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ARTICLE

SOX11 variants cause a neurodevelopmental disorder with infrequent ocular malformations and hypogonadotropic hypogonadism and with distinct DNA methylation profile

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ABSTRACT

Purpose: This study aimed to undertake a multidisciplinary characterization of the phenotype associated with *SOX11* variants.
Methods: Individuals with protein altering variants in *SOX11* were identified through exome and genome sequencing and international data sharing. Deep clinical phenotyping was undertaken by referring clinicians. Blood DNA methylation was assessed using Infinium MethylationEPIC array. The expression pattern of *SOX11* in developing human brain was defined using RNAscope.
Results: We reported 38 new patients with *SOX11* variants. Idiopathic hypogonadotropic hypogonadism was confirmed as a feature of *SOX11* syndrome. A distinctive pattern of blood DNA methylation was identified in *SOX11* syndrome, separating *SOX11* syndrome from other BAFopathies.
Conclusion: *SOX11* syndrome is a distinct clinical entity with characteristic clinical features and episignature differentiating it from BAFopathies.

Introduction

Coffin-siris syndrome (CSS) is a multiple congenital malformation syndrome, which is associated with variants in genes encoding subunits of the BAF complex (collectively termed BAFopathies).[1] The classical CSS phenotype consists of coarse facies, hypoplasia of the nail of the fifth digit, and developmental delay. Variants in *ARID1B* (OMIM 614556) are the most common cause of CSS, but *ARID1B* variants are also associated with nonsyndromic intellectual disability (ID).[2] *ARID1B* variants are among the most common causes of neurodevelopmental disorders (NDDs) identified in large scale sequencing studies.[3] Variants in other genes can be associated with CSS (eg, *ARID1A*, *DPF2*) but are considerably less frequent.[4,5]

Tsurusaki et al[6] reported de novo single nucleotide variants (SNVs) in *SOX11* in 2 children with CSS. Hempel et al[7] identified microdeletions at 2p25.2 (containing *SOX11*) and *SOX11* SNVs in a series of children with either nonsyndromal ID or CSS. The *SOX11* missense variants

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identified were located in the high-mobility group (HMG) DNA binding domain and were shown to impair activation of *SOX11* target genes in vitro. *SOX11* is a single exon gene with a single transcript, which is predicted to be haploinsufficient and loss-of-function intolerant.[7] The phenotype associated with *SOX11* variants was designated as CSS9 (OMIM 615866).

Proteins encoded by *SOX* genes are a family of transcription factors, which play crucial roles in multiple developmental processes.[8] All SOX protein family members contain an HMG box, which is the hallmark of these proteins. The HMG box binds to and regulates target genes. The HMG box also controls protein–protein interactions and trafficking of SOX proteins between cytoplasm and nucleus. Variants in a variety of *SOX* genes are associated with human developmental disorders (termed SOXopathies); examples include Waardenburg syndrome[9] (caused by pathogenic variants in *SOX10*) and *SOX2*-anophthalmia syndrome.[10] SOXopathies share some common features such as ocular malformations, ID, hypogonadotropic hypogonadism, and genital malformations. *SOX11* forms a peripheral component of the SWI/SNF complex, and thus, *SOX11*-associated syndrome (*SOX11* syndrome) may lie in the CSS spectrum. However, given the similarity in protein sequence and function between *SOX11* and other *SOX* gene family members, it is possible that the phenotypes associated with *SOX11* variants may be more congruent with SOXopathies.

In this article, we report a large series of individuals with *SOX11* variants identified via a genotype first approach in large scale exome and genome sequencing studies. We define the associated phenotypic and molecular genetic spectrum, including detailed developmental milestones. We identify ocular malformations and hypogonadotrophic hypogonadism as being features of *SOX11* syndrome. We report a unique peripheral blood DNA methylation signature as a diagnostic biomarker and phenotypic clustering analysis that distinguishes *SOX11* syndrome from BAFopathies.

Materials and Methods

Ascertainment of individuals with *SOX11* variants

Participants with protein altering SNVs in *SOX11* were identified through exome sequencing from the Deciphering Developmental Disorders (DDD) study and genome sequencing undertaken in the 100,000 Genomes Project.[11] The sequencing pipelines for DDD[3] and the 100,000 Genomes Project have been published elsewhere. Additional participants were identified via GeneMatcher (Paracel, Inc).[12] This study complied with the Declaration of Helsinki. Written informed consent was obtained from parents and guardians as appropriate, including for publication of photographs. Clinical and phenotypic data were gathered from medical records by the recruiting clinician. Kaplan-Meier analysis was performed (PASW, http://www.spss.com.hk/statistics/) to summarize acquisition of developmental milestones.

SOX11 variant classification

SOX11 variants (NM_003108.4) were classified using American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology[13] criteria applied using VarSome tool.[14]

In vitro analysis of *SOX11* transactivating activity

The *SOX11* open reading frame clone was purchased from Promega, and the mutant variants (c.1142_1143insT [p.Gly384Argfs*14], c.527C>A [p.Ala176Glu], and c.882C>G [p.Tyr294*]) of *SOX11* were generated through site-directed mutagenesis either with KOD-Plus-Mutagenesis Kit (Toyobo) (for c.1142_1143insT) or with PrimeSTAR Mutagenesis Basal Kit (Takara Bio) (for c.527C>A and c.882C>G). Wild-type and mutant *SOX11* complementary DNAs were amplified using polymerase chain reaction and cloned into p3xFLAG-CMV-14 expression vector (Sigma-Aldrich). The *GDF5* promoter 5′-flanking sequence (−448/+319, NM_000557.3, GRCh37/hg19) was polymerase chain reaction amplified and cloned into pGL3-basic vector (Promega). All constructs were verified using Sanger sequencing.

