GERMLINE PROKINETICIN RECEPTOR 2 (PROKR2) VARIANTS ASSOCIATED WITH CENTRAL HYPOGONADISM CAUSE DIFFERENTAL MODULATION OF DISTINCT INTRACELLULAR PATHWAYS.

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Germline Prokineticin Receptor 2 (PROKR2) Variants Associated With Central Hypogonadism Cause Differental Modulation of Distinct Intracellular Pathways

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Abstract

Introduction:

Defects of prokineticin pathway affect the neuroendocrine control of reproduction, but their role in the pathogenesis of central hypogonadism remains undefined, and the functional impact of the missense *PROKR2* variants has been incompletely characterized.

Material and Methods:
In a series of 246 idiopathic central hypogonadism patients, we found three novel (p.V158I, p.V334M, and p.N15TfsX30) and six already known (p.L173R, p.T260M, p.R268C, p.V274D, p.V331M, and p.H20MfsX23) germline variants in the *PROKR2* gene. We evaluated the effects of seven missense alterations on two different prokineticin receptor 2 (PROKR2)-dependent pathways: inositol phosphate-Ca^{2+} (G_q coupling) and cAMP (G_s coupling).

Results:
PROKR2 variants were found in 16 patients (6.5%). Expression levels of variants p.V158I and p.V331M were moderately reduced, whereas they were markedly impaired in the remaining cases, except p.V334M, which was significantly overexpressed. The variants p.T260M, p.R268C, and p.V331M showed no remarkable changes in cAMP response (EC_{50}) whereas the IP signaling appeared more profoundly affected. In contrast, cAMP accumulation cannot be stimulated through the p.L173R and p.V274D, but IP EC_{50} was similar to wt inp.L173R and increased by 10-fold in p.V274D. The variant p.V334M led to a 3-fold increase of EC_{50} for both cAMP and IP.

Conclusion:
Our study shows that single *PROKR2* missense allelic variants can either affect both signaling pathways differently or selectively. Thus, the integrity of both PROKR2-dependent cAMP and IP signals should be evaluated for a complete functional testing of novel identified allelic variants.

Research on the prokineticin system has revealed its involvement in several physiological mechanisms and pathological conditions. The knockout models for both ligand [prokineticin 2 (Prok2)] and the G protein-coupled receptor, Prokr2, revealed a role in olfactory bulb morphogenesis and sexual maturation, indicating *PROK2* and *PROKR2* as strong candidate genes for human GnRH deficiency (1, 2). The involvement of *PROKR2* in the pathogenesis of hypogonadotropic hypogonadism (HH) was first described in 2006 (3) and subsequently reported in several other patients series (4). Idiopathic HH is a rare disease with a complex pathogenesis but a strong genetic component. It is characterized by delayed or absent puberty secondary to gonadotropin deficiency and associated or not with olfactory defects in the Kallmann's syndrome (KS) or in the normosmic isolated HH (nIHH), respectively (4).

Very recently *PROKR2* variants have been also described in patients with idiopathic combined pituitary hormone deficits (CPHDs), including gonadotropin deficiency (5–7). Importantly, all previous in vitro studies on the identified allelic variants were showing variable impairments of PROKR2 function (5, 6, 8–11) either involving the expression level of the variant receptors or their ability to stimulate the Gq-dependent pathway. No direct data are so far available about the effect of these variants on the Gs-dependent cAMP signaling, despite evidence of its activation by nanomolar concentrations of PROK2 (12). Because a variable involvement of diverse transduction pathways by a single genetic variant has been reported for other G protein-coupled receptors (13), we decided to characterize the *PROKR2* genetic variations identified in a large Italian series of idiopathic central hypogonadism (CHg) patients by evaluating both either their direct expression on living-cell membrane and their ability to activate the two signal transduction pathways linked to the PROKR2.

Materials and Methods

Patients

A large cohort of Italian patients with CHg was collected (n = 197 males and 49 females) thanks to the Italian network for idiopathic CHg supported by the Italian Societies of Endocrinology, and of Pediatric Endocrinology and Diabetes. The CHg cohort includes KS (41%), nIHH (54%), or idiopathic CPHD patients (5%). The institutional Ethics Committee approved the study and all patients gave their informed consent for the genetic investigations. The patients' entire open reading frame of the human *PROKR2* gene was amplified by PCR (primers available upon request) and sequenced according to standard protocols.

