RESEARCH

The role of kisspeptin and MKRN3 in the diagnosis of central precocious puberty in girls

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Abstract

Objective: To evaluate the characteristics and significance of serum kisspeptin and makorin ring finger protein 3 (MKRN3) levels for the diagnosis of central precocious puberty (CPP) in girls.

Method: Thirty four individuals with CPP, 17 individuals with premature thelarche (PT), and 28 age-matched prepubertal girls as normal control (NC) were recruited in this case–control study. Physical measurements included BMI and tests for breast, bone, and sexual characteristics. Biochemical measurements included serum LH, FSH, estradiol, insulin-like growth factor-1, MKRN3, and kisspeptin. Blood samples were taken from individuals with CPP and PT before the gonadotrophin-releasing hormone stimulation test and at 30, 60, 90, and 120 min after injection with triptorelin.

Results: Serum kisspeptin levels were higher in the CPP group when compared to the NC group (P = 0.020), while serum MKRN3 levels were lower in the two groups (P = 0.028). There were no significant differences between the CPP and PT groups as well as the PT and NC groups (all, P > 0.05). The cut-off value of serum kisspeptin differentiating patients with CPP from those without CPP was 0.40 nmol/L, with 82.4% sensitivity and 57.1% specificity, while the cut-off value of serum MKRN3 was 0.33 pmol/L, with 79.4% sensitivity and 53.6% specificity. The area under the curves (AUCs) of both kisspeptin and MKRN3 for differentiating those girls with CPP from PT were less than 0.5.

Conclusions: Serum levels of kisspeptin and MKRN3 may play an auxiliary role in predicting CPP. However, the two measurements were not able to differentiate girls with CPP from PT and prepubertal control. This study emphasizes the need to search for markers to simplify the accurate diagnosis of CPP in girls.

Key Words

- kisspeptin
- makorin ring finger protein 3
- central precocious puberty
- premature thelarche
- gonadotropin-releasing hormone

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Introduction

In humans, the initiation of puberty is caused by reactivation of the hypothalamic-pituitary-gonadal (HPG) axis with re-emergence of pulsatile hypothalamic gonadotropin-releasing hormone (GnRH) secretion, leading to the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Central precocious puberty (CPP) is a common pediatric endocrine disease due to premature reactivation of the HPG axis leading to the development of secondary sexual characteristics before the age of 8 in girls and 9 in boys.

In Chinese girls, the incidence was about 1/5,000–1/10,000 (1); however, the most recent national survey on precocious puberty reported a six-fold increase in incidence for Danish girls (from 2.6/10,000 to 14.6/10,000) (2). In addition, early puberty in children has become a worldwide trend. There is also controversial debate as

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to whether the International Reference Age for puberty should be reset. The initiation of puberty depends on the interactions between inhibitory and stimulatory factors upon the HPG axis in individuals (3). Over the last 10 years, kisspeptin has emerged as an important excitatory regulator in the initiation of puberty (4); in recent years, makorin ring finger protein 3 (MKRN3) has also been considered as an inhibitor that plays a more critical role in regulating the initiation of puberty (5).

Up to now, the serum levels of kisspeptin in previous studies were reported to be higher in cases of CPP or lower in some cases (6, 7). The serum levels of kisspeptin in precocious puberty are inconsistent and this includes cases of premature thelarche (PT), which is defined as isolated budding of the breast in girls before 8 without other pubertal features. Additionally, there are only a few studies on the serum levels of MKRN3 in CPP and PT girls. How kisspeptin and MKRN3 are involved in the onset of puberty and their relationship to LH has yet not been clarified. The purpose of this study was to evaluate the characteristics of serum kisspeptin and MKRN3 levels and the significance of their use in the diagnosis of CPP and PT in girls.

Subjects and methods

Patients

Thirty four girls with CPP, 17 girls with PT, and 28 age-matched prepubertal girls as normal control (NC) were recruited into this case–control study. The subjects were recruited from September 2019 to January 2021 from the Department of Pediatrics, The First Affiliated Hospital of Guangxi Medical University. The study protocol was in line with the Declaration of Helsinki and was approved by the Scientific Ethics Committee of The First Affiliated Hospital of Guangxi Medical University. The informed consent of each participant was obtained.

