A novel MKRN3 missense mutation causing familial precocious puberty

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ABSTRACT: Central precocious puberty may be familial in about a quarter of the idiopathic cases. However, little is known about the genetic causes responsible for the disorder. In this report we describe a family with central precocious puberty associated with a mutation in the makinor RING-finger protein 3 (MKRN3) gene. A novel missense mutation (p.H420Q) in the imprinted MKRN3 gene was identified in the four affected siblings, in their unaffected father and in his affected mother. An in silico mutant MKRN3 model predicts that the mutation p.H420Q leads to reduced zinc binding and, subsequently, impaired RNA binding. These findings support the fundamental role of the MKRN3 protein in determining pubertal timing.

Key words: central precocious puberty / maternal imprinting / zinc finger / makinor RING-finger protein 3

Introduction

The timing of puberty is influenced by stimulating and restraining factors, many of which are still unknown. The study of pathological states of early and delayed puberty has provided valuable insight into those factors regulating GnRH activity.

Central precocious puberty (CPP), caused by early activation of pulsatile GnRH secretion, may result from hypothalamic tumors or lesions but in most cases is idiopathic. We have previously shown that idiopathic precocious puberty was familial in as many as 27.5% of cases but in most cases is idiopathic. We have previously shown that pulsatile GnRH secretion, may result from hypothalamic tumors or regulating GnRH activity.

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Central precocious puberty was familial in about a quarter of the idiopathic cases. However, little is known about the genetic causes responsible for the disorder. In this report we describe a family with central precocious puberty associated with a mutation in the makinor RING-finger protein 3 (MKRN3) gene. A novel missense mutation (p.H420Q) in the imprinted MKRN3 gene was identified in the four affected siblings, in their unaffected father and in his affected mother. An in silico mutant MKRN3 model predicts that the mutation p.H420Q leads to reduced zinc binding and, subsequently, impaired RNA binding. These findings support the fundamental role of the MKRN3 protein in determining pubertal timing.

Materials and Methods

Subjects

Four otherwise healthy siblings presented with idiopathic CPP (Fig. 1A). All had normal psychomotor development with high scholastic performance at school or in kindergarten. The non-consanguineous parents, of mixed Ashkenazi-Sephardic Jewish origin, are healthy and in both parents pubertal development was normal: maternal age at menarche was 12 years, and paternal age at first full-facial shaving was 16 years. The maternal grandmother had her menarche between 9 and 10 years of age and the paternal grandfather reported normal puberty. The two non-married paternal sisters were reported to have had normal puberty. None of the family members presented with fertility problems or other non-reproductive phenotypic features.

Pubertal stage was evaluated according to Marshall and Tanner (1969) and bone age according to Greulich and Pyle (1959). Growth velocity assessment at diagnosis was based on two height measurements taken at least 6 months apart.

All patients underwent a GnRH stimulation test. Serum basal LH > 0.1 U/l, peak GnRH-stimulated LH > 5.0 U/l, basal estradiol >3 pmol/l and basal testosterone >0.4 nmol/l were considered to be pubertal levels (Neely et al., 1995; de Vries et al., 2006).

Pituitary imaging was performed by magnetic resonance imaging (MRI). Transabdominal pelvic ultrasound scans were performed in all three sisters as previously described (de Vries et al., 2006).
Figure 1 Genetic analysis of a family with central precocious puberty and an in silico model of the mutated protein. (A) Pedigree of the reported family. Black symbols—clinically affected family members, symbols with black point inside—asymptomatic carrier. The MKRN3 genotype is shown for family members whose DNA was available for genetic studies. NM denotes non-mutated. (B) MKRN3 c.1260 T > G mutation analysis. Sequence chromatograph from genomic DNA of affected (M) and unaffected (WT) individuals. (C and D) An in silico structural model of the H420Q mutation. MKRN3 394–423 model structures of mutated (M) and wild-type (WT), presented as ribbons. C400, C410, C416 and H420 (Q in the mutant) are shown by sticks (Sulfur yellow, oxygen, red; nitrogen, blue). Zn ion is shown as Van der Waals sphere. Distances between Zn ion and cysteine’s sulfur atoms, histidine’s nitrogen atom and glutamine’s oxygen atom were measured in angstroms and presented in the figure. Q420 is predicted to be more distant from the Zn ion (4.778 Å) than H420 (1.846 Å). (E) By way of example, the X-ray structure of a zinc finger from human butyrate response factor 2 in complex with AU-rich RNA molecule (PDB 1RGO) is shown. The protein is shown by blue ribbons and solvent accessible surface, with the three Zn-binding cysteines in yellow sticks and histidine in red sticks. Zn is shown by a gray sphere. A phenylalanine residue, close to the Zn binding site and pointing towards the RNA, is also shown by red sticks. This residue was found to be important for MKRN3 function.
CPP was diagnosed on the basis of clinical signs of progressive pubertal development before the age of 8 years in the girls; GnRH-stimulated LH levels, advanced bone age, and normal brain MRI.