Expression and bioassays of PROKR2 variants

PROKR2 wild-type (wt) plasmid (Missouri S&T cDNA Resource Center) was used as a template to engineer the tested mutants (see Supplemental Material, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). PROKR2 variant expression was checked by fluorescence-activated cell sorter (FACS), immunocytochemistry and Western blot, whereas cAMP and inositol phosphate (IP)-1 bioassays were performed in transient transfection on human embryonic kidney (HEK)-293 or COS7 cells following previously described protocols (14) with specific modifications (see Supplemental Material).

Statistics

The data presented were from representative experiments. Differences between means were compared using an unpaired Student's *t* test and ANOVA with Dunnett post hoc test.

Results

Genetic analysis

The analysis of the *PROKR2* gene led to the identification of 16 patients carrying exonic variations of 246 CHg patients (6.5%). All the variants were present in the heterozygous state, except in one

case (Table 1). Nine different variants were identified: seven missense substitutions (p.V158I, p.L173R, p.T260M, p.R268C, p.V274D, p.V331M, and p.V334M) and two frame shift deletions generating premature stop codons (p.N15TfsX30 and p.H20MfsX23). Six variants had been previously identified (p.L173R; p.T260M, p.R268C, p.V274D, p.V331M, and p.H20MfsX23) (15, 16). All these variations affect well-conserved residues, and none of these variations was observed in 300 control alleles from the adult general population. The analysis of other candidate genes (*KAL-1*, *FGFR1*, *FGF8*, *GnRHR*, *GnRH1*, *TAC3*, *TAC3R*, *KISS1R*, *CHD7*, *PROK2*, and *SEMA3A*) allowed the identification of a second heterozygous variant in 4 of 16 patients (Table 1).

Clinical characteristics

The main clinical features of the 16 patients with *PROKR2* gene variations are reported in Table 1. No statistically significant correlations were found between the presence/absence of the genetic variations and clinical phenotypes and/or parameters such as LH/FSH and sex steroids levels (data not shown). All patients, but one, experienced a pubertal delay and the reversal of CHg was documented in three. Variable CPHDs were detected in three cases. Familial history revealed the recurrence of anosmia in an aunt of KS-1 carrying the wt-*PROKR2* allele and a pubertal delay in the heterozygous mother of KS-6. The *PROKR2* gene investigations in the families failed to show cosegregation with phenotype, as previously reported (5, 7, 11, 17). In KS-2, nIHH-1, and nIHH-7, the phenotype appeared in the family in association with the biallelic defect.

Functional and molecular studies

The two frame shift variants, p.N15TfsX30 and p.H20MfsX23, led to the generation of a protein lacking all the transmembrane and intracellular receptor domains, thus justifying the classification as mutations with a severe loss of function (LOF).

All missense mutants showed an impaired targeting on the cell surface, exception made for p.V334M, which is more efficiently expressed on the HEK293 membrane compared with the wt (Figure 1, A and E, and Supplemental Figure 1). Both FACS analysis after cell permeabilization and Western blotting showed a similar levels of receptor protein expression (Supplemental Figure 2, A and B), consistent with reproducible transfection efficiencies and the stability of translated proteins.

Two different bioassays were performed. Basal IP1 levels were similar for all PROKR2 variants. Differently, concentration-effect curves showed a variable right-shift modification of the EC_{50} in four tested variants (p.R268C, p.V274D, p.V331M, and p.V334M), whereas p.T260M had a borderline shift of the response curve (Figure 1, B and E) and a normal response was observed for the remaining two variants (p.V158I and p.L173R). The testing of the Gs-cAMP pathway did not reveal difference at baseline in any case but showed normal or statistically significant increase of the EC_{50} for two (p.V158I and p.V331M) or three variants (p.T260M, p.R268C, and .V334M), respectively. The remaining two variants, p.L173R and p.V274D displayed the complete lack of intracellular cAMP response, demonstrating a severe derangement of the Gs coupling (Figure 1, C and E).

