A new pathway in the control of the initiation of puberty: the *MKRN3* gene

Ana Paula Abreu, Delanie B Macedo, Vinicius N Brito, Ursula B Kaiser and Ana Claudia Latronico

Division of Endocrinology, Diabetes and Hypertension, Harvard Medical School, Brigham and Women’s Hospital, Boston, Massachusetts, USA

1Unidade de Endocrinologia do Desenvolvimento, Disciplina de Endocrinologia e Metabologia, Laboratório de Hormônios e Genética Molecular, LIM 42, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, Avenida Dr Enéas de Carvalho Aguiar, 255, 7º andar, sala 7037, CEP: 05403-900, São Paulo, Brazil

Correspondence should be addressed to A C Latronico

Email anacl@usp.br

Abstract

Pubertal timing is influenced by complex interactions among genetic, nutritional, environmental, and socioeconomic factors. The role of *MKRN3*, an imprinted gene located in the Prader–Willi syndrome critical region (chromosome 15q11–13), in pubertal initiation was first described in 2013 after the identification of deleterious *MKRN3* mutations in five families with central precocious puberty (CPP) using whole-exome sequencing analysis. Since then, additional loss-of-function mutations of *MKRN3* have been associated with the inherited premature sexual development phenotype in girls and boys from different ethnic groups. In all of these families, segregation analysis clearly demonstrated autosomal dominant inheritance with complete penetrance, but with exclusive paternal transmission, consistent with the monoallelic expression of *MKRN3* (a maternally imprinted gene). Interestingly, the hypothalamic *Mkrn3* mRNA expression pattern in mice correlated with a putative inhibitory input on puberty initiation. Indeed, the initiation of puberty depends on a decrease in factors that inhibit the release of GnRH combined with an increase in stimulatory factors. These recent human and animal findings suggest that *MKRN3* plays an inhibitory role in the reproductive axis to represent a new pathway in pubertal regulation.

Key Words

- hypothalamus and neuroendocrinology
- gonadotropins
- mutations
- secretion

Introduction

Puberty, a complex biological process involving sexual maturation and accelerated linear growth, is initiated when the pulsatile secretion of gonadotropin-releasing hormone (GnRH) increases after a quiescent period during childhood. The regulation of puberty initiation remains as one of the great mysteries of human biology and it is thought that a conjunction of factors plays a role to initiate puberty.

Environmental and metabolic factors are important regulators of pubertal development, but these influences are superimposed upon substantial genetic control. Similar timing of puberty shared by mothers and her children, and within racial groups, suggests a genetic component to the onset of puberty (Zacharias & Wurtman 1969, Herman-Giddens et al. 1997). Substantial efforts have been made to identify genes that are responsible for the initiation of puberty (Ojeda & Lomniczi 2014). The identification of these genes is critical for advancing the understanding of the neuroendocrine regulation of puberty initiation.
In an attempt to identify genes operating within the neuroendocrine brain that ultimately regulate the reproductive axis, researchers have been studying patients with pubertal disorders. The hypothalamic–pituitary–gonadal axis regulates puberty initiation and reproduction. GnRH is produced and secreted by the hypothalamus in a pulsatile manner during the embryonic and neonatal phases of life. GnRH secretion is actively inhibited during infancy and puberty begins with the reactivation of its secretion. GnRH deficiency results in hypogonadotropic hypogonadism, in which patients fail to develop puberty and are usually infertile. Conversely, early reactivation of GnRH secretion results in central precocious puberty (CPP). While several genes have been detected in association with GnRH deficiency and have contributed to the current knowledge of GnRH regulation (Bianco & Kaiser 2009, Semple & Topaloglu 2010), genes linked to CPP have until recently only been identified subsequent to their association with hypogonadotropic hypogonadism, such as \( \text{KISS1} \) and \( \text{KISS1R} \). However, only rare genetic defects in \( \text{KISS1} \) and its receptor have been identified in patients with CPP (Teles et al. 2008, Silveira et al. 2010).

