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The *De Novo* Q167K Mutation in the *POU1F1* Gene Leads to Combined Pituitary Hormone Deficiency in an Italian Patient

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ABSTRACT

The *POU1F1* gene encodes a transcription factor that is important for the development and differentiation of the cells producing GH, prolactin, and TSH in the anterior pituitary gland. Patients with *POU1F1* mutations show a combined pituitary hormone deficiency with low or absent levels of GH, prolactin, and TSH. Fourteen mutations have been reported in the *POU1F1* gene up to now. These genetic lesions can be inherited either in an autosomal dominant or an autosomal recessive mode. We report on the first Italian patient, a girl, affected by combined pituitary hormone deficiency. The patient was found to be positive for congenital hypothyroidism (with low TSH levels) at neonatal screening. Substitutive therapy was started, but subsequent growth was very poor, although psychomotor development was substantially normal. Hospitalized at 10 mo she showed hypotonic crises, growth retardation, delayed bone age, and facial dysmorphism. In addition to congenital hypothyroidism, GH and prolactin deficiencies were found. Mutation DNA analysis of the patient's *POU1F1* gene identified the novel Q167K amino acid change at the heterozygous level. The highly conserved Q167 residue is located in the *POU*-specific domain. No mutation was detected in the other allele. DNA analysis in the proband's parents did not identify this amino acid substitution, suggesting a *de novo* genetic lesion. From these data it can be hypothesized that the Q167K mutation has a dominant negative effect. **(*Pediatr Res* 54: 635–640, 2003)**

Abbreviations

CPHD, combined pituitary hormone deficiency
PRL, prolactin
FT_4, free thyroxine
T_4, total thyroxine
u-TSH, ultra thyroid-stimulating-hormone
US, upper segment
LS, lower segment
IGF-BP3, insulin growth factor-binding protein 3
FT_3, free triiodothyronine
POU-S, POU-specific
POU-D, POU-homeo

CPHD is a rare inborn heterogeneous disorder. Two transcription factors, the pituitary-specific transcription factor POU1F1 (MIM 173110, usually named PIT-1) and PROP-1, the Prophet of POU1F1, which is required for POU1F1 expression (MIM 601538), may be implicated. However, several other regulatory factors, some yet unknown, play a crucial role in the pituitary hormones' action, and they can also be the cause of pituitary disease (1–7). *POU1F1* acts as transactivator for GH, PRL, and β-TSH genes in the anterior pituitary gland in combination with other factors (8–11). POU1F1 recognizes the consensus binding sites within the regulation elements of the genes it controls. Deficiency of this transcription factor causes decreased levels of pituitary hormones with hypoplasia of the anterior pituitary and subsequently severe growth retardation (12). The human *POU1F1* gene has been mapped to chromosome 3p11 (13), and contains six exons spanning >17 kb (14). The 1050-bp *POU1F1* cDNA has been cloned (15), and it encodes a protein of 291 amino acids. This protein is characterized by two important regions: POU-S and POU-H domains, both of which are DNA binding regions. Its three-dimensional structure shows four α helices in the POU-S region and three α helices in the POU-H domain (16). The functional form of the human POU1F1 factor is an homodimer formed by interactions between the POU-S domain and the POU-H domain of two symmetrically related monomers (9, 16, 17). Dimerization occurs both by hydrophobic contacts and hydrogen binding between the two helices α_3 and α_4 of the POU-S domain in one monomer and the carboxyl terminus of the POU-H domain in another monomer (16, 17).

Two regulatory regions, which are responsible for transcriptional activation, autoregulation, and cell-specific expression of

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the human *POU1F1* gene, have been identified upstream of the human *POU1F1* gene, one distal (−11.8/−10.9 kb) and one proximal (−7.1/−2.3 kb). The distal regulatory region acts as a silencer, and the proximal region is a strong enhancer (18).