HeLa cells were cultured in Dulbecco's Modified Eagle Medium–high glucose supplemented with penicillin (50 units/mL), streptomycin (50 µg/mL), and 10% fetal bovine serum. Cells were plated in 24-well plates, 24 hours before transfection. Transfections were performed using TransIT-LT1 (Takara) with pGL3 reporter (500 ng per well), effector (250 ng per well), and pRL-SV40 internal control (6 ng per well) vectors. The cells were harvested for 24 hours after transfection, and luciferase activities were measured using the PicaGene Dual Sea Pansy Luminescence Kit (TOYO B-Net Co, Ltd). Wild-type and mutant SOX11 proteins were assessed using immunoblot analyses with monoclonal anti-FLAG M2 HRP antibody (1:3000 dilution; Sigma-Aldrich) following the manufacturer's instructions. Relative *GDF5* promoter activities were evaluated using *t* test. $P < .016$ was considered as significant (because 3 statistical comparisons were made and the *P* value was correct for multiple comparisons [.05/3 = .016]). Relative luciferase activities were compared with that of the empty vector and presented as mean ± SD for 2 independent experiments, with each experiment performed in triplicates.

Study of *SOX11* expression in fetal brain

Expression of *SOX11* transcripts in fetal structures were evaluated using RNAscope in situ hybridization (ISH) assay and compared with expression pattern of GnRHR. Methods are provided in the online Supplemental Methods.

Phenotype cluster analysis of *SOX11* syndrome and CSS

Human Phenotype Ontology (HPO)[15] terms were used to describe the phenotypes of individuals with *SOX11* or *ARID1B* variants in a standardized fashion. Individuals with pathogenic *ARID1B* variants were identified from the open access participants listed on DECIPHER. We selected HPO terms that were well defined and not reliant on subjective clinical evaluation (microcephaly <2 SD [HP 0000252], abnormal eye morphology [HP 0013272], oculomotor apraxia [HP 0000657], hypogonadotrophic hypogonadism [HP 0000044], and coarse facies [HP 0000280]).

Hierarchical cluster analysis was performed in Python using the Scikit-learn library and the AgglomerativeClustering object. The Euclidean distance and Ward parameters were selected to compute the linkage distance and cluster merge strategy. The dendrogram and heatmap were created using the Seaborn library and clustermap object. Input document is provided in Supplemental Methods. The script for the clustering test and plotting functions can be found on https://github.com/Eema-jawahiri/phenotypic-cluster-analysis.git

Identification of *SOX11* syndrome episignature

Full methods are provided in the Supplemental Methods. Peripheral blood DNA was extracted using standard techniques. Bisulfite conversion was performed, and samples were analyzed using Illumina Infinium MethylationEPIC BeadChips (Illumina). Details of DNA methylation data analysis and episignature discovery were previously described.[16-20] For mapping the episignature (probe and feature selection), MatchIt package[21,22] was used to randomly select controls matched for age, sex, and array type from the EpiSign Knowledge Database (EKD), providing a control sample size 5 times larger than that of the cases, resulting in 50 controls.

Methylation levels (β-values) were then transformed into M-values, which were used for linear regression modeling. Using the limma package, linear regression modeling was performed for the purpose of calculating the methylation differences between the case and the control groups, along with the corresponding *P* value for each probe. A total of 224 differentially methylated probes (DMPs) were identified and considered as the *SOX11* episignature.

Using the 224 DMPs, 2 binary support vector machine (SVM) classifiers with a linear kernel were constructed using the e1071 package as described previously.[16,17] The first classifier was trained using only the *SOX11* samples against the control samples, and then samples from 38 other Mendelian NDDs with an established episignature from the EKD were supplied into the model to assess the specificity of the model. Using the Platt's scaling method, the classifiers generate a methylation variant pathogenicity (MVP) score ranging from 0 to 1 for each sample, in which a score near 1 is indicative of similarity to the identified *SOX11* syndrome episignature, whereas a score near 0 shows that the sample has a methylation profile different from the *SOX11* syndrome episignature.

Results

Ascertainment of cohort

We identified 38 new patients with SNVs ($n = 34$) or deletions ($n = 4$) of *SOX11* (clinical and genomic data are summarized in Supplemental Tables 1, 2, and 3). Participants were identified through exome sequencing in the DDD study or genome sequencing in the 100,000 Genomes Project, with additional cases identified via GeneMatcher. We identified 15 published patients with *SOX11* SNVs (Supplemental Table 1).

Spectrum of *SOX11* variants in NDDs

We identified 29 distinct *SOX11* SNVs from these 38 new patients (clinical information is in Supplemental Tables 1 and 2, and genomic information in Supplemental Table 3). There were 25 unique missense variants (Figure 1), 4 protein truncating variants (PTVs), and 4 microdeletions. One sibling pair shared the same PTV, and 1 sibling pair shared the same missense variant. All PTVs were classified as pathogenic. In total, 5 missense variants were classified as likely pathogenic and 20 as pathogenic. De novo status was confirmed in 30 patients. In addition, 1 variant was inherited from a mosaic parent, and 2 affected sibships (4 participants) had a parent with ID who was presumed to be a *SOX11* variant heterozygote (but was not tested). No other likely pathogenic or pathogenic variants that might better explain the phenotype were identified. Case 24 had a *BPTF* variant (ACMG Class 3). Case 28 had a single variant in *IVD* (recessive isovaleric acidaemia). Case 36 had a *KATB* variant (ACMG class 3) inherited from an unaffected mother.

