Increased Transactivation Associated with *SOX3* Polyalanine Tract Deletion in a Patient with Hypopituitarism

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Backgound and Aims: Correct gene dosage of *SOX3* is critical for the development of the hypothalamo-pituitary axis. Both overdosage of *SOX3*, as a result of gene duplication, and loss of function resulting from expansion of the first polyalanine (PA) tract are associated with variable degrees of hypopituitarism, with or without mental retardation. The aim of this study was to further investigate the contribution of *SOX3* in the etiology of hypopituitarism and the mechanisms involved in the phenotypic variability.

Methods: We screened 154 patients with congenital hypopituitarism and an undescended posterior pituitary for mutations in *SOX3* and variability in the length of the first PA tract. In addition, 300 patients with variable septooptic dysplasia were screened for variability of the PA tract.

Results: We report a novel 18-base pair deletion (p.A243_A248del6, del6PA) in a female patient with hypopituitarism resulting in a 2-fold increase in transcriptional activation *in vitro*, compared with wild-type SOX3. We also identified a previously reported seven-alanine expansion (p.A240_A241ins7, +7PA) in two male siblings with isolated GH deficiency and a distinct phenotype, in addition to the nonsynonymous variant p.R5Q in an unrelated individual; this appears to have no functional effect on the protein. In contrast to +7PA, del6PA maintained its ability to repress β-catenin mediated transcription *in vitro*.

Conclusion: This is the first study to report that PA tract deletions associated with hypopituitarism have functional consequences *in vitro*, possibly due to increased activation of SOX3 target genes. In addition, we have expanded the phenotypic spectrum associated with PA tract expansion (+7PA) mutations to include panhypopituitarism or isolated GH deficiency, with or without mental retardation. **(*J Clin Endocrinol Metab* 96: E685–E690, 2011)**

SOX3 (OMIM 313430) is a single exon gene on chromosome Xq26-27; it is a member of the SOX (SRY-related high mobility group box) family of transcription factors, expressed in neuroepithelial progenitor and stem cells from the earliest stages of development. SOX3 contains a high mobility group DNA-binding domain conserved among SOX proteins, a short N-terminal domain, and a longer C-terminal domain containing four polyala-

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Abbreviations: CGH, Comparative genomic hybridization; EPP, ectopic posterior pituitary; IGHD, isolated GH deficiency; PA, polyalanine; rhGH, recombinant human GH; SOX, SRY-related high mobility group box.

nine (PA) tracts shown to be involved in transcriptional activation (1).

SOX3 dosage is critical for normal hypothalamopituitary development, and both duplications and loss-of-function PA expansions have been reported in pedigrees with variable phenotypes. Duplications at Xq26.1-27.3 have been reported in association with mental retardation and either isolated GH deficiency (IGHD) or panhypopituitarism (2, 3). We previously described a submicroscopic duplication encompassing *SOX3* (685.6 kb) in two siblings with variable hypopituitarism, no intellectual deficit, anterior pituitary hypoplasia, and an undescended or ectopic posterior pituitary (4). Expansion of the first PA tract by 11 alanine residues (p.A234_A235ins11, referred to as +11PA) had initially been reported in association with IGHD, mental retardation, and craniofacial abnormalities (5). We reported an expansion of the same tract by seven alanine residues (p.A240_A241ins7, +7PA) in three siblings with panhypopituitarism, anterior pituitary hypoplasia, and an undescended posterior pituitary, but no evidence of mental retardation or facial dysmorphism. The +7PA resulted in the loss of transcriptional activation, possibly due to reduced nuclear transport of the mutant protein (4). Recently, the +7PA has been described in a pedigree with IGHD and normal intelligence (6). A single in-frame deletion (p.A240_A248del9, del9PA) has been described in two brothers with mental retardation but without a clearly defined pituitary phenotype; the significance of this finding remains unknown because functional studies were not performed and the deletion was also observed in the unaffected maternal grandfather (5).

Correct dosage of *SOX3* is critical for normal hypothalamopituitary development. *Sox3* null mutant animals have a variable phenotype, showing poor growth, craniofacial defects, and variable endocrine deficits. They have a small anterior pituitary with additional clefts and dysgenesis of the corpus callosum (7).