Up to now, 14 different genetic lesions—11 missense (9, 19–26), two nonsense mutations (27, 28), and one deletion (22)—have been reported in the *POU1F1* gene. Figure 1 shows the schematic representation of the *POU1F1* gene and the relative mutations reported in the literature according to the nomenclature of Dunnen and Antonarakis (29). They are spread throughout the gene, usually occurring in exons 4 and 6, and they are inherited either in an autosomal dominant or in an autosomal recessive mode. The most common mutation, reported in patients with CPHD, is the R271W amino acid substitution, which has been demonstrated to have a dominant negative effect. This mutation alters the POU1F1 protein, which binds normally to DNA but inhibits transcription (19). Moreover, this mutation seems to be a hot spot for *POU1F1* mutations (30).

We report clinical and molecular studies on the first Italian patient, a girl, affected by CPHD. Molecular analysis identified a novel mutation (Q167 K), at the heterozygous status, in the patient's *POU1F1* gene.

Table 1. *Summary of endocrinologic evaluations*

	Patient	Normal range
Birth (neonatal screening)		
T_4 (μg/dL)	<0.01	>4
u-TSH (μU/mL)	0.03	<10
10 mo (replacement therapy with L-thyroxine)		
FT_3 (pg/mL)	3.0	1.6–4.8
FT_4 (ng/dL)	1.3	0.8–1.9
u-TSH (μU/mL)	0.055	0.4–4
IGF-I (ng/mL)	<2	3.7–150
IGF-BP3 (μg/mL)	<0.7	1.3–4.25
PRL (mUI/L, basal and after TRH stimulation test)undetectable		98–784
Peak GH concentration after stimulation tests with		
Arginine (ng/mL)	<0.05	>10
Clonidine (ng/mL)	<0.05	>10
GHRH (ng/mL)	<0.05	>10
18 mo (replacement therapy with L-thyroxine, 8 $\mu g \cdot kg^{-1} \cdot d^{-1}$ GH 0.6 $U \cdot kg^{-1} \cdot d^{-1}$)		
FT_3 (pg/mL)	1.8	1.6–4.8
FT_4 (ng/dL)	4.6	0.8–1.9
u-TSH (μU/mL)	0.08	0.4–4
IGF-I (ng/mL)	56.5	3.7–150
IGF-BP 3 (μg/mL)	2.5	1.3–4.25
PRL (mUI/L)	undetectable	98–784

Abbreviation used: GHRH, GH releasing hormone.

METHODS

The study was approved by the Institutional Review Board of the Department of Pediatrics, Florence University, and informed consent was obtained from parents.

Case report. SL, the first daughter of unrelated Italian parents, was admitted to our hospital at the age of 10 mo because of recurrent crises of hypotonia, pallor, drowsiness, and sweating. She was delivered by cesarean section at the 36th week of a first uncomplicated pregnancy. Her Apgar score was 5 and 7 at 1 and 5 min, respectively, her birth weight was 2400 g (35th centile), her length was 44 cm (15th centile), and her cranial circumference was 33 cm (60th centile). Immediately after birth cyanosis appeared and resuscitation procedures were instituted; neonatal screening led to suspected congenital hypothyroidism (Table 1), with the demonstration of a normal thyroid gland on ultrasound and no ^{99m}Tc-Pertec uptake on thyroid scintigraphy. Replacement therapy with L-thyroxine sodium (5 $\mu g \cdot kg^{-1} \cdot d^{-1}$) was started, but, in spite of this treatment, subsequent growth was very poor, although psychomotor development was substantially normal. Chromosome analysis was normal (46XX).

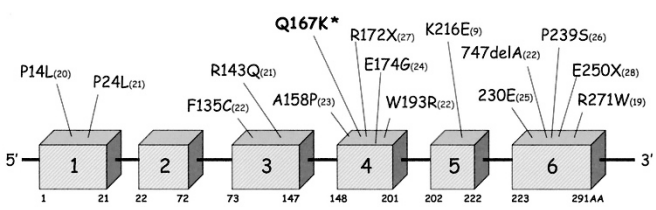

Figure 1. Schematic representation of the *POU1F1* gene listing the new and the known mutations identified. The new mutation is indicated with an asterisk. Designation of the new and previously published *POU1F1* gene mutations corresponds to the nomenclature of Dunnen and Antonarakis (29). Numbers in parentheses indicate reference citations.