Evidence that the *SOX11* HMG box could be a hotspot for pathogenic variants

The HMG box in SOX11 protein is a domain responsible for SOX11 binding to DNA and regulation of target genes. In addition, the HMG box regulates key protein–protein interactions and trafficking of SOX11 protein between cytoplasm and nucleus. Our previous in vitro studies showed that 4 missense variants in the HMG box impaired SOX11 transactivating activity (p.Lys50Asn, p.Pro120His, p. Ser60Pro, and p. Tyr116Cys). We could not perform functional analyses of all missense variants in the HMG box. It

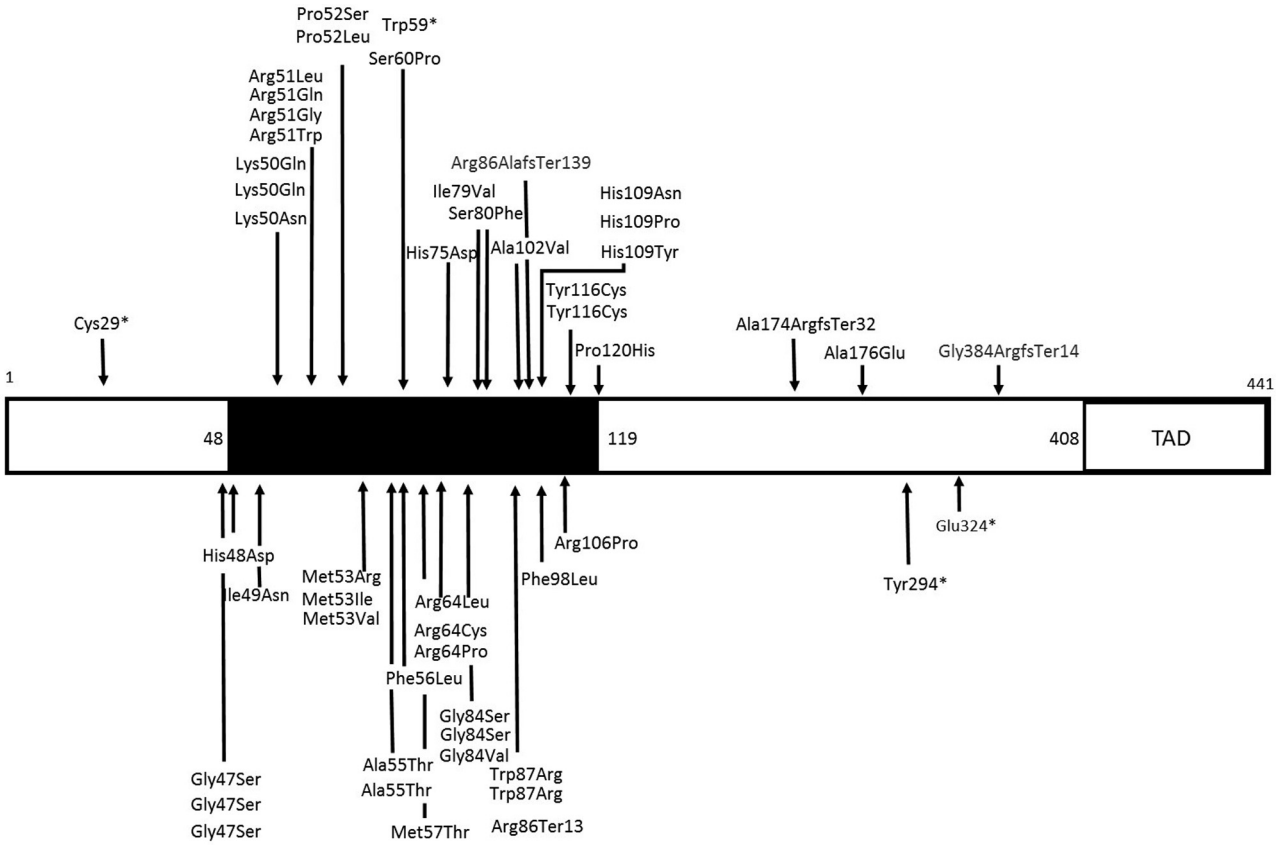

Figure 1 Schematic diagram of reported *SOX11* variants. Illustration of missense and protein truncating variants in *SOX11* in people with neurodevelopmental disorders. Both novel variants identified in this study and those identified in published patients are shown. The black box indicates the high-mobility group domain. The domain is not drawn to scale. Domain boundaries (amino acid number) as defined by Refseq (NP_003099.1), SwissProt (P35716.2), and International Nucleotide Sequence Database Collaboration (AAB08518.1). TAD, transactivating domain. Refseq, National Center for Biotechnology Information Reference Sequence Database.

should not be assumed that all HMG box missense variants are pathogenic. However, it is plausible that such variants could interfere with *SOX11* transactivating activity and have pathogenic potential.

First, none of the *SOX11* missense variants were present in Genome Aggregation Database (gnomAD), and only 8 HMG missense variants in *SOX11* were identified in 114,704 individuals in gnomAD v2.1.1 non-neuro data set. This strongly suggests that missense variants in this domain are not compatible with normal neurodevelopment. Second, if the HMG box is a pathogenic variant hotspot, then it should be relatively depleted of missense SNVs in cohorts without NDDs. Using gnomAD (v2.1.1 non-neuro data set) we identified that the percentage of residues with a missense SNV in the HMG box was significantly lower than in the N-terminal, central, or transactivating domains (Supplemental Figure 1). This suggests intolerance to variation in the HMG box. There is significant sequence homology between the HMG box domains of human SOX proteins. We reasoned that if pathogenic variants had been reported at a given residue in a SOX protein, then it could be taken as possible evidence of pathogenicity for the equivalent variant in *SOX11*. We therefore compared variants from DECIPHER and ClinVar between *SOX10* and *SOX11*. In total, 6 residues in *SOX11* had pathogenic variants (DECIPHER and ClinVar) at equivalent residues in *SOX10* (Supplemental Table 4). Several of these had identical amino acid change, eg, p.(Arg51Gly) in *SOX11* and p.(Arg106Gly) in *SOX10*.

Missense variants in regions of *SOX11* other than the HMG box could also interfere with protein function and have pathogenic potential. The p.(Ala176Glu) variant that we identified enabled us to investigate this. This variant significantly reduced SOX11 transactivating activity in vitro (Figure 2). This supports a potential pathogenic role for the variant.

