

Molecular analysis of the *PROP1* and *HESX1* genes in patients with septo-optic dysplasia and/or pituitary hormone deficiency

Análise molecular dos genes *PROP1* e *HESX1* em pacientes com displasia septo-ótica e/ou deficiência hormonal hipofisária

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ABSTRACT

Objective: The present study aimed at evaluating the *PROP1* and *HESX1* genes in a group of patients with septo-optic dysplasia (SOD) and pituitary hormone deficiency (combined – CPHD; isolated GH deficiency – GHD). Eleven patients with a clinical and biochemical presentation consistent with CPHD, GHD or SOD were evaluated. **Subjects and methods:** In all patients, the *HESX1* gene was analyzed by direct sequence analysis and in cases of CPHD the *PROP1* gene was also sequenced. **Results:** A polymorphism (1772 A > G; N125S) was identified in a patient with SOD. We found three patients carrying the allelic variants 27T > C; A9A and 59 A > G; N20S in exon 1 of the *PROP1* gene. Mutations in the *PROP1* and *HESX1* genes were not identified in these patients with sporadic GHD, CPHD and SOD. **Conclusion:** Genetic alterations in one or several other genes, or non-genetic mechanisms, must be implicated in the pathogenic process. Arq Bras Endocrinol Metab. 2010;54(5):482-7

Keywords

DNA mutational analysis; septo-optic dysplasia; pituitary hormonal deficiency

RESUMO

Objetivo: O presente estudo teve como objetivo avaliar os genes *PROP1* e *HESX1* em um grupo de pacientes com displasia septo-ótica (DSO) e deficiência hormonal hipofisária (combinada – DHHC; ou deficiência isolada de GH – DGH). Onze pacientes com apresentação clínica e bioquímica consistente com DHHC, DGH ou DSO foram avaliados. **Subjects and methods:** Em todos os pacientes, o gene *HESX1* foi analisado pelo sequenciamento direto e, nos casos de DHHC, o gene *PROP1* foi também sequenciado. **Resultados:** Um polimorfismo no gene *HESX1* (1772 A > G; N125S) foi identificado em um paciente com DSO. Foram encontrados três pacientes portadores da variação alélica 27T > C; A9A e 59 A > G; N20S no éxon 1 do gene *PROP1*. Mutações no gene *PROP1* e *HESX1* não foram identificadas nesses pacientes com DGH, DHHC e DSO esporádicos. **Conclusão:** Alterações genéticas em um ou diversos outros genes ou mecanismos não genéticos devem estar implicados nesse processo patogênico. Arq Bras Endocrinol Metab. 2010;54(5):482-7

Descritores

Análise mutacional do DNA; displasia septo-ótica; deficiência hormonal hipofisária

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INTRODUCTION

The pituitary gland is composed of the adenohypophysis, which is comprised of an anterior and intermediate lobe, and the neurohypophysis, which consists of projections of hypothalamic magnocellular neurons (1). During development, the oral ectoderm

invaginates to form the primordial rudimentary pituitary, also known as Rathke's pouch, which proliferates toward the third ventricle, where it merges with the posterior lobe (2). The posterior lobe is neural in origin, deriving from an evagination of the ventral hypothalamus and of the third ventricle (3).

At birth, the anterior pituitary is composed of five cell types that are defined by the hormones that they secrete: corticotrophs (ACTH), thyrotrophs (TSH), somatotrophs (GH), gonadotrophs (FSH and LH) and lactotrophs (PRL). A series of transcription factors such as Pit1 (*pituitary specific transcription factor 1*), PROP1 (*prophet of Pit1*), HESX1 (*Homeobox embryonic stem cell*), LHX3 (*Lim homeobox gene 3*), LHX4 (*Lim homeobox gene 4*), Pitx1/2 (*Paired-like homeodomain transcription factor 1/2*), and TPIT (*T Box transcription factor*), act in a temporally regulated manner to permit the progressive differentiation of these cell types (4).

Mutations in the genes encoding these factors are responsible for structural defects of the pituitary gland and hormonal deficiencies. Depending on the mutated transcription factor, these defects can be isolated, such as for example growth hormone deficiency (GHD), or they result in various forms of combined pituitary hormone deficiencies (CPHD) (5-7). The present study aimed at evaluating the *PROPI* and *HESX1* genes in a group of patients with septo-optic dysplasia (SOD), CPHD and isolated GHD.

