Functional SNPs within the Intron 1 of the **PROP1** Gene Contribute to Combined Growth Hormone Deficiency (CPHD)

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**Context:** Mutations within the **PROP1** gene represent one of the main causes of familial combined pituitary hormone deficiency (CPHD). However, most of the cases are sporadic with an unknown genetic cause.

**Objective:** The aim of this study was the search for low penetrance variations within and around a conserved regulatory element in the intron 1 of **PROP1**, contributing to a multifactorial form of the disease in sporadic patients.

**Methods and Patients:** A fragment of 570 bp encompassing the conserved region was sequenced in 107 CPHD patients and 294 controls, and an association study was performed with the four identified variants, namely c.109+435G>A (**rs73346254**), c.109+463C>T (**rs4498267**), c.109+768C>G (**rs4431364**), and c.109+915_917ins/ del **TAG** (**rs148607624**). The functional role of the associated polymorphisms was evaluated by luciferase reporter gene expression analyses and EMSA.

**Results:** A statistically significant increased frequency was observed in the patients for **rs73346254**A (**P** = 5 × 10⁻⁴) and **rs148607624**del **TAG** (**P** = 0.01) alleles. Among all the possible allele combinations, only the haplotype bearing both risk alleles showed a significantly higher frequency in the patients vs. controls (**P** = 4.7 × 10⁻⁴) and conferred a carrier risk of 4.19 (**P** = 1.2 × 10⁻⁶). This haplotype determined a significant decrease of the luciferase activity in comparison with a basal promoter and the other allelic combinations in GH4C and MCF7 cells (**P** = 4.6 × 10⁻⁶; **P** = 5.5 × 10⁻⁴, respectively). The EMSA showed a differential affinity for nuclear proteins for the alternative alleles of the two associated variations.

**Conclusions:** Variations with a functional significance conferring susceptibility to CPHD have been identified in the **PROP1** gene, indicating a multifactorial origin of this disorder in sporadic cases. (*J Clin Endocrinol Metab 97: E1791–E1797, 2012)*

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**The PROP1** gene (prophet of Pit; Mendelian Inheritance in Man 601538), encoding an anterior pituitary transcription factor, represents one of the most extensively studied genes in combined pituitary hormone deficiency (CPHD). In mice it is expressed exclusively in the embryonic pituitary, in which it is involved in the specification of Pit-1 dependent somatotrope, lactotrope, and caudomedial thyrotrpope lineages (1). A naturally occurring...
ring homozygous mutation within Prop1 was first described in the Ames dwarf mouse (2). These mice have less than 1% of the normal number of somatotrophs, with reduced lactotroph and thyrotroph numbers and expression of gonadotrophins.

To date, about 25 distinct causative mutations in PROPI have been detected in about 20–30% of familial cases of CPHD with an autosomal recessive mode of inheritance (3). The majority of these mutations are located within the DNA-binding sites and disrupt the ability of PROPI to bind and activate the PIT1 gene (3, 4).

However most CPHD patients, accounting for about 95% of the patients, present with no family history. The search in sporadic cases for mutations in PROPI as well as in other candidate genes failed to detect an appreciable frequency of causative mutations (5–7). The high proportion of sporadic cases with no detectable causative mutation might be explained by a multifactorial etiology determined by multiple low penetrance genetic factors rather than by the presence of high penetrance mutations in a single gene.

Because PROPI plays an important role in the hereditary forms of CPHD, we hypothesized that low penetrance mutations in this gene might contribute to the sporadic forms of panhypopituitarism.

In a previous report, Ward et al. (8) identified by interspecies genomic sequence comparison a conserved element within Prop1 intron 1 (IVS1). This element was sufficient to confer the correct spatial expression in a position-independent manner in the context of the transgene in mice, suggesting its functional role in the murine Prop1 gene regulation.

The identification of this conserved regulatory region prompted us to search for variations within and around the homologous human region and to evaluate their involvement in CPHD etiology in sporadic cases through a case-control association study.

A significant association with the disease of two polymorphisms within the PROPI IVS1 was detected. Functional analysis demonstrated that these variations influence the transcriptional activity of a basal promoter in vitro.

