jcrpe-2019-0191.R1

Original Article

DOI: 10.4274/jcrpe.galenos.2020.2019.0191

Mutations within The Transcription Factor *PROP1* in a Cohort of Turkish Patients with Combined Pituitary Hormone Deficiency

Short Title: *PROP1* Defects in Turkish Hypopituitarism Patients

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What is already known on this topic?
It is already known that *PROP1* gene product is a critical transcription factor for development and maintenance of proper functioning of anterior pituitary gland. So far, *PROP1* gene mutations are reported to be the most frequent genetic aetiology of combined pituitary hormone deficiency and it is responsible from progressive anterior pituitary hormone deficiencies.

What this study adds?
We have defined frequency of *PROP1* gene mutations in a Turkish cohort of combined pituitary hormone deficiency patients. Pathogenic mutations were detected in 11 patients, gross deletions were present. A novel variant was discovered in two siblings. We described patient characteristics and treatment responses.

Abstract
Objective: Mutations of genes encoding transcription factors **which** play important roles in pituitary morphogenesis, differentiation and maturation lead to combined pituitary hormone deficiency (CPHD). *PROP1* gene mutations are reported as the most frequent genetic aetiology of CHPD. The aim of this study is to describe phenotype of Turkish CPHD patients and define frequency of *PROP1* mutations.
Methods: Fifty-seven CPHD patients from 50 families were screened for *PROP1* mutations. The patients were affected by growth hormone and additional anterior pituitary hormone deficiencies.
Results: All patients had GH deficiency, 98,2% had central hypothyroidism, 45,6% had hypogonadotropic hypogonadism, 43,8% had **ACTH deficiency** and 7,1% had prolactin deficiency. Parental consanguinity rate was **50.9%**. **14** cases were familial. Mean height standard deviation score (SDS) and weight SDS were -3,8 (±1,4) and -3,1 (±2,0), respectively. Of 53 patients with available pituitary imaging, 32 showed abnormalities. None had extra-pituitary abnormalities. **8 index** patients had *PROP1* gene mutations. Five sporadic patients had homozygous c.301_302delAG (**p.Leu102CysfsTer8**) mutation, two siblings had exon 2 deletion, two siblings had complete gene deletion and two siblings had homozygous, novel c.353A>G (p.Q118R) mutation.
Conclusion: Phenotype of patients regarding hormonal deficiencies, pituitary morphology, presence of extra-pituitary findings, family history of CPHD and parental consanguinity are important to decide which pituitary transcription factor deficiency should be investigated. The frequency of the *PROP1* mutations was **16%** in our cohort. Mutation rate was higher in familial cases **compared to** sporadic cases (42,8% vs. 11,6%). *PROP1* mutation frequencies vary in different populations and its prevalence is high in Turkish CPHD patients.
Key words: Combined pituitary hormone deficiency, Hypopituitarism, Pituitary Transcription Factors, *PROP1* gene

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Submitted. 20-Nov-2019
Accept (10-Jan-2020)
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Introduction
Combined pituitary hormone deficiency (CPHD) is defined as deficiencies of growth hormone (GH), thyroid-stimulating hormone (TSH), the gonadotropins **-** luteinizing hormone (LH) and follicle-stimulating hormone (FSH), prolactin (PrL) and adrenocorticotropic hormone (ACTH). Worldwide prevalence of CPHD is estimated as 1/8000 (1).
Both in human and mice, pituitary organogenesis and maintenance of its proper functioning necessitates the appropriate expression of a cascade of signalling molecules and transcription factors which are crucial for organ commitment, cell proliferation, patterning and terminal differentiation (2-4).
The genes that are related to these transcription factors are *PROP1*, *POU1F1* (*PIT1)*, *LHX3*, *LHX4*, *HESX1*. In 1998, Wu et al. **(5)** identified homozygous or compound heterozygous inactivating mutations of *PROP1* gene **are associated with** CPHD. So far, it is defined as the most common genetic aetiology of CPHD in humans **(4-6)**. Prophet of PIT-1 (*PROP1*) is a paired-

like homeobox 1 gene, located on chromosome 5q35.3 and consists of three exons encoding for a 226–amino acid protein which is a late-expressed transcription factor (4). Mutations of *PROP1* gene cause autosomal recessively inherited CPHD and clinical phenotype includes GH, TSH, FSH/LH, PrL and rarely ACTH deficiencies and morphological pituitary anomalies (4,7).