Clinical examination on admission showed a prominent forehead, a depressed nasal bridge, anteverted nostrils, bluish sclerae, and a small facial skull: length was 56 cm (−5.25 SD), with an evident disproportion between the US and the LS, owing to an abnormal prevalence of the former (US/LS = 2.2, normal for age 1.6); weight was 4600 g (below the 3rd percentile), and head circumference was 41.5 cm (−2 SD).

Blood and urine routine analyses were normal, but several remarkable nonketotic hypoinsulinemic hypoglycemic events (minimum 36 mg/dL) were detected: cortisol, as well as FT_3 and FT_4 (Table 1), was normal for the patient's age. Basal LH and FSH levels (<0.7 and 0.6 mIU/mL, respectively) were low, but the GnRH stimulation test showed normal secretion patterns of both hormones (LH peak, 6.51 mUI/mL; FSH peak, 17.9 mUI/mL).

u-TSH was low and basal PRL was undetectable; moreover, basal IGF-I and IGF-BP3 were under normal range (Table 1). Bone age was between 0 and 3 mo, according to Greulich and Pyle (30a).

At magnetic resonance imaging the anterior pituitary height was 2.5 mm, at the very lower limit of normal size (31). The signal of the posterior pituitary gland showed a normal location. EEG was normal.

hGH stimulation tests (arginine, clonidine, and growth hormone releasing factor) all showed a total unresponsiveness, and a similar result was obtained by the TSH releasing hormone test, performed to evaluate TSH and PRL secretion patterns (Table 1).

Substitutive hGH therapy (0.6 UI/d) was begun, together with an increase of daily L-thyroxine sodium dosage up to 8 μg/kg with a complete and immediate disappearance of hypoglycemic events and a normalization of IGF-I and IGF-BP3 levels (Table 1).

After 6 mo follow-up height was 71 cm (−3.6 SD, with a mean monthly gain of 2.5 cm); US/LS ratio was 1.6 (normal for age, 1.52); weight was 6850 g; bone age (12 mo, chronologic age of 18 mo) showed a remarkable recovery.

Genomic DNA analysis of the* POU1F1 *gene. Genomic DNA was isolated from the patient's peripheral blood lymphocytes and from her parents' lymphocytes.

The genomic fragments covering all six exons and their flanking introns were amplified using a set of primers located in flanking intronic region sequences, (Table 2). PCR amplification was performed under the following conditions: denaturation at 94°C for 3 min followed by 30 cycles with denaturation at 94°C for 1 min, annealing temperature of 60°C for 1 min, and extension at 72°C for 1 min. The reaction mixture was carried out in a total volume of 25 μL containing 200 ng of template DNA, 2.5 U of Ampli*Taq* DNA polymerase (Polymed, Sambuca-Firenze, Italy), 30 pmol of each forward and reverse primer, 2.5 mM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, and 0.01% gelatin. To facilitate the amplification of exon 5, first a nested PCR with primers 4F and 6R was performed, followed by the 5F-5R amplification.

Genomic DNA analysis of the* PROP-1 *gene. The three exons and intronic boundary sequences of the *PROP-1* gene were amplified by PCR using primer and PCR conditions reported by Deladöey *et al.* (32).

DNA sequencing. PCR products were run on a 2% agarose gel containing ethidium bromide, and DNA was visualized with an UV transilluminator. DNA fragments were excised and purified using QIAQuick Gel Extraction kit (QUIAGEN, Hilden, Germany). Approximately 100 ng of purified amplifications was used in sequencing reactions. Both strands were sequenced with the same primers used for PCR amplification. The sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Ready Reaction Kit reagents (Applied Biosystems, Foster City, CA, U.S.A.). The reactions were run on an ABI 310 sequencer (Applied Biosystems) and were analyzed using Sequencing Analysis software, version 3.3. Full-length *POU1F1* and *PROP-1* DNAs and intron/exon boundaries were sequenced in both strands.

Restriction site analysis. To confirm the new mutation Q167K, a modified reverse genomic primer 167MUT CTGCAGATTTTCAAATCGGCAGATTGTTGTTC, which contained the c500A>G nucleotide substitution, was used, to generate a new *Taq*I restriction site. Four microliters of PCR product of fragment 167MUT-4F (Table 2) was incubated for 1 h at the temperature indicated by the manufacturer's instructions, with a reaction mixture containing 2 μL of 10× reaction buffer and 5 U of *Taq*I restriction enzyme. The total volume was brought up to 20 μL. Digested products were analyzed on a 2% agarose gel.