Materials and Methods

Subjects

A total of 107 patients (64 males and 43 females) with a median age at diagnosis of 10.9 yr (0.1–19.9 yr) were recruited from different Italian centers over a period of 8 yr and selected for this study according to the following criteria: 1) they presented with a clinical and hormonal evidence of childhood-onset GH deficiency combined with at least one other pituitary defect in the absence of an identified cause of hypopituitarism (e.g. cerebral tumors, cranial trauma, documented asphyxia, or other injuries at delivery), 2) they had a negative family history for pituitary dysfunction or apparent or declared consanguinity and were thus considered as sporadic cases, and 3) mutations in the coding sequences of genes associated with multiple pituitary hormone dysfunctions (PIT1, PROPI, HEXS1, LHX3, and LHX4) were excluded (data not shown).

Mean height SD score for chronological age was calculated according to Tanner et al. (9). The mean height of the patients at diagnosis was −2.26 SD score ± 2.3 SD.

Morphological evaluation of the hypothalamus-pituitary area and/or of the central nervous system was performed in 84 of 107 patients by magnetic resonance imaging, using precontrast coronal spin echo T1-weighted images followed by postgadolinium T1-weighted imaging. Abnormalities (ectopy of the neurohypophysis, pituitary hypoplasia, empty sella) were found in 76 of the evaluated patients (90%).

Patients or parents of the patients under 18 yr of age gave their written informed consent to participate to this study, which was approved by the local ethical committee of each contributing auxological center.

Two hundred ninety-four normal-stature Italian individuals matched for sex and age were included as controls in the genetic association analysis.

Hormonal investigations

Hormonal assays were performed using several commercial kits and normal values for each center were taken into account. Results of biochemical investigations at diagnosis were recorded including basal free T₄, TSH, cortisol, and ACTH levels and basal and peak levels of GH, TSH, LH, FSH, and cortisol in response to pituitary stimulation tests.

GH deficiency was diagnosed in the presence of low-normal IGF-I levels according to sex and age cutoffs and impaired response to two consecutive classical provocative tests (with arginine or clonidine or insulin; GH peaks <10 ng/ml) or one double stimulus with GHRH + arginine (GH peaks <20 ng/ml) (10). A diagnosis of TSH deficiency was made if serum free T₄ concentration was under the normal cutoff level(<10 pmol/liter) with normal or low TSH levels. ACTH deficiency, in presence of low or normal ACTH levels, was suspected when fasting morning serum cortisol was less than 193 nmol/liter and was confirmed by an impaired response to the 1-μg tetracosactide or insulin tolerance test (<4.97 nmol/liter). Gonadotroph axis was investigated only in patients of postpubertal age. FSH-LH deficiency was diagnosed on the basis of delayed or absent pubertal development and no increase in serum FSH and LH in response to the GnRH test.

Genetic analysis

The entire PROPI IVS1 fragment was amplified from genomic DNA with primers 5′-AGGAGGACACCCGAGAGAGAGCTGAGAGGACACCCGG-3′ (forward) and 5′-CTGACTCCAGCTGGTCCAATCG-3′ (reverse). PCR products were sequenced in a single reaction using the Big-Dye Terminator reaction kit on an automated ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).
The haplotypic combination of the four single-nucleotide polymorphisms (SNPs) was experimentally determined for each individual as reported in the Supplemental Material, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org.

Construction of luciferase reporter gene expression vectors

To generate the pGL3-TK vector the thymidine kinase (TK) minimal promoter was excised from the pRL-TK vector (Promega, Madison, WI) using BglII and HindIII and cloned into BglII/HindIII sites upstream of the luciferase gene in the pGL3-Basic vector (Promega) linearized with the same enzymes.

Four PROP1 IVS1 fragments bearing the four possible combinations for rs73346254 and rs148607624 alleles were used as template for a nested PCR with primers containing engineered KpnI sites [5’-CAGGTTACCTGGACGTTGAGCTCTAGG-3’ (forward); 5’-TCGGTACCTGGACCTGGACACTCATGACTGCTCT-3’ (reverse); KpnI sites are underlined]. The amplified products were cloned into the pMOS plasmid (pMOSBlue T-vector kit; Amersham/GE Healthcare, Wauwatosa, WI), controlled by DNA sequencing for polymerase-induced errors, released from pMOS by digestion with KpnI, purified from gel with the Gen Elute gel extraction kit (Sigma Aldrich, St. Louis, MO) and finally inserted into the KpnI site 5’ of the TK promoter in the luciferase reporter pGL3-TK vector.

