Testicular dysgenesis/regression without campomelic dysplasia in patients carrying missense mutations and upstream deletion of SOX9

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Abstract
SOX9 haploinsufficiency underlies campomelic dysplasia (CD) with or without testicular dysgenesis. Current understanding of the phenotypic variability and mutation spectrum of SOX9 abnormalities remains fragmentary. Here, we report three patients with hitherto unreported SOX9 abnormalities. These patients were identified through molecular analysis of 33 patients with 46,XY disorders of sex development (DSD). Patients 1–3 manifested testicular dysgenesis or regression without CD. Patients 1 and 2 carried probable damaging mutations p.Arg394Gly and p.Arg437Cys, respectively, in the SOX9 C-terminal domain but not in other known 46,XY DSD causative genes. These substitutions were absent from ~120,000 alleles in the exome database. These mutations retained normal transactivating activity for the Col2a1 enhancer, but showed impaired activity for the Amh promoter. Patient 3 harbored a maternally inherited ~491 kb SOX9 upstream deletion that encompassed the known 32.5 kb XY sex reversal region. Breakpoints of the deletion resided within nonrepeat sequences and were accompanied by a short-nucleotide insertion. The results imply that testicular dysgenesis and regression without skeletal dysplasia may be rare manifestations of SOX9 abnormalities. Furthermore, our data broaden pathogenic SOX9 abnormalities to include C-terminal missense substitutions which lead to target-gene-specific protein dysfunction, and enhancer-containing upstream microdeletions mediated by nonhomologous end-joining.
Intorduction

SOX9 (OMIM *608160) controls embryonic development by transactivating several genes such as COL2A1 involved in skeletal formation and AMH involved in testicular development. Known SOX9 mutations include various missense substitutions in the high-mobility group or dimerization domains, as well as several nonsense, frameshift, and splice-site mutations widely distributed in the coding region (Meyer et al. 1997; Bernard et al. 2003; Harley et al. 2003; Michel-Calemard et al. 2004; Staffller et al. 2010). Patients with SOX9 mutations manifest campomelia, hypoplastic scapulae, pelvic anomalies, micrognathia, and cleft palate, collectively referred to as campomelic dysplasia (CD), although a certain percentage of mutation-positive patients show a mild variant of CD that lacks campomelia (acampomelic CD: ACD) (Bernard et al. 2003; Michel-Calemard et al. 2004; Staffler et al. 2010). SOX9 mutations also result in complete or partial gonadal dysgenesis in individuals with 46,XY karyotype (Meyer et al. 1997; Michel-Calemard et al. 2004). As CD/ACD-compatible skeletal abnormalities were described in all patients with SOX9 mutations and disorders of sex development (DSD) were shared only by ~70% of 46,XY patients (Mansour et al. 1995), it seems that skeletal tissues are more vulnerable than testis to impaired SOX9 function. Kwok et al. (1996) suggested that SOX9 mutations are unlikely to underlie 46,XY DSD in the absence of skeletal abnormalities.

Recent studies have identified submicroscopic deletions in the SOX9 upstream region in six patients with isolated 46,XY DSD (Pop et al. 2004; Lecointre et al. 2009; Kim et al. 2015). These patients shared a 32.5 kb overlapping region of deletion at a position 607–640 kb upstream of the SOX9 start codon, which was designated as the XY sex reversal region (XYSR). Since SOX9 expression is regulated by multiple tissue-specific enhancers (Bagheri-Fam et al. 2006), XYSR likely contains a testis-specific enhancer. Considering the limited number of reported patients, further studies are necessary to clarify the phenotypic variability and mutation spectrum of SOX9 abnormalities. Furthermore, the genomic basis of SOX9 upstream deletions remains to be investigated. Here, we report three unique cases with SOX9 abnormalities.

Materials and Methods

Subjects

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. The study group consisted of 33 Japanese patients with 46,XY DSD. All patients showed genitai abnormalities at birth; of these, 29 had isolated DSD, whereas the remaining patients manifested DSD with additional clinical features. Eleven and 22 patients were raised as a female and male, respectively. Patients with apparent chromosomal abnormalities were excluded from this study.