RESULTS

Genomic analysis of the* POU1F1 *gene. Mutation analysis of the *POU1F1* gene was performed using genomic DNA sequencing and restriction enzyme in the patient, her parents, and control subjects.

The new Q167K mutation, which is caused by a C to A (c499C>A) transversion in exon 4, was found in the patient's genomic DNA. The mutation was present at the heterozygous level, and no other genetic lesion in the *POU1F1* gene was detected. The direct sequence performed in the patient's parents' DNA did not show any genetic lesion in their alleles, demonstrating that this mutation has occurred *de novo*.

Restriction analysis using the PCR fragment carrying an artificial amplification-created restriction site was performed. The c499C>A base change destroys a *Taq*I restriction site introduced by PCR amplification with the modified primer 167MUT. After *Taq*I digestion, the normal 197-bp sequence was cut into fragments of 161 bp and 33 bp, whereas the mutant fragment remained uncut (Fig. 2). Enzymatic digestion was used to screen 200 control chromosomes from unrelated individuals, and a benign polymorphism for the changed amino acid (Q167K) was excluded.

Genomic analysis of the* PROP-1 *gene. The *PROP-1* gene, which could be the cause of the CPHD phenotype, was also sequenced to exclude the possible presence of a genetic lesion responsible, in combination with the Q167K mutation in the *POU1F1* gene, for the patient's phenotype.

Genomic analysis of the *PROP-1* gene on the patient's DNA was performed, and all three exons of this gene were sequenced. No mutations in the entire coding sequence of the *PROP-1* gene and in the exon/intron boundaries were identified. However, two known polymorphisms were identified by direct sequencing: an intronic polymorphic site located at nucleotide 109+3 of intron 1 (IVS 1+3) and a T to C transition at the nucleotide 27 position (c27 T>C), which did not introduce any amino acid substitution (A9A) (32, 33). Both polymorphisms were detected at the homozygous level.

DISCUSSION

We report a female patient in whom the diagnosis of congenital hypothyroidism was made at neonatal screening. Low

Table 2. *Sequence of genomic primers used in PCR amplification*

Size	Exon	Name	Primer forward	Name	Primer reverse
330	1	1F	CTCAGAGCCTTCCTGATGTATA	1R	TCAAGATTCAAAGCATTCATTCTG
340	2	2F	CGAATGTGTCTTGAATCCTTATAC	2R	GAGCAGGAAACAAGAAGTGGTG
560	3	3F	GCTCTGGAGAAGGTAGAAGACA	3R	AACTACGTCCACAGTAGAGATG
391	4	4F	GTGTGTAATAGTTGACAAAGATAC	4R	CATCTCAAAGAGAAAAGGCGG
392	5	5F	CTGCGTTGAGATTTTTCTCTAAGG	5R	GACTGGTCTCGAGCTCCTGAC
651	6	6F	CCGTGACTCTCGTGTAACTCT	6R	AAAATAGATAATGTGGCTTCTGAG

The primer positions refer to the nomenclature published by Dunnen and Antonarakis 29.

* The 5 exon was obtained using 1 μL of the first PCR product as a template made with primers 4F and 6R.