SUBJECTS AND METHODS

Informed consent, patient assent, and approval of the hospital's Ethics Committee were obtained before initiating the study.

Patients

Eleven patients with a clinical and biochemical presentation consistent with CPHD, GHD or SOD were evaluated.

Clinical evaluation

The clinical data of patients were extracted from medical charts and included phenotypic characteristics, history of consanguinity, family history, auxological parameters and hormonal evaluation.

The criteria for inclusion were:

Growth hormone deficiency: Patients with a height less than two standard deviations (SD) below the mean, and two independent stimulation tests confirming growth hormone deficiency. The diagnostic criterion of GH deficiency was a peak GH level after stimulation < 7.0 ng/mL in the radioimmunoassay. In children with subnormal values of IGF-1 and IGFBP-3 associated with low growth velocity and deficiency of other pi-

pituitary hormones, the stimulation tests were not performed. Deficiency of two or more pituitary hormones was established by formal biochemical evaluation of all endocrine axes.

Septo-optic dysplasia was diagnosed based on two or more of the following findings: 1) hypoplasia of the optic nerve, 2) midline abnormalities (including agenesis of the corpus callosum and absence of the septum pellucidum, 3) pituitary hypoplasia with hormonal deficiencies.

We studied three patients with SOD, three patients with GHD and five patients with CPHD.

Hormone measurements

To assess the function of the anterior pituitary the following hormones and binding proteins were determined: TSH, free T4, PRL, LH, FSH, GH, IGF-1, IGFBP3, Cortisol and Testosterone.

Measurement of TSH, free T4, PRL, LH, FSH, Cortisol – Chemiluminescence assay using Immulite 2000 *Kits* (DPC, Los Angeles, CA).

Measurement of IGF-I: Immunoradiometric assay after ethanol extraction utilizing a specific monoclonal antibody kit (Diagnostic Systems Laboratories Inc. (DSL), Webster, TX, USA).

Measurement of IGFBP3 – Immunoradiometric assay kit (DSL, Webster, TX, USA).

Measurement of GH and free testosterone - Radioimmunoassay (RIA). A value of less than 7 ng/mL was considered as insufficient in the GH stimulation test.

Clonidine test: (oral administration of 100 µg/m² of body surface) with blood drawn for GH dosing at baseline, and at 30, 60, 90 and 120 minutes after administration of clonidine.

Insulin tolerance test: hypoglycemia was induced by i.v. administration of 0.1 U/kg of insulin. 200 µg of TRH and 100 µg of GnRH were administered at baseline for testing of the gonadal and thyroid axes. Peripheral blood was drawn for determining glucose, GH, cortisol, TSH, PRL, LH and FSH at the following points in time -15, 0, 15, 30, 45, 60, 90 and 120 minutes after administration of insulin.

Radiological evaluation

The brain and specifically the hypothalamic-pituitary region were imaged by magnetic resonance to obtain coronary and sagittal cuts at T1 with TR 350 and TE = 20 using a Tesla 0.5 magnet (Sigma GE, Milwaukee, Wisconsin, USA).

Molecular analysis

After obtaining informed consent from all the patients and their relatives, blood was collected for genetic testing. Analysis of the *HESX1* gene was performed using genomic DNA isolated from peripheral blood leukocytes. Exons 1, 2, 3 and 4 were amplified by PCR with the following primers:

- 1F: 5'AGCTGTTGCTCTGTGCAGACCACGAGA3';
- 1R: 5'ACAAAGAATTGAAACAATTAAGCTGTGGCA3';
- 2F: 5'TTGACCATCTAAGACAGGGC3';
- 2R: 5'AAGTGAGTGGGCTTTTGCTC 3';
- 3F: 5'GAGCAAAGCCCCTCACTT3';
- 4R: 5'CCACTGATTCTTCATGCTCT3'.

Amplification was performed with 100 ng of genomic DNA in a 25 µL reaction containing 20 pM of each primer, 10 X buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.0 mM MgCl₂, 0.2 mM of each dNTP, and 1.25 U Taq DNA Polymerase (Gibco-BRL). The following PCR conditions were used: initial denaturation for 5 minutes at 94°C, 30 cycles with 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a terminal extension at 72°C for 10 minutes.