Mutation analysis

After obtaining written informed consent from the patients or their parents, genomic DNA samples were collected from the patients. Mutation analysis was performed by next-generation sequencing (NGS). Genomic DNA samples were isolated from peripheral leukocytes. Target regions in the human genome were amplified with the SureSelect Target Enrichment system (G7531C or all exome v5; Agilent Technologies, Palo Alto, CA) and sequenced on a HiSeq 2000 sequencer (Illumina, San Diego, CA). Nucleotide alterations were called by Avadis NGS 1.3.1 (DNA Chip Research, Yokohama, Japan) or SAMtools 0.1.17 software (http://samtools.sourceforge.net/). In this study, we focused on protein-altering substitutions and splice-site mutations of 27 known causative genes for 46,XY DSD, that is, AKR1C2, AKR1C4, AMH, AMHR2, AR, ATF3, ATRX, BNC2, CYP11A1, DHH, DMRT1, GATA4, HSD3B2, HSD17B3, INSL3, INSR, LHCG, MAP3K1, NR5A1, POR, RXFP2, SOX9, SRD5A2, SRY, STAR, TSPYL1, and WT1. Nucleotide substitutions of allele frequency 1% or higher in the Japanese population (Human Genetic Variation Browser, http://www.genome.med.kyoto-u.ac.jp/SnpDB) were excluded as polymorphisms. SOX9 (NM_000346.3) mutations indicated by NGS were confirmed by Sanger sequencing using a primer pair: SOX9-exon3FW2 (5′-CAGGCACACGCTGA CCAC-3′) and SOX9-exon3RV (5′-CCTCTTTTCTCG GTTAT-3′). Furthermore, PCR products carrying the nucleotide alterations were subcloned into the TOPO TA cloning vector (Life Technologies, Carlsbad, CA) and the mutant and wild-type alleles were sequenced separately. Whenever possible, parental samples of mutation-positive patients were also subjected to molecular analysis.

Functional analyses of SOX9 substitutions

Conservation and functional consequences of SOX9 substitutions were predicted using Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al. 2010). Population frequencies of the substitutions were analyzed using the Exome Aggregation Consortium Browser (http://exac.broadinstitute.org/).
The transactivating activity of the substitutions was assessed by a previously reported method with modifications (Kelberman et al. 2006). Briefly, an expression vector for wild-type SOX9 was purchased from Origene Technologies (RC208944, Rockville, MD), and each SOX9 mutation was introduced into the expression vector by site-directed mutagenesis (PrimeSTAR Mutagenesis Basal Kit; Takara Bio, Ohtsu, Japan). In this study, we compared the transactivating activity of newly identified mutants to that of the known ACD-associated SOX9 mutant c.527C>T (p.Pro176Leu) (Michel-Calemard et al. 2004). We used a PGL3 reporter vector (Promega, Madison, WI) containing the murine Amh promoter sequence (from –231 to 0 to the transcription start site of Amh, NC_000076.6) and a PGL4 reporter vector (Promega) containing the murine Col2a1 enhancer sequence (from +1958 to +2485 to the transcription start site of Col2a1, NC_0000081.6). An expression vector for NRS5A1 was kindly provided by Professor Toshihiko Yanase (Fukuoka University, Fukuoka, Japan). Luciferase assays for the Amh promoter and for the Col2a1 enhancer were carried out using COS-1 (RIKEN, Ibaraki, Japan) and HEK293 cells (Health Science Research Resources Bank, Tokyo, Japan), respectively. The cells were seeded in 6-well dishes and treated with Lipofectamine 2000 Reagent (Life Technologies (RC208944, Rockville, MD), and each vector for wild-type SOX9 (Promega) was purchased from Origene Technologies). The deletion breakpoints resided in nonrepeat sequences and shared no homology (Fig. 1D). The fusion junction was accompanied by a short-nucleotide insertion of unknown origin [-AATG–3] that was indicative of an “information scar” of nonhomologous end-joining (NHEJ) (Lieber 2008). Patient 3 harboring the fusion junction. The products were generated using a primer pair: 5′-TTTTTTCTTGAAGTT AATG-3′ and 5′-AATGTAGTGCTATATATTGC-3′. Sizes and genomic positions of the deletions were analyzed using the UCSC genome browser (http://genome.ucsc.edu/; GRCh37/hg19) and the presence or absence of repeat sequences was examined with RepeatMasker (http://www.repeatmasker.org). We referred to the Database of Genomic Variants (http://projects.tcag.ca/variation/) to exclude known benign variants.

**Results**

**Mutation analysis**

We identified two heterozygous missense substitutions c.1180C>G (p.Arg394Gly) and c.1309C>T (p.Arg437Cys) in patients 1 and 2, respectively, in SOX9 (Fig. 1). These substitutions have not been reported previously. Patients 1 and 2 carried no mutations in the other genes examined or in other nucleotides of SOX9. The substitution of patient 2 was shared by the phenotypically normal mother, whereas parental samples of patient 1 were not available for genetic analysis. The p.Arg394Gly and p.Arg437Cys substitutions resided within the proline/glutamine/serine (PQS)-rich domain (also known as SPQ-rich domain) at the C-terminus (McDowall et al. 1999) (Fig. 1A).

