Supplemental Data: Detailed Characteristics of Patients with \textit{MKRN3} Mutations

Patient 1 was born after an uneventful pregnancy. She presented in our clinic at 7.8 years of age with a history of pubertal development since age 6.4 years. She had no neurological symptoms and no family history of precocious puberty. On physical examination, she had accelerated growth (height: 141.5 cm, SD: 2.89), advanced bone age (11 years) and the Tanner stage was 3 for breast development and 4 for pubic hair. No other clinical abnormalities were detected.

Hormonal evaluation revealed basal LH level (IFMA) of 1.0 U/L, basal FSH level (IFMA) of 5.9 U/L, LH after acute GnRH stimulation (IFMA) of 12 U/L, and basal estradiol level (FIA) of 15 pg/mL. Androgen precursors were normal. She was treated with a GnRH analog (depot leuprolide acetate, 3.75 mg monthly) with adequate control. A novel heterozygous variant in \textit{MKRN3}, c.482delC, p.Pro161Argfs*10, was identified in this patient. The deletion of cytosine 482 in the \textit{MKRN3} results in a frameshift mutation in proline at position 161 of the protein and in a premature stop codon, generating a truncated protein of 171 instead of 507 amino acids. Her father was carrier of the same mutation (c.482delC, p.Pro161Argfs*10), as was her older sister, who had a history of premature menarche at the age of 8.5 years and an advanced bone age (15 years) at first evaluation (at age 9 years). Their mother did not carry the mutation.

Patient 2 was first evaluated at our clinic at age 6.1 years. She had developed thelarche at age 5.4 years, without pubarche. At the first consultation, she was Tanner stage 3 for breast development and stage 1 for pubic hair, had a
height Z-score of 1.59 and a BMI Z-score of 0.25, with a bone age of 8.8 years (SD: 3.1). Her medical history was unremarkable and she had no family history of precocious puberty or consanguinity. Her mother’s menarche was reported to be at age 13 years. She has one younger sister (4 years old), who remains prepubertal to date. Hormonal investigation revealed basal LH and FSH concentrations (IFMA) of 1.3 U/L and 5 U/L, respectively, and a basal estradiol concentration of 36 pg/mL. She was treated with a GnRH analog (depot leuprolide acetate, 3.75 mg monthly) for 4.4 years, with adequate control. Treatment was withdrawn at age 10.6 years, with a bone age of 13 years (SD: 2.6). Her menarche was at age 11.9 years. At her last evaluation, she was 12.9 years old and a near final height was 158 cm, which is close to her target height (160 cm). Molecular analysis revealed a heterozygous variant, c.482_483insC, p.Pro161Argfs*16, in this patient. The insertion of a cytosine at position 482 of the \( MKRN3 \) gene results in a premature stop codon, generating a truncated protein of 177 instead of 507 amino acids. Her father and younger sister carried the same mutation (c.482_483insC, p.Pro161Argfs*16).

Patient 3 had a history of thelarche at age 6 years. She was diagnosed with CPP at the age of 7.9 years, at which time she was started on treatment with a GnRH analog (depot leuprolide acetate, 7.5 mg monthly). At the first visit, her height was 141 cm (Z-score: 2.3) and weight was 49.5 Kg (Z-score: 2.7). Her pubertal development was classified as Tanner stage 4 for breast development and Tanner stage 3 for pubic hair development, and she had an advanced bone age (11 years). Endocrine investigation revealed a basal LH level (IFMA) of 3.4 U/L, basal FSH level (IFMA) of 10 U/L and basal estradiol level (FIA) of 80 pg/mL.
Molecular study identified a heterozygous variant, c.482_483insC, p.Pro161Argfs*16, same as in Patient 2. DNA samples from her parents were not available.

