

## **Evaluation of serum makorin ring finger protein 3 (MKRN3) levels in girls with idiopathic central precocious puberty and premature thelarche**

**Running title:** Serum MKRN3 levels in girls with ICPP and PT

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## **Summary**

This study aims to investigate serum makorin ring finger protein 3 (MKRN3) levels in girls with idiopathic central precocious puberty (ICPP) and premature thelarche (PT), in order to determine whether circulating MKRN3 level is associated with ICPP and PT. A total of 90 girls were enrolled in the stud. 30 age-matched girls were allocated for each group (ICPP, PT and healthy controls [HC], respectively). The base LH (B-LH) and E2 levels were higher in ICPP girls than those in HC and PT girls. The peak LH (P-LH) levels and P-LH/P-FSH values were obviously higher in ICPP girls than those in PT girls, while higher peak FSH (P-FSH) levels were detected in PT girls when compared to those in ICPP girls. Kisspeptin levels were lower in HC girls than those in ICPP and PT girls. MKRN3 levels were the highest in HC girls among the three groups. There were relatively strong negative correlations among MKRN3, kisspeptin and P-LH/P-FSH. Circulating MKRN3 can have an important role in the onset of ICPP and PT. However, this should not be used as an independent diagnostic criterion for diagnosing ICPP or differentiating ICPP from PT, but should be used only as an adjunctive diagnostic biomarker.

## **Keywords**

makorin ring finger protein 3, kisspeptin, idiopathic central precocious puberty, premature thelarche, luteinizing hormone

## **Introduction**

Precocious puberty is a series of abnormal sexual development diseases characterized by the early appearance of adolescent development before eight years old in girls and nine years old in boys. In recent years, the incidence of this disease has

significantly increased, and it has become one of the most common endocrine system diseases in children, which seriously affects the physical and mental health of children (Atta et al. 2015). Precocious puberty can be divided according to different pathogeneses: gonadotropin releasing hormone (GnRH) dependent precocious puberty (GDPP) and GnRH independent precocious puberty (GIPP). The characteristic of GDPP is the early activation of the hypothalamus-pituitary-gonadal (HPG) axis that result in puberty development through the pulsed release of GnRH. GDPP without findable primary disease could be defined as idiopathic central precocious puberty (ICPP), which is the most common cause of precocious puberty.

The initiation of puberty development has been a hot topic in pediatric endocrinology research. Kisspeptin is a stimulating neuropeptide that can promote pubertal development *via* G-protein coupled receptor 54 (GPR54), which are present in GnRH neurons in the hypothalamus. It has an important role in HPG axis activation at the beginning of puberty. In recent years, gene mutation has provided researchers with a new perspective on puberty development. To date, researchers have discovered some genes that can regulate GnRH secretion, but only gain-of-function mutations of the KISS1 gene (encoded kisspeptin) (Silveira et al. 2010) and KISS1R gene (encoded GPR54) (Teles et al. 2008), and loss-of-function mutations of the makorin ring finger protein 3 (MKRN3) gene (Abreu et al. 2013) were defined as pathogenic variations founded in the family of central precocious puberty (CPP). In particular, the imprinted gene MKRN3 has been considered to play a crucial role in the regulation of puberty initiation. At present, studies on the MKRN3 gene have mainly focused on animal experiments and children with normal puberty. However, there are few studies in CPP children and puberty variant cases, such as premature thelarche (PT). The present study aimed to research the characteristics of serum MKRN3 levels in Chinese girls with ICPP and PT.

## **Materials and Methods**

### **Patients and controls**

This study was conducted in accordance with the declaration of Helsinki and the protocol was approved by the Scientific Ethical Committee of Qilu Hospital of Shandong University (No. KYLL-2016KS-002). A written informed consent was obtained from each patient or their guardian.