Because the variants behave differently in the three biological parameters examined, we considered the generation of a functional score integrating all these results. This score generates the ranking of PROKR2 variants (Supplemental Figure 3), with p.V274D, p.T260M, p.R268C, and p.L173R showing a complete or severe LOF; the remaining missense mutants (p.V331M, p.V334M, and p.V158I) conserve 50%–70% of wild-type activities.

Discussion

We report the frequency of *PROKR2* gene variants in about 6.5% of CHg patients belonging to the

largest Italian cohort so far investigated. This frequency appears comparable with that of the French population (3) but slightly higher in comparison with the US population (9). To understand their pathogenic role, we extensively characterized all the missense variants by testing the dual activation of Gq- and Gs-dependent pathways, and we identified variations with a predominant impact on one or the other downstream signal.

At variance with previous studies (8, 9), we used a direct method to test the expression of the mutated constructs at the cell surface. A hampered membrane targeting was detected in all cases, except the variant p.V334M, which exhibited an enhanced level of expression on the cell membrane. We therefore verified either the capacity for Gq or the Gs signal transduction pathway of wt and mutants (Figure 1, B, C, and E). Although the ability to stimulate the Gq pathway was affected in most of the cases, this function was well conserved for the p.V158I. The mutants p.R268C and p.V274D are both strongly lacking the ability to activate the Gq protein, whereas no activation of cAMP accumulation was detected in cells expressing the p.L173R and p.V274D constructs. Importantly, the severe LOF of the p.V274D mutant on both of the transduction pathways cannot be explained on only the basis of a poor membrane targeting because similar low surface expression levels were seen for both p.V274D and p.L173R mutants, but p.L173R was still able to normally activate the Gq-dependent pathway.

The functional evaluation of the p.L173R mutant represents another clear demonstration of the importance to evaluate the dual PROKR2 signaling. Although the membrane expression and the mechanisms leading to the Gq activation appear only partially compromised, the complete loss of the ability to stimulate the Gs-dependent pathway amplify the pathogenic impact of this particular variant. In addition, structural receptor models (Figure 1D) suggest two main messages on the molecular level for the tested substitutions (see the figure legend for details): 1) mutations like p.V274D [transmembrane helix (TMH)-6] or p.V334M and p.L173R modify the intrinsic signaling capacity of PROKR2 by modifying specific biophysical properties in structural parts participating significantly in receptor activation (eg, TMH6 and TMH7 interfaces); and 2) especially amino acid T260, R268 selectively influence Gq coupling and they are located at the intracellular transitions between intracellular loop (ICL)-3 and TMH5/TMH6, which is a potential direct G-protein contact region.

In light of our findings, the pathogenic role of mutations found in CHg patients can be misclassified if a single pathway is analyzed because the dual-signal ability of PROKR2 appears to be differentially affected by the variations detected in this large CHg cohort. The extensive functional analysis here reported (FACS, IP1, and cAMP assays) were therefore integrated in a formula that generates a score giving a comprehensive characterization of each variant (see Supplemental Figure 3). Then the p.V158I receptor represents a rather benign variation; instead p.L173R and p.V331M generate a severe damage of only one intracellular pathway (either Gq or Gs), whereas both of the transduction pathways were simultaneously affected to variable degrees in the remaining cases, p.V274D being the variant provided with an almost complete LOF.

Because the inactive p.V274D variant was found in the homozygous state in KS-6 case with spontaneous reversal of GnRH neuron function (18), our data indicate that a severe PROKR2 LOF can cause only a partial impairment of GnRH neuron function, which may be overcome by the plasticity of the neuronal network. In addition, the case CPHD-3 is carrying a mutation predicted to generate a truncated protein lacking all the transmembrane domains but came to the physician's attention because of typical hypogonadal manifestations at 58 years. He reported to have spontaneously fathered two daughters and transmitted the same heterozygous PROKR2 mutation to one of them, who spontaneously conceived and very recently delivered one baby. These data are consistent with a report describing the complex inheritance with highly variable penetrance among carriers of the ancient founder mutation p.L173R (17). The whole of these data indicates

that PROKR2 variants may indeed be found as rare polymorphisms in the general population and that the presence of *PROKR2* variations can partially weaken (likely to variable extents, depending on the severity of the functional alteration) the GnRH function and predispose to CHg and reproductive defects, but other genetic or acquired defects may be required to obtain the full-blown isolated HH phenotype. Consistently, the digenic defects were found here only in the KS or nIHH patients.