Cell culture and transient transfection assays

The transcriptional activity of PROP1 IVS1 haplotypes was evaluated by transient transfection experiments. Because a human gonadotrope cell line is not available, we used the rat anterior pituitary GH4C1 and the human breast adenocarcinoma MCF-7 cells.

GH4C1 and MCF7 cells (American Type Culture Collection, Manassas, VA) were respectively cultured in Ham’s F10 medium supplemented with 15% heat-inactivated fetal bovine serum, 2.5% heat-inactivated fetal bovine. They were maintained in a humidified 5% CO2 incubator at 37 C, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich). The day before transfection, cells were seeded into 24-well tissue culture plates in 1.0 ml medium and grown to 70–80% confluency. Transient transfections were carried out using 1 μg of each construct with Mirus reagent (Mirus Bio, Madison, WI) following the manufacturer’s instructions. After 48 h, the cells were lysed with the buffer of the luciferase assay system (Promega). Luciferase activities were measured using a luminometer (Anthos Lucy1; BioTek, Winoski, VT) and normalized with respect to protein concentration (bicinchoninic assay; Pierce, Rockford, IL). The pGL3-TK vector was determined as basal level.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from the GH4C1 cell line as previously described (11). Double-stranded oligonucleotides were labeled with [γ-32P]ATP using a T4 polynucleotide kinase (Promega Madison, WI) and purified on a Microspin G-25 column. Five micrograms of nuclear extract were incubated with 1 μg of poly (deoxyinosine-deoxycytosine) and 15 fmol of the 32P-labeled probes in binding buffer (50% glycerol; 10 mM Tris-HCl, pH 7.6; 500 mM KCl; 10 mM EDTA; and 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. For the competition studies, an increasing molar excess of unlabeled over the radiolabeled oligonucleotide was added to the binding reaction.

The reaction mixtures were then separated on a 5% non-denaturing polyacrylamide gel (19:1 acrylamide-bisacrylamide) and electrophoresed in a 0.25x Tris-boric buffer at 150 V for 1.5 h. The gel was dried on Whatman 3 mm paper (Whatman, Middlesex, UK) and radioactive bands were detected by autoradiography.

Statistical analysis

The association analysis was performed using the χ2 test with Yates’s correction. The strength of the association was evaluated by the odds ratio (OR) with 95% confidence intervals (CI).

Pairwise linkage disequilibria (LD) between SNPs were calculated by D’ and r2 using the Haploview program version 3.2 (http://www.broadinstitute.org). The same software was used to estimate haplotype structures and their frequencies from unphased genotype data.

The transfection experiment data were represented as mean ± SD of the mean. All the values are expressed as a percentage of the pGL3-TK vector mean value. The Mann-Whitney test was used to compare the relative luciferase activity between two groups (P < 0.05 was considered as significant).

Results

Genetic analysis of the PROP1 IVS1 variations

The human homologous conserved PROP1 IVS1 region defined trough interspecies alignment (http://genome.lbl.gov/vista/index.shtml) (12) encompassed a fragment extending from c.109 + 402 to c.109 + 616 displaying nearly 80% identity with the murine counterpart.

To identify polymorphisms with a possible functional role within and around this conserved region, we sequenced a portion of 570 bp, from c.109 + 378 to c.109 + 949, in 107 CPHD patients and detected four already known variations, namely c.109 + 435G>A (rs73346254), c.109 + 463C>T (rs4498267), c.109 + 768C>G (rs4431364), and the insertion/deletion polymorphism c.109 + 915_917insdelTAG (rs148607624). The c.109 + 435G>A and c.109 + 463C>T are located within the highly homologous region, whereas the others lie outside.

Pairwise LD values of the four SNPs were calculated separately in controls and in patients (Supplemental Fig. 1). A moderate LD was observed in controls between rs73346254 and rs148607624 (D’ = 0.77; r2 = 0.49), whereas it was complete in the patients (D’ = 1; r2 = 0.84).