In humans, both overdosage (by gene duplication) and underdosage (by PA tract expansion mutations) give rise to a similar phenotype of hypopituitarism and infundibular hypoplasia. To date, no missense or nonsense mutations have been reported (8).

The aim of this study was to further investigate the contribution of *SOX3* in the etiology of hypopituitarism. Because all patients described to date had an ectopic/undescended posterior pituitary (4), we screened a cohort of patients (n = 154) with hypopituitarism and an ectopic posterior pituitary (EPP) for mutations in *SOX3*. *SOX3* is also implicated in the etiology of septooptic dysplasia variant (9); we therefore screened 300 patients with variable septooptic dysplasia for mutations affecting only the PA tract because these seem to be more common and associated with a variable phenotype.

We report a novel deletion of six residues within the first PA tract (p.A243_A248del6, del6PA) and show that both the del6PA and the previously described del9PA (5) mutations affect the function of the SOX3 protein *in vitro*. This is the first report suggesting that PA deletions may be associated with hypopituitarism, possibly via a mechanism involving increased transcriptional activation of SOX3 target genes. We also extend the phenotypic spectrum associated with SOX3 PA tract expansion mutations to include panhypopituitarism or IGHD, with or without learning difficulties.

Patients and Methods

Patients and clinical evaluation

Patients were recruited from national and international centers; ethical committee approval was obtained from the University College London Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics committee. Hormonal assays and normal values for each center were taken into account. For patients recruited within our center, hormone evaluation and magnetic resonance imaging were performed as previously described (4).

Mutation analysis and detection of PA tract size

The single coding exon of *SOX3*, including 145 bp upstream and 121 bp downstream of the gene, was amplified from genomic DNA in five overlapping fragments and sequenced as previously described (4). To detect changes in the PA tract size, the corresponding fragment was amplified using a 5′-FAM-labeled forward primer, and patients were genotyped on the MegaBACE DNA analysis system (GE Healthcare, Amersham, UK) (see Fig. 2A).

Plasmid constructs

The *SOX3* coding region was amplified from patients' and control DNA and cloned into pcDNA3.1(+) (Invitrogen, Paisley, UK); the previously reported del9PA (5) was introduced by site-directed mutagenesis. A clone containing the full-length human β-catenin coding sequence (CTNNB1; IMAGE Consortium Clone ID 6151332) was obtained from Geneservice Ltd. (Cambridge, UK).

Dual luciferase assay experiments

SOX3 luciferase reporter assays were performed using Chinese hamster ovary (CHO) cells, cotransfected with 20 ng of a firefly luciferase reporter construct containing a 570-bp fragment of the *Hesx1* promoter encompassing SOX binding sites and varying amounts of *SOX3* expression vector. For the TOPFLASH assay (10), human embryonic kidney 293 cells were cotransfected with 20 ng TOPFLASH reporter, 30 ng β-catenin expression construct, and increasing amounts of wild-type and mutant *SOX3* expression constructs. Luciferase activity was measured using a BMG FLUOstar Optima multiplate reader (BMG LABTECH Gmbh, Offenburg, Germany), and experi-

TABLE 1. Summary of phenotypes in patients with *SOX3* mutations and the p.R5Q variant