**Functional analysis of SOX9 substitutions**

The c.1180C>G (p.Arg394Gly) and c.1309C>T (p.Arg437Cys) substitutions involved highly conserved amino acids, and were predicted as “probably damaging” by in silico analyses (Fig. 1B). These substitutions were absent from ~120,000 alleles of the exome database. The p.Arg394Gly and p.Arg437Cys mutants retained normal in vitro transactivating activity for the Col2a1 enhancer (relative fold activation: 1.14 and 1.17, respectively), but exerted impaired activity for the Amh promoter (relative fold activation: 0.74 and 0.81, respectively) (Fig. 1C). In contrast, the previously reported ACD-associated p.Pro176Leu mutant showed markedly reduced activity for both reporters (relative fold activation: 0.50 for the Col2a1 enhancer and 0.26 for the Amh promoter).

**Copy-number analysis**

Copy-number alterations were analyzed by comparative genomic hybridization using a catalog human array (4 × 180 k format) or a custom-made array (design ID, 031687) (Agilent Technologies). The deletion breakpoints were determined by direct sequencing of PCR products harboring the fusion junction. The products were generated using a primer pair: 5′-TTTTTTCTTGAAGTT AATG-3′ and 5′-AATGTAGTGCTATATATTGC-3′. Sizes and genomic positions of the deletions were analyzed using the UCSC genome browser (http://genome.ucsc.edu/; GRCh37/hg19) and the presence or absence of
carried no sequence alteration in all genes examined. The
SOX9 upstream deletion was identified in the phenotypi-
cally normal mother of patient 3.

**Clinical features of the mutation-positive patients**

Physical and hormonal findings of patients 1–3 are sum-
marized in Table 1. Blood inhibin B levels were not deter-
mined in these cases. Patient 1 was a 19-year-old
individual raised as a male. He manifested hypospadias
and bilateral cryptorchidism at birth and underwent sur-
gical intervention at 5 years of age. At 14 years of age, he
was subjected to endocrine evaluation because of a lack of
pubertal sexual development. Blood examinations revealed
increased levels of gonadotropins and mildly decreased
levels of testosterone, indicating testicular dysfunction. He
began to receive testosterone supplementation therapy at

![Figure 1. SOX9 abnormalities in patients 1–3. (A) Genomic and protein structures of SOX9/SOX9. The positions of the c.1180C>G (p.Arg394Gly)
and c.1309C>T (p.Arg437Cys) mutations are indicated by arrows. White and black boxes in the upper panel indicate the untranslated and coding
regions, respectively. Colored boxes in the lower panel indicate dimerization (codon 60–101) (Bernard et al. 2003), high-mobility group (HMG:
codon 101–184), proline/glutamine/alanine (PQA: codon 339–379), and proline/glutamine/serine-rich (PQS-rich: codon 386–509) domains
(McDowall et al. 1999). (B) Nucleotide substitutions detected in patients 1 and 2. Left panel: electro chromatograms of the mutations. The
mutated nucleotides are indicated by arrows. Right panel: in silico functional prediction of mutant proteins. (C) In vitro assays using reporters
containing the Col2a1 enhancer or Amh promoter. The transactivating activity of p.Arg394Gly and p.Arg437Cys mutants was compared to that
of the known ACD-associated SOX9 mutant p.Pro176Leu (Michel-Calemard et al. 2004). The results are expressed as the mean ± one standard
deviation. Relative transactivating activities of the SOX9 mutants against the wild-type are shown. Empty: empty expression vector; ns: not
significant. (D) SOX9 upstream deletion in patient 3. Upper panel: array-based comparative genomic hybridization analysis. The black, red,
and green dots denote signals indicative of the normal, increased (> +0.5) and decreased (< -1.0) copy-numbers, respectively. The blue and red boxes
represent previously reported XY sex reversal region (XYSR) (Kim et al. 2015) and SOX9 exons, respectively. Genomic positions refer to the UCSC
database (http://genome.ucsc.edu/; GRCh37/hg19). Lower panel: sequence of the fusion junction. The junction is accompanied by a short
nucleotide insertion of unknown origin (the red-shaded area).
age 14, and human chorionic gonadotropin and human menopausal gonadotropin therapy at age 16. He underwent surgical intervention for gynecomastia at 14 and 16 years of age. Abdominal ultrasound at 18 years of age showed small testes with focal microlithiasis. Mullerian duct derivatives were absent. He had no skeletal abnormalities except for spina bifida occulta, a relatively common neural tube anomaly of that has not been associated with SOX9 mutations (Greene and Copp 2014).