Written informed consent was obtained from all family members according to a study protocol approved by the institutional ethical committee.

Genetic analysis

Whole exome sequencing
Exonic sequences were enriched in the DNA sample of patient III.4 using SureSelect Human All Exon 50Mb Kit (Agilent Technologies, Santa Clara, CA, USA). Sequences were determined by HiSeq2000 (Illumina, San Diego, CA, USA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference, as previously described (Edvardson et al., 2012).

Sanger sequencing
We confirmed the identification of variants in the coding region of MKRN3 with the use of PCR amplification followed by sequencing of the products using the conventional Sanger method (Genetic analyzer 3130, Applied Biosystems, Foster City, CA, USA). Genotypes of all other family members were determined by Sanger sequencing.

In silico structural modeling
Template structure for residues 394–423 of the MKRN3 protein was found using HHpred: 4ii1 (chain A) and the wild-type (WT) structure was modeled using MODELLER (Sali and Blundell, 1993). 4ii1 is the protein data bank code for a solved structure for ZGPAT protein (zinc finger CCCH-type with G patch domain-containing protein).

Histidine 420 was mutated to glutamine using UCSF chimera swapaa command, and then minimized in the presence of a Zn ion, as for the WT model.

Results

Clinical characteristics

The proband (III.1) presented with thelarche at age 5.5 years, her sister (III.3) presented with thelarche and pubarche at age 5.5 years, and the youngest sister (III.4) presented with thelarche at age 4.5 years (Table I). Patient III.3 was followed by a pediatric endocrinologist for 11 months before referral to our center, during which period she progressed from breast Tanner 2 to 3. The parents brought in patient III.4 as soon as they noticed breast buds. The brother (III.2) presented at 9 years 10 months with advanced bone age, growth acceleration, testicular enlargement, penile length of 9.5 cm and Tanner stage 3 pubic hair. His first clinical presentation suggested the diagnosis of precocious puberty and his hormonal profile corroborated the diagnosis of CPP; puberty was estimated to have begun 2 years earlier.

All siblings had advanced bone age, accelerated growth velocity and pubertal levels of gonadal steroid and GnRH-stimulated LH.

Uterine width and volume were in the pubertal range (Herter et al., 2002; de Vries et al., 2006) in all three sisters.

All siblings were treated by GnRH analog (GnRHa) (3.75 mg of the long-acting GnRHa depot triptorelin) (Decapeptyl Depot, Ferring) and responded with regression of pubertal signs. The eldest sister discontinued...
therapy at age 11 years, had her menarche at 12.5 years and currently has
regular menses. The other three siblings are still being treated. The grand-
mother reported normal fertility and timely menopause.

Whole exome sequencing
The exome analysis of the DNA of patient III.4 yielded 65.00 million con-
fidently mapped reads. Following alignment to the reference genome, 124 793
variations were noted. We removed variations which were called
less than X8, were synonymous, present in the dbSNP (The Single Nucleotide Polymorphism Database) version 132, or in the Hadassah
in-house database, or predicted benign by Mutation Taster software
(Schwarz et al., 2010). A total of 195 variations, all heterozygous, sur-
vived this filtering process; among them were chr15: 23812189 T>G, p.His420Gln (H420Q) in the MKRN3 gene. The mutation was absent from
dbSNP138 and from the 6503 healthy individuals whose Exome
analysis results are available through the Exome Variant Server, NHLBI
Exome Sequencing Project, Seattle, Washington, USA (http://evs.gs.
washington.edu/ EVS-v.0.0.21) (accessed 28 January 2014). The mutation
was verified by Sanger sequencing in all family members and was
found to segregate with the disease in the family (Fig. 1A and B).