A total of 90 girls from the Department of Pediatrics, Qilu Hospital, Shandong University were enrolled in the present study from March 2016 to June 2017. Among these girls, 30 age-matched girls were allocated for each group (ICPP, PT and healthy controls [HC], respectively). The ICPP in girls was diagnosed by the presence of breast development before the age of eight years, advanced bone age (>1 year than chronological age), GnRH stimulation test, and pituitary magnetic resonance imaging (MRI). Triptorelin (Ferring GmbH, Germany) was used for the GnRH stimulation test, with an injected dose of 2.5 µg/kg (maximum dose, 100 µg), and luteinizing hormone (LH) and follicle stimulating hormone (FSH) were repeatedly measured before injection and at 30, 60 and 90 minutes after injection in the immunochemiluminometric assay (ICMA). According to the GnRH stimulation test, I basal LH (B-LH)  $\geq$  5.0 IU/L or II peaking LH (P-LH)  $\geq$  5.0 IU/L and P-LH/peaking follicle stimulating hormone (P-FSH)  $\geq$  0.6 after stimulation was considered to be CPP. All girls with ICPP had normal pituitary MRI and thyroid hormone. The PT in girls was diagnosed by the presence of only early breast development without other secondary sexual characteristics, which revealed a non-progressive self-limiting course follow-up for six months and no activation of the HPG axis according to the GnRH stimulation test. No organic cause was found in girls with ICPP and PT. HC girls without puberty development were enrolled from the child health

examination clinic in our hospital. There was no difference in age among the HC, PT and ICPP groups ( $7.16 \pm 0.35$  years old vs.  $7.13 \pm 0.48$  years old vs.  $7.20 \pm 0.41$  years old, respectively;  $F=0.229$ ,  $P>0.05$ ).

### **Evaluation of growth and development**

All subjects were instructed to take off their shoes, in order to allow for the accurate measurement of their height. The standard deviation score (SDS) for height was calculated using the following formula:  $SDS = (\text{measured value} - \text{average value}) / \text{standard deviation (SD)}$ . Body weight was expressed as body mass index (BMI), and was calculated using the following formula:  $BMI = \text{weight in kilograms} / \text{height in meters squared}$ . Physical and sexual development tests (including breast development, pubic hair, armpit hair, and external genitalia) were also performed. The wrist joint X-ray was evaluated for bone age (BA) using the Greulich-Pyle method. Gynecological ultrasound was performed to observe the ovary and uterus in children with ICPP and PT.

### **Biochemical analysis**

Blood specimens were drawn from girls with ICPP and PT before the GnRH stimulation test and at 30, 60 and 90 minutes after triptorelin injection. For HC girls, the blood specimens were drawn only once. The blood specimens without anticoagulant drawn from each girl in the three groups were centrifuged at  $2,500 \times g$  for 10 minutes, and the serum samples were extracted and stored at  $-80^{\circ}\text{C}$  until the reproductive hormone analysis was performed. The serum B-LH, B-FSH, P-LH, P-FSH and estradiol (E2) levels were measured by immunochemiluminescent assay (ICMA) using Roche original reagents and the Cobas e601 analysis series (Roche, Germany). The detection limits of LH, FSH and E2 were 0.1 mIU/ml, 0.1 mIU/ml and 5.0 pg/ml, respectively. The serum

MKRN3 and kisspeptin concentrations were detected by enzyme-linked immunosorbent assay (ELISA) using a human MKRN3 ELISA kit (MyBioSource, San Diego, CA, USA) and a human kisspeptin ELISA kit (Cusabio, WuHan, China) with a Model 450 microplate reader (Bio-Rad, USA), according to manufacturer's instructions. The sensitivity of the MKRN3 and kisspeptin ELISA kit were 7.8 pg/ml and 0.078 ng/ml, respectively.

### **Statistical analysis**

Statistical analysis was performed with the aid of the SPSS Statistics 20.0 software (SPSS Institute, Chicago, IL, USA). Data were presented as mean  $\pm$  standard deviation (SD) for normally distributed continuous variables, and medians with interquartile ranges (IQRs) for non-normally distributed variables. ANOVA and Kruskal-Wallis tests were used to evaluate the differences in normally and non-normally distributed continuous variables, respectively. The relationship between MKRN3 and other reproductive hormones were evaluated by Spearman's correlation. A two-tailed *P*-value of  $<0.05$  was considered statistically significant.