We then investigated the effect of PTVs on SOX11 transactivating activity. In vitro analysis of 2 PTVs showed significant impairment of *SOX11* transactivating ability (Figure 2). Only 2 PTVs are present in gnomAD (v2.1.1 non-neuro data set), and *SOX11* is predicted to be loss-of-function intolerant with probability of loss of function intolerance = 0.86 (observed/expected 0.09 [0.03-0.44]). In vitro evidence of impaired transactivation and depletion of PTV in gnomAD supports a pathogenic role for *SOX11* PTV. The mechanism through which PTV leads to reduction in SOX11 transactivating activity may relate to the loss of C-terminal transactivation domain.

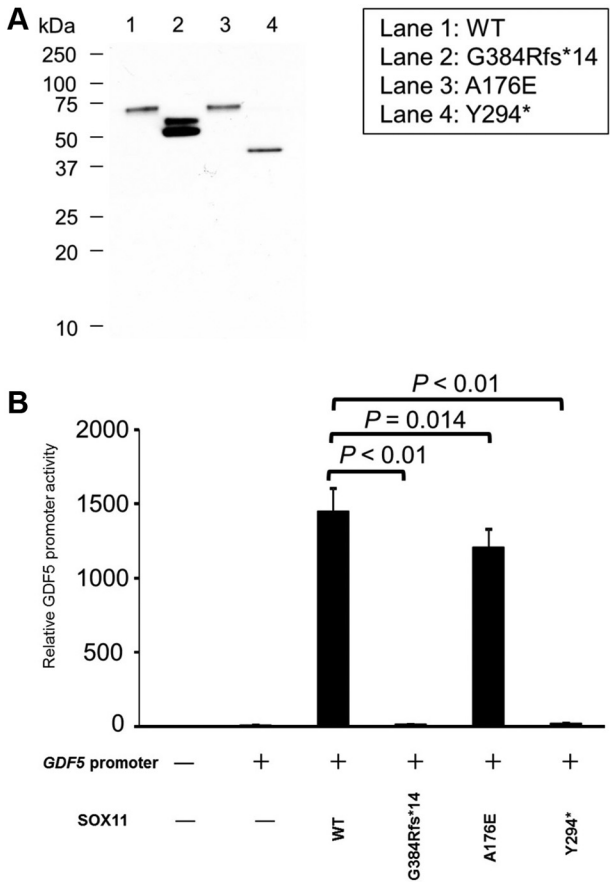

Figure 2 Assessment of *SOX11* variant impact on transactivation of target gene. A. Western blot showing lower molecular weight of G384Rfs*14 and Y294* *SOX11* variants than that of WT SOX11 protein. An anti-FLAG M2 HRP antibody (1:3000; Sigma-Aldrich) was used. B. Luciferase assay showing impaired activation of *GDF5* promotor by G384Rfs*14 and Y294* *SOX11* variants. A176E impairs SOX11 activity but to a much lesser extent than Y294* or G384Rfs*14. Data were taken from 2 separate experiments; each experiment was performed in technical triplicate. Activation of *GDF5* promotor was compared between WT SOX11 protein and 3 *SOX11* variants (G384Rfs*14, A176E, and Y294*) using *t* test. Correction for multiple comparisons was undertaken with a *P* value of .016 being taken as significant (.05/3 = .016). WT, wild type.

Clinical phenotype associated with *SOX11* variants

The clinical phenotypes are summarized in Supplemental Table 1. The mean age at examination was 9 years (range: neonate to 23 years). Microcephaly, short stature, and low body weight were common. There was a consistent facial dysmorphology across multiple ethnic groups (Supplemental Figure 2). All but 1 patient was reported to have developmental delay or ID. In total, 80% of patients had begun to sit by age 12 months (Supplemental Figure 3A), 70% were walking independently by age 30 months (Supplemental Figure 3B), and 80% had begun to speak by age 40 months (Supplemental Figure 3C). Internal organ malformations were uncommon, apart from renal anomalies (3 patients). Ocular involvement was infrequent, with oculomotor apraxia (4 patients), coloboma (2 patients), and microphthalmia (1 patient) being reported. Magnetic resonance imaging (MRI) of brain was performed in 20 (42%) patients and was abnormal in 12 (60% of imaged patients). Cerebellar malformations (4 patients), agenesis of the corpus callosum (4 patients), arhinencephaly (1 patient), Rathke's cleft cyst (1 patient), and small pituitary gland (2 patients) were reported. A total of 8 (21%) patients presented with hypogonadotropic hypogonadism, which was confirmed by endocrine testing (Supplemental Table 2). Investigations were prompted by delayed puberty, cryptorchidism, or genital malformations.

Detection and verification of an episignature for *SOX11* syndrome and classification of BAFopathy complex samples

An overall hypomethylation pattern was observed for most probes when comparing 10 *SOX11* cases (case details in Supplemental Table 5) and control samples (Supplemental Figure 4). A total of 224 DMPs (Supplemental Figure 5, Supplemental Table 6) were used for the purpose of constructing unsupervised and supervised classification models. To assess the robustness of the episignature in differentiating between cases and controls, hierarchical clustering (Supplemental Figure 6A) and MDS analysis (Supplemental Figure 6B) were performed, resulting in a clear separation between these 2 groups. BAFopathy complex samples were applied to the *SOX11* episignature classifier, but none of them were grouped with *SOX11* samples (Supplemental Figure 7A and B). Ten rounds of cross-validation on MDS plot were performed using 9 *SOX11* samples as training set and a single *SOX11* sample as testing set. In all steps, the testing samples were correctly clustered with the training samples, further providing evidence of a robust common DNA methylation signature for *SOX11* (Supplemental Figure 8).