Patient no.	1	2.1	2.2	3
Age at presentation (yr)	7.5	3.0	1.5	1.8
Height (cm)	113	85	73.7	63.5
Height SDS	−3.1	−3.0	−2.7	−7.0
Weight (kg)	NA	12.8		5.9
Weight SDS	NA	−1.2	−0.74	−4.2
Peak GH (μg/liter)	3.4[a]/4.9[b]	<0.3[a]	1.6[b]	<0.1[a]
Basal cortisol (μg/dl)	11.5	33.2	11.7	7.5
Peak cortisol (μg/dl)	17.1[a]	22.6[a]	20.3[a]	10.8[a]
TSH (mU/liter)	1.1	2.6	2.5	1.0
Free T_4 (ng/dl)			0.9 (0.7–1.8)	0.6 (0.8–2.0)
Total T_4 (μg/dl)	3.0 (5.3–11)	9.9 (4.5–5.6)		4.0 (4.0–12.0)
Prolactin (ng/ml)	8.9 (3.2–20) [basal]	27.7 [peak]	23.2 [peak]	0.9 [peak]
Age at GnRH test (yr)	17	3.0	1.5	1.8
LH basal (IU/liter)	1.4	<0.1	0.35	<0.1
FSH basal (IU/liter)	7.4	<0.1	0.4	<0.1
LH peak (IU/liter)	13.1	0.7	2.1	<0.1
FSH peak (IU/liter)	13.0	0.8	2.4	<0.1
Magnetic resonance imaging	Enlarged AP, NPP	NA	APH, EPP	APH, NPP, stalk intact
Age at start of rhGH treatment (yr)	12	4	2	1.6
Mean rhGH dose (mg/kg · d)	0.03	0.03	0.03	0.025
Puberty	Induced at age 17.2 yr	Normal	Normal	Followed up
Intelligence	Normal	Normal	Mild learning difficulties	Normal
Other	Turner-like habitus, 46,XX karyotype	No dysmorphism	No dysmorphism	Craniofacial disproportion
SOX3 mutation	del6PA	+7PA	+7PA	R5Q

AP, Anterior pituitary; APH, anterior pituitary hypoplasia; NA, not available; NPP, normal posterior pituitary; SDS, SD score. Normal ranges are indicated in *parentheses*.

The type of GH provocation test is denoted as follows: [a] insulin-induced hypoglycemia; [b] arginine and insulin stimulation.

ments were each repeated three times independently and performed in triplicate.

EMSA

SOX3 proteins were generated using the TNT Quick Coupled Transcription/Translation system (Promega, Southampton, UK), and EMSAs were performed as described previously (4).

X-inactivation studies and comparative genomic hybridization (CGH)

The X-chromosome inactivation pattern was determined using the androgen receptor gene methylation assay (11). CGH was performed using the Nimblegene 135K Whole Genome Tiling array and the InfoQuant CGHFusion software (Infoquant, London, UK).

Results

Mutation analysis

A total of 454 patients were screened for genetic variation within the first PA tract of *SOX3*, including 154 patients with hypopituitarism and an EPP screened for mutations in the entire *SOX3* coding sequence. All were negative for *HESX1* mutations. We identified a novel intragenic in-frame deletion of 18 base pairs, resulting in the loss of six alanine residues between codons 243 and 248 (p.A243_A248del6 or del6PA) in a female patient with GH, TSH, and gonadotropin deficiency (patient 1) and her unaffected father. She presented at the age of 7.5 yr with short stature, a Turner syndrome-like habitus, normal neurodevelopment, and a 46,XX karyotype. Results of investigations confirming GH insufficiency are shown in Table 1 and magnetic resonance imaging in Fig. 1. She was commenced on T_4, and the family decided to start recom-

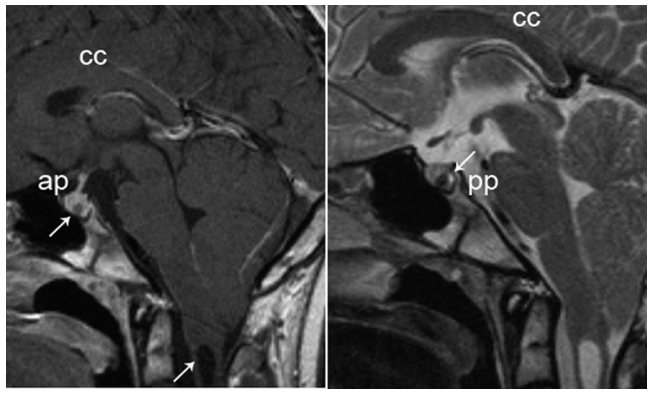

FIG. 1. Pituitary magnetic resonance imaging of patient with the del6PA deletion in *SOX3*, showing a small corpus callosum (cc) and enlarged anterior pituitary (ap) with a lesion (*arrow*) consistent with a cyst, microadenoma, or hematoma. The stalk and bright spot of the posterior pituitary (pp) have a normal appearance; there is descent of the cerebellar tonsils with syringomyelia (Arnold Chiari malformation type 2) (*arrow*).