The comprehensive diagnosis of CPP girls was based on breast development before 8 years of age, accelerated linear growth, progressive bone age (BA, >1 year than chronological age), GnRH stimulation test, and gynecological ultrasound according to Consensus Statement for the Diagnosis and Treatment of Central Precocious Puberty (2015) (1). GnRH stimulation test was performed by subcutaneously injecting 2.5 μg/kg (maximum dose, 100 μg) of triptorelin (Ferring GmbH), and LH and FSH were detected repeatedly before and at 30, 60, 90, and 120 min after injection. According to the GnRH stimulation test, peak LH(P-LH)/peak FSH(P-FSH) ≥ 0.6 levels and P-LH ≥ 5.0 IU/L after stimulation were considered to be CPP, whereas P-LH < 5.0 IU/L was considered to be PT in the subjects. Patients with organic lesions or syndromes were excluded. The prepubertal girls in the NC group were recruited from children undergoing physical examination in our hospital. A basal LH(B-LH) < 0.6 IU/L was considered as prepubertal hormone level (8).

Evaluation of growth and development

General physical examinations were recorded in all girls, including weight and height measurements, BMI, and Tanner staging of breast development. Sexual development tests (involving the appearance of pubic and armpit hair, as well as external genitalia) were also carried out. The left wrist joint was X-rayed and used to evaluate BA) according to the method of Greulich and Pyle. The ovaries and uteri of girls with CPP and PT were observed by gynecological ultrasound examinations.

Biochemical analysis

All blood specimens without anticoagulant were centrifuged at 4244 ***g*** for 10 min, and the resultant serum specimens were aliquoted and stored at –20°C. The serum LH, FSH, and estradiol (E2) levels were measured by chemiluminescence immunoassays (Mindray, CL-2000i, Shenzhen, China), with intra- and inter-assay CVs of LH, FSH, and E2 less than 10%. Insulin-like growth factor-1 (IGF-1) was tested by chemiluminescence (BIOBASE, MAGLUMI 4000 plus, Shandong, China). The serum MKRN3 and kisspeptin levels were measured by commercially available human MKRN3 and human KISS1 ELISA kits (Fine Test, Wuhan, China), with detection limits of 0.210 pmol/L and 0.008 nmol/L, respectively. The intra- and inter-assay CVs were less than 8% and 10%, respectively.

Statistical analysis

Statistical analysis was performed by using SPSS Statistics Version 23.0 software (IBM SPSS). Data were tested for normality using the Shapiro–Wilk test, and values greater than 0.05 were considered normal. For normally distributed continuous variables, the data were expressed as mean ± S.D.), and for non-normally distributed variables, they were expressed as the median and interquartile range (IQR). Student's *t*-tests and ANOVA tests were utilized to compare normally distributed continuous variables, and

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the Mann–Whitney and Kruskal–Wallis nonparametric tests were utilized to compare non-normally distributed variables. The relationships among MKRN3, kisspeptin, and anthropometric parameters were evaluated by using Pearson's correlation. The relationships among MKRN3, kisspeptin, and gonadotrophins were assessed by using Spearman's rank-order. *P* value less than 0.05 was considered to be statistically significant. A receiver operating characteristic (ROC) was constructed for kisspeptin and MKRN3 levels in order to differentiate CPP girls from the other two groups. The sensitivity and specificity were calculated based on cut-off values obtained by the ROC curves.

Results

Clinical and laboratory characteristics

No secondary sexual characteristics were found in girls of NC group. Bilateral breast development occurred in 82.35% (14/17) of girls in PT group, while other 17.65% (3/17) with unilateral breast development. Bilateral breast development occurred in all CPP girls, 50% (17/34) of these girls were in Tanner stage II, while other 50% (17/34) were in Tanner stage III. Only one CPP patient had progressed to menarche.