Construction of the binary prediction model

Two MVP plots were generated to confirm specificity of the classification model. In the first MVP plot in which the SVM was trained by comparing the 10 *SOX11* samples against controls, the classifier showed a high sensitivity for all *SOX11* samples, with all samples scoring high on the MVP axis (Figure 3A) further confirming the previous heatmap and MDS results. Some samples from other disorders that are in EKD that are part of the EpiSign V2 clinical assay, including autosomal dominant cerebellar ataxia, deafness, and narcolepsy, HVDAS_T, and Sotos syndrome, plus 1 sample from control (testing), Kabuki syndrome, and mental retardation, autosomal dominanttype 51 cohorts showed an elevated MVP score, suggesting levels of similarity in the DNA methylation profiles between these disorders.

To increase the specificity of the classifier, the SVM was trained by comparing the 10 *SOX11* samples against controls as well as 38 NDDs and congenital anomalies with

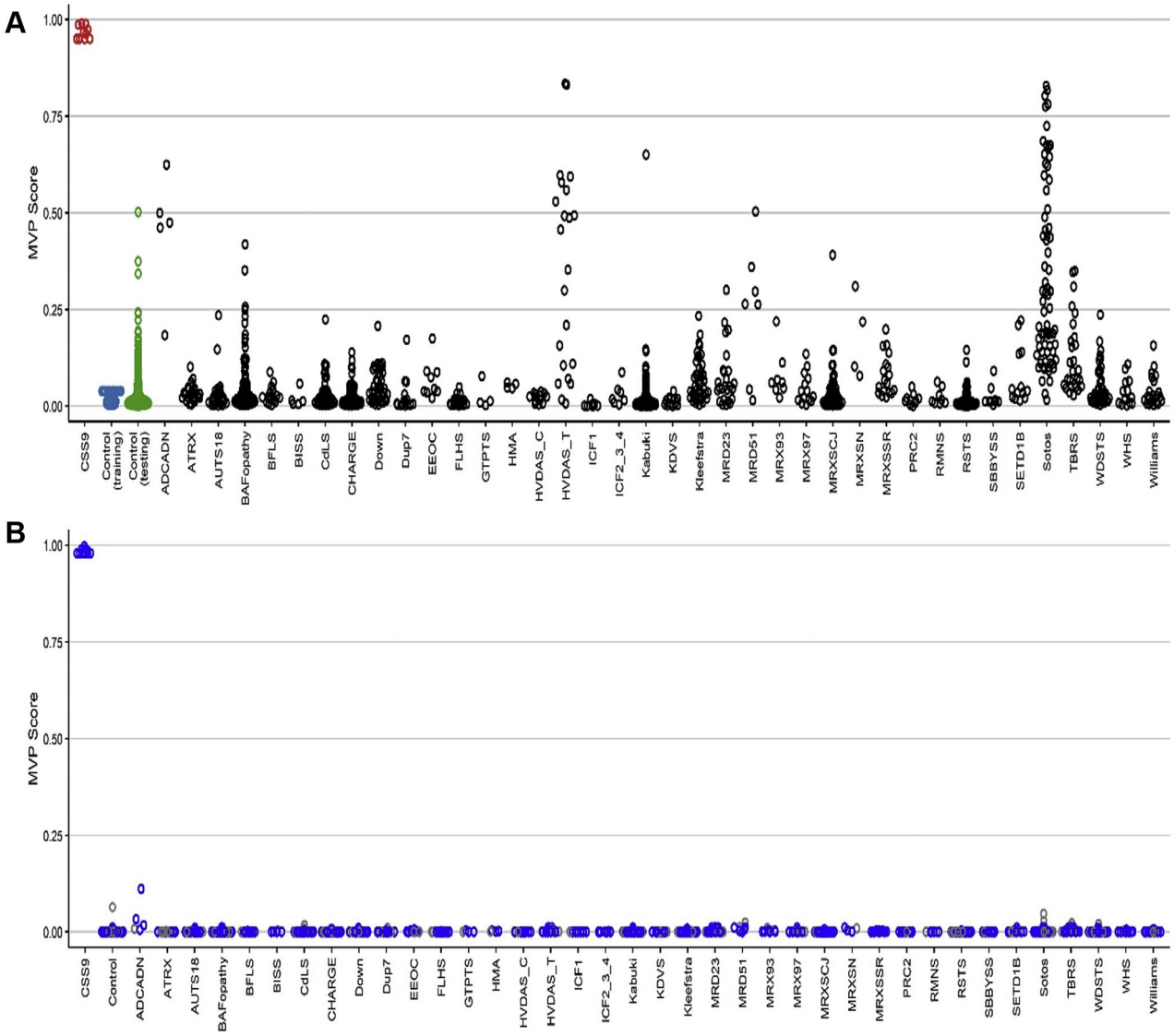

Figure 3 **MVP scores plot.** A. MVP scores was created using the support vector machine (SVM) trained by comparing 10 *SOX11* samples against controls. B. MVP scores were created using the SVM trained by comparing 10 *SOX11* samples against controls and 38 neurodevelopmental disorders and congenital anomalies available in the EpiSign Knowledge Database. The blue circles represent the training samples, and the gray circles represent the testing samples. MVP, methylation variant pathogenicity.

known episignatures present in the EKD. A high MVP score was seen in 10 *SOX11* samples with much improved specificity relative to other EpiSign conditions (Figure 3B).

Phenotype clustering separates *SOX11* syndrome from *ARID1B* CSS

We used a phenotype-based clustering analysis to show that *SOX11* syndrome and *ARID1B* CSS can be clinically distinguished (Figure 4). *SOX11* variant heterozygotes tended to be microcephalic, have oculomotor apraxia or abnormal eye morphology (cataract, microphthalmia), or have hypogonadotrophic hypogonadism. *ARID1B* CSS was distinguished by coarse facial features and the absence of the HPO terms prevalent in *SOX11* syndrome.