To assess a possible contribution of these variations to CPHD, a case-control association study was performed.
The allele and genotype frequencies, consistent with those expected from Hardy-Weinberg equilibrium, were compared between the 107 patients and 294 control individuals (Table 1).

The frequency of rs73346254A and rs148607624delTAG alleles was significantly higher in CPHD patients than in controls (*P* = 0.0005 and *P* = 0.01, respectively). Considering a dominant model, an OR value greater than 1 was observed for genotypes rs73346254 GA+AA (OR 3.53) and rs148607624 insTAG/delTAG+del/delTAG (OR 2.63) with respect to the homozygous GG and insTAG/insTAG, respectively (Table 1).

Considering all haplotype combinations of the four SNP (Supplemental Table 1 and Supplemental Material), the only haplotype carrying both associated alleles (A/C/ G/delTAG), showed a significantly higher frequency in the patients with respect to controls (*P* = 0.00047) and conferred a carrier risk of 4.19 (95% CI 1.9–9.39; *P* = 0.00012), being present in 16.8% (18 of 107) CPHD patients and 4.7% (14 of 294) control individuals. None of the other haplotype combinations showed a significantly different frequency in patients and controls. Haplotypes carrying only one of the two associated alleles were rare: the combination rs73346254G/rs148607624delTAG was detected in seven controls and none of the patients, whereas rs73346254A/rs148607624insTAG was detected in three patients and five controls.

### Functional analysis of the PROP1 IVS1 polymorphisms

To evaluate the contribution of each of the two variations, we performed a functional study by testing them separately and in combination.

We first investigated whether the PROP1 IVS1 polymorphisms affected the transcriptional activity of a reporter gene (luciferase) by cloning the fragment encompassing the variations upstream the TK minimal promoter and by testing its ability to modulate the promoter activity in two different cell lines. The luciferase activity induced by the plasmid bearing the two associated alleles (A/C/G/ delTAG) was compared with the activity of an haplotype differing at the two associated positions (G/C/G/insTAG) and with that of a plasmid bearing only the TK minimal promoter (pGL3-TKbasic, Fig. 1). The construct harbor- ing the two associated alleles exhibited a significantly decreased luciferase activity with respect to the pGL3-TK vector in both cell lines (0.4- to 0.3-fold induction, respectively). Conversely, haplotypes bearing only one of the two associated variants displayed a transcriptional activity comparable with that of the reference, suggesting that the decreased activity was mediated by the copresence of rs73346254A and rs148607624delTAG.

Transfection experiments were repeated by placing the same fragments in the antisense orientation. The A/C/G/ delTAG fragment conferred the same inhibitory effect as observed in sense orientation, with a reduction of 39% (*P* = 1.1 × 10⁻⁶) and 66% (*P* = 3.5 × 10⁻⁴) the activity of the basal TK promoter in GH4C and MCF7 cells, respectively.

To investigate the molecular mechanism underlying the inhibition of the transcriptional activity exerted by the two variants, we next determined whether the intronic sequences surrounding the two SNP could serve as a binding site for nuclear factors. An EMSA with oligonucleotide probes carrying the two alternative alleles for each of the two associated SNP was performed using nuclear extracts from GH4C cells (Fig. 2).

A strong difference was detected in the signal intensity of a DNA-protein complex for the two rs148607624 al-
leles (C1 and C2, Fig. 2A, lanes 1 and 6) and a slighter difference between the two rs73346254 alleles (complex C3, Fig. 2B, lanes 1 and 8), with the rs148607624delTAG and the rs73346254A allele probes showing lower affinity for a nuclear protein. The specificity and the affinity of these DNA-protein complexes were characterized by competition experiments with the unlabeled probes (Fig. 2).

**Discussion**

Fully penetrant loss of function mutations within the PROP1 coding sequence have been detected in approximately 20–30% of CPHD familial cases with an autosomal recessive inheritance (3). Conversely, the frequency of PROP1 mutations in sporadic cases is much lower, ranging in different studies from 0 to 22%, with a global prevalence of about 5% as calculated by a rough meta-analysis of all the studies (5–7, 13–17). We detected homozygous mutations within the PROP1 gene in 1.2% (two of 161) of the Italian CPHD sporadic cases (Giordano, M., unpublished data). Considering that mutations in the other disease genes (mainly PIT1 and HESX1) are rarer, at least 90% of the patients with sporadic CPHD remain unexplained.

