Central Precocious Puberty in a Girl and Early Puberty in Her Brother Caused by a Novel Mutation in the *MKRN3* Gene

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Context: Central precocious puberty (CPP), defined as the development of secondary sex characteristics prior to age 8 years in girls and 9 years in boys, results from the premature activation of the hypothalamic-pituitary-gonadal axis. Mutations in the imprinted gene *MKRN3* have been recently implicated in familial cases of CPP.

Objective: The objective of the study was to uncover the genetic cause of CPP in a family with two affected siblings.

Design and participants: The entire coding region of the paternally expressed *MKRN3* gene was sequenced in two siblings, a girl with CPP and her brother with early puberty, their parents, and their grandparents.

Results: A novel heterozygous missense variant in the *MKRN3* gene (p.C340G) was detected in the two affected siblings, their unaffected father, and the paternal grandmother. As expected, the mutated allele followed an imprinted mode of inheritance within the affected family. In silico analysis predicts the mutation as possibly damaging in all five software packages used. Furthermore, structural alignment of the ab initio native and mutant MKRN3 models predicts that the p.C340G mutation leads to significant structural perturbations in the 3-dimensional structure of the C3HC4 really interesting new gene motif of the protein, further emphasizing the functional implications of the novel *MKRN3* alteration.

Conclusions: We report a novel MKRN3 mutation (p.C340G) in a girl with CPP and her brother with early puberty. MKRN3 alterations should be suspected in all cases with familial CPP or early puberty, especially if male patients are also involved or the precocious puberty trend does not follow the usually observed mother-to-daughter inheritance. **(*J Clin Endocrinol Metab* 99: E647–E651, 2014)**

Central precocious puberty (CPP), defined as the development of secondary sex characteristics prior to the age of 8 years in girls and 9 years in boys, results from the premature activation of the hypothalamic-pituitary-gonadal axis. The meticulous study of CPP as a naturally occurring prototype has recently offered new insights into the mechanisms involved in puberty initiation. Teles et al (1) first identified a dominant kisspeptin receptor (KISS1R) mutation (p.R386P) leading to the prolonged activation of the intracellular signaling pathway and therefore CPP in a girl (2). After this report, Silveira et al (3) identified two missense mutations in the kisspeptin gene in patients with CPP, one of which (p.P74S) ensured prolonged protein action due to resistance to degradation.

In contrast to the activating mutations in the stimulatory pathway of the onset of puberty (ie, kisspeptin and KISS1R), Abreu et al (4) recently reported mutations in a factor that seems to exert an inhibitory effect on the ini-

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Abbreviations: CPP, central precocious puberty; KISS1R, kisspeptin receptor; MKRN3, makorin ring finger protein 3; PWS, Prader-Willi syndrome; RING, really interesting new gene; RMSD, root mean square distance; SNP, single-nucleotide polymorphism.

tiation of puberty: the product of the makorin ring finger protein 3 (*MKRN3*) gene. In the present study, we report two siblings with CPP associated with a novel mutation of the *MKRN3* gene.

Patients and Methods

Two siblings of Greek origin, a girl and a boy, were examined at age 7.1 years [bone age 10.5 y, height 128 cm (SD score 1.3, target height 160 ± 4.5 cm)] and 9.2 years [bone age 10 y, height 130.5 cm (SD score 1, target height 173 ± 4.5 cm)], respectively. At presentation, the girl showed breast development Tanner stage 3 and pubic hair Tanner stage 1 (breast development first noticed at age 6 y), and the boy had testes size 4 mL and pubic hair Tanner stage 1. Neither sibling was obese: body mass index in the girl was 18.9 kg/m^2 (75th percentile) and in the boy 17.5 kg/m^2 (25th to 50th percentile). Hormonal data indicated the diagnosis of CPP (Table 1) (5). Expected prepubertal levels for basal and peak levels of LH both in boys and girls are less than 0.15 and 5 IU/L, respectively (5). We consider prepubertal levels for T and estradiol to be less than 12 ng/dL and 15 pg/mL, respectively. The values of thyroid hormones and adrenal steroids were within normal limits for both siblings. Brain magnetic resonance imaging did not disclose any abnormalities in either child.

Due to the familial nature of the disorder, a genetic cause for the CPP was suspected (6). We focused our study on the *MKRN3* gene including the extended family. The study was approved by the Ethics Committee of the "Aghia Sophia" Children's Hospital. For the genetic analyses, assent was obtained from the children, and informed consent was obtained from the parents of the two siblings and the adult family members.