The clinical and biochemical characteristics of all girls were shown in Table 1. CPP patients had higher P-LH, P-LH/P-FSH ratios, and IGF-1 levels than PT girls (all, $P < 0.001$), and no difference was found in P-FSH levels ($P = 0.156$). CPP girls had higher serum B-LH and B-FSH levels than PT and NC groups (all, $P < 0.001$); however, there were no differences between PT and NC groups, respectively (all, $P > 0.05$). Serum kisspeptin levels were higher in the CPP group compared to the NC group ($P = 0.020$), while serum MKRN3 levels were lower in the CPP group compared to the NC group ($P = 0.028$). No differences were found between the CPP and PT groups as well as the PT and NC groups (all, $P > 0.05$).

Correlations

Serum kisspeptin levels were correlated with B-LH and basal FSH(B-FSH) as shown in Fig. 1 ($r = 0.325$, $P = 0.004$; $r = 0.278$, $P = 0.013$; respectively). No other correlations were found between serum kisspeptin and MKRN3, P-LH, P-FSH, P-LH/P-FSH, IGF-1, E2 (all, $P > 0.05$). Serum MKRN3 level was inversely correlated with BMI as illustrated in Fig. 2 ($r = -0.247$, $P = 0.028$). However, there were no correlations between MKRN3 levels and the reproductive hormones (all, $P > 0.05$).

Table 1 Clinical and biochemical characteristics of the NC, PT, and CPP girls.

Items	NC ($n = 28$)	PT ($n = 17$)	CPP ($n = 34$)
CA (years)	7.58 ± 0.74	7.39 ± 0.89	7.80 ± 0.69
BA (years)	–	7.29 ± 0.95	8.96 ± 1.23[b]
BA/CA	–	0.996 ± 0.13	1.149 ± 0.14[b]
Height (cm)	123.15 ± 6.71	123.82 ± 5.45	133.44 ± 8.79[a,b]
BMI (kg/m^2)	14.63 ± 1.07	15.90 ± 1.46[a]	17.29 ± 2.04[a,b]
Length of the uterus (cm)	–	1.85 ± 0.41	2.41 ± 0.59[b]
Ovarian volume (ml)	–	1.96 ± 0.95	3.36 ± 2.44b
B-LH (IU/L)	0.12 (0.07, 0.16)	0.11 (0.06, 0.17)	1.41 (0.56, 2.33)[a,b]
P-LH (IU/L)	–	3.64 (2.89, 4.22)	17.38 (11.43, 34.92)[b]
B-FSH (IU/L)	1.85 (1.48, 2.26)	2.07 (1.35, 2.58)	3.96 (2.50, 5.92)[a,b]
P-FSH (IU/L)	–	13.34 (10.70, 17.95)	16.77 (11.96, 22.68)
P-LH/P-FSH	–	0.27 (0.18, 0.35)	1.35 (0.83, 1.94)[b]
IGF-1 (nmol/L)	–	32.02 ± 7.44	48.53 ± 14.18[b]
E2 (pmol/L)	23.56 (6.17, 50.49)	91.64 (38.42, 111.20)[a]	100.54 (75.42, 147.45)[a]
Kisspeptin (nmol/L)	0.12 (0.10, 0.22)	0.17 (0.14, 0.33)	0.17 (0.14, 0.25)[a]
MKRN3 (pmol/L)	13.35 (4.10, 31.06)	2.44 (1.06, 33.72)	5.06 (1.27, 10.89)[a]

The median and interquartile ranges (IQR) were shown except for age, height, BMI, length of the uterus, ovarian volume, and IGF-1 levels which were expressed as means ± s.d.s. Student's *t* tests were performed for BA, BA/CA, length of the uterus, ovarian volume, and IGF-1 levels, and the Mann–Whitney U tests were performed for P-LH, P-FSH, P-LH/P-FSH. The ANOVA tests were used for age, height and BMI, and the Kruskal-Wallis tests were used for all other parameters.

