

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 chorionic 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 accounted 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 hypomasculinized 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*, *FGFR1*, *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 Genetics (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]. HeLa 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 Lipofectamin 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 orchidometer 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 chorionic 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

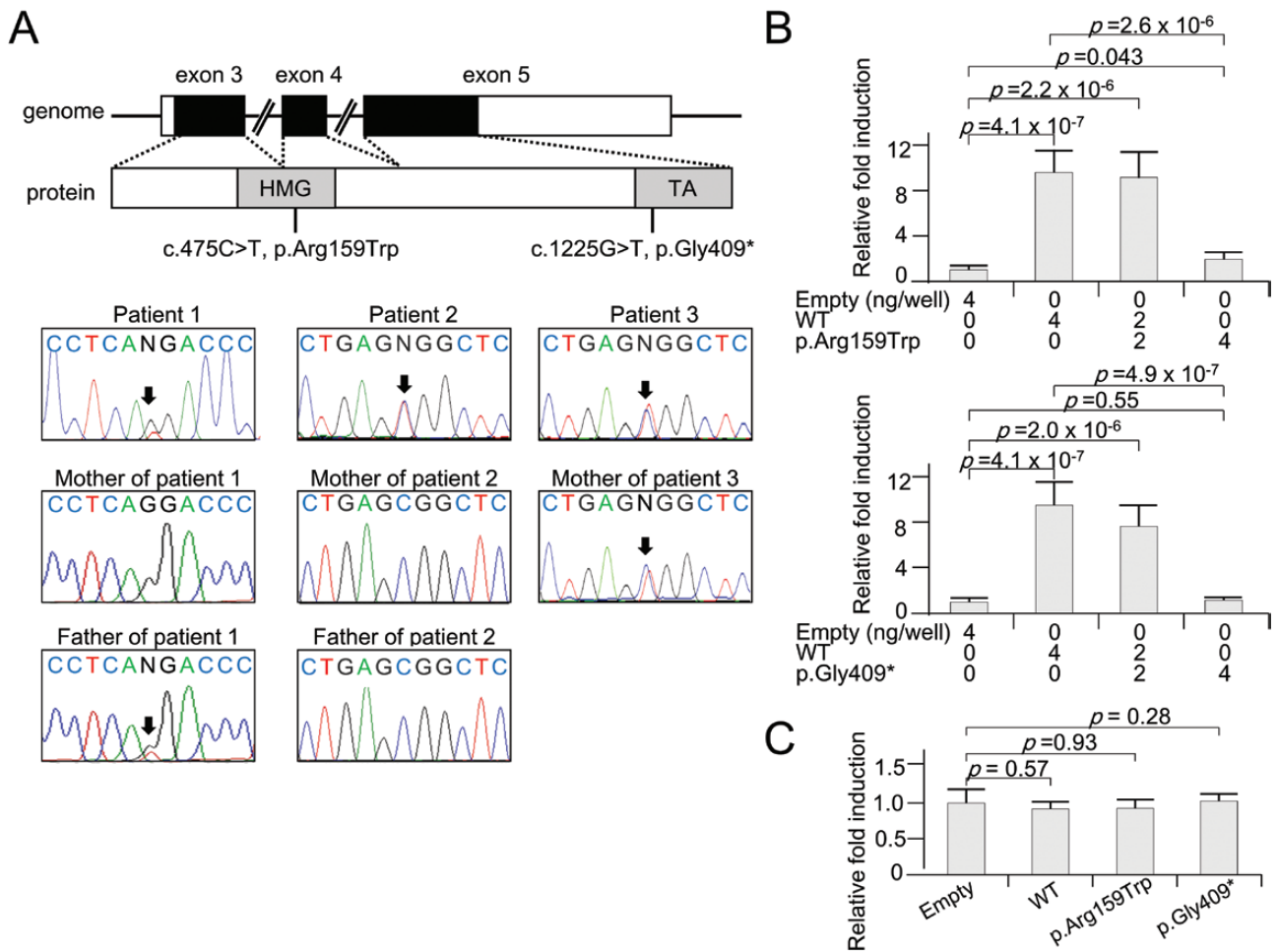


Figure 1. *SOX10* variants identified in this study. **A:** The positions and chromatographs of the c.475C > T and c.1225G > T variants. The black and white boxes in the upper panel depict translated and untranslated regions, respectively. Altered nucleotides are indicated by black arrows. **B:** Representative results of in vitro assays. Relative fold inductions for the *MITF* promoter-containing reporter (the mean \pm 1 SD) by the empty expression vector (Empty) and the expression vectors for the wildtype *SOX10* and 2 variants (p.Arg159Trp and p.Gly409*) are shown. **C:** Transfection efficiency of the expression vectors. Relative fold inductions for the pCMV-PRL vector (the mean \pm 1 SD) co-expressed with the empty vector or the expression vectors for WT *SOX10* or 2 variants are shown. Abbreviations: HMG, the high-mobility group domain; SD, standard deviation; TA, the transactivating domain; WT, wildtype.

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%,

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

Variant		ClinVar ^a	dbSNP ^b	Allele Frequency in the General Population			In Silico Analysis				
cDNA	Protein			1000G ^c	gnomAD ^d	HGVD ^e	Poly Phen-2 ^f	SIFT ^g	CADD ^h	M-CAP ⁱ	Mutation Taster ^j
c.1225G > T	p.Gly409*	No data	No data	No data	No data	No data	No Data ^k	No Data ^k	38.0	No Data ^k	Disease causing
