMtools 0.1.18 software (http://samtools.soursefrge.net/). In this study, we analyzed protein-altering mutations and splice-site variations in genes that have previously been associated with human sex development [Bashamboo and McElreavey, 2013; Ono and Harley, 2013; Eggers et al., 2014; Baxter et al., 2015].

Nucleotide alterations identified in patients 1 and 2 were analyzed *in silico*. We

examined the population frequency of the substitutions in the Exome Aggregation Consortium (ExAC) Browser (http://exac.broadinstitute.org/), dbSNP (http://www.ncbi.nlm.nih.gov/snp/), the 1000 Genomes Browser (http://ncbi.nlm.nih.gov/variation/tools/1000genomes/), and Human Genetic Variation Browser (HGVB; http://www.genome.med.kyoto-u.ac.jp/SnpDB). DNA samples obtained from eight healthy Japanese females were used as control. Nucleotide substitutions whose frequency in the general population is more than 1.0% were excluded as polymorphisms. In addition, mutations shared by our control samples or by the mothers of patients 1 or 2 were considered as non-pathogenic. The functional consequences of the substitutions were predicted by Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/), and SIFT (http://sift.jcvi.org/), by using the default parameters. Three-dimensional structures of mutants were predicted by PyMOL (http://www.pymol.org). Putative pathogenic mutations were confirmed by Sanger sequencing. Primer sequences are available upon request.

To assess the pathogenicity of an *NR5A1* mutation identified in patients 1 and 2, we performed sequence analysis of 200 healthy Japanese controls (100 males and 100 females). The samples were obtained from the Human Science Research Resources Bank (Tokyo, Japan; present distributer, National Institute of Biomedical Innovation, Osaka, Japan). Furthermore, to examine whether the *NR5A1* mutations in patients 1 and 2 share a common genetic origin, we genotyped several single nucleotide polymorphisms in the *NR5A1* locus in the patients and their family members.

Submission of mutation data to a database

An *NR5A1* substitution identified in this study was submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-j.html; accession number, LC037393).

Copy-number analysis

Copy-number changes in the genome of patients 1 and 2 were analyzed by array-based comparative genomic hybridization using a catalog human array (4 × 180 k format; Agilent Technologies). We referred to the Database of Genomic Variant (http://dgv.tcag.ca/dgv/app/home) to exclude benign copy-number polymorphisms.

Plasmids

Expression vectors for wildtype human *NR5A1* and *FOXL2* were purchased from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). An expression vector for the mutant *NR5A1* and *FOXL2* were generated by mutagenesis using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Otsu, Shiga, Japan). An expression vector for *NR0B1* and a reporter vector containing the *SOX9* testis enhancer sequence core element (TESCO) were generated in our previous studies [Okuhara et al., 2008; Sekido and Lovell-Badge, 2008]. An expression vector for human *SOX9* was purchased from OriGene Technologies (MD, USA). The pRL-null vector (Life Technologies, CA, USA) was used as an internal control for transfection.

Luciferase assays

Transactivation activity of wildtype and mutant NR5A1 on *SOX9* TESCO was determined by luciferase reporter assays. Chinese hamster ovary (CHO)-K1 cells and murine Leydig tumor cells (MLTC1) (ATCC, VA, USA) were seeded in 12-well plates (1.0×10^5 cells/well) and transiently transfected with 100 ng *NR5A1* expression vector, 100 ng *SOX9* expression vector, 500 ng *SOX9* TESCO reporter vector, and 100 ng pRL-null control vector, along with increasing doses of the *NR0B1* expression vector (6.25, 25, 100, and 400 ng/well). Lipofectamine 3000 (Life Technologies) was used for transfection. Relative luciferase activity was measured 48 hours after transfection, using the Dual Luciferase Reporter Assay System (Promega, MD, USA). Luciferase activity was measured in triplicate in a single assay and all assays were repeated three times. Results are expressed as the mean ± standard

deviation, and statistical significance was determined by *t*-test. *P*-values less than 0.05 were considered significant.

We also analyzed the effects of wildtype *FOXL2* and the p.Arg349Gly mutant on NR5A1- and SOX9-induced *SOX9* TESCO activity. In this experiment, CHO cells were transfected with expression vector for *FOXL2* (100 ng/well), along with *NR5A1* expression vector (100 ng/well), *SOX9* expression vector (100 ng/well), *SOX9* TESCO reporter vector (500 ng/well), and pRL-null control vector (100 ng/well). Relative luciferase activity was measured as described above.