Patient 4 had a history of thelarche and increased growth velocity at the age of 3 years and pubarche at age 6 years. She was Tanner stage 3 for breast development and stage 1 for pubic hair at her first evaluation at age 6.7 years. Her height Z-score was 2.1, BMI Z-score was 1.5 and she had a bone age of 8.8 years. Her medical history was unremarkable and she had no family history of precocious puberty or consanguinity. The mother reported her menarche at the age of 13 years. The patient has one healthy older brother. Hormonal evaluation showed basal LH and FSH concentrations (IFMA) of 1.8 and 4.9 IU/L, respectively, a GnRH-stimulated LH of 62.5 IU/L, and estradiol level was 60 pg/mL. She was treated with a GnRH analog (depot leuprolide acetate), initially with 3.75 mg monthly and later with 11.25 mg every 12 weeks, until the age of 10 years, when her menarche occurred due to non-compliance with treatment. She has now reached near final height within her target height (156 cm). This patient had a heterozygous deletion of a cytosine at position 482 (c.482delC, p.Pro161Argfs*10) of the MKRN3, same as in Patient 1. Familial segregation analysis showed that her father was a carrier of the same variant whereas her mother and older brother were wild-type for MKRN3.

Patient 5 was a 6.8-year-old girl referred because of breast development since age 4 years. Birth occurred at term without complications. Her medical history was unremarkable. The mother reported menarche at age 12 years and denied a family history of precocious puberty. At first examination, the girl was
137.2 cm tall (SD: 3.22) and had an advanced bone age (12 years). Her pubertal
development was classified as Tanner stage 4 for breast and Tanner stage 3 for
pubic hair development. Endocrine investigation revealed a basal LH level (IFMA)
of 6.1 U/L, basal FSH level (IFMA) of 4.7 U/L and basal estradiol level (FIA) of 62
pg/mL. She was treated with a GnRH analog (depot leuprolide acetate, 7.5 mg
monthly) for 3 years with inadequate control due to non-compliance. The treatment
was withdrawn when the bone age was 13.5 years. She had menarche at age 10
years and reached a final height of 158 cm at a chronological age of 17 years. She
had a BMI of 34 kg/m² at the age of 18 years. A heterozygous insertion of an
adenosine, c.675_676 insA, p.Gln 226Thrfs*6, was identified at position 675 of
MKRN3. This insertion results in a truncated protein of 232 amino acids. The
parents were not available for molecular study.

Patient 6 was referred to our clinic at 6.6 years of age with a history of
thelarche and pubarche since age 6 years. She was born at term after an
uneventful pregnancy. She had no neurological symptoms and no exposure to sex
steroids. Physical examination showed mild syndromic features, including high-
arched palate, abnormal positions of her teeth, hand clinodactyly and
hyperlordosis. Her height was 120 cm (SD: 0.5), with advanced bone age (7.8
years), and the Tanner stage was 3 for breast development and stage 2 for pubic
hair. Hormonal evaluation revealed a basal LH level (IFMA) of 1.6 U/L, a basal
FSH level (IFMA) of 4.6 U/L and a basal estradiol level (FIA) of 20 pg/mL. She was
treated with a GnRH analog (depot leuprolide acetate, 11.25 mg every 3 months)
with adequate control. This patient with apparently sporadic idiopathic CPP had a
heterozygous deletion of an adenosine at position 766 (c.766delA,
p.Glu256Glyfs*36) of MKRN3, leading to a premature stop codon and a truncated protein of 292 amino acids instead of 507. Unfortunately, no relatives were available for molecular study.

Patient 7 had a history of thelarche at 6 years of age and was Tanner stage 3 for breast and stage 1 for pubic hair development at first evaluation at age 7.1 years. Her height Z-score was 0.94, BMI Z-score 0.2 and she had a bone age of 8.8 years at diagnosis (SD: 2.1). Her medical history was unremarkable and she had no family history of precocious puberty or consanguinity. Hormonal evaluation showed basal LH and FSH concentrations (IFMA) of 0.37 and 4.4 U/L, respectively, a stimulated LH peak of 7.5 U/L, and basal estradiol level was 44 pg/mL. She was treated with a GnRH analog (depot leuprolide acetate, 3.75 mg monthly) from 7.2 to 11.1 years with satisfactory control. Her menarche occurred at age 12 years and her final height is 162 cm (Z-score-0.17). She is now the mother of a healthy 3-year-old boy. A novel missense mutation, c.1249T>A, p.Phe417Ile, was identified in this patient. This variant is predicted to affect protein function in four different in silico analyses (PolyPhen-2, SIFT, Mutation Taster, Panther). Molecular study revealed that her father was an asymptomatic carrier whereas her mother and son did not carry the mutation.