No rare or pathogenic variants were found in the KISS1R, KISS1, TAC3
and TACR3 (encoding tachykinin 3 and its receptor, respectively) genes.
We reviewed all 195 potential single-nucleotide polymorphisms that
were found, for genes that could be associated with the phenotype.
MKRN3 was the only common gene found when comparing the list of
variants with the list of genes known to be associated with puberty from
In addition, an analysis of the list of genes with the DAVID bioinformatics
tool (http://david.abcc.ncifcrf.gov/) failed to identify any gene sup-
pessedly involved in tumor suppression or puberty.

In silico structural modeling of the mutation
In the protein modeling the distances between zinc ion and cysteine’s
sulfur atoms, histidine’s nitrogen atom and glutamine’s oxygen atom
were measured in angstroms (Fig. 1C and D). The Q420 mutated
MKRN3 is predicted to be more distant from the Zn ion (4.778 Å)
than the WT protein (1.846 Å).

To further analyze the possible effect of the mutation on the function of
the MKRN3 protein, an X-ray structure of a similar zinc finger from
another RNA binding protein, the human butyrate response factor 2,
in complex with AU-rich RNA molecule (PDB 1RGO), was prepared
(Fig. 1E). The Zn-binding site forms a pocket for the RNA, and the histi-
dine is in close proximity with the zinc. Changing the histidine to glutam-
ine is predicted to have a deleterious effect on the formation of this
pocket, leading to dysfunction of the protein.

Discussion
This report describes a novel, heterozygous missense mutation in
the maternally imprinted MKRN3 gene in four affected siblings, their
unaffected father and their affected paternal grandmother.
MKRN3 encodes makorin RING-finger protein 3, which is involved in
ubiquitination and cell signaling. This gene is maternally imprinted and
thus only the paternal allele is expressed (Jong et al., 1999). The inherit-
ance pattern in the studied family is in accordance with the expected
mode of inheritance. The MKRN3 protein includes two copies of a
C3H motif in the N-terminal, followed by a novel Cys–His configura-
tion, a C3HC4 RING zinc finger, and a final C3H motif. Previously identified
mutations in this gene consisted of seven frameshift mutations and four
missense mutations: p.R36S (Abreu et al., 2013), and p.C340G
(Settas et al., 2014) at the C3HC4 RING domain which are responsible
for the ubiquitin ligase activity of the protein. Both missense mutations
are predicted to disrupt protein function as an E3-ubiquitin ligase.
Another missense mutation, F417I (Macedo et al., 2014), was found
in the last C3H1 domain, not far from the missense mutation described
in the present study. The fourth mutation is a nonsense mutation at
codon p.Glu111* at the first C3H motif (Schreiner et al., 2014).
As C3H zinc finger motifs have been implicated in RNA binding, we
predict that the mutation found in the present study is responsible for dis-
turbed RNA binding. To further elucidate the mechanism by which the
mutation leads to a deleterious effect, we used an in silico structural model.
We demonstrated that substituting histidine 420 with glutamine in
the MKRN3 protein is predicted to cause a zinc-to-ligand distance
longer than the longest distance estimated for bond lengths (Laitaoja
et al., 2013); the average length of the zinc-histidine bond is 2.09 Å
and the longest distance found was 4.12 Å. Thus, the change is predicted
to reduce the affinity between the Zn ion binding site and the relevant
Zn, disrupting the binding pocket. In zinc finger proteins, the zinc ion
is required for correct folding of the polypeptide chain, and removal of
the zinc ion causes the finger to unfold (Krishna et al., 2003).

In a study of the conserved C3CH zinc finger using a model of
Euryarchaeotes (mesophilic archaean Methanosaricina acetivorans),
the N-terminal deletion mutant contained zinc at a level comparable to
the WT protein level while the C-terminal deletion mutant was devoid
of zinc (Lin et al., 2005). The latter study supports our hypothesis regard-
ing the mutation effect. This model brings us one step further towards
understanding the as yet unknown mechanism by which MKRN3 is
involved in the process of pubertal onset.

Puberty represents the reactivation of the suppressed GnRH pulse-
generator characteristic of late infancy and childhood, leading to
increased amplitude and frequency of GnRH pulsatile discharges. This
change is related to an increase in stimulatory factors and decreased in-
hibitory hormone, which are controlled by gene expression (Terasawa
and Fernandez, 2001; Plant and Barker-Gibb, 2004).