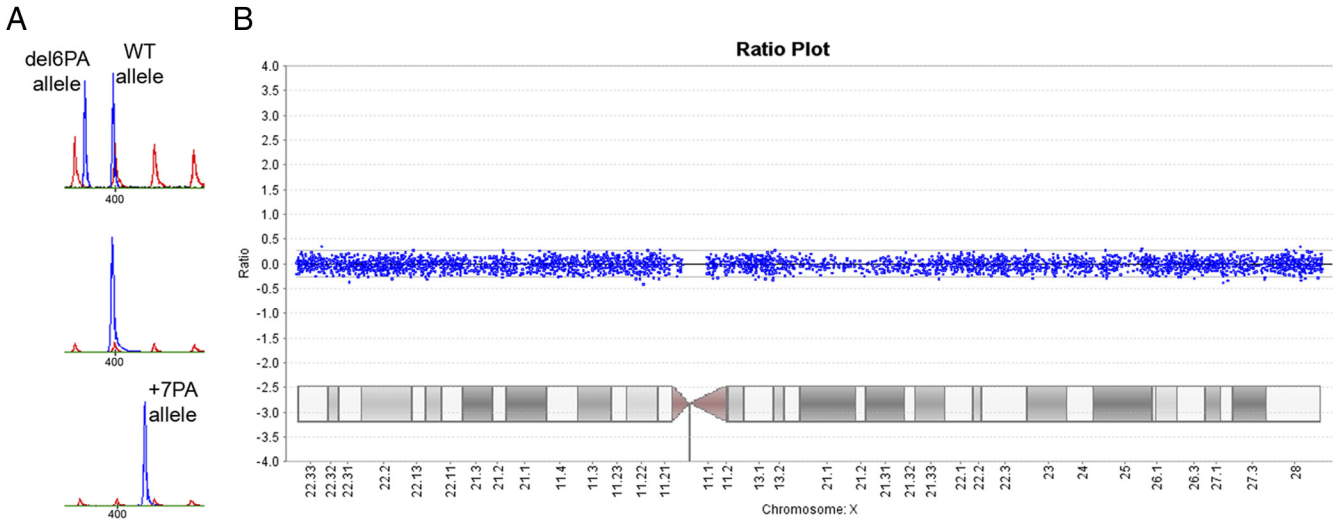

FIG. 2. A, Electropherogram showing results of genotyping for the length of the first PA tract in *SOX3* (*blue*), compared with ET-550R size standard peaks. The female patient 1 was heterozygous for the del6PA mutation in *SOX3* (*top panel*), compared with normal control (*middle panel*). Expansion of the PA tract in one of the male siblings (patient 2.2) is shown in the *bottom panel*. B, Whole genome array comparative genomic hybridization and detailed analysis of the X-chromosomes for patient 1 showed no significant copy number changes.

binant human GH (rhGH) treatment at the age of 12 yr. She failed to enter puberty, and puberty was therefore induced at 17.2 yr. There was no evidence of skewed X-inactivation in genomic DNA extracted from peripheral blood, and there were no significant copy number changes on CGH array (Fig. 2B).

The previously reported +7PA (p.A240_A241ins7) was identified in two male siblings with IGHD (patients 2.1 and 2.2); their phenotypically normal mother was a carrier. The older sibling presented at the age of 3 yr with short stature and micropenis; he had normal intelligence, IGHD, and gonadotropin response to GnRH test consistent with his age. His younger brother presented at the age of 18 months with IGHD and mild learning difficulties. Both siblings progressed normally through puberty.

A novel sequence variant (c.14G>A, p.R5Q), was found in a boy from Brazil who presented at 1.8 yr with hypopituitarism and a single central maxillary incisor (patient 3); his normal-height mother was heterozygous for the mutation; his father was negative. This variant was not identified in 108 ethnically matched controls.