Patient 8 was born after an uneventful pregnancy. She presented in our clinic at 6.9 years of age with a history of thelarche at age 5.9 years, with pubarche since age 6.6 years. She denied neurological symptoms. She had a reported family history of precocious puberty (her father and her youngest paternal aunt). Her mother’s menarche was reported to be at age 12 years. At the first visit, her height was 127.1 cm (Z-score 1.62) and weight was 32.2 Kg (Z-score 3.04). Her pubertal
development was classified as Tanner stage 3 for breast development and stage 2 for pubic hair development, and she had an advanced bone age (10.8 years). Hormonal investigation at the age of 6 years revealed basal LH and FSH concentrations of 1.5 and 6.5, respectively, and a basal estradiol concentration of 47 pg/mL. She was treated with a GnRH analog (depot leuprolide acetate, 3.75 mg monthly for the first 3 months and then 11.25 mg every 3 months) for 2.2 years, with adequate control. Treatment was withdrawn at age 9.7 years, with a bone age of 11 years. Her menarche was at age 11.2 years. At her last evaluation, she was 12.4 years old and a near final height was 151 cm, which is close to her target height (153 cm). Molecular study identified a heterozygous MKRN3 variant, c.482_483insC, p.Pro161Argfs*16, same as Patients 2 and 3. Her father, who had a history of premature sexual development, had the same heterozygous variant (c.482_483insC, p.Pro161Argfs*16). DNA from her affected aunt was not available for genetic analysis.

MRI of central nervous system of these 8 patients showed no abnormalities.
Supplemental Methods:

Genetic Studies

PCR amplifications of the entire coding region of MKRN3 were performed as follows: 94°C denaturation, 30 seconds; 58°C annealing, 30 seconds; 72°C extension, 1min; 35 cycles. Amplified DNA fragments were sequenced by the Sanger method. Primers were developed for amplification and sequencing. Both flanking and internal primers were used for sequencing (Table S1). All MKRN3 variants were confirmed in two independent PCR products and sequencing reactions of both strands.

Table 1. Primers used for PCR amplification and sequencing reactions.

<table>
<thead>
<tr>
<th>Flanking primers (PCR and sequencing)</th>
<th>Internal primers (sequencing)</th>
</tr>
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<tbody>
<tr>
<td>1F: 5´GCCTCAAGCCCATAAAGAAA3´</td>
<td>1R: 5´AGCCATCTGCTTCCTCTCAG´</td>
</tr>
<tr>
<td>3R: 5´GGGAAAACAGGCAATAGCAG3´</td>
<td>2F: 5´GGCATTTGGACAAAGCAGA3´</td>
</tr>
<tr>
<td></td>
<td>2R: 5´CACTGGGAATGACCAATTC3´</td>
</tr>
<tr>
<td></td>
<td>3F: 5´CCAATTGCAACCATTCCCTTC3´</td>
</tr>
</tbody>
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Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

Genomic DNA for each sample tested (20 ng) was denatured and hybridized overnight with the MLPA probe mix according to the manufacturer’s protocol. The samples were split equally into two aliquots. The first aliquot underwent ligation only for copy number variation analyses, whereas the second underwent ligation plus HhaI digestion for methylation analyses, after which both aliquots were subjected to PCR amplification using primers provided with the MS-MLPA kit. Amplicons were run on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the LIZ® size standard. The peak area for each fragment was measured with GeneMapper analysis software V.5 (Life Technologies) and normalized by dividing the signal of each probe by the signal of all reference probes in that sample (“intra-sample normalization”).
The intra-sample normalized value and data analysis were established according to the manufacturer’s protocol (SALSA MS-MLPA Kit ME028, MRC Holland, Amsterdam, Netherlands). A reduced relative peak height (or area) of the undigested amplification product of a particular probe by 35-50% was indicative of a heterozygous deletion of the corresponding region of the genomic DNA. Methylation patterns were determined by comparing the peak height of the digested amplification product of a particular probe to the undigested product - the absence of a reduction in peak height after digestion indicated that both copies of genomic DNA corresponding to that probe were methylated (and thus resistant to $HhaI$ digestion), whereas an ~50% reduction in peak height indicated that one copy was methylated, and a complete loss of a peak indicated that neither copy was methylated. A genomic DNA sample from a patient with typical Prader Willi syndrome due to maternal uniparental disomy (confirmed by microsatellite analysis) was used as a positive control for methylation abnormalities (see Supplemental Figure S2).
Supplemental Results:

**Table 2.** Clinical and hormonal characteristics of 215 CPP patients and comparative analysis between patients with and without *MKRN3* mutations examined in the current study.

