Clinical Research Article

**SOX10 Mutation Screening for 117 Patients with Kallmann Syndrome**

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**Abbreviations:** ACMG, the American College of Medical Genomics; hCG, human chronic gonadotropin; HH, hypogonadotropic hypogonadism; HMG, high-mobility group; KS, Kallmann syndrome; PCWH, peripheral demyelinating neuropathy, central demyelination, Waardenburg syndrome, and Hirschsprung disease; T, testosterone; WS, Waardenburg syndrome.

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**Abstract**

**Introduction:** Kallmann syndrome (KS) is a genetically heterogeneous condition characterized by hypogonadotropic hypogonadism (HH) and olfactory dysfunction. Although **SOX10**, a causative gene for Waardenburg syndrome (WS) and peripheral demyelinating neuropathy, central demyelination, WS, and Hirschsprung disease (PCWH) has previously been implicated in KS, the clinical significance of **SOX10** variants as the cause of KS remains uncertain.

**Patients and Methods:** A total of 117 patients with KS underwent mutation screening of **SOX10** and 14 other causative genes for KS/HH. Rare **SOX10** variants were subjected to in silico and in vitro analyses. We also examined clinical data of the patients and their parents with **SOX10** variants.

**Results:** Sequence analysis identified 2 heterozygous variants of **SOX10** (c.1225G > T, p.Gly409* and c.475C > T, p.Arg159Trp) in patients 1–3, as well as in the parents of patients 1 and 3. The variants were assessed as pathogenic/likely pathogenic, according to the American College of Medical Genomics guidelines. Both variants lacked in vitro transactivating activity for the **MITF** promoter and exerted no dominant-negative effects. Patients 1–3 carried no pathogenic variants in other genes examined. The patients presented with typical KS, while such features were absent in the parents of patients 1 and 3. None of the 5 variant-positive individuals exhibited hypopigmentation, while 1 and 2 individuals exhibited complete and partial hearing loss, respectively.
Conclusion: These results provide evidence that SOX10 haploinsufficiency accounts for a small percentage of KS cases. SOX10 haploinsufficiency is likely to be associated with a broad phenotypic spectrum, which includes KS without other clinical features of WS/PCWH.

Key Words: gonadotropin deficiency, hypogonadotropic hypogonadism, mutation, puberty, Waardenburg syndrome

Kallmann syndrome (KS) is a rare congenital disorder characterized by hypogonadotropic hypogonadism (HH) and olfactory dysfunction [1, 2]. KS primarily arises from the defective development of neural crest cells [2–4] and is frequently associated with additional clinical features, such as hearing impairment and craniofacial anomalies [2, 3]. KS is a genetically heterogeneous condition; more than 30 genes have been implicated in KS and/or HH [2, 5, 6]. Monoallelic, biallelic, and oligogenic mutations of these genes account for about half of the previously reported patients with KS/HH [5–7], indicating that several causative genes for KS/HH may remain unidentified.

SOX10 (NM_006941) encodes a transcription factor involved in neural crest cell development [4, 8]. Heterozygous loss-of-function variants in SOX10 are known as the cause of Waardenburg syndrome (WS), characterized by hearing loss and hypopigmentation of hair, iris, and skin [8–10]. Furthermore, SOX10 variants can result in a more complex phenotype consisting of peripheral demyelinating neuropathy, central demyelination, WS, and Hirschsprung disease (PCWH) [8, 10]. Previous studies revealed that a certain percentage of patients with WS/PCWH due to SOX10 variants exhibit KS [4, 11–13]. More recently, SOX10 pathogenic variants were identified in multiple KS patients with and without typical clinical features of WS/PCWH [6, 14–17]. Thus, SOX10 abnormalities are likely to be associated with broad phenotypic variations including WS, PCWH, and KS. However, since previous SOX10 analyses mostly focused on WS/PCWH patients and their family members, the significance of SOX10 abnormalities in the etiology of KS is not fully established. To address this issue, we performed mutation screening of SOX10 for 117 patients clinically diagnosed with KS.