## **Results**

### **Clinical growth and development indexes**

No secondary sex characteristic was detected in HC girls. In girls with PT, all patients only had Tanner stage-II breast development without other secondary sex characteristics. Bilateral breast development was found in 56.67% (17/30) of the PT girls, while unilateral breast development was found in 43.33% (13/30) of the PT girls. For girls with unilateral breast development, 46.15% (6/13) of the girls had left breast development, while 53.85% (7/13) of the girls had right breast development. All ICPP

girls had bilateral breast development. Among these girls, 40% (12/30) were in Tanner stage-II, while 60% (18/30) were in Tanner stage-III. For ICPP girls, pubic hair, ovary and uterus development were observed as other secondary sex characteristics. None of the ICPP girls had menarche. There were no statistically significant differences in BMI among the HC, PT and ICPP girls ( $15.48 \pm 0.72 \text{ kg/m}^2$  vs.  $15.71 \pm 0.73 \text{ kg/m}^2$  vs.  $16.02 \pm 1.03 \text{ kg/m}^2$ , respectively;  $P > 0.05$ ) (Table 1). None of the girls were overweight or obese. There were no significant differences detected between the HC and PT groups, in terms of BA, BA/CA ratio and height SDS (all,  $P > 0.05$ ; Table 1). The BA, BA/CA ratio and height SDS were higher in ICPP girls, when compared to HC and PT girls (all,  $P < 0.05$ ; Table 1).

### **Laboratory biochemical indicators assay**

The B-LH and E2 levels were higher in ICPP girls, when compared to HC and PT girls (all,  $P < 0.05$ ), but there were no statistically significant differences between HC and PT girls ( $P > 0.05$ ). Furthermore, there were no significant differences among HC, PT and ICPP girls, in terms of B-FSH levels ( $P > 0.05$ ). After the GnRH stimulation test, the P-LH levels and P-LH/P-FSH were obviously higher in ICPP girls, when compared to PT girls ( $P < 0.05$ ). Higher P-FSH levels were detected after the GnRH stimulation test in PT girls, when compared to ICPP girls, which is consistent with the feature of PT ( $P < 0.05$ ). However, kisspeptin levels were lower in HC girls, when compared to ICPP and PT girls (all,  $P < 0.05$ ), but no statistically significant differences were found between ICPP and PT girls ( $P > 0.05$ ). MKRN3 levels were the highest in HC girls among the three groups (all,  $P < 0.05$ ), but there were no significant differences between ICPP and PT girls ( $P > 0.05$ ). All the above data are presented in Table 1.

### **Correlations between MKRN3 and other biochemical indicators**

There were relatively strong negative correlations between MKRN3, and kisspeptin and P-LH/P-FSH ( $\rho = -0.525$ ,  $P < 0.001$ ;  $\rho = -0.405$ ,  $P < 0.001$ ; respectively; Table 2). The weak negative correlations were detected between MKRN3 and B-LH, P-LH, P-FSH and E2 ( $\rho = -0.231$ ,  $P = 0.029$ ;  $\rho = -0.365$ ,  $P < 0.001$ ;  $\rho = -0.263$ ,  $P = 0.012$ ;  $\rho = -0.310$ ,  $P = 0.003$ ; respectively; Table 2). There was no significant correlation between MKRN3 and B-FSH ( $\rho = -0.020$ ,  $P = 0.850$ ; Table 2).

### **Discussion**

Although the pathogenesis of CPP remains not fully understood, genetic factors have been considered as an important cause of CPP in recent years (Shin 2016). To date, a series of genes have been found to be involved in regulating HPG axis function and puberty development. However, merely the KISS1, KISS1R and MKRN3 genes have been observed in patients with CPP, and CPP patients with the KISS1 or KISS1R gene mutation are very few. The pathogenicity of other candidate genes that regulate GnRH secretion, such as GABRA1, LIN28B, NPY-Y1R, TAC3 and TACR3, remains to be confirmed in patients with CPP (Bulcao et al. 2014).