Patient 2 was a 5-year-old male individual. At birth, he showed male-type external genitalia; however, bilateral testes were not palpable in the scrotum or in the inguinal region. At 2.6 years of age, laparoscopic examination detected possible gonadal remnants with spermatic cord in the bilateral inguinal canals. Histological examination of biopsied samples showed that the possible remnants were fibrous tissues without germ cells. At 2.8 years of age, he was referred to our clinic for further evaluation. He showed normal skeletal features and borderline micro penis. Endocrine analyses revealed increased gonadotropin levels and undetectable levels of testosterone and anti-Mullerian hormone. Abdominal imaging did not detect a uterus or gonads. Thus, this patient was diagnosed with testicular regression. At 5 years of age, he was capable of voiding in a standing position.

Patient 3 was a 22-year-old individual with a female phenotype. Her growth and development were uneventful until pubertal age. At 13 years of age, she visited our clinic because of a lack of pubertal sexual development. She showed normal skeletal features and female-type external genitalia. Endocrine evaluation indicated severe gonadal dysfunction. Abdominal magnetic resonance imaging detected a uterus of a prepubertal size. Estrogen supplementation therapy from 14 years of age successfully induced breast budding and vaginal bleeding. Gonadectomy was performed at 17 years of age. Histological examination revealed bilateral dysgenic gonads with seminoma.

Discussion

This study provides several notable findings. This is the first report documenting the association between SOX9 intragenic mutations and isolated 46,XY DSD. In vitro assays confirmed the target-specific functional impairment of the c.1180C>G (p.Arg394Gly) and c.1309C>T (p.Arg437Cys) mutants, which were not observed in the known ACD-associated mutant c.527C>T (p.Pro176Leu). Notably, unlike other known pathogenic SOX9 missense mutations, p.Arg394Gly and p.Arg437Cys resided within the C-terminal PQS-rich domain. As the PQS-rich domain is required for SOX9 interaction with other proteins (Tsuda et al. 2003), the two mutations may affect SOX9-mediated protein–protein interactions in the developing testis. Physical and hormonal findings of patients 1 and 2 with these mutations were indicative of impaired testicular development, although blood inhibin B levels, a sensitive marker for the function of the testis (Grinspon et al. 2012), were not determined in these patients.

It is worth mentioning that patient 2 manifested bilateral testicular regression, a rare form of 46,XY DSD that probably occurs as a result of the disturbance of developmental processes during testicular tubule formation (Mizuno et al. 2012). The genetic basis of testicular regression remains unknown, with the exception of NR5A1 muta-
|                   | Patient 1                          | Patient 2                          | Patient 3                          |
|-------------------|-----------------------------------|------------------------------------|------------------------------------|
| **Karyotype**     | 46,XY                             | 46,XY                              | 46,XY                              |
| **Molecular defects in SOX9 (NM_000346.3)** | c.1180C>G (p.Arg394Gly)            | c.1309C>T (p.Arg437Cys)             | Upstream deletion                   |
| **External genitalia** | Male-type genitalia with hypospadias and unpalpable testes | Male-type genitalia with micropenis and unpalpable testes | Complete female-type genitalia |
| **Physical findings at later ages** | | | |
| Age at exam. (year) | 19                                | 2.8                                | 13                                 |
| Penile size (cm)   | 5.7 (8.6–10.0)                    | 2.5 (3.0–3.6)                      | 22                                 |
| Gonadal histology  | 5 (right), 3–4 (left)             | Not palpable                       | Not examined                       |
| Uterus             | Absent                            | Fibrous tissues                    | Not palpable                       |
| Additional findings | Spina bifida occulta              | Absent                            | Streak gonad with seminoma         |
| **Hormonal findings** | | | |
| Age at exam. (year) | 19                                | 2.8                                | 13                                 |
| LH (mIU/mL)        | 37.5 (0.5–5.0)                    | 2.8 (<0.3–1.3)                     | 13.8 (<0.2–2.1)                    |
| FSH (mIU/mL)       | 17.7 (0.8–4.4)                    | Not analyzed                       | 119.4 (1.3–3.9)                    |
| Testosterone       | 5.3 (2.5–11.0)                    | 109.8 (8.4–1.5)                    | 82.5 (<0.3–3.0)                    |
| (ng/mL)            |                                    | Not analyzed                       | 135.8 (1.3–3.9)                    |
| AMH (ng/mL)        | Not analyzed                      | <0.03 (<0.06–0.16)                 | 0.09 (0.68–1.22)                   |
|                    |                                    | <0.03 (<0.2)                       | **0.16 (2.96–5.58)**               |

The conversion factor to the SI unit: LH 1.0 (IU/L), FSH 1.0 (IU/L), testosterone 3.47 (nmol/L), and AMH 7.14 (pmol/L). Penile size and hormone values below the reference range are boldfaced, and hormone values above the reference range are italicized. B, basal; S, stimulated; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone.

