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

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Background 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, /H11001 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 /H11001 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 polyalanine...
nine (PA) tracts shown to be involved in transcriptional activation (1).

SOX3 dosage is critical for normal hypothalampituitary 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, referred to as +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 hypothalampituitary 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 overdoseage (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-
EMSAs

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 T4, and the family decided to start recom-

<table>
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<th>Patient no.</th>
<th>1</th>
<th>2.1</th>
<th>2.2</th>
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<td>Height (cm)</td>
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<td>Weight (kg)</td>
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<td>1.6^a</td>
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<td>Basal cortisol (µg/dl)</td>
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<td>11.7</td>
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<td>Peak cortisol (µg/dl)</td>
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<td>Free T4 (ng/dl)</td>
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<td>Total T4 (µg/dl)</td>
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<td>Prolactin (ng/ml)</td>
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<td>FSH basal (IU/liter)</td>
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<td>0.03</td>
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<td>SOX3 mutation</td>
<td>del6PA +7PA +7PA R5Q</td>
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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.

AP, ANTERIOR PITUITARY; APH, ANTERIOR PITUITARY HYPOPLASIA; NA, NOT AVAILABLE; NPP, NORMAL POSTERIOR PITUITARY; SDS, SD SCORE. NORMAL RANGES ARE INDICATED IN PARENTHESES.

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).
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 /H11001 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 /H11022 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-
reduced transactivation, as we previously reported (4). Data are presented as mean ± 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+/−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 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).

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 < 0.001). The +7PA mutant protein results in significantly reduced transcription, 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, consistent with β-catenin-mediated activation of a target gene compared with β-catenin alone. Columns represent the mean relative luciferase activity as determined in three experiments performed in triplicate ± SD (mean ± SD).
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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