In conclusion, routine assessment of the impact of PROKR2 variants on both the cAMP and IP pathways is warranted to avoid absolving mutations that selectively compromise one signaling branch while leaving the other intact and thereby to avoid erroneously predicting a reduced risk of disease in the variant-carrying progeny of such patients.

Abbreviations:

CHg	central hypogonadism
CPHD	combined pituitary hormone deficit
FACS	fluorescence-activated cell sorter
HEK	human embryonic kidney
HH	hypogonadotropic hypogonadism
ICL	intracellular loop
IP	inositol phosphate
KS	Kallmann's syndrome
LOF	loss of function
nIHH	normosmic isolated hypogonadotropic hypogonadism
Prok2	prokineticin 2
Prokr2	prokineticin receptor 2
TMH	transmembrane helix
wt	wild type.

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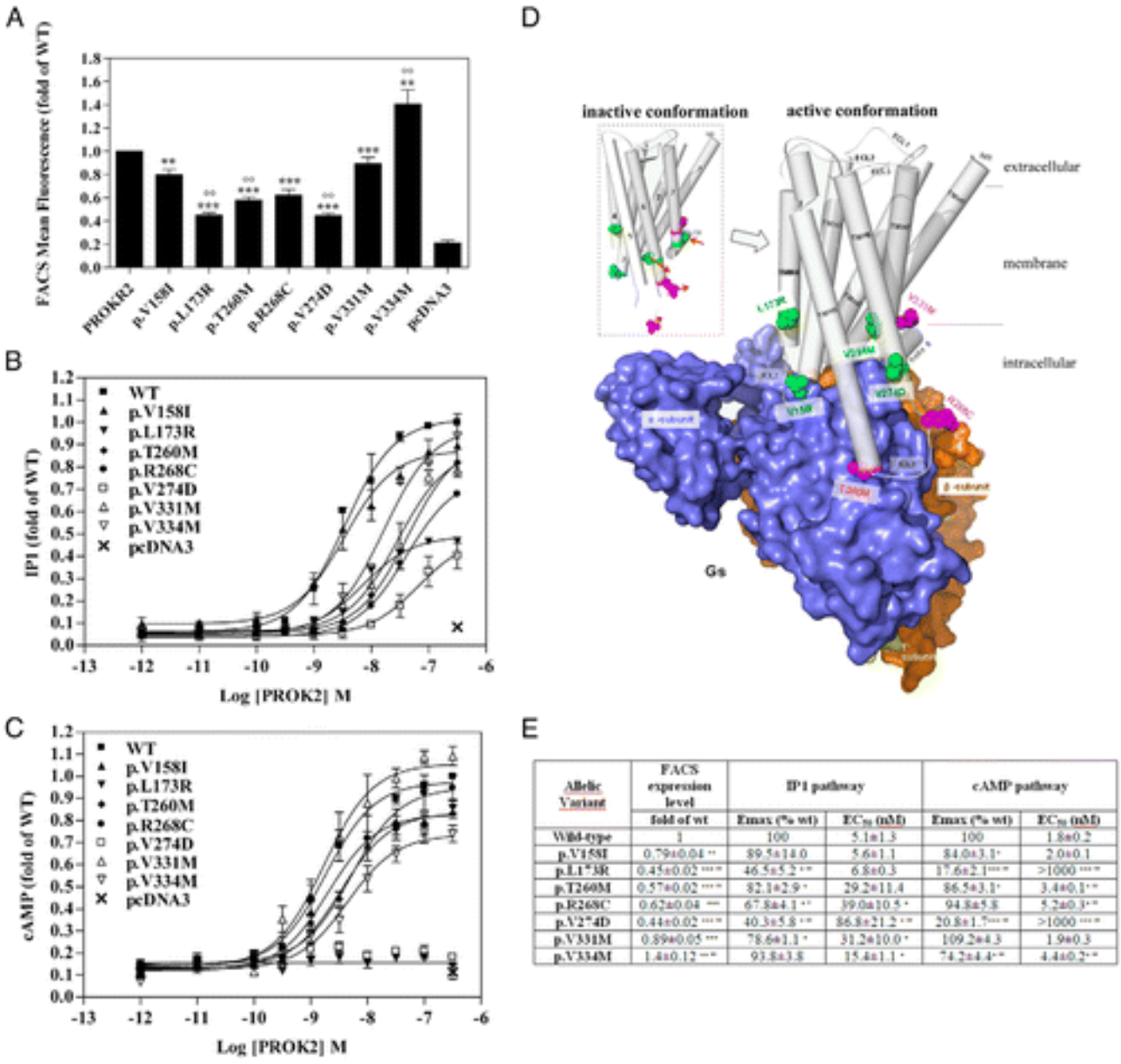
Figure 1.
A, Flow citofluorometry analysis, FACS, of intact HEK293 cells transiently transfected with wt PROKR2 or the identified variants. Results are expressed as fold of wt. The Prism computer program (GraphPad Software, Inc) was used for statistical analysis: Student's t test in comparison with the wt: ***, $P < .0001$, **, $P < .001$; ANOVA with Dunnett post hoc test: °°, $P < .01$, °, $P < .05$. The figure illustrates the integrated results (mean ± SE) of three independent experiments. B and C, Concentration-effect curves for the PROKR2 variants under stimulation of increasing concentrations of human recombinant PROK2. HEK293 cells transiently transfected with the various constructs were stimulated, and intracellular IP1 (B) or cAMP (C) values were determined. The results are expressed as fold of wt. The Prism computer program (GraphPad Software) was used for curve fitting and for EC50 determination. The figure shows the integrated results (mean ± SE) of six independent experiments. D, Homology models of the inactive (boxed window) and active PROKR2 conformations. The receptors (helices and loops) are visualized by cartoon representation (white) and the three subunits of Gs are shown with different colors. On these structural PROKR2 models, wt positions (sticks) of investigated single side-chain substitutions are highlighted. The colors encode specific functional effects of these variants: green, Gs and Gq signaling were both decreased; cyan, slightly effected expression level; magenta, selectively impaired Gq signaling. During receptor activation-transition between inactive and active signaling state, the transmembrane helices (TMHs) 5, 6, and 7 are predicted to undergo significant structural rearrangements (indicated by red arrows) with