Analysis of the *PROPI* gene was performed using genomic DNA isolated from peripheral blood leukocytes. Exons 1, 2 and 3 were amplified by PCR with the following primers:

- 1F: 5'ACCTACACACACATTCAGAGACAG3';
- 1R: 5'TGGAGCCTATGCTTTCAGC3';
- 2R: 5'AAAGACTGGAGCAGCACAGGACGCA3';

- 2R: 5' CTCAATGCAGTTGCTCCGATG3';
- 3F: 5'GCCTTGTGGAAGAGCTTTACTCC3';
- 3R: 5' ATTTCTAATCGGTGAGCTGACCC3'.

Amplification was performed with 100 ng of genomic DNA in a 25 µL reaction containing 20 pM of each primer, 10 X buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.0 mM MgCl₂, 0.2 mM of each dNTP, and 1.25 U Taq DNA Polymerase (Invitrogen). The following PCR conditions were used: initial denaturation for 3 minutes at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, 2 minutes at 72°C and a terminal extension at 72°C for 5 minutes.

PCR products were analyzed on 1% agarose gels, stained with ethidium bromide and visualized with UV light. After PCR amplification, both strands were sequenced using a Genetic Analyzer 3100 Sequencer (Applied Biosystem, Foster City, CA, USA). Sequencing was performed in the Molecular Biology Laboratory, Department of Clinical Medicine, Botucatu School of Medicine – Unesp.

RESULTS

The 11 patients were divided into the following groups according to the clinical presentation (Table 1):

1. Septo-optic dysplasia (patients 1, 2, 3).
2. Isolated GH deficiency (patients 6, 7, 8).
3. Combined pituitary hormone deficiency (patients 4, 5, 9, 10, 11).

Table 1. Clinical data, Laboratory Measurements and Radiological Evaluation of Patients

Patient	Gender	Age	Z score (height)	ft ₄ (ng/dL) (0.8-1.9)	TSH uU/mL (0.4-4)	FSH/LH mUI/mL	Cortisol ug/dL	ftesto pg/mL	IGF1/IGFBP3 ng/mL	GH - ITT ng/mL	MRI
1	M	3 y 3 m	-0.4	0.77	6.05	0.52/0.17	3.52	15.07	27/1610	#	APH/EN/ONH
2	F	1 y 3m	-0.85	0.55	6.47	#	5.8	#	8.8/1086	#	APH/EN/ONH
3	M	3y 5m	-5.8	1.00	2.69	<0.1/0.1	7.74	0.01	#	#	APH/CCM/Coloboma
4	M	3y 5m	-3.48	0.7	1.9	<1.0/0.6	3.9	0.7	#	0.5	APH
5	F	13y 10m	-5.7	0.535	6.5	0.32/0.1	1.89	#	#	0.54	Normal
6	M	14y 3m	-2.6	1.3	2.6	4.13	10.98	0.34	#	6.5	Normal
7	M	18y5m	-6.4	1.13	2.88	6.7	18.6	9.74	#	0.33	Normal
8	M	14y3m	-3.0	1.65	3.74	18.1	14.5	0.96	#	0.73	Normal
9	F	16y9m	-6.0	0.71	3.02	#	2.57	#	#	#	APH
10	F	9y3m	-4.49	0.63	0.005	22	<1	#	#	<0.25	APH/EN
11	M	14y	-2.9	0.71	1.33	8.77	15.5	0.84	#	5.6	APH

#: unavailable data; M: male; F: female; APH: anterior pituitary hypoplasia; EN: ectopic neuro-hypophysis; ONH: optic nerve hypoplasia; CCM: corpus callosum malformations.

Reference values: LH: M: 0.8-7.6 mUI/mL; F: 1.1-11 mUI/mL, FSH: M: 0.7-11.1 mUI/mL; F: 2.8-11.3 mUI/mL, Cortisol: 5-25 ug/dL, Free testosterone (ftesto): 8.8-27 pg/mL, IGF1: 20-200, IGFBP3: 1400-4250 ng/mL, GH peak after ITT (insulin tolerance test) < 7 ng.

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Sequence analysis

All the exons of the *HESX1* gene were amplified at the expected sizes. In the SOD patient group, sequence analysis revealed an allelic variant in patient 2, consisting of a 1772 A > G transition (Figure 1) that results in replacement of an asparagine with a serine at position 125 (N125S). In the GHD patient group no sequence alterations could be identified. In the CPHD patient group, no mutations were found in the *HESX1* gene. In patients 4 and 5, a monoallelic variant (27 T > C; A9A) was found in exon 1 of the *PROP1* gene (Figure 2) and patient 10 was homozygous for this variant. (Figure 3). A further biallelic variant (59 A > G; A20S) was found patients 4, 5 and 10 (Figure 4).