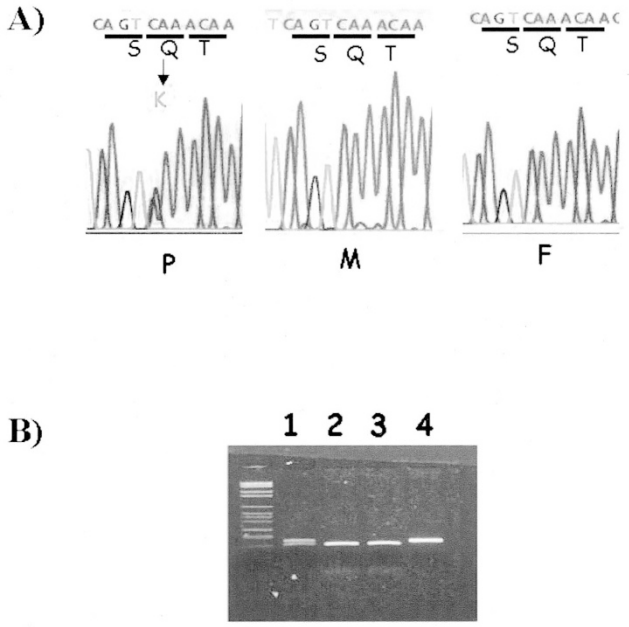

Figure 2. Molecular analysis of family's DNA. *A,* partial nucleotide sequence of the patient's *POU1F1* gene showing the new c499C>A transversion leading to Q167K amino acid substitution identified at heterozygous level. The direct sequence performed in the patient's parents' DNA did not identify any genetic lesion, showing that this mutation has occurred *de novo* (P, patient; M, mother; F, father). *B,* enzymatic restriction analysis for detection of Q167K using genomic DNA. A *Taq*I restriction site was generated in the wild-type PCR fragment using the 4F-167Mut primers. *Lane 1,* Patient; *Lane 2,* Mother; *Lane 3,* Father; *Lane 4,* PCR control not digested.

values of basal T_4 with a low u-TSH level led to the diagnosis of secondary hypothyroidism. Hormonal replacement therapy with L-thyroxine sodium allowed normal psychomotor development, but severe growth retardation was not sufficiently taken into consideration. She was referred to our clinic at the age of 10 mo, after a first episode of hypotonia, pallor, drowsiness, and sweating. During hospitalization, other hypotonic crises with concomitant hypoglycemia occurred. These episodes, as well as growth failure and low TSH levels, led us to suspect CPHD, which was confirmed by the endocrinologic evaluations (Table 1). Among reported CPHD patients central hypothyroidism varies in severity and age of onset. However, in many cases hypothyroidism was found before GH deficiency (34).

The size of the pituitary gland at magnetic resonance imaging was at the lower limit of the normal range. In CPHD patients, the size of the pituitary can vary enormously from small to normal to enlarged (4, 35, 36). In the *POUF1* defect anterior pituitary size is generally small or normal (36). However, it has also been hypothesized that the hypoplasia of the pituitary gland could be a late-onset and age-dependent manifestation (37),.

Several transcriptional factors (POU1F1, PROP-1, LHX3, PTX2, HESX1, and so forth) are involved in the development of the pituitary gland and in the proliferation of pituitary cell populations (9). A deficiency in one of these factors leads to the CPHD phenotype. Most molecular studies carried out in patients affected by CPHD with GH, PRL, and TSH deficiencies have shown genetic lesions in the *PROP-1* gene (20, 32, 36), even if Brown *et al.* (38) reported that approximately half of CPHD cases can be attributed to mutations in the *POU1F1* gene.

To clarify a possible molecular defect leading to CPHD in this patient, the *POU1F1* gene was sequenced. A novel missense mutation, Q167K, of the *POU1F1* gene at the heterozygous level was identified. The mutation was not detected in the patient's unaffected parents.

Comparison with other proteins of the POU family in several species has shown that the Q167 amino acid is a highly conserved residue (Fig. 3). This amino acid is the first residue of the third α helix (α_3) of the POU-S domain. In particular, the three residues, Q167, T168, and R173, lying within helix α_3 of the POU-S domain, form hydrogen bonds with DNA base pairs (16). Crystallographic analyses have shown that this helix α_3 has the most contacts with the major grooves of DNA in the consensus binding sites of the target genes (16, 17). The Q167K causes a change in polarity. A neutral glutamine residue is changed with a basic lysine residue, and the decreased hydrophobicity could affect the correct protein folding.

In our patient, despite the sequencing of the entire coding region and the exon/intron boundaries, the only Q167K genetic lesion was detected at the heterozygous level.