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Supp. Table S1. *In silico* functional prediction of *NR5A1*, *FOXL2* and *POR* substitutions

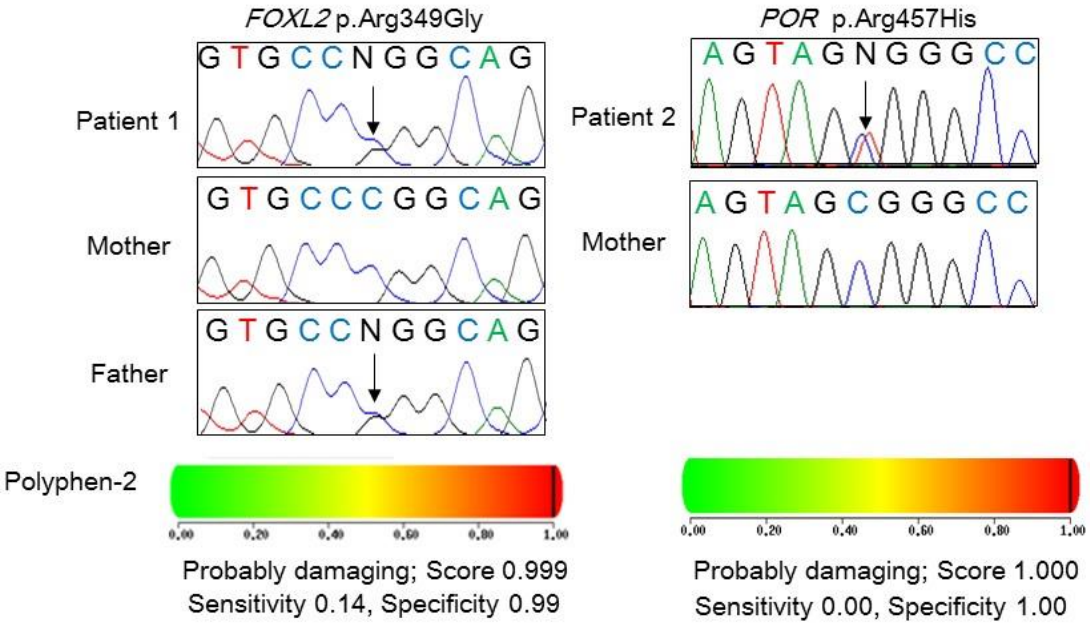
Gene	cDNA	Protein	MutationTaster[a]		Polyphen-2[b]		SIFT[c]	
			Score	Prediction	Score	Prediction	Score	Prediction
NR5A1	c.274C>T	p.Arg92Trp	0.999	disease causing	1.000	probably damaging	0.00	damaging
FOXL2	c.1045C>G	p.Arg349Gly	0.999	disease causing	0.999	probably damaging	0.02	damaging
POR	c.1370G>A	p.Arg457His	0.999	disease causing	1.000	probably damaging	0.00	damaging

In silico analyses were performed by using the default parameters.
[a]MutationTaster (http://www.mutationtaster.org/). Current version: MutationTaster2, GRCh37/Ensembl 69.
[b]Polyphen-2 (http://genetics.bwh.harvard.edu/pph2), Current version: 2.2.2, GRCh37. Scores between 0.909 and 1, between 0.447 and 0.908 and below 0.446 denote probably damaging, possibly damaging and benign, respectively.
[c]SIFT (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html). Current version: Aug. 2011; GRCh37/Ensembl 63. Scores of less than 0.05 were assessed as damaging.

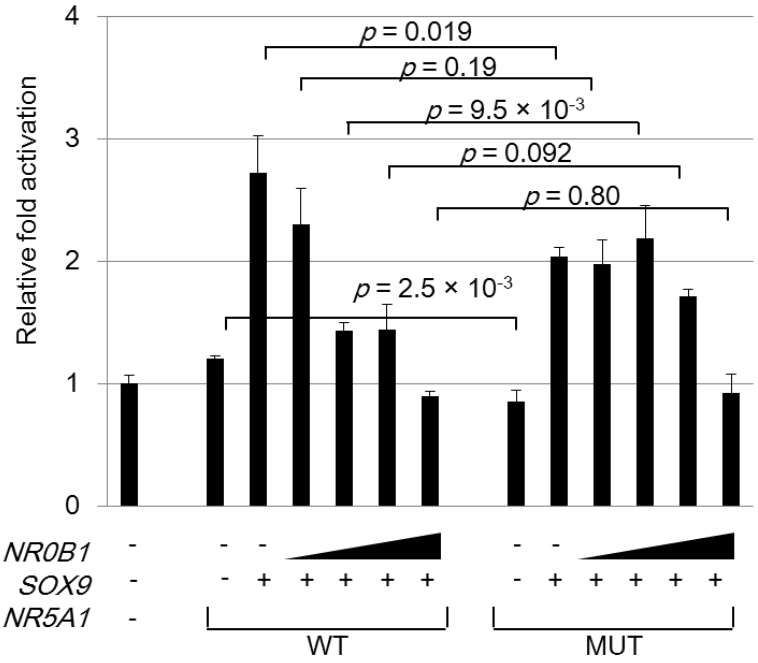
Supp. Table S2. Genotypes of single nucleotide polymorphisms in the *NR5A1* locus