Phenotypes associated with *PROP1* **gene mutations** can be highly variable. Deficiencies of **all** pituitary hormones **can** be seen in different severity and at different ages. But in all cases anterior pituitary function deteriorates over time (4,8). TSH and GH deficiencies have a tendency to occur at early childhood, whereas gonadotropin and corticotropin deficiencies manifests later in life (4,8). As, PROP1 is a "later-acting transcription factor", extra-pituitary manifestations are not observed (8). Magnetic resonance imaging (MRI) of the anterior pituitary gland shows normal or enlarged gland in early stages and pituitary involution in later stages. Whilst, size and location of posterior pituitary is normal and pituitary stalk interruption is not observed (1,4). Rarely, pituitary masses associated with *PROP1* gene mutations are reported (4,9,10).

Point mutations, small and large deletions and insertions in *PROP1* gene have been reported so far, but there are not any specific variants linked to specific regions or ethnicities (11-13).

In this study, we aimed to define patient characteristics and to identify *PROP1* gene mutations in our patient cohort with combined pituitary hormone deficiency.

Materials and Methods

Study design and patient selection

This retrospective cohort study was conducted in Çukurova University Research and Education Hospital between January 1997 and August 2019 and included 57 patients with combined anterior pituitary hormone deficiency. Exclusion criteria for the patients were isolated GH deficiency, brain tumour, central nervous system surgery, cranial–neck irradiation, systemic chronic illnesses or chromosomal abnormalities.

Fifty-seven patients from 50 families (39 males and 18 females) diagnosed with CPHD were analysed for *PROP1* mutations. The patients included to the study were affected by GH deficiency and additional one or more anterior pituitary hormone deficiencies including TSH, gonadotropins, ACTH or PrL. Diagnosis was based on clinical, laboratory and imaging investigations. Patients with additional chronic diseases or surgery involving pituitary gland were excluded. Serum growth hormone, Insulin-Like Growth Factor-1 (IGF-1), Insulin-like growth factor-binding protein-3 (IGFBP-3), FSH, LH, oestradiol, cortisol, TSH, free thyroxin (fT4) and PrL and plasma adrenocorticotropic hormone levels were analysed by commercial kits based on solid-phase, two-site sequential, or competitive chemiluminescent immunometric assay or electrochemiluminescence immunoassay.

Genomic DNA was isolated from peripheral blood cells. *PROP1* gene **(transcript ID: ENST00000308304.2 and protein ID: O75360)** was screened by polymerase chain reaction (PCR) amplifications of exons and neighbouring intronic regions. The PCR products were purified and directly sequenced using the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif., USA) in an ABI PRISM 3130 automatic sequencer. DNA sequence data analyses were evaluated with DNA Sequencing Analysis Software Sequencher 5.0 programme (http://genecodes.com/). All of the variants were investigated using 1000 genomes browser database (http://browser.1000genomes.org/index.html) **and the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/clinvar)** whether they are novel or previously reported. Subsequently, mutant variants were interpreted by *in silico* prediction tools such as Mutation Taster, SIFT and PolyPhen-2 **(14-16).**

Segregation analysis was performed only for the family of patients with the novel variant. We were not able to test the parents of the patients with known pathogenic variants due to financial limitations.

PCR amplification of certain exons of *PROP1* gene had failed for DNA of nine patients. Four patients from two family had gave consent for further testing and multiplex ligation dependent probe amplification (MLPA) assays were performed only for these patients. **The other five patients were not included in the calculation of mutation frequency.**

The patients who are from the same family are indicated with the same superscript letter.

The Ethics Committee of the Çukurova University Faculty of Medicine approved this study (approval #TF2013LTP24), and written informed consent was obtained for each patient from their legal guardians.

Statistical Method

Data obtained from this study were analysed using SPSS statistical software package program (Version 23.0 for Windows; Armonk, NY: IBM Corp.). Descriptive statistics were presented as mean ± standard deviation. Frequency distributions and percentages were given for categorical variables.