Description of phenotypes and growth charts are provided in Supplemental Data (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Molecular analysis of *SOX3* mutations

Luciferase reporter assays using a reporter construct containing a fragment of *Hesx1* promoter to which SOX3 can bind and activate transcription *in vitro* (4) showed that both the del6PA and del9PA SOX3 mutant proteins resulted in an approximately 2-fold increase in transcriptional activation compared with wild-type SOX3 ($P <$ 0.001) (Fig. 3A). There was no difference in transcriptional activation between p.R5Q and WT-SOX3 in this assay (Supplemental Fig. 1). In DNA binding assays, both the del6PA and del9PA mutations, as well as the p.R5Q variant, were capable of binding to a SOX consensus DNA probe (Fig. 3B). Previous studies have revealed that some SOX proteins, including *Xenopus* Sox3, are capable of associating with β-catenin and repressing the activity of a β-catenin responsive promoter containing TCF/LEF binding sites (10). We cotransfected the TOPFLASH reporter with an expression construct containing human β-catenin, resulting in increased activation of the reporter. Cotransfection with WT-SOX3 led to a dose-dependent reduction in β-catenin-induced activation. The del6PA, del9PA mutations, and the p.R5Q SOX3 variant were capable of repressing β-catenin-mediated transcription of the reporter to the same extent as the wild-type SOX3 (Fig. 3C and Supplemental Data) (10); none of the SOX3 proteins can bind the TCF sites (data not shown). Repeated experiments with the +7PA failed to repress β-catenin-mediated transcription (Fig. 3C and Ref. 12).

Discussion

PA repeats are present in a number of transcription factors and play a role in protein-protein interaction, DNA binding, and transcriptional activation. PA expansions in an increasing number of genes are associated with autosomal or X-linked disorders (13). We report the association of +7PA in two siblings with IGHD, in contrast to panhypopituitarism, with and without learning difficulties, fur-