It is possible that a variable proportion of these cases are phenocopies resulting from unrecognized acquired causes. However, the rarity of causative mutations in sporadic cases in comparison with the higher incidence in the familial forms suggests that, at least in some of these patients, the disease has a multifactorial etiology being determined by DNA variations with smaller effect, possibly in conjunction with environmental or stochastic developmental defects.

We investigated by a case-control study the possible contribution to CPHD of PROP1 intronic SNPs located within and around a conserved regulatory region. Our study had a power 80% or greater to detect with P ≤ 0.05 a frequency difference between patients (n = 107) and controls (n = 294) for sequence variations conferring an OR of 2.5 and a minor allele frequency between 0.04 and 0.40. We detected a significant association with rs73346254A and rs148607624delTAG, with an OR of 2.63 and 3.53, respectively, increasing to an OR of 4.2 for individuals carrying their haplotypic combination. The association with this particular allele combination could reflect the presence of an undetected causative variation carried by the same haplotype. However, their combination drastically reduced the transcription of a basal promoter, whereas the two alleles did not elicit the same inhibitory effect when they were tested separately (Fig. 1), suggesting a functional cooperation between the two positions located about 500 bp apart.

Among those in the control group, there was one normal-stature individual homozygous for the CPHD-associated haplotype (rs73346254A/rs148607624delTAG). He was not investigated for the presence of pituitary hormone deficiencies because he was apparently healthy, but we cannot exclude pituitary hormonal defects because they might be present also in normal-stature subjects.

The EMSA results indicated that both sequences surrounding the two SNP bind nuclear proteins with differential affinity, depending on the allele. This was particularly evident for the rs148607624 alleles with the
formulation of two complexes (Fig. 2A) in the presence of the insTAG probe that were almost completely absent with the delTAG probe.

On the basis of these functional data, it can be hypothesized that the sequences around the two SNPs serve as binding sites either for common nuclear protein(s) or for different interacting proteins and that the copresence of A at position c.109 + 435 and delTAG at c.109 + 915_917 determines a globally reduced affinity for these putative regulatory factors. Searching in silico for the possible nuclear factors interacting with these sequences revealed that the sequence surrounding c.109 + 435 (rs73346254) can interact with two common factors, aptor protein and glucocorticoid receptor-α, regardless of the G or A alleles, whereas a binding site for c-Jun was recognized only for the A variant. The c.109 + 915_917 delTAG (rs148607624) allele can represent a binding site for c-Myb, whereas the c.109 + 915TAG allele failed to predict a possible binding site. This prediction was not in agreement with the intense band visualized by the EMSA experiments with the TAG probe.

Our experiments support a regulatory function for the conserved PROPI intronic sequence. However, the in vitro transfection experiments failed to demonstrate an enhancer activity in this region for haplotype rs73346254G/rs148607624insTAG. In fact, none of the constructs exhibited a statistically significant increased transcriptional activity with respect to the basal promoter activity (Fig. 1). This may be attributable to the experimental system: GH4C and MCF7 represent differentiated cell types and may not express the transcription factors and cofactors necessary for the correct activity of all PROPI enhancer elements because PROPI is significantly expressed only during early pituitary development.

In summary, we have identified a combination of two IVS1 SNPs of the PROPI gene associated with CHPD influencing the transcription efficiency in vitro through allelic differences in the affinity for unidentified transcription factor(s). Our data suggest that in vivo the CPHD-associated variants of rs73346254 and rs148607624 might down-regulate the expression of PROPI influencing correct pituitary development and hormone secretion.

Thus, analogous to other diseases (18), it appears that, although familial CPHD results from rare, highly penetrant, loss-of-function pathogenic mutations, low pen-
etradence, noncoding, common variants, mainly with a regulatory function, may contribute to the risk of sporadic CPHD. These results should be replicated in an independent set of CPHD patients and controls.

Acknowledgments

We are grateful to the patients and their parents who participated in this study.

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This work was supported by grants from the Eastern Piedmont University, Regione Piemonte (Ricerca Scientifica Applicata, bando 2008bis), and “Cariplo” Foundation (project 2009-2009).

Disclosure Summary: None of the authors declared any conflict of interest.

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