DNA sequencing and mutational analysis of genomic DNA

Genomic DNA was isolated using the Maxwell 16 Instrument (Promega). Specific amplification of the intronless *MKRN3* gene was performed in two overlapping fragments by PCR using the following pairs of primers: GAGATGCACACTTCCCCCAG, TCTCCCCACGAAAGCAAACTCC and TTGAGTTTGTTC-CAGGGCAGC, CAGAAGCACTGCCTCAACAGC. The PCR products were purified (ExoSAP-IT reagent; Affimetrix-USB Products) and sequenced bidirectionally (ABI 3500; Applied Biosystems), using the same primers as for the PCR. Reference sequence was obtained from Ensembl (ENST00000314520).

Nonsynonymous single-nucleotide polymorphism (SNP) in silico analysis

For the prediction of the pathogenic nature of the nonsynonymous SNP substitution found and its evolutionary conservation, an in silico analysis was performed using the following software packages: Mutation taster (http://www.mutationtaster.org/); SNPs&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html); PolyPhen-2 (Polymorphism Phenotyping version 2; http://genetics.bwh.harvard.edu/pph2/); Sorting Intolerant From Tolerant (SIFT Human Protein; http://sift.jcvi.org/www/SIFT_enst_submit.html); and CONsensus DELeteriousness score of missense SNVs (Condel) (http://bg.upf.edu/condel/home).

Ab initio modeling

I-Tasser was used for the ab initio modeling of the native and the mutated MKRN3 deduced amino acid sequences of the novel p.C340G and the previously reported p.R365S (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (4, 7–9). Predicted models were evaluated using http://modbase.compbio.ucsf.edu/modeval/ for discrete optimized protein energy and root mean square distance (RMSD) score (used to measure average distance between the backbones of the superimposed proteins), whereas the TM score (used to assess topological similarity) was calculated using TM-Align (10–12). The chosen 3-dimensional structures of the native and the mutated structures were further analyzed using PyMOL molecular graphics system (DeLano Scientific; http://pymol.sourceforge.net/).

Results

Pertinent clinical and hormonal data are depicted in Table 1.

Table 1. Clinical and Hormonal Data of the Two Siblings With the p.C340G MKRN3 Mutation: A Girl With CPP and Her Brother With Early Puberty

						GnRH Test					
						LH		FSH			
Sex	Age, y	Bone Age, y	Height	Breast	Testes	Basal	Peak	Basal	Peak	T	E2
F	7.1	10.5	128	Tanner 3	–	1.3	38.8	3.8	25	2	23
M	9.2	10	130.5	–	4	0.7	13.9	1.3	3.6	41	–

Abbreviations: E2, estradiol; F, female; M, male. Age and bone age are reported in years, height is reported in centimeters, testes volume is reported in milliliters, LH and FSH are reported in international units per liter, T is reported in nanograms per deciliter, and estradiol is reported in picograms per milliliter. GnRH stimulation test was conducted using 100 μg of a GnRH and sampling was done at 0, 30, and 60 minutes. Dashes indicate that data are not applicable. Expected prepubertal levels for basal and peak levels of LH both in boys and girls are less than 0.15 IU/L and 5 IU/L, respectively (5). We consider prepubertal levels for T and estradiol to be less than 12 ng/dL and 15 pg/mL, respectively.

DNA sequencing disclosed a novel heterozygous missense variant in the intronless *MKRN3* gene (c.1018T>G, TGT>GGT, p.C340G) in the two affected siblings, their father, and the paternal grandmother. All other family members who participated in our study (mother and both maternal grandparents) carried nonmutated alleles (Figure 1, A and B).

Nonsynonymous SNP in silico analysis

Software packages used for the in silico analysis predicted the pathogenicity of the p.C340G alteration of MKRN3 as follows (prediction in parentheses): PolyPhen-2 (damaging), Mutation Taster (disease causing), SIFT (damaging), SNPs&GO (disease), and Condel (deleterious).

Ab initio Modeling

For the novel p.C340G, the TM score of the alignment was 0.86795 and the RMSD value was 2.81, indicating potential pathogenicity for the identified missense mutation. RMSD values greater than 0.15 are considered as an indicator for significant structural perturbations, which could have functional implications for the protein (13). The 3-dimensional effect of the p.C340G mutation [located in the C3HC4 really interesting new gene (RING) motif of the MKRN3 protein)] was defined using ab initio modeling. As shown in Figure 1, C and D, the alignment of the ab initio native and mutant models reveals that the mutation structurally disrupts the tertiary protein structure. For the previously described p.R365S, the TM score was 0.87352 and the RMSD value was 3.13 (comparison with native and p.C340G in Figure 1, E–G) (4).