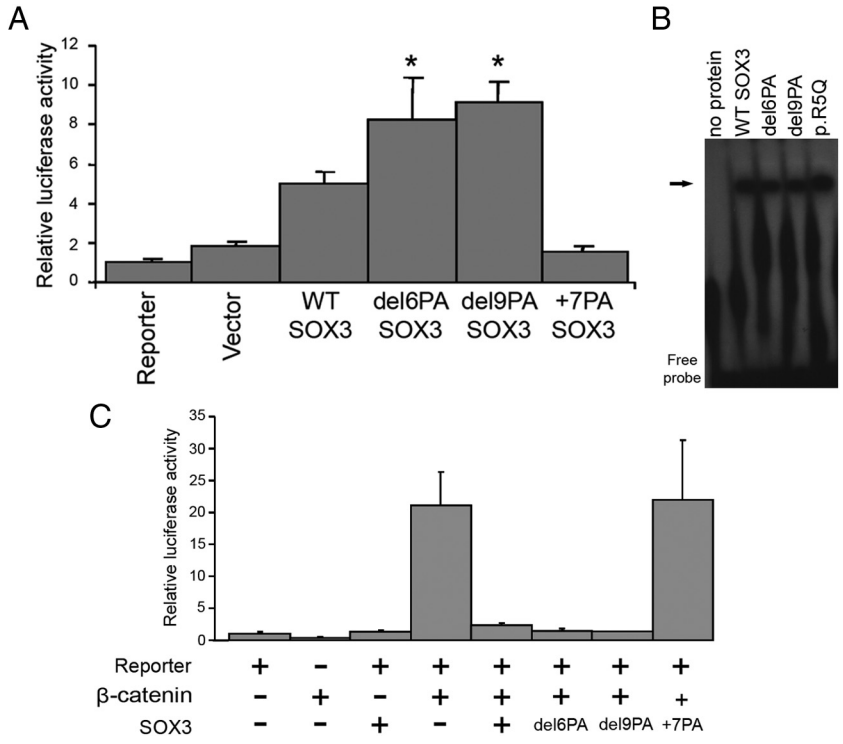

FIG. 3. A, Luciferase reporter assay, using a reporter construct containing a fragment of the *Hesx1* promoter to which SOX3 can bind and activate transcription *in vitro,* shows that both the del6PA SOX3 and del9PA SOX3 mutant proteins result in an approximately 2-fold increase in transcriptional activation compared with wild-type (WT). *Asterisks* denote significant difference from WT ($P \leq 0.001$). The +7PA mutant protein results in significantly reduced transactivation, as we previously reported (4). Data are presented as mean ± SD. B, EMSA using *in vitro* translated SOX3 proteins demonstrates that both the del6PA and del9PA SOX3 proteins, as well as the p.R5Q variant, were capable of binding to a SOX consensus DNA probe (*arrow*). C, Human embryonic kidney 293 cells were cotransfected with a TOPFLASH reporter construct that contains TCF/LEF binding sites, and SOX3 proteins individually with β-catenin. Wild-type SOX3 is capable of repressing β-catenin-mediated activation of the reporter. Both deletion mutations (del9PA, del6PA) retain the ability to repress the activity of β-catenin, whereas the PA expansion (+7PA) results in loss of repression of β-catenin-mediated reporter gene activation, showing levels of activation comparable to cotransfection with β-catenin alone. *Columns* represent the mean relative luciferase activity as determined in three experiments performed in triplicate ± SD (mean ± SD).

ther supporting the phenotypic variability associated with *SOX3* mutations.

In contrast to PA expansions, deletions have rarely been described (13). We report a del6PA mutation in a female patient with GH, TSH, and gonadotropin deficiency. Intriguingly, we have identified a mutation that appears to result in gain of function in a female patient whose unaffected father was a carrier. In previously described pedigrees with *SOX3* duplications (3, 4) or large deletions (14), the female carriers were unaffected and showed preferential inactivation of the abnormal X-chromosome. Although in our proband we did not find evidence of skewed X-inactivation in peripheral lymphocytes (data not shown), the possibility of tissue- or developmental stage-specific skewing in the hypothalamopituitary region cannot be excluded (15). She had a distinct pituitary appearance with an enlarged anterior pituitary and a normally placed posterior pituitary. Pituitary enlargement has previously been reported in association with mutations in *PROP1* (16) and *LHX3* (17), although screening for mutations in these genes in our patient was negative. Large copy number changes on the X-chromosome have been excluded by whole genome CGH array analysis. This supports the contribution of the del6PA mutation to the phenotype of this patient, although a polygenic effect cannot be excluded.

Murine data suggest the presence of structural defects in the hypothalamopituitary region and abnormal pouch morphology in three of four female adult XX; $Sox3^{+/\Delta gfp}$ mice without growth failure, reflecting incomplete penetrance (7). Thus, the finding of a hypothalamopituitary phenotype in human females with heterozygous *SOX3* mutations would not be entirely unexpected.

The del6PA and del9PA mutations resulted in increased transcriptional activation of a luciferase reporter construct *in vitro*, suggesting that PA deletions may result in increased target gene activation, which would have a similar effect to duplicated dosage of the protein (4). The mechanism underlying the phenotypic variability remains unknown. We could speculate that it may result from changes in dosage of interacting proteins such as β-catenin, or variable effects of *SOX3* on as yet unknown downstream targets. The Wnt-β-catenin signaling pathway is important for hypothalamopituitary development (9) (18). *Xenopus* XSox3 (19) as well as murine and human SOX2 (20) are capable of repressing the activity of a β-catenin responsive reporter (19). In this study, we show that human SOX3, as well as the del6PA and del9PA mutant proteins, repress β-catenin mediated activation *in vitro*; the +7PA was unable to repress β-catenin, consistent with a recent report (12). The failure to repress β-catenin may be another effect of the +7PA mutant *in vivo*, in addition to the impaired nuclear translocation (4, 12). Although reporter assays are an artificial *in vitro* system, we have demonstrated different effects between SOX3 PA tract expansion and deletion mutations in assays that affect direct transcriptional activation of a target gene compared with that mediated by interaction with a putative partner protein. Given the redundancy in the function of the SOXB1

proteins, we cannot exclude the possibility that specific effects of mutations of the PA tract may be compensated by other factors.

Variability in the size of the PA tract in *SOX3* is uncommon, at least in this cohort, and missense mutations are even rarer. We suggest that PA mutations, in combination with different interacting proteins, may have diverse effects on downstream targets. Further work is required for their identification, which will lead to better understanding of the molecular mechanisms by which PA tract mutations affect normal development.

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