1Reference ranges are shown in parentheses.
2After hormone replacement therapy.
3After surgical interventions.
4Gonadotropin releasing hormone stimulation test (100 μg/m², max. 100 μg bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 min).
5Human chorionic gonadotropin stimulation test (3000 IU/m², max. 5000 IU i.m. for three consecutive days; blood sampling on days 1 and 4).
tions that account for a minor fraction of cases (Philibert et al. 2007). It has been suggested that testicular regression and gonadal dysgenesis are a continuum of a disorder (Marcantonio et al. 1994). Indeed, NR5A1 mutations are known to underlie both conditions (Ferraz-de-Souza et al. 2011). Animal studies suggested that SOX9 plays a role in testicular tubule differentiation through the interaction with SOX8 (Barriónuevo et al. 2009). Moreover, SOX9 plays a critical role not only in testicular development but also in the maintenance of differentiated status of the testes (Sekido and Lovell-Badge 2009; Veitia 2010). Thus, testicular regression may be a rare manifestation in patients with SOX9 mutations. However, this notion is based on the findings of a single individual, and therefore awaits further investigation.

One may argue against p.Arg394Gly and p.Arg437Cys being responsible for the severe DSD in patients 1 and 2 because these mutations resulted in only modest decrease in the transactivating activity for the AMH promoter. The discrepancy between the phenotypic severities and the results of in vitro assays can be explained by assuming that some SOX9 target genes other than AMH are more sensitive to defective function of SOX9. Actually, a number of testicular genes are known to be regulated by SOX9 (Sekido and Lovell-Badge 2009; Veitia 2010). Alternatively, in the developing testis, the p.Arg394Gly and p.Arg437Cys mutations may disrupt the synergic interaction between SOX9 and certain cofactors. It is known that SOX9 synergizes with other proteins to transactivate target genes (Sekido and Lovell-Badge 2009; Veitia 2010). On the other hand, we cannot exclude the possibility that genetic variations in other genes or some environmental factors affected sexual development in patients 1 and 2, although mutations in known 46,XY DSD causative genes were excluded in these patients. Further studies, including in vitro assays using reporter vectors containing various SOX9 target promoters and expression vectors for several SOX9 cofactors, and whole exome sequencing of patients 1 and 2, will clarify the precise functional consequences of the mutants.

The findings in patient 3 support the notion that the 32.5 kb YYSR contains a DNA element(s) essential for testicular development. Moreover, exclusion mapping indicates that SOX9 enhancers for skeletal tissues and craniofacial regions are located outside of the −491 kb region deleted in patient 3 (Fig. 2) (Pop et al. 2004; Hill-Harfe et al. 2005; Velagaleti et al. 2005; Lecointre et al. 2009; Jakubiczka et al. 2010; Ledig et al. 2010; Kim et al. 2015). In addition, the results of this study, together with those of previous studies (Pop et al. 2004; Lecointre et al. 2009; Kim et al. 2015) provide evidence of the genomic heterogeneity of SOX9 upstream deletions. The breakpoint sequences in patient 3 suggest that the deletion resulted from NHEJ. Actually, genomic regions around SOX9 are not enriched with repeat sequences that serve as substances of nonallelic homologous recombination (Fig. 2). Thus, NHEJ may be the major cause of SOX9 upstream microdeletions, although other mechanisms such as microhomology-mediated replication errors may also be involved in the development of such deletions. Since all known pathogenic deletions in the SOX9 upstream region, including that in our patient, were transmitted from phenotypically normal mothers of the patients (Kim et al. 2015), de novo occurrence of such deletions seems to be a rare event.

In summary, the results indicate that the phenotypic consequences of SOX9 mutations are broader than previously reported and include testicular dysgenesis and regression without skeletal dysplasia. Furthermore, our data suggest that DSD-associated SOX9 abnormalities include C-terminal missense substitutions that lead to target-specific protein dysfunction, and NHEJ-mediated upstream microdeletions encompassing YYSR.

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Conflict of Interest

None declared.

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