Patients and Methods

SOX10 mutation screening for KS patients

The study was approved by the Institutional Review Board Committee at the National Center for Child and Development in Japan. Written informed consent was obtained from the participants or their parents. We studied 117 unrelated male and female individuals clinically diagnosed with KS. These patients were identified mostly by hypomasaclized external genitalia and/or delayed puberty. Patients with chromosomal abnormalities or combined pituitary hormone deficiency were excluded. None of these individuals exhibited PCWH or hypopigmentation in hair, iris or skin.

Genomic deoxyribonucleic acid samples were extracted from peripheral leukocytes. We sequenced coding exons and their flanking introns of SOX10, together with those of other major causative genes for KS and HH, ie, CHD7, FGF8, FGFRI, GNRH1, GNRHR, ANOS1, KISS1R, PROKR2, TACR3, IGSF1, KISS1, PROK2, TAC3, and WDR11 [2, 5–7].

These targeted genes were amplified by multiplex-PCR or by using the Haloplex system (Agilent Technologies, Santa Clara, CA, USA) and sequenced on a next-generation sequencer (Illumina, San Diego, CA, USA). Rare variants were called by previously reported methods [5, 18]. Allele frequencies of the variants in the general population were examined using the 1000 Genomes Database (https://www.internationalgenome.org/1000-genomes-browsers/), the gnomAD browser (https://gnomad.broadinstitute.org/), and the Human Genetic Variation Database (https://www.hgvd.genome.med.kyoto-u.ac.jp/). Functional outcomes of the variants were predicted by in silico analysis using Polymorphism Phenotyping v2, (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant, (http://provean.jcvi.org/index.php), Combined Annotation Dependent Depletion, (https://cadd.gs.washington.edu/), Mendelian Clinically Applicable Pathogenicity, (http://bejerano.stanford.edu/mcap/) and MutationTaster (http://www.mutationtaster.org/). We called nonsynonymous coding variants and splice site substitutions, whose allele frequencies in the public databases are less than 1%. Identified rare variants were visually confirmed by the Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/). Furthermore, rare SOX10 variants were confirmed by Sanger sequencing. Parental samples of patients with rare SOX10 variants were also subjected to Sanger sequencing. The pathogenicity of each SOX10 variant was assessed according to the American College of Medical Genomics (ACMG) guidelines [19].

In vitro functional assays for SOX10 variants

To examine the in vitro function of SOX10 variants, we performed previously reported reporter assays with slight modifications [13]. A Halo-tag-containing expression vector
for wildtype SOX10 was purchased from the Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). Expression vectors for the variants were generated by site-directed mutagenesis. We used a luciferase reporter vector containing the MITF promoter sequence (-2253 to +97), as previously described [20, 21]. HEK293 cells seeded in 96-well plates were transiently transfected with 4 ng/well of the empty vector or SOX10 expression vectors (wildtype or variant) and 80 ng/well of the luciferase reporter vector using Lipofectamidin 3000 (Thermo Fisher Scientific, Waltham, MA, USA). At 48 hours after transfection, the cells were harvested and subjected to luciferase analysis using the ONE-Glo Luciferase Assay System (Promega, Madison, WI, USA). These experiments were carried out in quadruplicate within a single experiment, and the experiment was repeated 3 times. Statistical significance was determined by the t-test. Then, to confirm the transfection efficiency of the SOX10 expression vectors, we performed co-transfection assays using a control plasmid with a Renilla luciferase construct. We transfected 40 ng/well of the empty or SOX10 expression vectors, together with 5 ng/well of the pCMV-PRL control vector (Promega). At 48 hours after transfection, the cells were harvested and analyzed by the dual luciferase reporter assay system (Promega). This experiment was carried out in triplicate.

Clinical analysis for individuals with SOX10 variants

We analyzed clinical information of the patients with a pathogenic or likely-pathogenic SOX10 variant. Testicular volume was measured by using an orichdometer or by ultrasonography. Olfactory function was assessed by intravenous injection of combined vitamins (Takeda Pharmaceutical, Tokyo, Japan) or by a smell identification test using odor sticks (Daiichi Yakuhin Sangyo, Tokyo, Japan). We also analyzed clinical data of patients’ parents who carried the same SOX10 variant as the proband.