<table>
<thead>
<tr>
<th></th>
<th>Entire cohort (215 patients)</th>
<th>MKRN3 mutation (8 patients)</th>
<th>No MKRN3 mutation (207 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at initial pubertal signs (years)</strong></td>
<td>6.0 (0.1 – 9.0)</td>
<td>6.0 (3.0 – 6.4)</td>
<td>6.0 (0.1 – 9.0)</td>
</tr>
<tr>
<td><strong>Height Z-score</strong></td>
<td>1.4 (-2.0 - 7.6)</td>
<td>1.9 (0.5 – 3.2)</td>
<td>1.2 (-2.0 – 7.6)</td>
</tr>
<tr>
<td><strong>Tanner stage</strong></td>
<td>3 (2 - 5)</td>
<td>3 (2 – 4)</td>
<td>3 (2 – 5)</td>
</tr>
<tr>
<td><strong>∆ (BA-CA)</strong></td>
<td>3.5 (-1.0 - 8.8)</td>
<td>2.5 (1.2 – 5.2)</td>
<td>3.5 (-0.4 – 8.8)</td>
</tr>
<tr>
<td><strong>Basal LH (UI/L)</strong></td>
<td>1.1 (0.1 - 7.0)</td>
<td>1.7 (0.3 – 6.1)</td>
<td>1.1 (0.1 – 7.0)</td>
</tr>
<tr>
<td><strong>GnRH-stimulated LH (UI/L)</strong></td>
<td>14.0 (3.9 - 138)</td>
<td>14.3 (7.5 – 62.5)</td>
<td>14.0 (3.9 - 138)</td>
</tr>
<tr>
<td><strong>Basal FSH (UI/L)</strong></td>
<td>3.8 (1.0 - 10)</td>
<td>4.9 (4.4 – 10)*</td>
<td>3.6 (1.0 – 9.8)</td>
</tr>
</tbody>
</table>

* *p* < 0.05, using the Mann-Whitney U test for comparison between patients with and without *MKRN3* mutations; ∆: Difference; BA= bone age; CA=chronological age; #: clinical and hormonal data at first evaluation. Only one member (proband) from each family was considered in this analysis.

**Supplemental Appendix - Figure Legend 1:**

Schematic illustration of MKRN3 domains and the mutations identified in patients with CPP. The protein has five zinc finger domains: three C3H motifs (dark blue squares), one C3HC4 RING motif with presumed ubiquitin ligase activity (blue square), and a MKRN-specific Cys-His domain (green square). The numbers correspond to the amino acid positions in the protein. Mutations shown in red correspond to the locations of the novel frameshift mutations and
the one in green corresponds to the novel missense mutation. Mutations in black represent the previous variants described (9).

**Supplemental Appendix- Figure Legend 2:**

Representative MLPA peak patterns of the 15q11 locus, with and without *HhaI* digestion (Left Column).

Histograms showing representative data from one subject, normalized to the results of three control individuals and to internal controls (Right Column). Normalized relative values within a confidence interval of 0.7 to 1.3, which was established by control sample variations, correspond to the presence of two gene copies.

**a.** Four *SNRPN* probes. **b.** *MKRN3* probe (exon 1; not digested by *HhaI*). **c.** *NDN* probe. The site recognized by this probe has a tendency to be overdigested, and may therefore appear to be only 30% methylated. *HhaI* digestion positive controls.

**A0.** Normal subject (undigested). **A1.** Normal subject (digested with *HhaI*). This sample shows an approximately 50% reduction in the height (or area) of the peaks corresponding to the four *SNRPN* probes (a) and the *NDN* probe (c), indicating methylation of one allele, as expected. **B.** Subject with Prader Willi syndrome due to maternal uniparental disomy (digested with *HhaI*). This subject did not have a reduction of the peak height of the four *SNRPN* probes, indicating methylation of both alleles. **C.** Subject with CPP without *MKRN3* mutation (digested with *HhaI*). This subject had a peak height pattern similar to the control subject; no abnormal methylation pattern was detected in this area.