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2021

Varimo , T , Iivonen , A-P , Känsäkoski , J , Wehkalampi , K , Hero , M , Vaaralahti , K ,
Miettinen , P J , Niedziela , M & Raivio , T 2021 , ' Familial central precocious puberty : two
novel MKRN3 mutations ' , Pediatric Research , vol. 90 , pp. 431-535 . <https://doi.org/10.1038/s41390-020-01270-z>

<http://hdl.handle.net/10138/340896>

<https://doi.org/10.1038/s41390-020-01270-z>

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Familial central precocious puberty: two novel *MKRN3* mutations

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Funding: This work was supported by the Foundation for Pediatric Research, Sigrid Juselius Foundation, the Academy of Finland, and Helsinki University Hospital Research Funds.

Financial disclosure: The authors have nothing to disclose

Conflict of Interest: The authors have no conflict of interest

Category of the study: Clinical study

Author Contributions: TV, A-PI, JK, KW, KV were included in acquired and analyzed the data, and participated in the manuscript drafting. MH, PJM did the critical analysis of the manuscript. MN, TR made the concept and design of the study, and drafted and critically analyzed the manuscript. All authors approved the final version of the manuscript.

Impact

- We describe the genetic cause for central precocious puberty (CPP) in two families.
- This report adds two novel *MKRN3* mutations to the existing literature. One of the mutations, p.(Ile313Met) affects the RING finger domain of MKRN3, which has been shown to be important for repressing the promoter activity of *KISS1* and *TAC3*.
- We describe the first long-term observation of a male patient with CPP due to a paternally inherited *MKRN3* loss-of-function mutation. Without GnRH analog treatment he achieved an adult height that was in accordance with his mid-parental target height.

ABSTRACT

BACKGROUND: Paternally inherited loss-of-function mutations in *MKRN3* underlie central precocious puberty (CPP). We describe clinical and genetic features of CPP patients with paternally inherited *MKRN3* mutations in two independent families.

METHODS: The single coding exon of *MKRN3* was analyzed in three patients with CPP and their family members, followed by segregation analyses. Additionally, we report the patients' responses to GnRH analog treatment.

RESULTS: A paternally inherited novel heterozygous c.939C>G, p.(Ile313Met) missense mutation affecting the RING finger domain of MKRN3 was found in a Finnish girl with CPP (age at presentation 6 years). Two Polish siblings (a girl presenting with B2 at the age of 4 years and a boy with adult size testes at the age of 9 years) had inherited a novel heterozygous *MKRN3* mutation c.1237_1252delGGAGACACATGCTTTT p.(Gly413Thrfs*63) from their father. The girls were treated with GnRH analogs which exhibited suppression of the hypothalamic-pituitary-gonadal axis. In contrast, the male patient was not treated yet he reached his target height.

CONCLUSIONS: We describe two novel *MKRN3* mutations in three CPP patients. The first long-term data on a boy with CPP due to an *MKRN3* mutation questions the role of GnRH analog treatment in augmenting adult height in males with this condition.

INTRODUCTION

Environmental and genetic factors affect the timing of puberty (1, 2). Precocious activation of the hypothalamic-pituitary-gonadal axis, *i.e.* central precocious puberty (CPP), results in testis growth in boys before the age of 9 years and in breast development in girls before the age of 8 years. The incidence of CPP has been estimated to be 37 per 100,000 (3). Early puberty is associated with increased risk of cardiovascular disease and delinquent behavior in adulthood (4, 5). In the UK biobank study, earlier timing of normal puberty was associated with 48 adverse health outcomes such as breast cancer, short stature, and depression (6). Our understanding of the CPP genetics was expanded in 2013, when Abreu *et.al* showed that paternally inherited mutations in the makorin ring finger protein 3 (MKRN3) underlie CPP (7). *MKRN3* encodes a putative ubiquitin ligase, and MKRN3 protein interacts with proteins implicated in the timing of puberty and congenital hypogonadotropic hypogonadism (8). The mechanisms by which MKRN3 regulates puberty are rapidly emerging (9-11). The latest major advance in the field shows that, in mice, *Mkfn3* is coexpressed in kisspeptin neurons, human MKRN3 binds to *KISS1* and *TAC3* promoters, and mutations affecting the RING finger domain of the protein reduce MKRN3 ubiquitination activity and compromise the ability of MKRN3 to repress *KISS1* and *TAC3* promoter activity (11). Describing the phenotype, penetrance, and long-term follow-up of CPP patients with loss-of-function *MKRN3* mutations are thus expected to increase our understanding on the elusive functions of the *MKRN3* and to reveal possible genotype phenotype correlations.