Results

All of the patients included to the study were affected by growth hormone deficiency and diagnosed in childhood. 56 patients (98,2%) had central hypothyroidism, 26 (45,6%) had hypogonadotropic hypogonadism, 25 (43,8%) had ACTH deficiency and 4 (7,1%) had PrL deficiency. There were more male patients (68,4%). Age at diagnosis was **7,9 (±4,8)** years (min-max: 3 months - 19,8 years). Delayed bone age at diagnosis was **3,3 (±2,4)** years. **29 patients (50,9%)** had parental consanguinity. **14** patients were familial cases. There was no history of perinatal asphyxia or difficult birth. None of the patients had any major dysmorphic findings. Height standard deviation score (SDS) at diagnosis was -3,8 (±1,4). Weight SDS at diagnosis was -3,1 (±2,0). IGF-1 SDS at diagnosis was -3,0 (±1,5). Mean age at the start of treatment was **8,6 (±4,8)** years. All of the patients received appropriate treatments for their hormonal deficiencies. 12 patients achieved their final height and mean final height SDS for these cases was **-1,0 (±0,7)**. Final height and target height values for *PROP1* mutated patients are listed in Table 1.

Pituitary magnetic resonance imaging (MRI) was available for 53 patients. 21 had normal pituitary MRI, 17 had pituitary hypoplasia, eight had hypoplasia of the adenohypophysis, three had ectopic neurohypophysis and three had pituitary adenoma. Patient 22 had pituitary adenoma which resolved on follow-up and transform into anterior pituitary hypoplasia. None had extra-pituitary abnormalities on MRI.

Patients 14, 22, 41, 46 and 57 had homozygous deletion of c.301_302delAG in exon 2 of *PROP1* gene. This mutation resulted in frame-shift and premature stop codon **(p.Leu102CysfsTer8)**. These five patients had different combinations of

anterior pituitary hormone deficiencies. All had GH and TSH deficiencies at the time of diagnosis. Four of these patients who have reached the age of puberty showed clinical and laboratory findings of hypogonadotropic hypogonadism. Only one had ACTH deficiency and none had PrL deficiency (Table 1). Patients 14, 22, 41 and 46 responded quite well to the GH and levothyroxine supplementations and appropriate hormone replacement to induce secondary sex characteristics. Patient 57 was newly diagnosed and he was recently started GH replacement.

PCR amplification of second and third exons of *PROP1* gene had failed for DNA of patients 1[a]-2[a], 7[d]-8[d], 9[e]-10[e] and 15 all of whom had parental consanguinity. Whereas, pathogenic mutations were not detected within exon 1 for these patients. MLPA assays could be performed only for patients 1[a] and 2[a] from the same family and homozygous deletion of exon 2 of *PROP1* gene were detected in both siblings. These two brothers have GH deficiency at the time of diagnosis and developed TSH deficiency after approximately one or two years. Both had delayed pubertal development and lack of secondary male sex characteristics due to hypogonadotropic hypogonadism. **Eventually**, both developed ACTH deficiency (Table 1).

PCR amplification of whole *PROP1* gene had failed for DNA of patients 58[f]-59[f]. MLPA assays detected complete gene deletion in these siblings. The elder sister showed GH deficiency in early childhood and developed TSH deficiency 4 years later. When she reached the age of puberty, she developed both ACTH deficiency and hypogonadotropic hypogonadism. Whereas younger brother showed both TSH and GH deficiencies at diagnosis and currently he is prepubertal and he is not affected by **ACTH** insufficiency (Table 1). Pituitary imaging revealed pituitary adenoma in patient 58[f] and normal in 59[f]. Adenoma did not exhibit progression and remained stable.

Patients 3[b] and 4[b] from the same family with the same phenotype had homozygous c.353A>G (p.Q118R) variant in exon 3 of *PROP1* gene (Figure 1). This novel variant was predicted to be disease-causing by *in silico* predictive tools such as Mutation Taster, SIFT and PolyPhen-2 **due to splice site changes and possibly affected protein features (14-16).** Both parents, who were consanguineous, and **a healthy sister** were heterozygous for the same mutation. Both siblings have GH deficiency at the time of diagnosis and a few years later they developed TSH deficiency. They showed hypogonadotropic hypogonadism and PrL deficiency in adolescence (Table 1). On physical examination, decreased body hair growth and pubic hair growth were marked in both siblings. Patient 4[b] had pituitary adenoma on pituitary MRI. On follow-up, she had visual impairment, so she had undergone pituitary surgery.