The advances in sequencing methods permitted the detection of new genes implicated in the neuroendocrine regulation of puberty. Using exome sequencing analysis and studying familial cases of CPP, genetic defects in a gene with no previous link to the hypothalamic–pituitary–gonadal axis, Makorin ring finger 3 (\( \text{MKRN3} \)), were identified as the cause of premature sexual development in one-third of the families (Abreu et al. 2013). \( \text{MKRN3} \) is located on the long arm of chromosome 15 in the Prader–Willi syndrome (PWS) critical region and is maternally imprinted (discussed later). Subsequently, we showed that mutations in \( \text{MKRN3} \) are also the cause of CPP in apparently sporadic cases (Macedo et al. 2014). These findings were significant contributions to the field and will advance the understanding of the pubertal process. We will review the genetic defects identified in patients with CPP and their clinical implications, and discuss herein the possible role of \( \text{MKRN3} \) within the reproductive axis.

**Loss-of-function mutations of \( \text{MKRN3} \) cause familial CPP**

The role of \( \text{MKRN3} \) in pubertal initiation was first described in 2013 after a comprehensive genetic study of several families with CPP (Abreu et al. 2013). In this study, the authors investigated 40 members of 15 families with CPP from different ethnic groups (12 Brazilian, two American, and one Belgian families), and applying whole-exome sequencing four deleterious \( \text{MKRN3} \) mutations – three frameshift and a missense mutation (Fig. 1) – were detected in five of these families (33%). Both sexes were equally affected by \( \text{MKRN3} \) mutations (eight girls and seven boys) (Table 1).

More recently, Macedo et al. (2014) studied 215 unrelated children (207 girls and eight boys) with CPP from three different University Medical Centers and identified five novel heterozygous mutations in eight unrelated Brazilian girls, including four frameshift variants and one missense variant (Fig. 1 and Table 1). No family history of premature sexual development was

Figure 1

MKRN3 protein structure and mutations identified in patients with central precocious puberty. Zn, zinc; H, histidine; C, cysteine. The three C3H zinc finger motifs are shown in red, the C3HC4 RING finger motif is in blue, and the MKRN-specific Cys–His domain is shown in green. The numbers correspond to the amino acid positions in the protein. Blue mutation labels and arrows indicate the location of frameshift mutations; red mutation labels and arrows indicate the location of the missense mutations.
### Table 1  
MKRN3 mutations identified in children with CPP. (Gonadarche or testicular enlargement. Breast/Genital development according to Tanner stage and bone age assessed at the time of diagnosis)

<table>
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<th>Origin</th>
<th>Patient number</th>
<th>Sex</th>
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<th>Characteristics at time of diagnosis</th>
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F, female; M, male.

*Bone age at 7.4 years.
reported in the majority of these studied patients. However, segregation analysis was performed for five of these eight girls, and in all cases it has been demonstrated that the mutant allele was paternally inherited in all families with MKRN3 mutations.

In order to investigate whether the CPP phenotype could arise from loss of MKRN3 expression by the paternal allele due to a de novo deletion, maternal uniparental disomy, or an imprinting defect, Macedo et al. (2014) investigated 52 patients with familial and sporadic CPP, without known MKRN3 sequence defects, by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) of chromosome 15q11. No copy number changes or methylation abnormalities were detected in the 15q11 locus, suggesting that epigenetic defects involving this locus are likely rare mechanisms in this disorder (Macedo et al. 2014).

Recently, other investigators have also reported MKRN3 defects associated with familial CPP. Settas et al. (2014) described a novel heterozygous missense mutation (p.Cys340Gly) in MKRN3 in two Greek siblings, a girl with CPP and a boy with early puberty. Soon thereafter, Schreiner et al. (2014) identified two heterozygous MKRN3 mutations (p.Glu111* and p.Ala162Glyfs*14) in affected members of two German families. Finally, de Vries et al. (2014) identified a novel missense mutation (p.His420Gln) in four siblings, including a boy, from an Ashkenazi–Sephardic Jewish family (Fig. 1 and Table 1). These studies further expanded the MKRN3 mutational spectrum.

Clinical features of CPP associated with MKRN3 mutations

All patients with CPP carrying loss-of-function mutations in MKRN3 exhibited typical clinical and hormonal features of premature activation of the reproductive axis, including early pubertal signs, such as breast, testes, and pubic hair development, accelerated linear growth, advanced bone age, and elevated basal and/or GnRH-stimulated LH levels (Table 1). Except for two related patients (one girl and her brother) who presented with esotropia (Abreu et al. 2013), which is a minor criterion for PWS, no other signs of PWS were detected in any patient with CPP caused by MKRN3 defects. Another female patient had mild nonspecific syndromic features, including a high-arched palate, dental abnormalities, clinodactyly, and hyperlordosis (Macedo et al. 2014). Regarding the therapeutic response of CPP patients with MKRN3 defects, six of eight patients have demonstrated adequate responses to conventional treatment with depot GnRH agonists to date (Macedo et al. 2014).