It has been shown that the expression of Mkrn3 in mice of both sexes
was highest at post-natal day 10 and declined significantly just before the
onset of puberty (Abreu et al., 2013), suggesting that the MKRN3 protein
may be involved in the restraint mechanism during childhood. We
suggest that a low affinity to Zn leads to inadequate folding of the zinc
finger, resulting in a reduced inhibitory effect of MKRN3 on GnRH secre-
tion and earlier activation of puberty. The exact role of the MKRN3
protein and how it interacts with other inhibitory and excitatory neuro-
transmitters regulating puberty is unknown and definite confirmation
that the missense variant causes premature loss of the inhibitory tonus
acting on GnRH secretion awaits the availability of a functional assay
for MKRN3. To date, there has been no direct evidence for a MKRN3/
GnRH interaction or any biological testing for the consequences of the
mutation on function of the MKRN3 protein. Moreover, there has
been no documentation of the profiles of expression of MKRN3 in
other reproductive tissues. Thus, the suggested role of MKRN3
remains only speculation and the possibility of additional actions at other levels of
the reproductive axis cannot be ruled out.

In the present study, we used whole exome sequencing as a
hypothesis-free approach to identify genetic variants. Among the 195
variants that survived the filtering process there was the intronless MKRN3 gene, recently found to be associated with familial CPP, and we focused on this gene in our study. We also reviewed the other 194 variants for mutations putatively affecting known pathways regulating pubertal onset, such as LIN28B (Ong et al., 2009). We did not find any gene that is known to be associated with precocious puberty, or with the pubertal process, apart from MKRN3.

It is not yet clear why clinical presentation occurs only at the age of 4 or 5 years and not earlier. The mutated MKRN3 protein may provoke an imbalance between stimulatory and inhibitory factors. It may be that the increase in stimulatory factors during childhood is gradual, and that when the inhibitory mechanism is impaired, as with MKRN3 mutation, the ’threshold’ for puberty activation is lower. Conversely, it may be that the physiological decline in inhibitory factors is gradual, and that the switch occurs earlier in the absence of adequate MKRN3 activity. Clinically, it seems that the MKRN3 protein plays a role in determining pubertal timing, but not tempo, as observed in patient III.3, and possibly her brother (patient III.2). This is further supported by the normal course of pubertal progression observed in the eldest sibling (patient III.1, now 19 years old) following treatment cessation and the reported pubertal progression in the grandmother. Our observation is substantiated by the previous reports (Abreu et al., 2013; Macedo et al., 2014), showing that in some of the cases there was a significant lapse of time between initial clinical puberty signs and the diagnosis of Tanner stage 2 or 3. For instance, in the manuscript of Abreu et al. (2013) a male patient whose pubertal signs appeared at the age of 5.9 years was at Tanner stage 3 at the age of 8.1 years, and two girls with two different mutations who had thelarche at the ages of 3.0 and 4.0 years were at Tanner 3 when diagnosed at the ages of 6.7 and 6.8, respectively (Macedo et al., 2014). This observation may have clinical implications when considering GnRH agonist treatment for a child with precocious puberty associated with a MKRN3 mutation.

Although the timing of pubertal onset in the boy is uncertain, it seems that his degree of precocity was not as striking as in the girls. This gender dimorphism in the clinical presentation of the mutation in the MKRN3 gene is consistent with that found in recent studies (Abreu et al., 2013; Settas et al., 2014). The mechanism for such a gender difference is yet to be revealed.

In conclusion, a novel mutation, predicted to cause inadequate folding of the last zinc finger, is associated with familial precocious puberty. We suggest that MKRN3 signaling is a critical element in the restraint mechanism on the GnRH pulse-generator, maintaining the timing of the pubertal process.

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Authors’ roles

L.d.V. developed the idea for the study, participated in the study design, collected the DNA samples, performed pelvic ultrasound, participated in interpretation of data and writing of the manuscript. G.G.-Y. performed analyses and participated in interpretation of data as well as writing of the manuscript. N.D. provided patients for the study and contributed to the analysis and interpretation of data and critical revision. A.S. contributed to the study design, interpretation of data and critical revision. M.P. contributed to the study design, interpretation of data and critical revision. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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