Discussion

In this study, we have detected *PROP1* gene mutations in **8 index** patients from a cohort of 57 CPHD patients **from 50 families**. **Segregation analysis of the variants in the pedigree revealed 3 patients with the same pathogenic *PROP1* mutations**. More than half of the patients with mutation were familial cases and positive mutation frequency was higher in familial cases compared to sporadic cases (**3/7** familial cases versus 5/43 sporadic cases). There are several cohorts defining genetic aetiology of CPHD from different parts of the world. *PROP1* gene mutations are reported to be the most frequent amongst both sporadic and familial CPHD patients (4,6,8,17). But the frequency was reported to be in between 0% and 70,1% from different populations (10,**18-21**). *PROP1* mutation frequencies among CPHD patients are highest in Eastern European populations especially Lithuanian, Polish and Hungarian, also Portuguese, Russian and Brazilian cohorts (3,10,12,**22-28**). Contrarily, *PROP1* mutation rates are usually low in Western and Southern European countries, Australia and in cases with Asian origin especially in sporadic CPHD patients (3,6, **18-21, 29**). *PROP1* gene mutations are not rare among Turkish CPHD patients (13,**30**). In 2014, Baş et al. screened 76 Turkish CPHD patients and frequency of *PROP1* mutations was 21,8% (**30**). ***PROP1* mutation frequency in this study was similar to our study.** Kandemir et al. reported *PROP1* mutations in 2 familial patients and 51 sporadic CPHD patients were mutation negative (13). **In our study, we detected *PROP1* mutations in 16% patients. Interestingly, Kandemir et al. detected lower *PROP1* mutation prevalence compared to our study. This might be attributed to dissimilarities in ethnicity, parental consanguinity rate and frequency of familial cases between these three Turkish cohorts. Overall evaluation of Turkish CPHD patients from previous studies together with the patients from our study elucidate that frequency of *PROP1* gene mutations is 16,6% amongst Turkish CPHD patients.** In addition to their study, De Rienzo et al. reviewed all CPHD cases retrospectively and postulated that *PROP1* gene mutations are responsible for 11,2% of all CPHD cases (6).

PROP1 mutation prevalence is higher in familial patients compared to sporadic cases in all cohorts (3,6,13,**22,24,26,29-32**). Parental consanguinity is known to increase the risk for autosomal recessive conditions. Also, parental consanguinity is an apparent risk factor for *PROP1* mutations (12,**22,30**). Likewise, in our study, overall parental consanguinity rate was **50,9**% whereas it was 81,8% amongst *PROP1* mutated patients. If the cases are sporadic, meaning there is a single affected individual in a family, and do not have parental consanguinity, the aetiology is more likely to be acquired rather than genetic causes (1,3,4).

c.301_302delAG mutation was reported to be one of the most prevalent mutations of *PROP1* gene (2,8,10,11,**26**). This mutation is a two base pair deletion results in a frameshift and early termination of the protein at **codon 109**. Dusatkova et al. investigated this variant and they suggest that the reason of its high occurrence may be a founder effect rather than a variant hot spot (2). **This assumption was made by the haplotype analyses and the geographic distribution of the c.301_302delAG variant that it has an ancestral origin (2).** Five of our patients had this variant and showed variable hormone deficiencies. We detected large deletions in 4 patients. Many studies, in which CPHD patients from different populations including Turkish patients were screened for PROP1 deficiency, reported homozygous deletions of the entire gene or particular exons (7,**30,33**). For this reason, MLPA analysis should be a routine part of genetic investigation.

Previously unreported p.Q118R substitution is interpreted as likely pathogenic considering the concordance of phenotype, parental consanguinity and segregation analyses of the variant. This variant is anticipated to be important as it is highly conserved in different orthologues. Also, it is located in homeobox domain (5). In 1998 Wu et al (5), identified p.F117I and p.R120C substitutions and they postulated that these variants allowed protein binding but with reduced affinity. As, p.Q118R variant is present in between these variants, it is assumed that this variant is also associated with pathogenicity due to altered protein function. *In silico* analyses with Mutation Taster, SIFT and PolyPhen-2 also point out alteration of protein features and splice site changes (14-16).

Patients with *PROP1* mutations typically have clinical manifestations of GH deficiency in early childhood. TSH and PrL deficiencies often coexist at the time of diagnosis. At the onset age of puberty, patients usually do not exhibit secondary sexual characteristics due to hypogonadotropic hypogonadism. Rarely, some patients show pubertal changes and hypogonadotropic hypogonadism may develop later in adulthood. ACTH deficiency occur variably as the patient grows older (1,4). As a result, these patients should be carefully monitored for occurrence of other anterior pituitary hormone deficiencies. It is postulated that this phenomenon of progressive hormone deficiency is due to dysfunction of PROP1 in initiating pituitary stem cell migration and differentiation (**34**). Patients with *PROP1* mutations lack extra-pituitary manifestations (6,8,**31**).