Discussion

Idiopathic central precocious puberty is a rather common endocrine problem, especially in girls, with a percentage as high as 27.5% being familial (6). Nevertheless, in extremely few cases has the underlying molecular defect been disclosed. Specifically, CPP has been linked to mutations in three genes: *KISS1*, *KISS1R*, and *MKRN3* (1, 3, 4). The latter was recently reported by Abreu et al (4) in an elegant whole-exome sequencing study that uncovered three frameshift mutations and one missense mutation (p.R365S) that was expected to be "probably damaging" on the basis of PolyPhen2 prediction software. Our study in two siblings (a girl with CPP and a boy with early puberty) revealed a novel missense *MKRN3* mutation in both patients, their father, and the paternal grandmother (Figure 1, A and B). The mutation (c.1018T>G, TGT>GGT, p.C340G) resides in the C3HC4 RING motif of MKRN3, which is responsible for ubiquitin ligase ac-

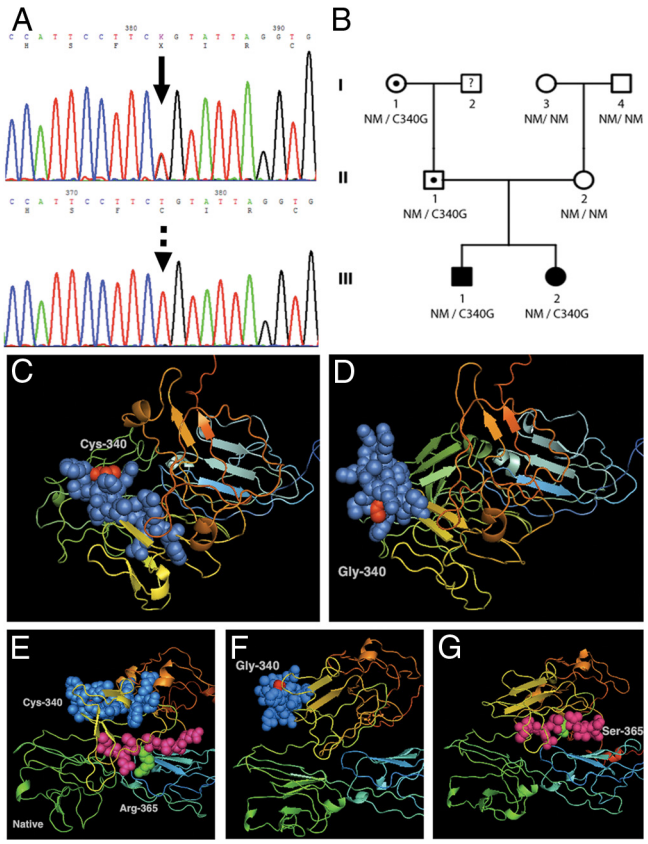

Figure 1. A, Part of the sequencing chromatographs of the *MKRN3* gene showing the novel heterozygous missense mutation (c.1018T>G, TGT>GGT, p.C340G) detected in the two siblings, a girl with CPP and a boy with early puberty (above). The same mutation was uncovered in the father and the paternal grandmother. Solid arrow points to the T>G transversion. The nonmutated *MKRN3* alleles of their mother are depicted below. Broken arrow points to the wild-type nucleotide at c.1018. B, Pedigree of the family with the novel p.C340G MKRN3 mutation. Squares indicate male family members, circles female family members, black symbols the two siblings with CPP (girl) and early puberty (boy), symbol with black point inside the asymptomatic carriers, and the symbol with question mark family member whose DNA was not available. NM, nonmutated allele. *MKRN3* is a maternally imprinted gene, and therefore, one can be affected only if his/her paternal allele is mutated. In the family described herein, the unaffected paternal grandmother conveyed the p.C340G allele to the unaffected father, and subsequently he conveyed the mutant allele to his two children, who developed CPP (girl) and early puberty (boy). Native (C) and mutant (D) p.C340G structure of the produced MKRN3 protein using ab initio modeling (please see *Patients and Methods* and *Results* for details). The residues 334–347 are depicted as blue spheres. The red spheres represent the residue at position 340: Cys in the native structure and Gly in the mutant structure. Images are focused in the affected area. According to the prediction model, the mutation disrupts the 3-dimensional structure of the protein and therefore affects protein function: note that the residues-spheres lay in a line in the native structure and form a curved ball in the mutant structure. Also note marked changes in areas in green color. A different point of view of the predicted 3-dimensional protein structures is chosen to allow direct visual comparison between the native MKRN3 protein product (E) and the novel p.C340G (F) and the previously reported p.R365S (see Reference 4) (G) mutants. Both p.C340G and p.R365S are missense mutations residing in the same MKRN3 domain (C3HC4 RING motif). In the native and p.C340G structures, the residues 334–349 are depicted as blue spheres with the residue at position 340 highlighted as red spheres (Cys in the native

tivity. The p.C340G variant described herein is predicted to disrupt the protein function by all five software packages used (see *Patients and Methods* and *Results* for details). Moreover, the structural alignment of the ab initio native and mutant MKRN3 models predicts that the C340G mutation leads to significant structural perturbations in the 3-dimensional structure of the C3HC4 RING motif, further supporting the functional implications of the novel *MKRN3* mutation (Figure 1, C-D, and comparison with previously described p.R365S in Figure 1, E–G) (13). Nevertheless, specific functional relevance of the p.C340G mutation depends on further in vitro studies.