Genomic position[a]	dbSNP ID	Region	Patient 1	Mother of patient 1	Father of patient 1	Patient 2	Mother of patient 2
chr9:127244955	rs915034	UTR	A/A	A/A	N.A.	G/A	A/A
chr9:127245412	rs7037254	intronic	C/C	C/C	C/T	C/C	C/C
chr9:127253308	N.D.	intronic	A/A	A/A	A/A	C/A	A/A
chr9:127255448	rs2297605	intronic	G/A	G/A	G/A	A/A	A/A
chr9:127255611	rs76274669	intronic	G/C	C/G	G/G	G/G	G/G
chr9:127262802	rs1110061	exonic	C/G	G/C	C/C	C/C	C/C
chr9:127262965	N.D.[b]	exonic	A/G	G/G	A/G	A/G	G/G
chr9:127263084	rs1889311	intronic	G/G	G/G	G/G	G/G	G/G
chr9:127265286	rs115601896	intronic	C/C	C/C	C/C	C/C	C/C
chr9:127265775	rs76584717	intronic	G/A	A/G	G/G	G/G	G/G

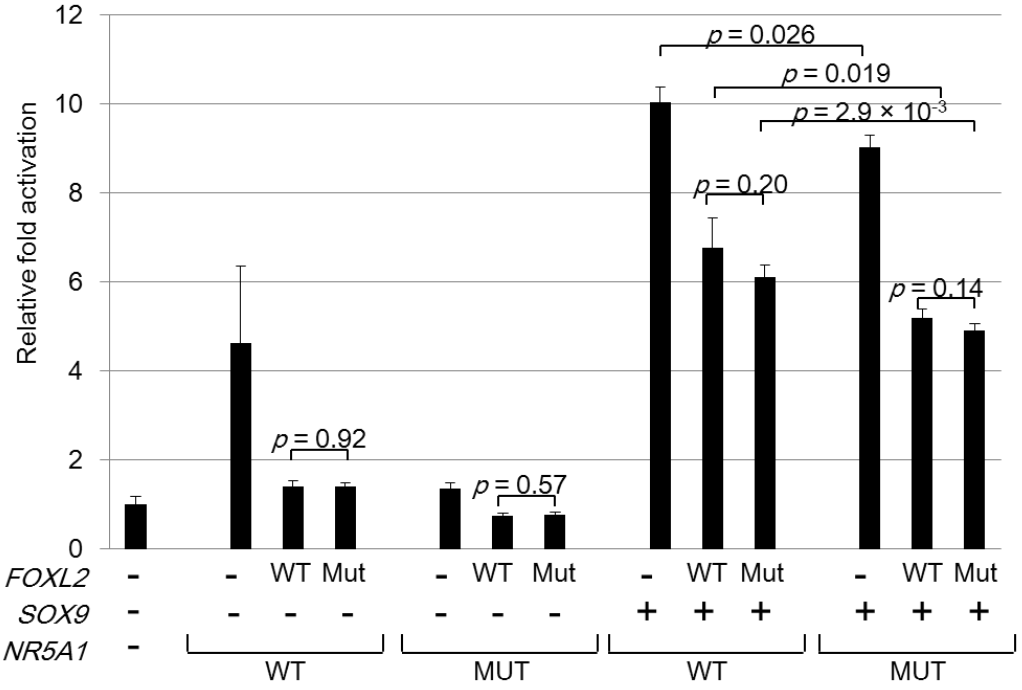
N.A., not analyzed; N.D., no data; UTR, untranslated region.
[a] Physical position referred to Human Genome (GRCh37/hg19).
[b] p.Arg92Trp



Supp. Fig. S1. Substitutions of *FOXL2* and *POR*.
Mutated nucleotides are indicated by black arrows. The p.Arg457His mutation in *POR* has been linked to fetoplacental androgen overproduction, but not to testicular development in genetic females.



Supp. Fig. S2. Representative results of luciferase assays using murine Leydig tumor cells (MLTC1).
Expression vectors for *NR5A1* and *SOX9* were transfected into the cells, along with the *SOX9* TESCO reporter vector and various doses of *NR0B1* expression vector (6.25, 25, 100, and 400 ng/well). Results are expressed as mean ± standard deviation. WT, wildtype *NR5A1*; MUT, the p.Arg92Trp mutant.



Supp. Fig. S3. Representative results of luciferase assays using *FOXL2*.
Expression vectors of wildtype *FOXL2* or the p.Arg349Gly mutant (100 ng/well) were transfected into CHO cells, along with the *SOX9* TESCO reporter vector and the expression vectors for *NR5A1* and *SOX9*.
WT, wildtype *NR5A1* or *FOXL2*; MUT, p.Arg92Trp of *NR5A1* or p.Arg349Gly of *FOXL2* .