SOX11 expression in fetal central nervous system and pituitary gland

ISH showed widespread expression of *SOX11* in fetal cranial structures (Figure 5). Use of positive and negative controls confirmed the specificity of this finding (Supplemental Figure 9). At all Carnegie stages examined, *SOX11* was strongly expressed in the cerebral cortex and hindbrain (Supplemental Figure 10). Expression within the developing retina and optic nerve was also noted, particularly in Carnegie stage 23. Of interest, *SOX11* expression was noted in the developing pituitary, lining the lumen of the adenohypophysis, and also within the neurohypophysis (Figure 5). There was no clear difference observed in spatial localization between *SOX11* expression and *GnRHR* expression (Supplemental Figure 11).

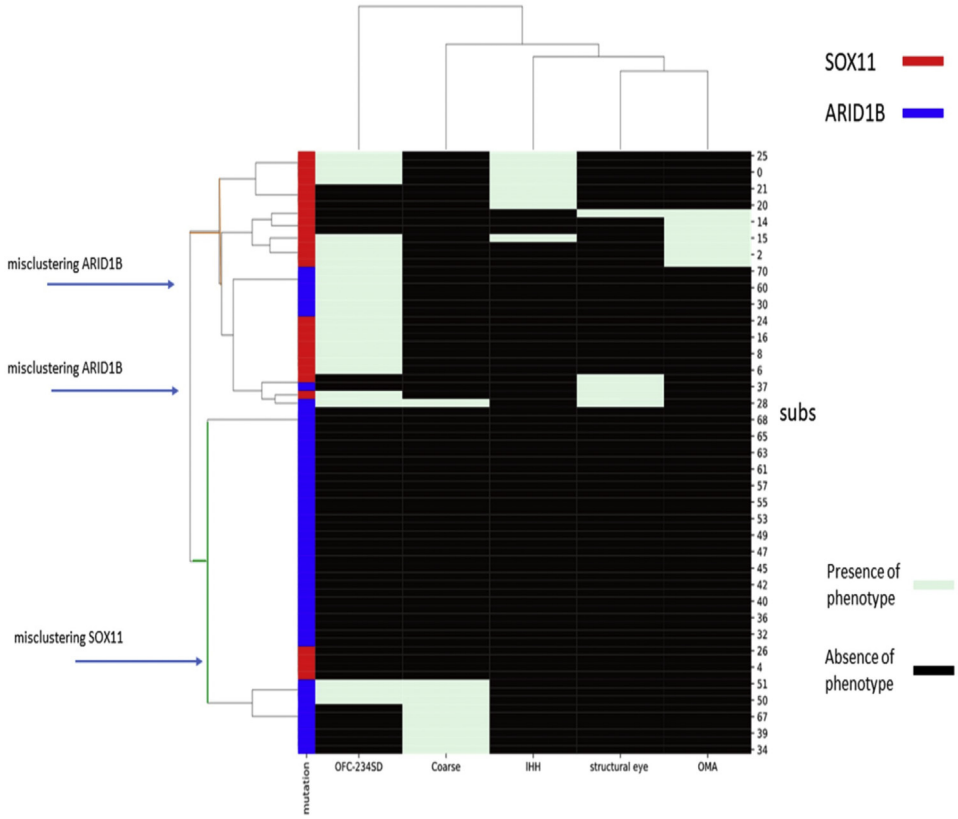

Figure 4 Phenotype-based clustering analysis of *SOX11* and *ARID1B* phenotypes. Hierarchical cluster analysis was performed in Python. Euclidean distance and Ward parameters were used to compute linkage distance and cluster merge strategy. *SOX11* and *ARID1B* variant heterozygotes lie in different clusters. OFC indicates OFC < 2 SD. Coarse indicates coarse facial features. Structural eye indicates structural eye disease. Subs indicates individual subject. IHH, idiopathic hypogonadism; OFC, orbitofrontal circumference; OMA, oculomotor apraxia.

Discussion

In this article, we report a large cohort of people with *SOX11* pathogenic variants and define the molecular genetics and clinical spectrum of the disorder. Most pathogenic and likely pathogenic variants were missense. These were all located within or next to the HMG box, a common motif in all SOX proteins that activate target genes via DNA binding.[8] The HMG box was significantly depleted of missense variants in samples from gnomAD, suggesting that it does not tolerate variation in healthy individuals. This shows that the HMG box could be classified as a variant "hot spot" for *SOX11*, further supported by the recurrent variants found at several residues in this domain. Most *SOX11* variants in our cohort were de novo, in keeping with a severe syndrome that impairs reproductive fitness. We identified 1 instance of transmission from a mosaic mother. This shows that recurrence could be possible due to mosaicism.

In addition, we identified a single de novo missense variant, p.(Ala176Glu), that was not in the HMG box. This variant reduced SOX11 transactivating ability. We have not identified other affected individuals with missense variants in this region (or other SOX11 protein regions that are not the HMG box). The individual (case 11) died early in the neonatal period. They had cerebellar hypoplasia and microcephaly, which is compatible with *SOX11* syndrome. However, it was not possible to ascertain whether they had other features of *SOX11* syndrome. It is possible that missense variants in different regions of *SOX11* might be pathogenic, as shown by case 11. This warrants further investigation through identification of more cases and functional analyses.