In parallel, all of the *PROP1* mutated patients in our cohort have GH and TSH deficiency at the time of diagnosis in early childhood. Nine patients have hypogonadotropic hypogonadism when puberty should start and the other two patients were prepubertal. Two siblings with the novel mutation have markedly low amount of body and pubic hair. ACTH deficiency was observed in half of the patients and the patients without ACTH deficiency are ongoingly monitored as usually it is the last occurring hormonal deficiency. Onset age of progressive hormonal deficiencies differ in patients with the same mutations and even in familial cases in our cohort. A clear phenotype-genotype correlation has not proposed in the literature, since progressive hormonal deficiencies occur at different chronology even in individuals with the same genotype (4,**17**,**24**). Response to GH treatment was satisfying in our patient cohort similar to the literature (32). Final height was achieved in nine of the *PROP1* mutated patients. All of whom have final height SDS in between their mid-parental target height SDS. This result was in agreement with the previous reports (10, **35,36**).

MRI of hypophysis often reveal pituitary hypoplasia or aplasia but occasionally pituitary hyperplasia evolving to hypoplasia and pituitary masses are reported. (1,4,6,**37,38**). On the other hand, ectopic posterior lobe and stalk abnormalities have not been observed (4). Interestingly, anterior pituitary MRI were normal in six patients and three patients had adenoma, two had hypoplasia and one had adenoma in the beginning which evolved into pituitary hypoplasia. Pituitary morphology can change during follow-up of patients with *PROP1* gene mutation (9). None of our patients showed extra-pituitary manifestations on neuroimaging. Patients with adenoma have different genotypes; two had the common homozygous c.301_302delAG mutation, one had a novel mutation and one had complete gene deletion. Interestingly, two of these cases were familial and their siblings had normal pituitary gland upon MRI. With the exact genetic aetiology, patients with pituitary adenoma have the opportunity to avoid unnecessary invasive procedures (1).

Study Limitations:

Five of the patients with the failure of PCR amplification were not available for further testing with MLPA analysis. It is the limitation of our study because there is a high probability that a large deletion may exist in *PROP1* gene of these familial CPHD cases with parental consanguinity**. In this study, we were not able to test the parents of all patients with pathogenic mutations due to financial limitations.** For future studies, patients without any mutations identified in *PROP1* gene may be screened for the other genes of pituitary transcription factors and gene panels may be more cost-effective for this purpose.

Conclusion

It is crucial to screen GH deficiency patients regularly for other anterior pituitary hormone deficiencies. With the exact genetic aetiology, the family is able to receive genetic counselling, unnecessary laboratory testing can be avoided and at the same time with the opportunity of predicting the typical phenotype, hormonal deficiencies can be detected earlier. Especially if the patients are familial and have parental consanguinity, genetic testing would be more cost-effective.

Ethics

Ethics Committee Approval: The Ethics Committee of the Çukurova University Faculty of Medicine approved this study (approval #TF2013LTP24)

Informed Consent: Written informed consent was obtained for each patient from their legal guardians.

Authorship Contributions

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Disclaimer statements

Funding Source: The authors received financial support from Scientific Research Projects Coordination Unit of Çukurova University for the publication of this article.

Financial Disclosure: The authors have no financial relationships relevant to this article to disclose.

Conflict of Interest: The authors have no potential conflicts of interest to disclose.

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Figure 1. Sequencing electropherogram of patients 3[b] and 4[b]

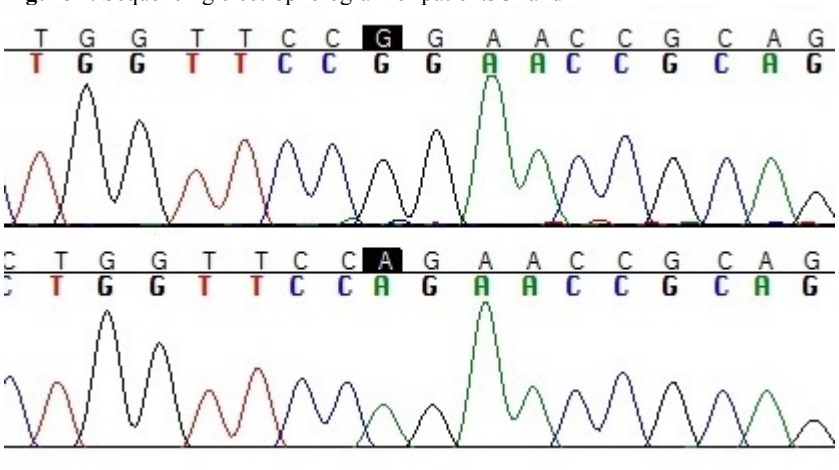

Table 1. Clinical features and genotype of the patients with *PROP1* gene mutations