It has been demonstrated that there is a striking reduction in MKRN3 levels immediately before puberty in the arcuate nucleus of mice (4). Hence, it is possible that the mutation herein described leads to MKRN3 deficiency, mimicking, albeit in an untimely manner, the waning inhibitory milieu that normally leads to GnRH pulsatile secretion and therefore premature initiation of puberty.

MKRN3 is located on chromosome 15q11.2, in the Prader-Willi syndrome (PWS) critical region, and is unmethylated on the paternal but methylated on the maternal allele (14). Due to maternal imprinting, only the paternal allele is expressed, and therefore, CPP can be expected only if the mutated (or deleted) *MKRN3* allele comes from one's father, through maternal uniparental disomy, or from chromosomal translocations (15, 16). In accordance with the expected mode of inheritance, our two heterozygous patients with CPP received the mutated gene from their father. Nevertheless, their father did not enter puberty prematurely. In fact, he recalls as "entering puberty late with respect to his classmates." Our study revealed that the father inherited the mutated *MKRN3* allele from his mother and was therefore expected to be an asymptomatic carrier (Figure 1B).

Since *MKRN3* mutations cause CPP, one would expect that the deletion of the entire gene, as in the case of PWS (del15q11–13), would exert the same effect. However, PWS patients are usually characterized by incomplete, delayed, or disturbed pubertal development, which has been attributed to hypothalamic dysfunction. Therefore, PWS is an inadequate model for *MKRN3* deletions. Nevertheless, it must be underlined that there have been rare reports of CPP in PWS patients that can be attributed to the variability in the deletion spectrum observed within PWS patients (17–18). Interestingly, in a patient with a paternal deletion of the *MKRN3*, *MAGEL2*, and *NDN* genes, CPP was documented (19).

In general, studies of familial cases of idiopathic CPP aiming at investigating the mode of inheritance have suggested an autosomal dominant transmission with incomplete, gender-dependent penetrance (6). Indeed, proven CPP-causing mutations in the *KISS1* and *KISS1R* genes are inherited as a dominant trait. Moreover, CPP is far more frequent in girls than in boys both in sporadic (93%) and familial cases (97.6%) (6). In contrast, CPP caused by *MKRN3* mutations follows an imprinted mode of inheritance and seems to have an equal gender distribution (4). Nevertheless, although the number of reported MKRN3 cases in the study by Abreu et al (4) is small, it must be noted that the MKRN3 alterations seem to affect girls more severely than boys: the median age at the onset of puberty was −2.25 years in girls and only −0.9 years in boys with respect to the lower age limit for normal onset of puberty (ie, 8 and 9 y, respectively). This seems to hold true in our two patients: the girl entered puberty at a relatively younger age than her brother with respect to what is normally expected for each gender. Her breast development was first noticed by her parents as early as 6 years, whereas puberty initiation in the boy could be designated as early (and not precocious) at evaluation (age 9.2 y). Therefore, gender dimorphism, which characterizes the physiological pubertal process, is also manifested in the MKRN3 defect cases (20).

Conclusions

The study of unique natural prototypes and deviations has helped disentangle and understand complex physiological processes. The study of patients with idiopathic familial central precocious puberty recently uncovered the role of MKRN3 in puberty initiation. Herein we report a novel MKRN3 mutation (p.C340G) in two siblings, a girl with CPP and a boy with early puberty, further expanding the mutational spectrum and confirming the imprinted mode of inheritance. MKRN3 mutations affect both genders equally but seem to have a greater effect on girls with respect to the timing of puberty. MKRN3 alterations should be suspected in all familial CPP and early puberty cases, especially if male patients are also involved or the precocious puberty trend does not follow the usually observed mother-to-daughter inheritance.

Figure 1 (Continued). structure and Gly in the mutant structure). In the native and p.R365S structures, the residues 361–369 are depicted as magenta spheres with the residue at position 365 highlighted as green spheres (Arg in the native structure and Ser in the mutant structure). Images are focused in the affected area. The two mutations disrupt the 3-dimensional structure of the protein in a different manner with respect to the native structure. Nevertheless, predicted 3-dimensional changes cannot predict functional difference and allow direct functional comparison of the mutant structures.

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