All but 1 of the patients in this cohort had ID or developmental delay. Speech was particularly affected, with Kaplan-Meier analysis suggesting that 20% of the patients may not attain speech. Renal malformations were the only common internal organ malformation. This is in keeping with murine studies indicating that loss of *SOX11* results in a spectrum of congenital anomalies of the kidney and urinary tract.[23] In mice, these included duplex kidney, malpositioned kidneys, and hydroureter, which overlaps with the renal anomalies in our cohort. We confirm that ocular malformations—coloboma, lens abnormalities, and microphthalmia—occur in *SOX11* syndrome.[24-27] These are recapitulated in *sox11* null zebrafish, confirming the specificity of the finding.[28] In our cohort, 10% of patients had oculomotor apraxia. Diagnosis of oculomotor apraxia requires specialized neuro-ophthalmological evaluation, and therefore, the true prevalence of this feature is likely to be

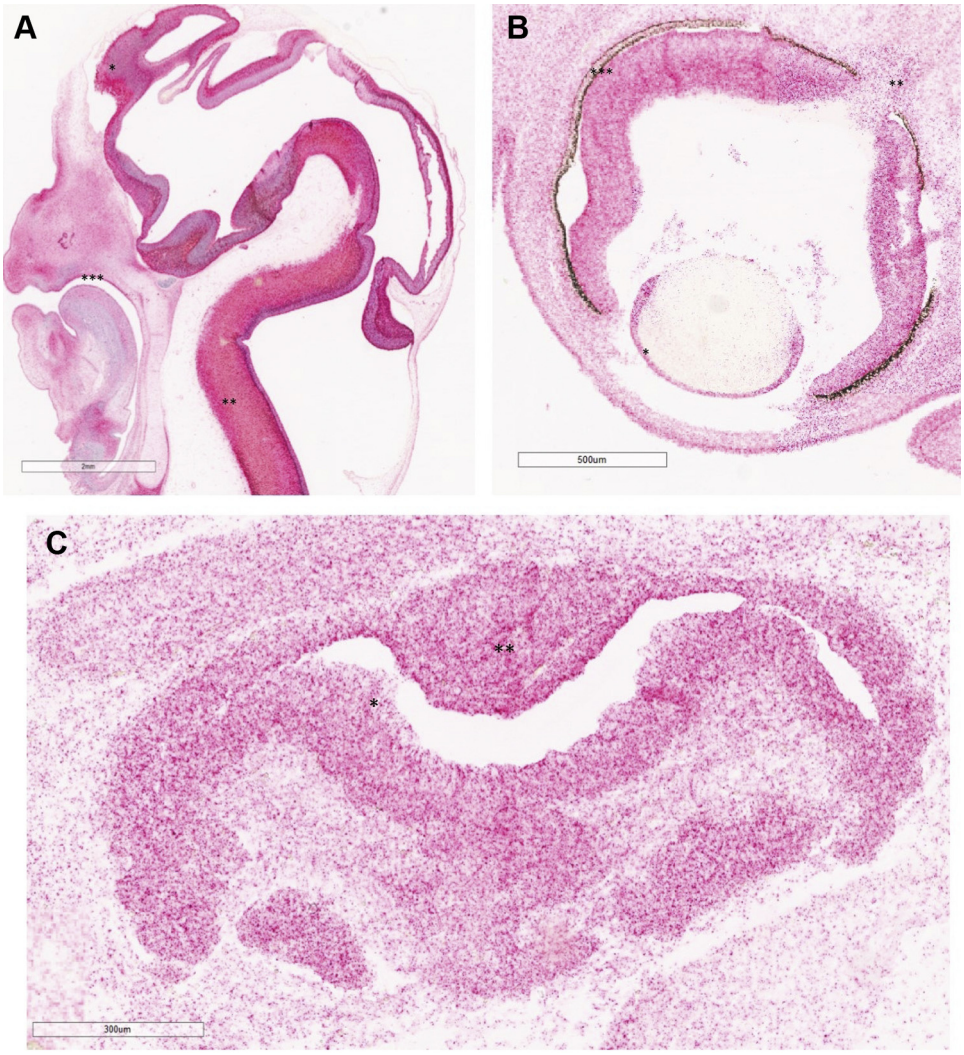

Figure 5 RNAscope images of *SOX11* expression using probes to *SOX11*. A. Saggital image of Carnegie stage (CS) 21 (CS21) (around 51 days after conception, 2× magnification). Note, diffused staining in central nervous system (red signal). *SOX11* expression in frontal cortex (*), spinal cord (**), and palate (***). *SOX11* probe is labeled red. No counterstain was used. B. *SOX11* expression in developing eye at CS23 (around 56 days after conception, 4×) in lens (*), optic nerve (**), and neuroretina (***). *SOX11* probe is labeled red. No counterstain was used. C. *SOX11* expression in pituitary at CS20 (around 49 days after conception, 10×) lining lumen of adenohypophysis (*) and also in neurohypophysis (**). *SOX11* probe is labeled red. No counterstain was used.

higher. Our study confirms that *SOX11* syndrome should be part of the differential diagnosis of oculomotor apraxia.

The extent of brain malformation in *SOX11* syndrome is revealed by our work. Of the patients who were imaged, 60% had an abnormality on brain MRI, and the true prevalence is likely to be higher given that children with ID are often not imaged. The most striking findings were those of cerebellar hypoplasia. In *Sox11* null mice, there is generalized reduction in size of the cerebrum and cerebellum.[29] In this current report, humans with *SOX11* variants have microcephaly and cerebellar hypoplasia. The combined human and murine findings indicate that *SOX11* has a general role in brain development, rather than a predominant role in the cerebrum or cerebellum. Beyond neurodevelopmental delay, there is little evidence that brain malformations in *SOX11* syndrome are associated with neurological disorders. Only 2 participants had epilepsy, and there was no clearly defined ataxia in association with the cerebellar findings on imaging.

In our cohort, 21% of the patients had hypogonadotrophic hypogonadism. A role for *SOX11* in hypogonadotrophic hypogonadism is plausible given that other *SOX* genes (*SOX2, SOX10*) cause hypogonadotrophic hypogonadism in humans.[9,10] In males with *SOX11* syndrome presentation, genital malformations at birth was reported, but in both sexes delayed puberty was the principal manifestation. Hypogonadotrophic hypogonadism with anosmia is termed Kallman syndrome[30] and is associated with failure of hypothalamic gonadotrophin releasing hormone (GnRH)–releasing neurons to migrate correctly into the hypothalamus. Given the neurodevelopmental delay in *SOX11* syndrome, formal assessment of olfaction is not possible. However, 1 participant was reported to have

anosmia with hypoplasia of the olfactory nerves on MRI, and the *Sox11* null mouse has small olfactory bulbs.[29] Based on current evidence, it is not possible to state whether *SOX11* syndrome is associated with Kallman syndrome or normosmic hypogonadotrophic hypogonadism.