DISCUSSION

Monoallelic and biallelic mutations in the *HESX1* gene are associated with a large spectrum of clinical phenotypes. At present, eleven mutations of the gene have been described. The patients presented with midline defects, optic nerve hypoplasia, neuro-pituitary ectopia

and pituitary hypoplasia associated with hormonal deficiencies (8). Individuals homozygous for the recessive mutation R160C presented with septo-optic dysplasia (SOD/Morsier's syndrome) with agenesis of the corpus callosum and CPHD (9). Nevertheless, individuals with monoallelic mutation presented with milder phenotypes suggesting that they result from haploinsufficiency of the *HESX1* protein (10).

The reported incidence of SOD is 1/10,000 live births, and it is equally prevalent among males and females. Despite the generally sporadic nature of the condition, familial cases have been described. To date, Dattani and cols., have tracked more than 800 patients with SOD and pituitary hormone deficiency and identified mutations in less than 1% of individuals, confirming the rarity of mutations in *HESX1* (11).

The frequency of mutations in the *HESX1* gene is low, mutations in other genes are involved in the pathogenesis of SOD. For example, mutations in the

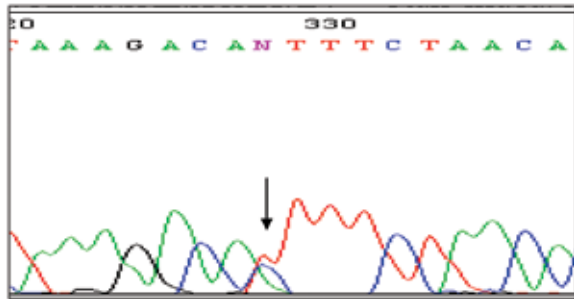


Figure 1. Segment of the chromatograms illustrating a *HESX1* gene allelic variant in patient 2, consisting of a 1772 A > G transition that results in the replacement of an asparagine by a serine at position 125 (N125S).

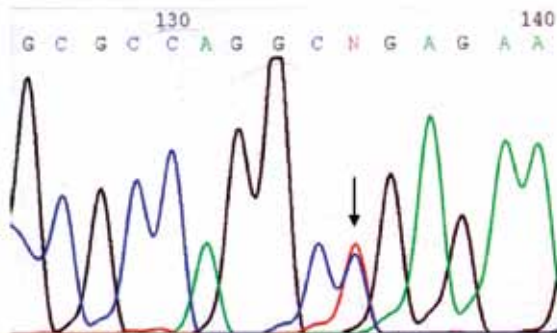


Figure 2. Segment of the chromatograms illustrating a *PROP1* gene allelic variant in patients 4 and 5, consisting of a monoallelic variant (27 T > C, A9A).

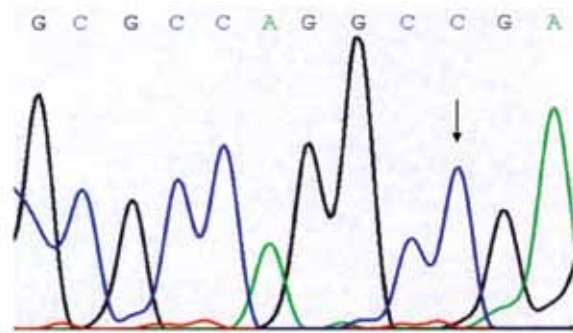


Figure 3. Segment of the chromatograms illustrating a *PROP1* gene allelic variant in patient 10, consisting of a monoallelic variant (27 T > C, A9A) in homozygose.

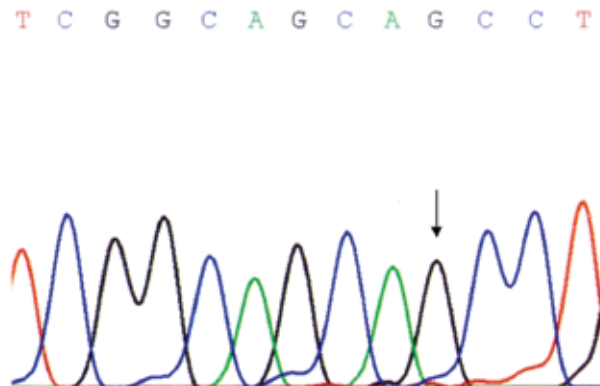


Figure 4. Segment of the chromatograms illustrating a *PROP1* gene allelic variant in patients 4, 5 and 10, consisting of a biallelic variant (59 A > G, A20S).