[a]$P < 0.05$ when compared to the NC group; [b]$P < 0.05$ when compared to the PT group.

NC, normal control; PT, premature thelarche; CPP, central precocious puberty; CA, chronological age; BA: bone age; BMI, BMI; s.d., standard deviation; B-, base-; P-, peak-; LH, luteinizing hormone; FSH, follicle stimulating hormone; IGF-1, insulin-like growth factor-1; E2, estradiol; MKRN3, makorin ring finger protein 3.

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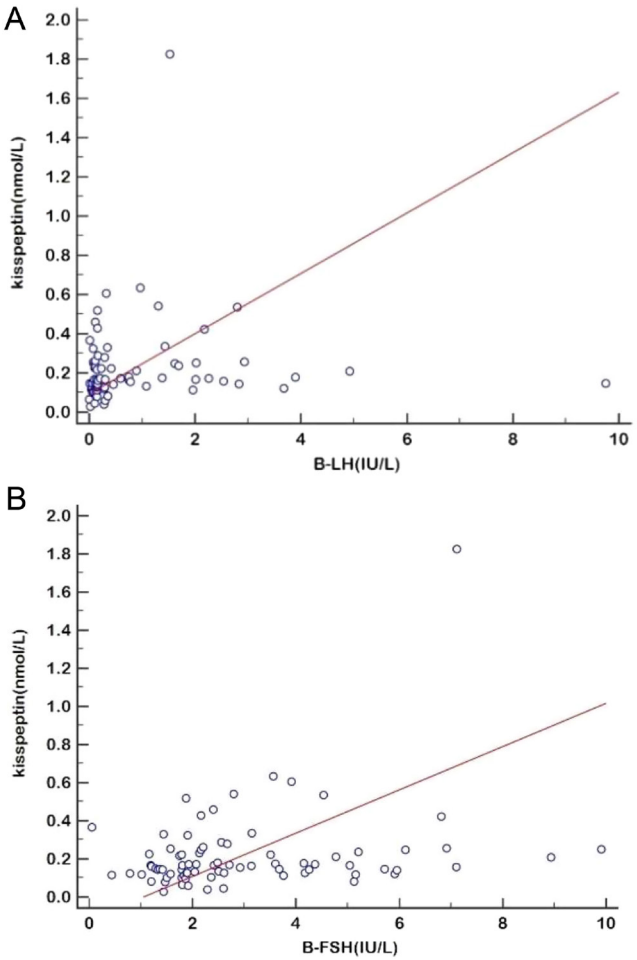

Figure 1
Positive correlations between circulating kisspeptin levels and (A) B-LH and (B) B-FSH in patients with the CPP, PT, and NC groups. (A, $r = 0.325$, $P = 0.004$; B, $r = 0.278$, $P = 0.013$).

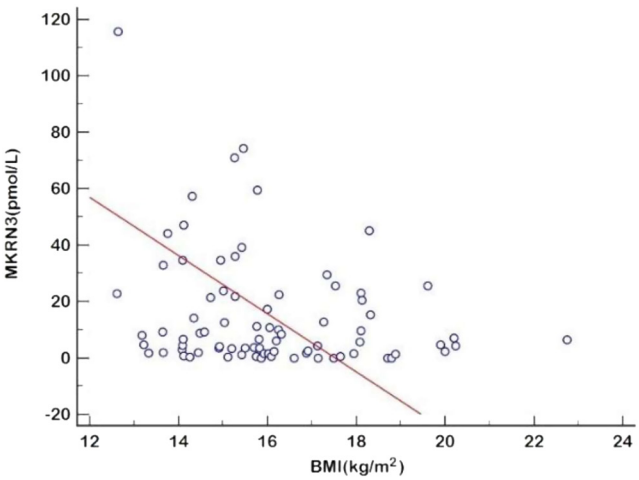

Figure 2
Inverse correlations between circulating MKRN3 levels and BMI in patients from the CPP, PT, and NC groups. ($r = -0.247$, $P = 0.028$).