c.475C > T	p.Arg159Trp	No data	No data	No data	No data	No data	0.999	0.000	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/>).

^dthe gnomAD 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.

	Arg ¹⁵⁹								
Human	Q	M	R	L	R	E	A	E	E
Rhesus	Q	M	R	L	R	E	A	E	E
Mouse	Q	M	R	L	R	E	A	E	E
Dog	Q	M	R	L	R	E	A	E	E
Elephant	Q	M	R	L	R	E	A	E	E
Chicken	Q	M	R	L	R	E	A	E	E
X_tropicalis	Q	M	R	L	R	E	A	E	E
Zebrafish	Q	K	R	L	R	E	A	E	E

	Gly ⁴⁰⁹								
Human	G	Y	Y	P	G	S	P	Q	H
Rhesus	G	Y	Y	P	G	S	P	Q	H
Mouse	G	Y	Y	P	G	S	P	Q	H
Dog	G	Y	Y	P	G	S	P	Q	H
Elephant	G	Y	Y	P	G	S	P	Q	H
Chicken	S	Y	Y	P	G	S	P	Q	H
X_tropicalis	S	Y	Y	S	S	S	P	Q	H
Zebrafish	A	Y	Y	S	G	S	A	Q	H

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/>).

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-terminus. 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* haploinsufficiency. Such a broad phenotypic spectrum has been reported in other disorders due to haploinsufficiency of transcription factor genes [18, 32]. It is possible that the phenotype of *SOX10* haploinsufficiency is affected by various factors, including the expression levels of the wildtype *SOX10* allele and polymorphisms in other genes. However, considering that some previously reported patients with *SOX10* pathogenic variants developed hearing impairment or sex

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.	9.5 years	29 years	12 years
FSH (IU/L)			
Basal	0.9 (0.6–3.0)	0.4 (2.2–8.4)	1.6 (4.8–10.4)
Stimulated ^a	4.8 (6.3–15.6)	2.7 (> 4.4)	9.0 (8.3–20.0)
LH (IU/L)			
Basal	0.1 (0.0–0.4)	0.1 (1.8–12.0)	0.4 (0.4–4.1)
Stimulated ^a	2.1 (0.4–6.0)	2.9 (> 9.0)	4.8 (8.5–15.5)
Testosterone (nmol/L)			
Basal	<0.10 (0.49–0.76)	0.84 (7.97–29.99)	–
Stimulated ^b	4.44 (5.06–6.93)	–	–

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

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

hormone deficiency at later ages (eg, 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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Additional Information

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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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