Results

SOX10 mutation screening for KS patients

We identified 2 rare heterozygous SOX10 variants in 3 patients (patients 1–3) (Fig. 1A). Patients 1–3 carried no further rare variants in SOX10 or in the other genes examined. Of the remaining 114 patients, 34 and 27 carried pathogenic/likely-pathogenic variants and rare variants-of-unknown significance (VUS) in the 14 tested genes, respectively. Two of these patients had both pathogenic/likely-pathogenic variants and rare VUS.

The SOX10 variant in patient 1 was c.1225G > T, which creates a premature termination codon at the 409th position (p.Gly409*), whereas the variant shared by patients 2 and 3 was c.475C > T, which leads to a missense substitution in the high-mobility group (HMG) domain (p.Arg159Trp) (Fig. 1A). The p.Arg159Trp variant was assessed as probably damaging by all in silico analyses used in this study (Table 1). The Arg159 residue was highly conserved among species (Fig. 2). The c.1225G > T variant has not been reported previously, whereas the c.475C > T variant was previously identified in a Chinese family with a partial phenotype of WS and KS [17]. The variants in patients 1 and 3 were shared by their father and mother, respectively, while that in patient 2 was de novo. According to the ACMG guidelines, the c.1225G > T and c.475C > T variants were classified as pathogenic (PVS1 and PS3) and likely-pathogenic (PS3 and PM2), respectively.

In vitro functional assays for SOX10 variants

While the wildtype SOX10 protein significantly transactivated the MITF promoter, both of the 2 variant proteins exerted no effects on the promoter activity (Fig. 1B). No dominant-negative effects were observed for the 2 variants. The transfection efficiency was comparable among the empty vector and the wildtype and variant SOX10 expression vectors (Fig. 1C).

Clinical analysis for individuals with SOX10 variants

Clinical features of patients 1–3 and the parents of patients 1 and 3 with SOX10 variants are summarized in Tables 2 and 3. These 5 individuals were otherwise healthy and had no intellectual disability.

Patient 1 was a boy with bilateral sensory deafness and dysgeusia. At 9.5 years of age, he was referred to our clinic for the evaluation of small testis. He showed small testis (right, 0.5 mL; left, 0.7 mL; reference, 1.0–3.5 mL), but normal stretched penile length (4.5 cm; reference, 3.8–5.2 cm). A smell test using intravenous vitamin injection induced no response. Blood examination revealed that testosterone levels were low at baseline and responded poorly to human chronic gonadotropin (hCG) stimulation. FSH and LH levels were within prepubertal ranges both at baseline and after GnRH stimulation. Brain magnetic resonance imaging (MRI) showed olfactory bulb agenesis, but no abnormalities in the hypothalamus or pituitary. The father of patient 1 with the same SOX10 variant exhibited no clinical abnormalities.

Patient 2 was an adult male with right sensory deafness. At 29 years of age, he visited our clinic because of a lack of pubertal signs. Physical examinations showed small testes (right, 3.0 mL; left, 3.0 mL; reference, 11.4–20.3 mL), short
stretched penile length (3.0 cm; reference, 9.6–10.3 cm), gynecomastia, and the lack of pubic hair. A smell test using odor sticks revealed partial impairment. Blood examination revealed a low testosterone level. LH levels were within the prepubertal range both at baseline and after GnRH stimulation. Brain MRI showed olfactory nerve hypoplasia.

Patient 3 was a girl with cleft lip. At 12 years of age, she was referred to our clinic because of the lack of pubertal signs. She had no breast budding or pubic hair. Intravenous vitamin injection induced delayed response of smell. Pure tone audiometry showed normal results for both ears. Blood examination revealed a low estrogen level at baseline. LH levels were within the prepubertal range and responded poorly to GnRH stimulation. The mother of patient 3 with the same SOX10 variant had a normal phenotype, except for mild hearing impairment and mild constipation.