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SOX3 and *SOX2* genes in combination with environmental factors such as very young maternal age, have been identified in individuals with SOD (8). Furthermore, mutations in other transcription factors such as *PROP1*; *PIT1*, *LHX3* and *LHX4* can cause CPHD, whereas mutations in the growth hormone releasing receptor (*GHRHR*) gene are responsible for the majority of cases of GHD (12,13).

To date, 22 mutations have been described in the *PROP1* gene in more than 170 patients suggesting that mutations in this gene may be the most common cause of CPHD accounting for approximately 50% of all familial cases. The incidence of *PROP1* mutations in sporadic cases is much lower (14,15). In 2005, Turton and cols. (16), performed a mutational screen in a cohort of CPHD patients, consisting of 189 sporadic cases and 44 familial cases. The prevalence of mutations in the *PROP1* gene in sporadic cases was very low (1.1%) compared to familial cases (29.5%) (16). In the same year, Rainbow and cols. (17), studied the *PROP1* gene in 27 children with CPHD from 26 families (there were only 3 families with more than 1 member affected); they did not find any mutations in the *PROP1* gene, but a high incidence of a silent polymorphism (27 T > C; A9A). The authors attributed these latter findings to the ethnic background of the studied patients and to the probable presence of a founder effect in certain regions with a high number of affected patients (17). However, it cannot be excluded that the observed polymorphism is in linkage disequilibrium with an undiscovered disease-causing mutation in a regulatory or intronic region.

In familial cases, all affected individuals are homozygous for inactivating mutations. The majority of the mutations described to date are located in the homeodomain of the DNA binding site, which forms a highly conserved region in mice and humans, with approximately 91% homology among the nucleotides. The loss of function in these mutations is due to a lack of DNA-binding and transactivation capacity. The most common mutation in the *PROP1* gene, corresponding to 50%-72% of all familial cases, is the deletion of 2 base pairs in exon 2, resulting in a *frameshift* mutation in codon 101 and introduction of a stop codon at position 109 (14).

As an exception to the usual location of *PROP1* mutations, Reynaud and cols. (18) described a mutation *downstream* from the homeodomain, leading to substitution of a tryptophan residue and introduction of a stop codon at position 194 in the transactivation do-

main. This truncated protein retains only 34% of transcriptional activity when compared to the wild-type (18).

In 2000, Osório and cols. (19) described a novel 263T > C transition in exon 2 of the *PROP-1* gene, resulting in the substitution of phenylalanine 88 by serine. F88, which corresponds to position 20 of the homeodomain in transcription factors of this class, constitutes the hydrophobic core of the first α -helix forming the homeodomain. This finding underscores the critical role of this highly conserved hydrophobic phenylalanine in the structure and function of the homeodomain of PROP-1 (19).

In 2009, Dattani and cols. (20) ascertained three pedigrees with *PROP1* mutations from a large cohort of patients with variable degrees of CPHD and described four novel mutations in *PROP1* gene (20). The phenotypic variation observed in association with *PROP1* mutations both within and between families, together with the evolving nature of hormone deficiencies and sometimes changing pituitary morphology indicates a need for continual monitoring of these patients (20).

We did not find any mutation of the *PROP1* gene in the five CPHD patients studied. Our results are in accordance with literature, since the incidence of mutation in sporadic cases is very low. We confirmed three patients had allelic variant A9A and N2S, which has already been described as polymorphism (21).

Among the patients with SOD, we found one individual with a monoallelic variant 59 A > G; N125S, a polymorphism previously described in the Afro-Caribbean population. Brickman and cols. (19), analyzing 461 patients, found 5 patients of Afro-Caribbean descent with variable severity of SOD with this allelic variant (9). Analyzing the DNA of 42 normal individuals of Afro-Caribbean background, they encountered 5 individuals homozygous for the wild-type allele, 17 individuals homozygous and 20 heterozygous for the 59 A > G; N125S allelic variant. Brickman and cols. (9) suggested that this variation may not be entirely silent, because the replacement of a serine with a proline at position 18 corresponds to the Prop1 homeodomain mutation implicated in the phenotype of the Ames dwarf mouse (9).

The absence of *HESX1* mutations in SOD patients is consistent with the findings of other mutational screens. The finding suggests that mutations in one or several other genes, possibly in conjunction with environmental factors or stochastic developmental defects, are involved in the pathogenesis of SOD phenotypes (21).

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