This study describes genotypic and phenotypic features of two independent CPP families with paternally inherited *MKRN3* mutations as well as their growth parameters, responses to GnRH analog treatment, and summarizes the reported mutations in *MKRN3*. While the majority of the *MKRN3* mutations described to date lead to truncated proteins,

missense mutations affecting the RING finger domain are of particular interest (11). Also, such mutations pose a diagnostic challenge since they are easily classified as variants of unknown significance, whereas the demonstration of paternal inheritance of such a mutation to a patient with CPP is the key to set the correct diagnosis.

MATERIALS AND METHODS

Patients

We analyzed phenotypes and *MKRN3* carrier status of two independent families who originated from Finland (one female CPP proband, investigated in the New Children's Hospital at the Helsinki University Hospital, Finland) and from Poland (brother and sister who were evaluated at the Karol Jonscher's Clinical Hospital, Poznan, Poland). The detailed clinical phenotypes of the patients and their hormonal data are described in the Results section.

Hormone assays, radiological studies and clinical measurements

In the Finnish patient, serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured with immunoelectrochemiluminometric assay on an automated immunoanalyser (Modular Analytics E170 Roche Diagnostics, Mannheim, Germany). The detection limit of the LH assay was 0.1 IU/l, intra-assay coefficient of variation (CV) was less than 2% and inter-assay CV was less than 6%, whereas the detection limit of the FSH assay was 0.1 IU/l with intra-assay CV less than 3% and inter-assay CV less than 5%. GnRH stimulation test was performed by intravenous injection of a GnRH analogue (3.5 µg/kg [max. 100 µg], Relefact® LH-RH 0.1 mg; Aventis Pharma, Frankfurt, Germany).

In Polish patients, LH, FSH and E2 were assessed with chemiluminescent microparticle immunoassays (CMIA) by ARCHITECT (Abbott Laboratories, Lisnamuck, Ireland). The detection limit of the LH assay was 0.03 IU/l, intra-assay CV was less than 3.6 % and inter-assay CV was less than 8.7%. The detection limit of the FSH assay was 0.02 IU/l with intra-assay CV less than 3.3% and inter-assay CV was less than 4.6%, whereas the detection limit of the E2 assay was 36.7 pmol/l, intra-assay CV varied from 1.4 % to 6.4% and inter-assay CV varied from 1.8 % to 7.4 %. Testosterone was determined with TESTO-RIA-CT (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) with the lowest detection limit of 0.1 nmol/l, intra-assay CV was less than 8.9% and inter-assay CV varied from 5.2% to 11.6%. GnRH stimulation test was conducted with an intravenous injection of 2.5 µg/kg of GnRH (Relefact LH-RH®, Sanofi-Aventis, Frankfurt, Germany) (max. 100 µg) i.v. with blood drawn prior to the test and at 30 minutes and at 60 minutes after injection.

Bone age was determined from X-ray of the left hand and wrist with an automated bone age assessment (BoneXpert) or by a pediatric endocrinologist (12). Brain magnetic resonance imaging was performed with 1.5 or 3T platforms.

To calculate height SDS, we used the most recent national reference data (13, 14). In all patients, the age-adjusted BMI values (ISO-BMI) were calculated from documented growth measurements by using the Finnish reference data (13).

Genetic testing

The genetic evaluation of the Finnish index patient was performed with Blueprint Genetics MKRN3 single gene test Plus. Peripheral blood or saliva samples were collected from her parents and from the Polish family for DNA extraction. The coding exon and exon-intron

boundaries of *MKRN3* (ENSG00000179455, ENST00000314520, Ensembl release 97) were then PCR-amplified. The PCR conditions and primers are available upon request. The PCR products were purified with ExoProStar treatment (GE Healthcare Life Sciences, Chicago, IL, USA), and sequenced from both directions using the ABI BigDyeTerminator Cycle Sequencing Kit (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). The DNA sequences were aligned and read with Sequencher® 4.9 software (Gene Codes Corporation, AnnArbor, MI, USA).

Allele frequencies of the identified variants were validated from the Genome Aggregation Database (gnomAD) (<http://gnomad.broadinstitute.org/>) and SISu Database (<http://sisuproject.fi>) (15, 16). The gnomAD contains whole genome sequencing and exome data from 141,456 individuals including 12,562 Finnish samples. Effects of the identified variants on transcripts were predicted with SIFT (<http://sift.jcvi.org/>) (17), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and MutationTaster (<http://www.mutationtaster.org/>) *in silico* tools (18, 19).

Ethics

The study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. Written informed consents were obtained from the patients and their parents.