participation of specific amino acids. Helix 5 is extended in the active conformation compared to the inactive state and TMH6 is shorter at the intracellular site. The mutations described here are located at different spatial receptor regions. I, Variant p.V158I (slightly decreased cell surface expression level) is located at the junction between the transmembrane helix 3 (TMH3) and intracellular loop 2 (ICL2) and sticks into the cytoplasm without interior intramolecular contacts. II, The side chain of substitution p.L173R (positively charged) in TMH4 points toward TMH3 (to A148). This mutant leads to decreased levels of cell surface expression, decreased maximal IP, and impaired cAMP signaling. This supports that the arginine substitution (large, branched, positively charged) is not tolerated in a hydrophobic cage at TMH3 instead of a large and branched hydrophobic wt leucine side chain. III) The selectively inactive (Gq) p.T260M variant is located in the ICL3 but is part of the junction between TMH5 and ICL3 in the active-state conformation with an extended helix 5 that is of importance for receptor/G protein contact and activation. IV) The mutation p.R268C is located at the transition between TMH6-ICL3 and therefore might have a more direct impact on the receptor/Gq contact. V) According to the functional characterization of the p.V274D substitution, V274 is a key player for signal transduction. The drastic change from hydrophobic to hydrophilic side-chain properties likely impedes the helix-movement between TMH5 and TMH6 that is a general prerequisite for G protein activation. VI) Mutants p.V331M and p.V334M were characterized to inhibit Gq signaling, and they are located at the TMH7, close to the intracellular site. In the inactive state, side chains of V331 and V334 point toward the membrane, whereby V334 is predicted to rotate slightly toward TMH1 during receptor activation (Gs). It cannot be excluded that they might participate in intermolecular contacts, as previously suggested (19). Further experiments based on the cotransfection of wt and variant PROKR2 will be worthy to confirm such possibility. Structure images were produced using PyMOL software (The PyMOL molecular graphics system, version 1.3; Schrödinger LLC). E, Summarized data indicating the functional results of the PROKR2 allelic variants. Data are expressed as mean ± SE of three FACS or six independent signaling experiments, respectively. ***, $P < .0001$ vs wt;

**, P < .001 vs wt; *, P < .01 vs wt using Student's t test; °, P < .05 vs wt, °°, P < .01 vs wt using ANOVA with Dunnett post hoc test.