In patients with CPP due to MKRN3 defects, the median age of puberty onset was 6.0 years in girls (ranging from 3.0 to 7.5 years) and 8.25 years in boys (ranging from 5.9 to 9.0 years) (Abreu et al. 2013, Macedo et al. 2014, Schreiner et al. 2014, Settas et al. 2014, de Vries et al. 2014), suggesting that the MKRN3 mutations may affect girls more severely than boys (Table 1). Given the median age of pubertal onset of affected patients with MKRN3 mutations, it is speculated that the prepubertal inhibitory tonus on GnRH secretion took place normally, but was lost prematurely in patients with MKRN3 mutations. This clinical observation suggests that MKRN3 may not be crucial for GnRH suppression after the mini-puberty of early infancy, but that the downregulation of MKRN3 plays a relevant role for the re-emergence of GnRH pulses in the pubertal onset (Macedo et al. 2014).

MKRN3 mutations and polymorphisms

Currently, 12 distinct loss-of-function mutations of MKRN3 have been described in 30 patients (22 girls and eight boys) with CPP from 17 families from different ethnicities (Fig. 1 and Table 1). Remarkably, eight of these mutations encode either premature stop codons or frameshift mutations (Table 1). The four missense mutations (p.Cys340Gly, p.Arg365Ser, p.Phe417Ile, and p.His420Gln) were located within a zinc finger motif or a RING finger motif (Fig. 1), regions predicted to be involved in RNA binding and ubiquitin ligase activity of the protein respectively, and essential for protein function. All missense mutations are predicted to be pathogenic by in silico analysis. Additionally, ab initio modeling of the mutations p.Arg365Ser and p.Cys340Gly predicts that these mutations lead to significant structural perturbations in the 3D structure of the RING finger motif (Settas et al. 2014). The substitution of histidine 420 with glutamine in the MKRN3 protein is predicted to reduce the affinity between the Zn ion-binding site and the relevant Zn, disrupting the binding pocket leading to unfolding of the finger (de Vries et al. 2014). Interestingly, most of the MKRN3 mutations (64%) were located in the amino-terminal region of the protein, which is encoded by a poly-C-rich sequence, suggesting that this area may be a potential hotspot.

The important role of MKRN3 in human puberty initiation has been reinforced recently by large genome-wide and custom-genotyping arrays in up to 182,416 women of European descent (57 studies) (Perry et al. 2014).
MKRN3 was first cloned in 1999 by Jong et al. (1999a) during a study of the Prader–Willi/Angelman syndrome (PW/AS) critical region. They identified a cDNA in the PW/AS region encoding a zinc finger protein, initially named zinc finger protein 127 (ZNF127) and later renamed MKRN3. The functional and physiological relevance of MKRN3 is not known and despite its location in the PWS critical region, its role in this syndrome is also unclear. An antisense transcript was concomitantly identified and named ZNF127AS (MKRN3-AS1). The antisense gene is not translated and is expressed weakly during fetal development and at very low levels in adult brain regions (Jong et al. 1999a). Similar to other antisense genes, ZNF127AS may regulate the expression of the ‘sense’ gene (MKRN3).

MKRN3 is located on human chromosome 15q11–13 (chromosome 7C in mouse) in a region that contains a cluster of imprinted genes associated with two different neurobehavioral disorders (Lalande 1996, Nicholls et al. 1998). Mutations or loss of expression of the maternally expressed gene UBE3A lead to Angelman syndrome (AS), while PWS is thought to be a contiguous gene syndrome arising from the loss of expression of multiple paternally expressed genes, including MKRN3. The report of two patients with PWS with a deletion of the 15q11–13 locus that did not include MKRN3 suggested that this gene is not required for the development of the syndrome, but we still cannot rule out a role for MKRN3 in some of the clinical features of PWS (Kanber et al. 2009).