RESULTS

The Finnish index patient (Patient 1) was born at term and was appropriate for gestational age. Her early developmental milestones were unremarkable. At the age of 6 years, she presented with breast development and was referred at the age of 6.4 years to Pediatric Endocrine Outpatient clinic. Her mother's age for menarche was 13 years and she was 171 cm tall (+0.5 SDS), whereas the father had a history of precocious puberty. His pubertal growth had ended at the age of 12 years, and his adult height was 160 cm (-3.4 SDS). He had another daughter (half-sister to the index patient) who also had been diagnosed and treated for CPP at the age of 5.5 years. The pedigree is presented in **Figure 1A**.

Due to her familial CPP, *MKRN3* was analyzed and a heterozygous missense c.939C>G, p.(Ile313Met) variant that was predicted to be deleterious according to the used *in silico* tools (SIFT score 0.01, "deleterious"; Polyphen-2 score 1.000, "probably damaging" and Mutationtaster score 29, "disease-causing") was found. The mutation was absent from the gnomAD database and from SISu (the national Finnish variant database). Her father with CPP carried the same mutation, whereas the mother did not (**Figure 1A**). Index patient's half-sister and cousin presented early puberty; however, their genotypes were not available. In physical examination, the index patient had Tanner breast stage 2 and pubic hair stage 1; otherwise, her physical examination was unremarkable. She displayed accelerated growth (growth velocity 7.8 cm/yr, **Figure 2**) and her bone age at the calendar age of 6.4 was 6.8 years. Her baseline serum LH was 0.3 IU/l and FSH concentration 5.2 IU/l. GnRH-stimulation test revealed a clearly pubertal LH response (max. 10.4 IU/l). Brain MRI scan showed normal pituitary region. She was started on GnRH analog (Leuprorelin 3.75mg every 4 weeks) at the age of 6.5 years, and, at the age of 7.9 years, she had breast stage 1 and pubic hair stage 1. Her linear growth and ISO-BMI curves before and during the treatment are

shown in **Figures 2** and **3**. Her weight was normal before and after the initiation of the treatment.

The Polish family included two siblings who both had CPP (**Figure 1B**). Their mother had menarche at the age of 13 years and her adult height was 170 cm (+0.7 SDS). The father was 186 cm (+1.2 SDS) tall and had normal pubertal timing. However, because both the children presented with familial CPP, *MKRN3* gene was sequenced in all family members. The father and both children had a novel heterozygous deletion in *MKRN3* that was predicted to lead to frameshift and premature stop codon (c.1237_1252delGGAGACACATGCTTTT p.(Gly413Thrfs*63)). The variant was absent from the gnomAD database. The boy (patient 2) was diagnosed with CPP at the age of 9 years and 3 months when he presented with adult-sized testes (20 ml) and tall stature with not observed acceleration of his growth velocity (**Figure 2**). He had pubarche at the age of 7 years and axillarche at the age of 9 years. His testicular ultrasound were normal, testosterone level was 3.6 nmol/L, and GnRH-stimulated maximal LH and FSH responses were 8.9 IU/l and 1.7 IU/l, respectively. His brain MRI showed an incidental small pineal cyst. His bone-age was 11.2 years, and his mid-parental target height was 184.5 cm (+1.0 SDS). First signs of pubertal spurt were noticed at the age of 9 years and 9 months. He did not receive treatment for the CPP. He was followed-up until the age of 15.4 years, when he had reached adult height of 180.5 cm, which was 4 cm below his mid-parental target (**Figure 2**). During follow-up, he was overweight, based on the ISO-BMI values (**Figure 3**).

His younger sister (Patient 3, **Figure 1B**) was diagnosed with CPP at the age of 4.5 years. She presented with thelarche and tall stature with no acceleration of her growth velocity at the age of 4 and 4.5 years, respectively (**Figure 2**). She was of overweight prior to the initiation of the treatment, and thereafter ISO-BMI values were within normal limits

(Figure 3). Her bone age was 5 years, and her mid-parental target height was 171.5 cm (+1.0 SDS). Her maximal LH response (12.6 IU/l) to GnRH stimulation test was consistent with CPP. Her serum estradiol level was 91.8 pmol/l. Brain MRI scan was unremarkable, and no tumor was found in transabdominal ultrasound. She was started on GnRH analogue (Triptorelin 3.75 mg every 4 weeks) at the age of 4.5 years, and at age of 5.3 years the breast examination was Tanner I showing regression of breast tissue. GnRHa treatment is continued until now.