E

Allelic Variant	FACS expression level	IP1 pathway		cAMP pathway	
	fold of wt	Emax (% wt)	EC_{50} (nM)	Emax (% wt)	EC_{50} (nM)
Wild-type	1	100	5.1±1.3	100	1.8±0.2
p.V158I	0.79±0.04 °°	89.5±14.0	5.6±1.1	84.0±3.1°	2.0±0.1
p.L173R	0.45±0.02 °°°	46.5±5.2 °°	6.8±0.3	17.6±2.1°°°	>1000 °°°
p.T260M	0.57±0.02 °°°	82.1±2.9°	29.2±11.4	86.5±3.1°	3.4±0.1°°
p.R268C	0.62±0.04 °°°	67.8±4.1 °°	39.0±10.5°	94.8±5.8	5.2±0.3°°
p.V274D	0.44±0.02 °°°	40.3±5.8 °°	86.8±21.2 °°	20.8±1.7°°°	>1000 °°°
p.V331M	0.89±0.05 °°°	78.6±1.1°	31.2±10.0°	109.2±4.3	1.9±0.3
p.V334M	1.4±0.12 °°	93.8±3.8	15.4±1.1°	74.2±4.4°°	4.4±0.2°°

Table 1. Clinical and Genetic Parameters of the CHg Patients With PROKR2 Allelic Variants

Patients	Sex and Age at Diagnosis, y	cDNA Mutation	Protein Mutation	Zygosity	Associated Gene Variants[a]	MRI Olfactory Structure	Sense of Smell	Familial Manifestations	Phenotypic Details
KS-1	M, 14	c.472 G>A	p.V158I	Hetero	None	Hypoplastic	H	Anosmia in aunt with wt PROKR2	—
KS-2	M, 15	c.518 T>G	p.L173R	Hetero	FGFR1: c.IVS17-6 C>T	Hypoplastic	A	—	—
KS-3	M, 16	c.518 T>G	p.L173R	Hetero	None	Hypoplastic	A	—	—
KS-4	M, 20	c.779 C>T	p.T260M	Hetero	None	Absent	A	—	—
KS-5	M, 18	c.802 C>T	p.R268C	Hetero	None	Absent	A	—	—
KS-6	M, 19	c.820 T>A	p.V274D	Homo	None	Absent	A	Pubertal delay in hetero mother	CHg reversal
nIHH-1	M, 14	c.518 T>G	p.L173R	Hetero	FGFR1: p.Q89R	Normal	N	—	Partial empty sella
nIHH-2	M, 20	c.518 T>G	p.L173R	Hetero	None	Normal	N	—	—
nIHH-3	M, 18	c.802 C>T	p.R268C	Hetero	None	Normal	N	—	—
nIHH-4	M, 12	c.1000 G>A	p.V334M	Hetero	PROK2: p.C383FfsX1	Normal	N	—	—
nIHH-5	F, 16	c.1000 G>A	p.V334M	Hetero	None	Normal	N	—	—
nIHH-6	M, 21	c.56delC	p.H20MfsX23	Hetero	None	Normal	N	—	CHg reversal
nIHH-7	M, 18	c.43insACTTT	p.N15TfsX30	Hetero	GNRHR: p.Q106R	nd	N	—	CHg reversal
CPHD-1	M, 22	c.779 C>T	p.T260M	Hetero	None	Normal	N	—	Adult GHD; OB
CPHD-2	M, 37	c.991 G>A	p.V331M	Hetero	None	Normal	N	—	CHa; T2DM; OB
CPHD-3	M, 58	c.56delC	p. H20MfsX23	Hetero	None	Normal	N	—	Adult-onset CHt; T2DM; OB

Abbreviations: A, anosmia; CHa, central hypoadrenalism; CHt, central hypothyroidism; F, female; GHD, GH deficit; H, hyposmia; Hetero, heterozygosity; M, male; MRI, magnetic resonance imaging; N, normal; nd, not determined; OB, obesity; T2DM, type 2 diabetes mellitus; —, none of interest.
[a]Associated genetic variants found in heterozygosity.