The MAKORIN family

The MKRN gene family encodes putative ribonucleoproteins with a distinctive array of zinc-finger motifs including several C3H zinc fingers, a makorin-specific Cys–His arrangement, and a RING zinc finger (Jong et al. 1999a, b). In particular, MKRN3 has a centrally located RING finger motif (C3HC4), two amino-terminal C3H zinc finger motifs followed by the unique pattern of conserved Cys–His residues called a Makorin zinc finger motif, and a carboxy-terminal C3H zinc finger motif (Jong et al. 1999a, b; Fig. 1). MKRN3 is highly conserved among species, and the mouse and human MKRN3 amino acid sequences share 69% identity and 82% similarity (Jong et al. 1999a). Mice and humans usually do not have conserved UTRs, yet the MKRN3 3′-UTR has 90% identity between these two species, implying a functional significance to this region of MKRN3. MKRN3 is ubiquitously expressed in
adult human tissue, with the highest expression levels in the testis. In the fetus, it is highly expressed in the CNS and is expressed in post-meiotic sperm germ cells, particularly in round spermatids (Jong et al. 1999a).

The characteristic arrangement of cysteine (Cys or C) and histidine (His or H) residues in the zinc finger proteins in MKRN3 can allow some predictions of its function. C3H zinc fingers have been identified in RNA-binding proteins (Barabino et al. 1997, Murray et al. 1997). RING zinc fingers have been shown to mediate protein:protein interactions (Schwabe & Klug 1994). More recent evidence suggests that the RING zinc finger is a signature domain for E3 ligases, a category of enzymes mediating the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to target protein substrates (Deshaies & Joazeiro 2009). Ubiquitination can have diverse effects on the protein substrate, varying from proteasome-dependent proteolysis to modulation of protein function and/or localization (Deshaies & Joazeiro 2009, Behrends & Harper 2011). The multiple types and number of zinc finger motifs in makorin proteins suggest possible multiple cellular functions for this protein.

Makorin proteins share a highly homologous amino acid sequence, particularly in the zinc finger domains (Fig. 2), suggesting that they may share similar functions or regulatory mechanisms. Although there are not many studies on MKRN3 function to date, MKRN1 function has been better explored. A possible important cellular function for MKRN1 is supported by the high identity (92%) between the human and murine orthologs and the ubiquitous protein expression in human and mouse tissues (Gray et al. 2000). MKRN1 acts as an E3 ubiquitin ligase, inducing degradation of human telomerase reverse transcriptase (hTERT), viral capsid proteins, p53 and p21 cell cycle regulators, and peroxisome proliferator-activated receptor gamma (PPARγ) (Kim et al. 2005, 2014, Lee et al. 2009).

An alternative mechanism of action for MKRN1 was described when it was identified as a repressor of c-Jun transcriptional activity using a yeast assay. Through regulation of RNA polymerase II-dependent transcription, MKRN1 can have either negative or positive effects on gene expression (Omwancha et al. 2006). Interestingly, it has been demonstrated that the disruption of ubiquitin ligase activity of MKRN1 did not affect its inhibitory transcriptional activity, suggesting that this function is independent of protein ubiquitination (Omwancha et al. 2006).

MKRN2 is also ubiquitously expressed (Gray et al. 2000). Similar to other members of this family, MKRN2 is highly conserved throughout evolution. It has been shown that mkrn2 negatively regulates neurogenesis via PI3K/Akt signaling in Xenopus embryos; however, the detailed molecular mechanisms of this effect and the potential functions of mammalian MKRN2 remain yet to be studied (Yang et al. 2008).

MKRN3 presumptively possesses ubiquitin-protein isopeptide ligase (E3) activity, intimated by the presence of the highly conserved C3HC4 RING finger domain. The tandem repeat of C3H zinc fingers may provide C3H motifs, in yellow the MKRN-specific Cys–His motif, and in green the RING finger domain. MKRN1 NCBI Reference Sequence NP_038474, UniProtKB/Swiss-Prot: Q9UHC7.3. MKRN3 NP_005655.1 and UniProtKB/Swiss-Prot Q13064.1.