DISCUSSION

This report describes two families with precocious puberty and novel *MKRN3* variants: a Finnish family with a c.939C>G, p.(Ile313Met) missense variant and a Polish family with a c.1237_1252delGGAGACACATGCTTTT p.(Gly413Thrfs*63) frameshift variant. These families underwent genetic testing since both pedigrees contained multiple affected persons. Since only paternally inherited loss-of-function mutations in *MKRN3* cause CPP, segregation analysis was of crucial importance. Indeed, both novel *MKRN3* variants reported herein were inherited from the fathers to patients 1-3, and were absent from gnomAD. The Finnish family variant p.(Ile313Met) was also absent from the national Finnish variant database SISu. Both variants were predicted to be deleterious according to the used *in silico* tools. The p.(Ile313Met) variant is located in the C3HC4 RING zinc finger motif where at least seven previous variants, two of which affecting nearby codons, have been linked to CPP (**Figure 4**) (7, 20-24). It was recently shown that *MKRN3* mutations that affect the RING finger domain exhibit reduced E3 ubiquitin ligase activity and disrupt the ability of MKRN3 to transrepress *KISS1* and *TAC3* promoters (11). We speculate that these molecular mechanisms also cause CPP in patient 1. In turn, the Polish family variant c.1237_1252delGGAGACACATGCTTTT, p.(Gly413Thrfs*63) is located in the last C3H1 zinc finger motif of the MKRN3 protein, likewise known to contain at least four previous variants associated with precocious puberty (**Figure 4**) (25-28). The frameshift mutation is predicted to lead to a premature stop codon and to produce a truncated protein.

In our report, the boy with the *MKRN3* mutation presented advanced signs of puberty at the age of 9 years, whereas the girls had thelarche at the ages of 6 and 4.5 years. This sex difference in the timing of the puberty is in agreement with previous patient series (20, 23,29), and that the initiation of puberty in girls is approximately 2 years earlier than in the

male mutation carriers (23, 29). To the best of our knowledge, this is the first report on the long-term effects of an *MKRN3* mutation in a male patient. Interestingly, patient 2 did not receive any treatment for the condition, and yet reached adult height that was in accordance with his mid-parental target height. We speculate that his favorable height outcome reflects the relatively mild initial advancement of pubertal timing. To this end, his bone age was only approximately two years ahead of his chronological age, and his growth had not accelerated at the time of diagnosis. This was rather unexpected, since he displayed adult-size testes at the age of 9 years, suggesting rapid tempo of sexual maturation after the loss of central gonadotropin secretion restraint. On the other hand, the father of the Finnish family, who carried the missense *MKRN3* mutation, had also CPP but was relatively short. Of note, two of the patients were overweight in early childhood, and previous studies have reported high BMI values at the time of puberty onset in CPP patients with or without *MKRN3* mutations (10, 30), and that in mice *Mkfn3* functions independently of leptin action (31). At the same time, we acknowledge that our study included four patients with CPP and an *MKRN3* mutation and that detailed phenotypes were available only in three of them.

In conclusion, we describe two CPP families with multiple members carrying paternally inherited *MKRN3* mutations. The long-term health consequences of *MKRN3* mutations, if any, are currently unknown, and further investigations are needed to evaluate whether they bear predictive value in terms of clinical management of these patients.

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FIGURE LEGENDS

Figure 1. Pedigrees of the two families harboring novel heterozygous *MKRN3* mutations. Filled symbols denote central precocious puberty. Panel A, the Finnish pedigree; panel B, the Polish pedigree. *Arrow*, index patient. N/A: phenotype not available.

Figure 2. Height standard deviation scores (SDS) of the Finnish (Patient 1, girl) and Polish (Patient 2, boy; Patient 3, girl) patients with central precocious puberty with paternally inherited *MKRN3* mutations. *Arrows*, initiation of the GnRH analogue treatment. *TH*, mid-parental target height.

Figure 3. Age-adjusted body mass index (ISO-BMI) curves of Finnish (Patient 1, girl) and Polish (Patient 2, boy; Patient 3, girl) patients with central precocious puberty due to a paternally inherited *MKRN3* mutation. ISO-BMI values were calculated by using the Finnish reference data for all patients. *Arrows*, initiation of GnRH analogue treatment.

Figure 4. Schematic of the human *MKRN3* protein showing the locations of so far described mutations (not in scale). C3H1: C3H1-type zinc finger motif, *MKRN3* CYS-HIS: Makorin-type Cys-His-motif; C3HC4 RING: C3HC4 RING-type zinc finger motif. Adapted from Valadares *et.al* (20) and Chen *et.al* 2019 (32). The promoter region mutations were reported in Ramos *et.al* (9), Fanis *et.al* (33) and Lu *et.al* (34), and are indicated in relation to the translation initiation codon.