Because only one mutation at the heterozygous status was identified in the *POU1F1* gene, and taking into account that mutations in the human *PROP-1* gene lead to a form of CPHD with the same symptomatology before the age of puberty (39), the patient's *PROP-1* gene was analyzed. Direct sequencing of the *PROP-1* gene did not show any genetic alteration associated with the patient's CPHD phenotype. The *PROP-1* gene is required for *POU1F1* activation, and its deficiency involves the production of GH, PRL, and TSH hormones and later of FSH and LH. According to the molecular data, the patient's gonadotropin secretions, in spite of low basal levels, were normal, as demonstrated by the gonadotropin releasing hormone stimulative test. Mutations in the *POU1F1* gene, reported in the literature, can cause CPHD of both autosomal recessive and dominant inheritance. Up to now, 14 different genetic lesions have been reported in the *POU1F1* gene, and four of them (K216E, R271W, P14L, and P24L) have been shown to be inherited as a dominant trait (9, 19–21). The pathogenic mechanism of dominant inheritance has been extensively explored for only two mutations, R271W and K216E (9, 19). R271W occurs near the end of the POU-H domain. This mutant protein shows a greater affinity for DNA binding sites of the target genes than the wild-type protein. Therefore,

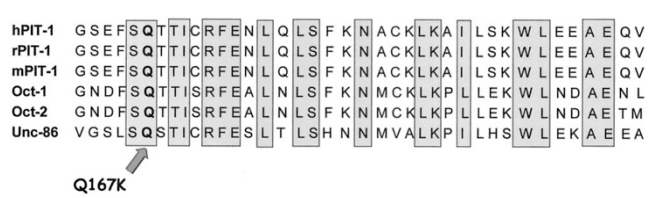

Figure 3. Alignment of a POU protein fragment with similar domain. hPIT-1 (*POU1F1* human), rPIT-1 (rat), mPIT-1 (mouse), Oct-1 and Oct-2 (human transcription factors), and Unc-86 (POU protein of the *Caenorhabditis elegans*). Amino acids that are highly conserved are boxed.

the dominant mechanism, probably caused by competitive inhibition of the mutant homodimer and/or the mutant/wild type heterodimer as opposed to the wild-type homodimer, leads to a block in transcription (19). The other well-characterized dominant effect has been studied for the K216E mutation (9). The K216E mutant protein acts on the distal *POU1F1* gene promoter, which contains the retinoic receptor binding site and the POU1F1 protein binding site. The latter is necessary for autoregulation at negative feedback. The activation of transcription needs both factors as well as retinoic acids. The K216E mutant protein is unable to form a complex with retinoic acid and its receptor, causing a block of the *POU1F1* gene transcription. The decreased levels of *POU1F1* mRNA and subsequently of the POU1F1 protein give rise to a CPHD phenotype (9).

Based on these data and considering that a dominant mechanism can occur in this gene, we hypothesize that the Q167K amino acid substitution in the *POU1F1* gene causes a dominant molecular defect. This defect can be responsible for a *POU1F1* expression reduction by inhibition of the *POU1F1* autoregulation or a decrease in DNA binding affinity. This genetic lesion must be considered pathogenic as it was not detected in 200 normal chromosomes. Besides, the Q167K involves a highly conserved amino acid (Fig. 3), located within the important third α helix of the POU-S domain. The absence of this mutation in the patient's parents supports the hypothesis of a *de novo* mutation with a dominant negative effect.

Nevertheless, a second genetic lesion outside the *POU1F1* coding sequence or intron/exon boundaries tested (promoter, enhancer, and so forth) or a deletion larger than one of the sequenced fragments cannot be excluded. Moreover, a combined effect by an alteration in another correlated gene, even if it has never been reported in CPHD cases, could be possible.

CONCLUSIONS

In conclusion, we report a patient affected by CPHD, in whom central hypothyroidism was detected at neonatal screening thanks to simultaneous TSH and T_4 determination. This allowed early replacement therapy, with a subsequent normal psychomotor development. Nevertheless, GH deficiency caused hypoglycemic crises and severe growth retardation. Further biochemical and molecular investigations led to a CPHD diagnosis owing to a defect of the transcriptional factor POU1F1 with a possible dominant effect. In patients with central congenital hypothyroidism, attention should be paid to linear growth to avoid a late diagnosis of GH deficiency. Moreover, a GH and ACTH evaluation is advisable because hypoglycemia and subsequent brain damage may occur in young children before failure to thrive.

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