How *SOX11* variants might result in hypogonadotrophic hypogonadism is unclear. GnRH neurons are a central part of the hypothalamic-pituitary-gonad axis.[30] GnRH neurons originate in the olfactory neuroepithelium and migrate to the hypothalamus. *SOX11* variants could disrupt this process. In support of this, *SOX11* has been shown to be expressed in GnRH hypothalamic neurons in mice and has been shown to stimulate expression and secretion of GnRH in vitro.[31] However, in an induced human pluripotent cell model of GnRH neurons, *SOX11* expression was not enriched.[32] A non–mutually exclusive hypothesis is that *SOX11* plays a role in pituitary gonadotropes. A single-cell RNA-sequencing study of murine pituitary showed significant enrichment of *SOX11* in gonadotropes.[33] Our ISH study showed *SOX11* expression in developing human pituitary and hypothalamus, compatible with a direct role in the development of both. *SOX11* may play multiple roles in development of the hypothalamic-pituitary-gonadal axis, which requires further study.

We describe the pattern of *SOX11* transcript expression in human development using ISH. As expected, *SOX11* is widely expressed in the developing central nervous system including the cerebrum, cerebellum, and brainstem. This confirms previous work using RNA-sequencing and microarray data from the BrainSpan atlas of the developing brain. *SOX11* was also observed in the developing palate. This suggests a direct role of *SOX11* in human palatogenesis and *SOX11* loss-of-function in the cleft palate observed in some patients of our cohort. Cleft palate has been described in *Sox11*-null mice.[34] This was associated with reduced proliferation of cells in the palatal shelves but also mandibular hypoplasia, with the suggestion that the cleft resulted from a Pierre-Robin sequence. *SOX11* was also expressed within the lumen of the developing adenohypophysis and the neurohypophysis. This supports a role for *SOX11* in the development of the pituitary gland, as discussed earlier. *SOX11* expression in the retina and optic nerve confirms the importance of *SOX11* for development of these structures in humans.

Unique genomic DNA methylation patterns, referred to as episignatures, are promising alternatives to diagnose NDDs and overgrowth/ID syndromes. Our group and others have shown the diagnostic utility of genome-wide DNA methylation analysis using peripheral blood samples.[14,15,17,35] In this study, we showed a highly sensitive and specific blood-derived episignature with small number of DMPs for *SOX11* syndrome, using a relatively small number of patient samples. Many of these DMPs have regulatory roles in neural differentiation and are associated with NDDs (ie, family with sequence similarity 160 member B1 [*FAM160B1*][36] and *FMN2*[37]). Some DMPs have regulatory role in the epigenetic machinery, such as *DPF1*[38] and *AHCTF1*.[39] This highlights the fact that aberrations in the expression/methylation status of *SOX11* affects expression/methylation status of genes involved in neural differentiation and/or epigenetic machinery, in agreement with the observed global hypomethylation seen in Supplemental Figure 5. It also shows the utility of DNA methylation profiling as a useful biomarker for clinical diagnosis of *SOX11*-related disorders.

DNA methylation is an epigenetic modification affecting molecular mechanisms, including chromatin assembly and gene transcription. Recent advances in sequencing and array technologies, capable of scrutinizing genome-wide DNA methylation patterns, gave unexpected novel insights into the identification of epigenetic biomarkers. This study focuses on DNA methylation as a clinical diagnostic biomarker, enabling interpretation of genetic variation in *SOX11*. Future studies focusing on integrating epigenomic and gene expression profiles in patients with *SOX11*-related disorders may provide insights into how epigenetic alterations lead to NDDs. The plastic nature of epigenomic profiles may offer an opportunity to study the use of chromatin and epigenomic targeting agents as a potential therapeutic avenue.

Individuals with *SOX11* syndrome were initially reported as having a CSS phenotype. Our phenotype driven clustering analysis shows that, based on HPO terms, *SOX11*n-syndrome is clinically distinct from *ARID1B*-related CSS. Particular differentiating features were presence of oculomotor apraxia, ocular malformations, and idiopathic hypogonadotrophic hypogonadism in *SOX11* syndrome. This is further supported by methylation analyses of peripheral blood DNA, which shows that *SOX11* syndrome and BAFopathies have distinct episignatures (Figure 3). Taken together, this confirms that *SOX11* syndrome should be considered as a distinct clinical entity from CSS.

Data Availability

For the episignature work, summarized and anonymized data for each subject are described in the study. The source DNA methylation data are available from the authors upon request. Software used in this study is publicly available, and the detailed analytical methodology is as previously reported (PMID: 34087165). The images from RNAscope (*SOX11* and *GnRHR* probes) experiment are available from Jasmin Turner or Alisdair McNeill. Exome sequencing data from Deciphering Developmental Disorders study are available through application to the Deciphering Developmental Disorders study management committee (https://www.deciphergenomics.org/). Genome sequencing data from the 100,000 Genomes Project is available in the Genomics England Research Environment https://re.extge.co.uk/ovd/

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Ethics Declaration

A relative or carer gave written consent for publication of clinical details and photographs of the participants in this study. Exome sequencing in the Deciphering Developmental Disorders study has United Kingdom Research Ethics Committee (REC) approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). Genome sequencing undertaken in the 100,000 Genomes Project has approval from the East of England—Cambridge South REC (REC Ref 14/EE/1112). The Human Developmental Brain Resources has approval from the North East—Newcastle and North Tyneside 1 REC (18/NE/0290). The study was approved by the Western University Research Ethics Board (REB 106302 and 116108).

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

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