The mutations of the MKRN3 gene, which was first discovered by Jong *et al.* (1999) according to the investigation of the Prader-Willi syndrome critical region in 1999, were detected in familial CPP through whole-exome sequencing analysis by Abreu *et al.* (2013). To date, a total of 21 different MKRN3 gene mutations had been found (including eight frameshift mutations, 10 missense mutations, and three nonsense mutations) in patients with CPP. In the Asian population, the frequency of MKRN3 mutations was relatively lower in sporadic and familial CPP cases, when compared to people in south America, due to different genetic backgrounds (Bessa et al. 2017; Jeong et al.. 2017; Lee



et al. 2016; Macedo et al. 2014). The first Japanese MKRN3 mutation causing CPP in an 8-year-old girl was identified (Nishioka et al. 2017). More and more evidences have indicated that the non-functional mutation of the MKRN3 gene is an important reason for human CPP. The MKRN3 gene is the key factor in regulating sexual development and reproductive function (Simon et al. 2016). The MKRN3 has been considered as a gatekeeper of puberty onset, based on the consensus of researchers. In healthy children, circulating MKRN3 levels were detected to decline before clinical pubertal onset and through puberty (Busch et al. 2016; Hagen et al. 2015; Varimo et al. 2016). Grandone *et al.* (2018) reported that girls with CPP had lower serum MKRN3 levels, when compared to age-matched controls, and that these negatively correlated to LH, FSH, E2 and BMI, even if no MKRN3 gene mutation was detected. The present findings were similar to Grandone's research, and may reveal that the involvement of MKRN3 in CPP is not only in children with familial CPP. These results may confirm the advanced decline in MKRN3 level in children with ICPP, when compared to normal controls. However, it remains to be determined whether the advanced reduction in MKRN3 is the initial factor or secondary change of ICPP.

Kisspeptin/neurokinin B/dynorphin (KNDy) neuropeptides, especially kisspeptin, have been recognized as important factors that promote GnRH pulse release and play an important role in pubertal development, which is expressed in KNDy neurons (Merkle et al. 2012). de Vries (2009) and Rhie *et al.* (2011) found that serum kisspeptin levels were significantly higher in girls with CPP, when compared to age-matched prepubertal controls. Demirbilek *et al.* (2012) reported that the circulating kisspeptin levels of girls with CPP before treatment were higher, when compared to age-matched controls, and these significantly declined after gonadotropin releasing hormone analogue (GnRHa) effective treatment. The data of the present study supports a previous research, in which

the serum kisspeptin levels of girls with ICPP in the present study were also higher than prepubertal controls. The present study may be the first study that has correlated MKRN3 with kisspeptin in ICPP patients. The negative relationship between these reveals the advanced decline in MKRN3 and rise in kisspeptin would be an important physiopathologic mechanism of ICPP.

The early manifestation of girls with ICPP and PT were both breast development, but it was difficult to distinguish at the early stage. At present, the differential diagnosis was mainly based on clinical examination, BA and GnRH stimulation tests, but there were apparent limitations. For example, ICPP girls in the early stage may have not presented an advanced BA, growth acceleration and typical GnRH stimulation test response due to the short course of disease. In this case, ICPP may be misdiagnosed as PT. The change in serum kisspeptin in ICPP girls remains under dispute. Akinçi *et al.* (2012) and Kurnaz E *et al.* (2017) reported that plasma kisspeptin levels were higher in girls with PT, when compared to age-matched controls. Abacı *et al.* (2015) found that the serum kisspeptin levels were significantly higher in ICPP and PT girls, when compared to controls, but there was no significant difference between ICPP and PT girls. These results were similar to those in the present study. The present study also found that serum MKRN3 levels were lower in ICPP and PT girls, when compared to HC girls, which were accompanied by higher levels of kisspeptin, but there was no significant difference between ICPP and PT girls. Negative relationships were detected between MKRN3, and kisspeptin, gonadotropins and E2. These results may partially prove the hypothesis that PT may have resulted from the temporary and incomplete activation of central signals. The overlap of MKRN3 and kisspeptin levels among ICPP, PT and HC suggests that MKRN3 or kisspeptin level alone cannot be reliable indicators for the diagnosis of ICPP, or the differentiation between ICPP and PT.