Discussion
We identified pathogenic/likely-pathogenic SOX10 variants in 3/117 patients clinically diagnosed with KS. In vitro assays revealed that, although the expression vectors of the 2 SOX10 variants retained normal transfection efficiency, they lacked in vitro transactivating activity for the MITF promoter and exerted no dominant-negative effects. The 3 patients carried no additional rare variants in SOX10 or in the 14 other major causative genes for KS/HH. In our cohort, the overall frequency of pathogenic/likely-pathogenic variants and rare VUS in the 14 tested genes was 64%,
which was almost comparable to previous reports [5–7, 22, 23]. These results indicate that SOX10 haploinsufficiency accounts for a small percentage of the etiology of KS.

The results of this study expand the mutation spectrum of SOX10. First, the c.1225G > T variant was hitherto unreported. This variant encodes a truncated protein lacking 57 amino acids at the C-terminal end. Since the stop codon of this variant resides within the last exon, the truncated messenger RNA (mRNA) is likely to escape nonsense mediated mRNA decay [24]. Hence, the lack of in vitro transactivating activity of this variant suggests that the 57 amino acids at the C-terminal end is indispensable for SOX10 function. Indeed, this region was reported as the transactivating domain [10]. Second, the c.475C > T variant is a previously reported substitution in the HMG domain. The results of in vitro assays imply that amino acid sequences of the HMG domain are critical for the activity of SOX10. Lastly, the c.475C > T variant in patient 2 was de novo, whereas the same variant in patient 3 was inherited from her mother. Considering that this variant has already been identified in a Chinese family [17], the cytosine at the 475th position may be susceptible for de novo nucleotide substitution. Consistent with this, previous studies suggested that CG dinucleotides within exons of genes are relatively prone to develop de novo substitutions [25].

Clinical analysis of the variant-positive individuals also provided notable findings. First, the results of this study support the notion that phenotypes of SOX10 haploinsufficiency are highly variable even in individuals with the same variant [9, 13, 15, 26–31]. Indeed, the phenotypes of the parents of patients 1 and 3 highlight incomplete penetrance and variable expressivity of clinical features of SOX10. Lastly, the c.475C > T variant in patient 2 was de novo, whereas the same variant in patient 3 was inherited from her mother. Considering that this variant has already been identified in a Chinese family [17], the cytosine at the 475th position may be susceptible for de novo nucleotide substitution. Consistent with this, previous studies suggested that CG dinucleotides within exons of genes are relatively prone to develop de novo substitutions [25].

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### Table 1. Rare SOX10 variants identified in this study

| Variant   | ClinVara | dbsNPb | Allele Frequency in the General Population | In Silico Analysis |
|-----------|----------|--------|------------------------------------------|-------------------|
|           |          |        | 1000Gc | gnomADd | HGVDd | Poly Phen-2f | SIFTg | CADDh | M-CAPI | Mutation Tasteri |
| c.1225G > T | p.Gly409* | No data | No data | No data | No data | No Datak | 38.0 | 0.999 | 0.000 | Disease causing |
| c.475C > T | p.Arg159Trp | No data | No data | No data | No data | No Datak | 25.5 | 0.543 | Disease causing |

Scores indicative of a damaging variant are boldfaced.

aClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).
bdbSNP (https://www.ncbi.nlm.nih.gov/snp/).
cthe 1000 Genomes Database (https://www.internationalgenome.org/1000-genomes-browsers/).
dthegnomAD browser (https://gnomad.broadinstitute.org/).
eThe Human Genetic Variation Database (https://www.hgvd.genome.med.kyoto-u.ac.jp/).
fPolymorphism Phenotyping v2 (http://genetics.bwh.harvard.edu/pph2/).
gSorting Intolerant From Tolerant (http://provean.jcvi.org/index.php).
hCombined Annotation Dependent Depletion (https://cadd.gs.washington.edu/).
iMendelian Clinically Applicable Pathogenicity (http://bejerano.stanford.edu/mcap/).
jMutationTaster (http://www.mutationtaster.org/).
kIn silico prediction is available only for missense variants.