Case	Sex	Parental cons.	Age at Dx (years)	Current age (years)	Peak GH, stim. (µg/L)	Onset of hormonal def. (years)					MRI of anterior pituitary	GH dose (mg/kg/week)	Height SDS (before Tx)	Growth vel. SDS (1st year of Tx)	Growth vel. SDS (2nd year of Tx)	Final Height (cm [SDS])	Target Height (cm [SDS])	Mutation
						GH	TSH	Gn.	ACTH	PrL								
1[a]	M	+	5,25	20,67	0,9	4	5,25	12	13,5	N/A	Normal	0,36	-4,39	10,83	6,13	168,7 [-1,09]	167,5 [-1,28]	Homozygous deletion of exon 2
2[a]	M	+	6,6	22,25	0,08	4	6	13	14,25	N/A	Normal	0,35	-4,77	9,97	5,15	166,2 [-1,4]	167,5 [-1,28]	Homozygous deletion of exon 2
3[b]	F	+	9,1	20,33	0,1	9,1	11,75	13	12	13	Normal	0,27	-3,77	4,08	2,18	164,5 [0,24]	164 [0,15]	**Homozygous c.662A>G (p.Q118R)**
4[b]	F	+	4,25	17	0,1	4,25	8,5	14	9	10	Adenoma	0,27	-5,11	6,09	3,51	159,2 [-0,66]	164 [0,15]	**Homozygous c.662A>G (p.Q118R)**
14	F	+	8,83	22	0,4	8,83	8,83	15	N/A	14,5	Normal	0,25	-4,1	3,44	1,3	160,4 [-0,41]	166,1 [0,51]	Homozygous c.301_302delAG (p.Leu102CysfsTer8)
22	F	-	7,75	20,67	0,01	7,75	7,75	14	13,1	N/A	Adenoma/Hypoplasia	0,29	-3,67	4,92	2,34	162,2 [-0,15]	158,5 [-0,78]	Homozygous c.301_302delAG (p.Leu102CysfsTer8)
41	F	+	6	18,42	2,3	6	6	14	N/A	N/A	Hypoplasia	0,3	-6,04	8,33	2,02	164,7 [0,32]	154,5 [-1,46]	Homozygous c.301_302delAG (p.Leu102CysfsTer8)
46	F	-	7,75	19,75	0,5	7,75	7,75	15	N/A	13,5	Normal	0,31	-4,88	11,51	5,08	157,5 [-0,95]	164,5 [0,24]	Homozygous c.301_302delAG (p.Leu102CysfsTer8)
57	M	+	4,33	4,5	0,6	4,33	3	N/A	N/A	N/A	Adenoma	N/A	-2,9	N/A	N/A	N/A	174,5 [-0,28]	Homozygous c.301_302delAG (p.Leu102CysfsTer8)
58[f]	F	+	6,3	13	0,07	2,5	6,5	12	12	N/A	Normal	0,24	-1,23	5,86	5,54	N/A	168,5 [0,92]	Homozygous complete deletion of *PROP1* gene
59[f]	M	+	6	10,8	1,1	5	5	N/A	N/A	N/A	Adenoma	0,32	-2,93	7,85	4,39	N/A	181,5 [0,86]	Homozygous complete deletion of *PROP1* gene

Abbreviations: ACTH=adrenocorticotropic hormone, cons.=consanguinity, def.=deficiency, Dx=diagnosis, FSH=follicle-stimulating hormone, GH=growth hormone, Gn.=gonadotropins, LH=luteinizing hormone, MRI=magnetic resonance imaging, N=normal, N/A=not applicable, PrL=prolactin, SDS=standard deviation score, stim.=stimulated, TSH=thyroid-stimulating hormone, Tx=treatment, vel.=velocity

*Novel mutations are shown in bold.