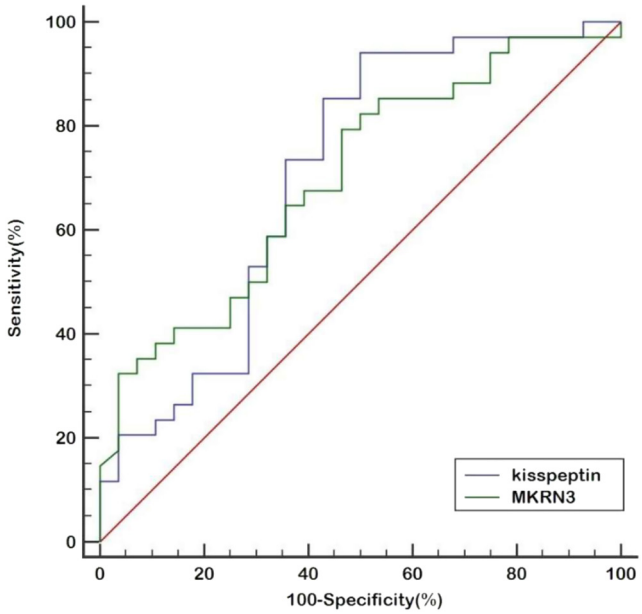

Figure 3
The receiver operating characteristic curves of kisspeptin and MKRN3 levels. The areas under curve for identifying the patients with or without CPP were 0.707 (95% CI: 0.572–0.842, $P = 0.005$) and 0.699 (95% CI: 0.568–0.829, $P = 0.008$), respectively. The cut-off value of serum kisspeptin differentiating patients with CPP from those without CPP was 0.40 nmol/L, with 82.4% sensitivity and 57.1% specificity, while the cut-off value of serum MKRN3 was 0.33 pmol/L, with 79.4% sensitivity and 53.6% specificity.

ROC analysis

ROC analysis was used to predict the significance of kisspeptin and MKRN3 levels for the diagnosis of CPP as shown in Fig. 3. The AUC for identifying the patients with or without CPP were 0.707 (95% CI: 0.572–0.842, $P = 0.005$) and 0.699 (95% CI: 0.568–0.829, $P = 0.008$), respectively. The cut-off value of serum kisspeptin differentiating patients with CPP from those without CPP was 0.40 nmol/L, with 82.4% sensitivity and 57.1% specificity, while the cut-off value of serum MKRN3 was 0.33 pmol/L, with 79.4% sensitivity and 53.6% specificity. According to ROC curve analysis, the AUC for Kisspeptin combined with MKRN3 levels to identify CPP was 0.793 (95% CI: 0.671–0.885, $P < 0.0001$) with 94.1% sensitivity and 60.7% specificity. The AUC of both kisspeptin and MKRN3 for differentiating those patients with CPP from PT was less than 0.5.

Discussion

In this study, we sought to investigate how serum kisspeptin and MKRN3 levels change and to test the

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predictive value of serum kisspeptin and MKRN3 levels in the diagnosis of girls with CPP. First, our data indicated that the serum kisspeptin levels were significantly higher and the serum MKRN3 levels were lower in girls with CPP than in age-matched prepubertal controls. The changes of serum kisspeptin and MKRN3 levels support the concept that kisspeptin and MKRN3 play indispensable roles in the onset of puberty. We also found that serum kisspeptin levels were positively correlated with B-LH and B-FSH. However, no correlations were observed between gonadotropins and MKRN3 levels. Moreover, this study revealed the predictive significance of serum kisspeptin and MKRN3 levels in CPP girls.