**Figure 2.** Conservation of the mutated amino acids. Mutated residues are shown in red. Data were obtained from the UCSC genome browser (https://genome-asia.ucsc.edu/).
hormone deficiency at later ages (e.g., ages 14 and 17 years), long-term follow-up is necessary for patients 1–3 and their parents with SOX10 variants. Second, patient 3 exhibited KS but no other features of WS/PCWH. Previous studies have shown that the majority of patients with KS due to SOX10 abnormalities exhibit hearing impairment, indicating that the auditory system is highly sensitive to compromised function of SOX10 [4, 16]. Yet, the results of patient 3 provide evidence that SOX10 haploinsufficiency may cause KS as a sole discernible clinical abnormality. Actually, we expected FGFR1 variants in patient 3, because this patient showed a cleft lip, which is typically seen in patients with FGFR1 haploinsufficiency [2, 3, 7]. Our data indicate that SOX10 should be included in mutation screening for KS patients, even when the patient had no hearing loss or other clinical features of WS/PCWH. Moreover, since patient 3 was only 12 years of age, we cannot exclude the possibility that she has delayed puberty but not HH. It remains to be clarified whether delayed puberty is one of the clinical manifestations of SOX10 haploinsufficiency. Lastly, this study demonstrated that pathogenic SOX10 variants can occur de novo and be transmitted from a phenotypically normal parent. Thus, sequence analysis for parental samples is essential for genetic counseling of families with SOX10 abnormalities.

In summary, we identified pathogenic/likely-pathogenic SOX10 variants in 3/117 patients clinically diagnosed with KS. This study highlights the broad mutation spectrum and phenotypic variations of SOX10 haploinsufficiency. SOX10 should be included in molecular diagnoses for KS patients, even when the patient had no hearing loss or other clinical features of WS/PCWH.

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Disclosures: The authors have nothing to disclose.

Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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| Table 2. Molecular and clinical data of five individuals with SOX10 variants |
| --- |
| **Age (yr)** | **Sex** | **SOX10 variant** | **Clinical Features** |
| **DNA** | **Protein** | **Puberty** | **Sense of Smell** | **Olfactory Bulb** | **Auditory Function** | **Other Features** |
| Patient 1 | 9.5 | Male | c.1225G > T | p.Gly409* | Prepubertal | Anosmia | Bilateral agenesis | Bilateral hearing loss | Dysgeusia |
| Father of patient 1 | Adult | Male | c.1225G > T | p.Gly409* | Normal | Normal | No data | Normal | No |
| Patient 2 | 29 | Male | c.475C > T | p.Arg159Trp | Delayed | Partial impairment | Left agenesis | Right hearing loss | No |
| Patient 3 | 12 | Female | c.475C > T | p.Arg159Trp | Possibly delayed | Delayed response | No data | Normal | Cleft lip |
| Mother of patient 3 | Adult | Female | c.475C > T | p.Arg159Trp | Normal | Normal | No data | Mild impairment | Mild constipation |

| Table 3. Endocrine data of patients 1–3 |
| --- |
| **Patient 1** | **Patient 2** | **Patient 3** |
| **Age at exam. (yr)** | 9.5 | 29 | 12 |
| **FSH (IU/L)** | 0.9 (0.6–3.0) | 0.4 (2.2–8.4) | 1.6 (4.8–10.4) |
| Basal | 0.1 (0.0–0.4) | 0.1 (1.8–12.0) | 0.4 (0.4–4.1) |
| Stimulated* | 4.8 (6.3–15.6) | 2.7 (> 4.4) | 9.0 (8.3–20.0) |
| LH (IU/L) | Stimulation test (100 μg/m2, bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 minutes). |
| Basal | 2.1 (0.4–6.0) | 2.9 (> 9.0) | 4.8 (8.5–15.5) |
| Stimulated* | 4.44 (5.06–6.93) | – | – |
| Testosterone (nmol/L) | Basal | <0.10 (0.49–0.76) | 0.84 | – |
| Stimulated* | 4.44 (5.06–6.93) | – | – |

*GnRH stimulation test (100 μg/m2, bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 minutes).

hCG stimulation test (3000 U/m2/day i.m. for 3 consecutive days; blood sampling at the 4th day.)
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