Some studies have had different results. The study conducted by Yang *et al.* (2015) demonstrated that plasma kisspeptin levels were higher in ICPP girls, when compared to PT girls and normal controls, but there was no difference between PT and controls. Özgen *et al.* (2016) reported that plasma kisspeptin levels were higher in ICPP girls, when compared to HC girls, but there was no difference between either PT and HC, or ICPP and PT. The difference in these results should be mainly due to the small sample size of present studies.

In conclusion, lower serum MKRN3 levels were detected in girls with ICPP and PT, when compared to age-matched healthy controls. Furthermore, there were negative relationships between MKRN3, and kisspeptin, gonadotropins and E2. The increase in serum kisspeptin level and decline in serum MKRN3 level suggests that these could take part in pubertal development and play an important role in the onset of ICPP and PT. The present study suggests that serum MKRN3 levels may not be used as an independent diagnostic criterion alone for diagnosing ICPP, or for differentiating ICPP from PT, but only as an adjunctive diagnostic biomarker. The present studies all had a small sample size. Hence, further large-scale studies are needed to clarify the role of MKRN3 in the occurrence of ICPP, and confirm the usefulness of MKRN3 as a diagnostic biomarker.

### **Conflicts of interest**

All authors declare that they have no conflict of interest to disclose.

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Table 1 Clinic and laboratory indicators among HC, PT and ICPP girls

	HC (n=30)	PT (n=30)	ICPP (n=30)
CA (years)	7.16±0.35	7.13±0.48	7.20±0.41*
BA (years)	7.27±0.38	7.42±0.47 <sup>▲</sup>	8.48±0.62**
BA/CA	1.02±0.04	1.04±0.03 <sup>▲</sup>	1.18±0.06**
Height SDS	0.25±0.44	0.43±0.40 <sup>▲</sup>	1.07±0.37**
BMI (kg/m <sup>2</sup> )	15.48±0.72	15.71±0.73	16.02±1.03*
B-LH (mIU/ml)	0.12(0.10-0.28)	0.19(0.10-0.45) <sup>▲</sup>	0.86(0.38-1.25)**
P-LH (mIU/ml)	--	3.82(2.12-5.10)	11.27(7.44-16.41)**
B-FSH	2.35(1.96-3.39)	3.58(1.94-4.68)	3.38(1.85-5.86)*
(mIU/ml)			
P-FSH	--	22.89(17.74-34.17)	11.57(10.03-17.57)**
(mIU/ml)			
P-LH/P-FSH	--	0.17(0.11-0.21)	0.77(0.66-1.21)**
E2 (pg/ml)	7.57±1.71	8.05±3.19 <sup>▲</sup>	21.01±8.81**
kisspeptin	1.31±0.19	1.73±0.18 <sup>▲▲</sup>	1.82±0.16***
(ng/ml)			
MKRN3	978.0(897.5-1069.8)	834.0(663.5-967.8) <sup>▲▲</sup>	680.0(519.8-983.5)***
(pg/ml)			

HC, healthy controls; PT, premature thelarche; ICPP, idiopathic central precocious puberty; CA, chronological age; SDS, standard deviation score; BMI, body mass index; B-, base-; P-, peak-; LH, luteinizing hormone; FSH, follicle stimulating hormone; E2,



estradiol; MKRN3, makorin ring finger protein 3; \* among three groups  $P > 0.05$ ; \*\* v.s.

PT group  $P < 0.05$ ; \*\*\*v.s. PT group  $P > 0.05$ ; ▲ v.s. HC group  $P > 0.05$ ; ▲▲ v.s. HC group

$P < 0.05$

Table 2 Correlations between MKRN3 and other reproductive endocrine indicators

	B-LH	P-LH	B-FSH	P-FSH	P-LH/P-FSH	E2	kisspeptin
$\rho$	-0.231	-0.365	0.020	-0.263	-0.405	-0.310	-0.525
P	0.029	<0.001	0.850	0.012	<0.001	0.003	<0.001

MKRN3, makorin ring finger protein 3; B-, base-; P-, peak-; LH, luteinizing hormone;

FSH, follicle stimulating hormone; E2, estradiol