The increased levels of kisspeptin and the decreased levels of MKRN3 in girls with CPP in our study is congruent with those previous reports showing there were changes of serum kisspeptin and MKRN3 levels during the peripubertal period (9, 10, 11, 12). It has been demonstrated in mice that the expression of KISS-1 mRNA in the anteroventral periventricular nucleus was induced by the onset of puberty (13), while the kisspeptin mRNA-expressing neurons in the arcuate nuclei appeared earlier than those in the anteroventral periventricular nuclei in rats (14). Activating mutations in genes KISS1 (encoding kisspeptin) and KISS1R (encoding kisspeptin receptor) and inactivating mutations in imprinted gene MKRN3 (encoding protein MKRN3) are monogenic causes of CPP. MKRN3 mutations are the most common genetic etiology associated with CPP, especially in familial cases, while KISS1/KISS1R mutations are very rare. Teles *et al*. (15) were the first to identify a KISS1R mutation (Arg386Pro) in a girl with CPP. It was revealed that an increased and prolonged cellular response to kisspeptin stimulation led to the release of an increased amplitude pulse of GnRH and accelerate the maturation of the reproductive axis. This was similar to the mechanism of the first KISS1 heterozygous mutation (Pro74Ser) discovered by Silveira *et al*. (16), which identified that the Pro74Ser mutation had a greater capacity to stimulate signal transduction than the wild type, leading to greater kisspeptin bioavailability. In addition to an increase in excitatory inputs, a reduction in inhibitory influence seemed to be a prerequisite for re-emergence of pulsatile GnRH secretion (17). Abreu *et al*. (5) were the first to suggest that a mutation of MKRN3 causing its inactivation was the reason for CPP in humans. Levels of MKRN3 mRNA were shown to be high in the arcuate nuclei of prepubertal mice, and this appeared to decrease immediately before puberty and then remained low after puberty (5). A study by Hagen *et al.* (11) in Danish girls came to the same conclusion that serum levels of MKRN3 declined by 15% preceding pubertal onset and MKRN3 levels were lower in early maturing girls compared to age-matched prepubertal girls. However, it revealed that genetic variants near MKRN3 did not correlate with the serum levels of MKRN3. Recently, Li *et al*. (18) found that MKRN3-mediated ubiquitin signaling could control both transcriptional and post-transcriptional switches of mammalian puberty initiation by epigenetically regulating the transcription of GNRH1. Still, the function of MKRN3 and the mechanism by which MKRN3 mutations trigger puberty are not yet known.

Our findings of decreasing serum levels of MKRN3 are in line with the findings in previous studies which have showed declining levels of MKRN3 preceded pubertal onset and undetectable or low MKRN3 levels were observed in a subgroup of patients with early onset of puberty (11). These recent human and animal findings suggest that MKRN3 plays an inhibitory role in the reproductive axis which represents a novel pathway in pubertal regulation (19). However, kisspeptin and MKRN3 levels in PT girls were not statistically different from those in the CPP and NC groups in our study, which was slightly different from the results of previous studies. Akinci *et al*. (20) showed that kisspeptin levels in the PT group were higher compared to the levels in the control group. Kisspeptin levels of CPP girls were higher than the PT group in Yang *et al.*'s study (21), while no difference was found between the CPP and PT groups in Abaci *et al*.'s study (22). Ge *et al*. (23) showed kisspeptin levels were the lowest, while MKRN3 levels were the highest in the control girls among three groups, but there also were no significant differences between girls in the CPP and PT groups. Although PT acting as partial CPP with a not fully activated HPG axis, serum kisspeptin and MKRN3 levels in PT group were not simply between the levels of CPP and NC group. Therefore, whether kisspeptin and MKRN3 are switches for full activation of this axis, or whether they operate as effectors of up-stream regulatory factors requires further investigation.

It is known that kisspeptin is a potent GnRH-dependent stimulator of LH secretion (24). We showed positive correlations between serum kisspeptin levels and B-LH, B-FSH. However, there was no correlation between kisspeptin levels and either P-LH or P-LH/P-FSH ratios in our study, which was inconsistent with the findings of a previous study which showed that serum kisspeptin was positively correlated with P-LH, and P-LH/P-FSH ratios during the GnRH stimulation test (9). Furthermore, we found no correlation between MKRN3 levels and either B-LH, B-FSH or P-LH/P-FSH ratios. In addition, there was no correlation between kisspeptin and MKRN3 levels.