**Figure 2**
MKRN1 and MKRN3 protein sequence alignment. The two protein sequences share high homology and similarity, especially in the RING finger domains. Bold letters represent conserved amino acids, and squares similar amino acids. Sections highlighted in red represent the three zinc fingers.
high-specificity RNA binding (Hudson et al. 2004), and the unique Cys–His makorin motif has been suggested to be a DNA-binding domain. Taken together, the studies of MKRN1 and MKRN2 imply that MKRN3 can similarly act as an E3 ligase, based on the high homology of the RING finger domain (Fig. 2). A previous study on mice demonstrated that Mkrn3 is highly expressed in the hypothalamic arcuate nucleus during the infantile and early juvenile periods, with a reduction in expression at postnatal days 12–15, before puberty initiation (Abreu et al. 2013). The arcuate nucleus is the site of expression of critical regulators of GnRH secretion, such as kisspeptin, neurokinin B, and dynorphin (Navarro et al. 2011). Puberty is initiated with a loss of inhibitory inputs and a gain in excitatory inputs (Ojeda & Lomniczi 2014). The Mkrn3 expression pattern in the hypothalamic arcuate nucleus suggests that this peptide plays a role in the inhibition of GnRH secretion during the prepubertal quiescent period (Fig. 3). The decrease in Mkrn3 expression is hypothesized to be associated with an increase in GnRH stimulatory factors and/or GnRH expression, and it can be postulated that MKRN3 may be acting at the hypothalamic level as an E3 ligase to inhibit stimulatory inputs, so that loss-of-function mutations of MKRN3 result in early activation of the hypothalamic–pituitary–gonadal axis, expressed phenotypically as CPP. It is also possible that MKRN3 can act as a transcriptional regulator, as has been demonstrated for MKRN1. Data from the Human Protein Atlas indicate that MKRN3 is located primarily in the plasma membrane and cytoplasm, and also in the nucleus (www.proteinatlas.org). Based on its location in the plasma membrane, we can speculate that MKRN3 may also be involved in endocytosis and downregulation of receptors, as has been demonstrated for some other E3 ligases (Hershko & Ciechanover 1998). The genetic findings from patients with CPP are in agreement with the hypothesis that MKRN3 may act as a ‘brake’ or inhibitor of GnRH secretion during childhood (Hughes 2013). Further studies are needed to elucidate the precise mechanism(s) of action of MKRN3.

Conclusion

Age of puberty initiation is associated with causes of substantial morbidity and mortality. Early age of menarche is associated with breast cancer and cardiovascular disease (Kvale 1992, Lakshman et al. 2009). Therefore, it is important to understand the mechanisms controlling puberty initiation. In an attempt to identify genes that will broaden the knowledge on GnRH regulation to bring new genetic screening, and diagnostic and treatment tools, researchers have been trying to identify genes associated with CPP for several years. By studying individuals with hypogonadotropic hypogonadism, a genetic disorder caused by absence or deficiency of GnRH secretion, we learned about important stimulators of GnRH secretion (Bianco & Kaiser 2009, Semple & Topaloglu 2010). Owing to the complexity of the mechanisms involved in puberty initiation and the initial failure to identify genes associated with CPP, it has been hypothesized that CPP was not caused by mutations in a single gene, but rather was a consequence of complex interactions among environment, metabolic factors, and polygenic defects. However, with the development of new sequencing methodologies, the combination of whole-exome sequencing analysis with detailed phenotypic characterization and the careful selection of the correct cohort of patients led to the identification of MKRN3 as an important regulator of pubertal development. MKRN3 is the first gene with a probable inhibitory effect on GnRH secretion, with mutations identified within humans. To date, MKRN3 is the most common genetic defect associated with CPP. The identification of loss-of-function mutations in this gene can contribute to the diagnosis of CPP, especially in boys in whom the signs of puberty initiation are not easily detectable, thereby helping to make the diagnosis earlier and facilitating treatment decisions. In addition, the presence of MKRN3 mutations can contribute to early diagnosis of CPP in familial cases and guide genetic counseling. It is not clear if there is a sex difference in the effect of MKRN3 mutations, but it
is evident that mutations in this gene accelerate puberty initiation in both sexes. Although the precise mechanism of regulation of GnRH secretion by MKRN3 is not yet understood, its importance in the hypothalamic–pituitary–gonadal axis is indisputable. The studies of MKRN3 expression in the hypothalamic arcuate nucleus of mice support the findings that loss-of-function mutations in humans lead to early puberty initiation and strengthen the hypothesis that MKRN3 acts as an inhibitor of GnRH secretion during childhood (Fig. 3). Ultimately, the recent GWAS findings linking MKRN3 with the age of menarche have consolidated the involvement of this gene in pubertal timing.

The identification of a putative inhibitory factor in the hypothalamic–pituitary–gonadal axis has opened an exciting new arena in the neuroendocrine field. Further studies will elucidate the precise mechanism of action of this important regulator of GnRH secretion.

Declaration of interest
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