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These were also inconsistent with the findings of previous studies which showed that there were inverse correlations among MKRN3, kisspeptin, and gonadotropins (12, 23). The reason for this inconsistency may be an evident overlap between the levels of kisspeptin and MKRN3 in girls with the CPP and PT groups when compared to controls. Another plausible explanation for the inconsistency may be the small sample size of our study or the absence of the data with respect to the GnRH stimulation test in our control group, which were not performed due to ethical considerations.

Of note, we found serum MKRN3 levels were inversely correlated with BMI, but there was no correlation between kisspeptin levels and BMI. Although this correlation was also found in some previous studies (6, 9, 20, 25), the effects of BMI on the results of the GnRH stimulation test and on kisspeptin levels should be taken into account in girls with precocious puberty in recent studies (26, 27). Similarly, the inverse correlation between MKRN3 levels and BMI was in line with another previous study (12), and no correlation was found in at least one study (28). The adipose hormone and leptin are known to play the essential roles in the metabolic control of puberty and fertility (29). The inverse relationship with BMI suggests that MKRN3 in girls may be regulated by nutritional factors and/or adipokines such as leptin (12). Leptin may act as a permissive factor, rather than a trigger, that allows puberty to proceed only if sufficient body energy reserves are available (29). However, a recent report showed that the peripubertal decline in MKRN3 expression was independent of leptin action in a leptin-deficient (ob/ob) mouse model (30). Thus, interactions and relationships between neuroendocrine factors and adipokines in the onset of puberty have yet to be fully elucidated.

To date, the GnRH stimulation test is considered to be the best available biochemical parameter for diagnosing CPP (31). But its clinical application is limited by the disadvantages such as requiring repeated sampling, time consuming, high cost, and poor patient compliance. Looking for simplified methods to diagnose CPP is a focus of current research in the field. So far, numerous studies on kisspeptin and/or MKRN3 levels have showed that these parameters may be useful as adjunctive tools in diagnosing CPP (6, 9, 10, 12, 28). However, none of these studies have evaluated the predictive value of kisspeptin and MKRN3 levels in combination for diagnosis of CPP. This study showed that the predictive value of kisspeptin combined with MKRN3 levels in diagnosing CPP was not significant when compared to using the levels of these metabolites separately. Although the method is highly sensitive, its specificity is low (94.1% sensitivity and 60.7% specificity), which limits its use as a single diagnostic tool until further data can be obtained from conducting larger studies. Perhaps an improvement to diagnosis can be achieved by incorporating measurements for another metabolite, such as serum levels of neurokinin B (32).

In this study, we also found values in the CPP group were significantly higher than the PT group for BA, height, ovarian volume, uterine length, and circulating IGF-1 levels. This suggests that the differential diagnosis of PT and CPP may be based on the comprehensive analysis of clinical indicators. The serum E2 concentration was significantly higher in girls with PT than prepubertal controls and similar to that of the CPP group. Transient E2 secretion by small follicular cysts may be attributable to partial and transient activation of the HPG axis and increased sensitivity of breast tissue to minute amounts of E2, leading to increased E2 levels in PT girls (33). Serum E2 levels correlated well with the stage of sexual development and might be of diagnostic and prognostic use in girls with sexual precocity (33).

In conclusion, our findings that there are significant differences in the serum levels of kisspeptin and MKRN3 between the CPP group and prepubertal controls suggest that these two markers may play an auxiliary role in predicting CPP. However, these markers are not able to differentiate patients with CPP from those with PT and prepubertal controls owing to the evident overlap observed in the three groups. This may be ascribed to the complex mechanisms involved in the onset of precocious puberty, including the interactions of genetic and nutritional status, as well as epigenetic factors (34). There is still a need to elucidate the pathogenesis of precocious puberty and to seek reliable biochemical markers to aid and simplify the diagnosis of CPP in girls.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D L and M L participated in study design and data analysis, and together with J Zho drafted the manuscript. M L and Y Che completed the experimental part. B Lia and J T participated in data collection. All authors approved the final version.

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