No association of the Arg51Gln and Leu72Met polymorphisms of the ghrelin gene and polycystic ovary syndrome

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BACKGROUND: Ghrelin plays a role in regulating glucose metabolism and energy balance. Polymorphisms in preproghrelin and ghrelin gene could be responsible for obesity, insulin resistance and low ghrelin levels observed in some individuals. The objective of this study was to evaluate the influence of two single-nucleotide polymorphisms (SNPs) of ghrelin gene on the clinical, the hormonal and metabolic features in women with polycystic ovary syndrome (PCOS) in a Chinese population.

METHODS: A large sample of Chinese PCOS (n = 271) women and a control group (n = 296) of healthy women matched for age were studied. Hormone and metabolic profiles were measured and blood samples were collected for genotype and allelic frequency analysis. Non-synonymous SNPs in the coding region (exon 2) of the preproghrelin gene (Arg51Gln (346 G > A) and Leu72Met (408 C > A) were studied using PCR and restriction fragment length polymorphism analysis.

RESULTS: The polymorphism Arg51Gln was not found in the cohorts studied. The distribution of Leu72Met was similar in PCOS group and in healthy controls. There was no significant difference in age, BMI, waist-hip-ratio and levels of FSH, LH, estradiol, testosterone and prolactin between PCOS patients with different genotypes, and the level of plasma glucose and insulin was also similar.

CONCLUSIONS: No association was found between Leu72Met and Arg51Gln polymorphisms in the ghrelin gene and PCOS in Chinese population.

Key words: polycystic ovary syndrome / single nucleotide polymorphism / Ghrelin / gene / glucose metabolism

Introduction

Polycystic ovary syndrome (PCOS) is a common complex and heterogeneous endocrine disorder affecting women in their reproductive years. It is characterized by oligomenorrhea or amenorrhea, hyperandrogenism and multiple small subcapsular cystic follicles in the ovary on ultrasonography. Its clinical manifestations include insulin resistance (IR), an increased prevalence of obesity and abdominal obesity, the metabolic syndrome, impaired glucose tolerance and type 2 diabetes mellitus (T2DM) (Norman et al., 2001; Hart et al., 2001).
Several polymorphisms of ghrelin gene have been described, including the single base substitutions G152A, with Gln replacing Arg at position 72 in the preproghrelin C-terminal tail, hence their important function. Some studies found that these two polymorphisms were associated with obesity and obesity-related phenotypes (Ukkola et al., 2001, 2002, 2003; Korbonits et al., 2002; Miraglia et al., 2004; Kuzuya et al., 2006). The Met72 allele seems to be protective against fat accumulation (Ukkola et al., 2002) and associate with an earlier age of self-reported onset of obesity in several studies (Ukkola et al., 2001, Miraglia et al., 2004). Some studies demonstrated a positive genetic association between Leu72Met and IR, T2DM, metabolic syndrome (Poykko et al., 2003a, b; Steinle et al., 2005). Others explored the relationship of variants of ghrelin gene with plasma ghrelin level. Arg51Gln polymorphism was found to be associated with lower plasma ghrelin levels (Ukkola et al., 2002, 2005) and obesity (Ukkola et al., 2001).

Since obesity, particularly the central phenotype, is frequently associated with PCOS (Pasquali and Casimirri, 1993; Gambineri et al., 2002), and hyperinsulinemia in PCOS has been suspected to play a pivotal role in the development and/or maintenance of the syndrome (Poretsky, 1991; Dunai, 1997), studies exploring the association of polymorphisms in Ghrelin gene with PCOS are much warranted.

In this study, we focused on non-synonymous single-nucleotide polymorphisms (SNPs) in the coding region of the preproghrelin gene (Arg51Gln (346 G>A) and Leu72Met (408 C>A) in exon 2 because they result in amino acid changes and thus are most likely to affect the biological function of the gene. We examined the distribution of these two polymorphisms in PCOS and healthy controls, and evaluated the association of polymorphism in Ghrelin gene with PCOS and with PCOS-related phenotypes in the Chinese population.

Materials and Methods

Subjects

Pcos patients

Patients with PCOS were consecutively recruited between 1 January 2004 and 30 August 2006 from the reproductive center clinic at the Shandong Provincial Hospital in Jinan. None had used hormonal preparations, including oral contraceptives, for at least 3 months preceding the study. According to the revised diagnostic criteria, announced in the 2003 American Society for Reproductive Medicine/European Society for Human Reproduction and Embryology (ASRM/ESHRE) Rotterdam consensus, PCOS is diagnosed when the phenotypes of patients are satisfied by two of the three criteria [oligomenorrhea or amenorrhea, clinical or biochemical hyperandrogenism and ultrasonographic polycystic ovary (PCO) morphology], and other causes, such as non-classic congenital adrenal hyperplasia, are excluded (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). We recruited 271 PCOS women.

Controls

296 healthy age-matched women with normal cycles, also recruited during this period and evaluated consecutively, served as the controls. All the controls were carefully evaluated to avoid any selection bias. Each of them had normal ovulatory menstrual cycles, absence of hirsutism and other manifestations of hyperandrogenism, and absence of sonographic signs of PCOS. None of them had sign of galactorrhea and thyroid dysfunction or personal or family history of diabetes. They had normal hormonal status, and had not received oral contraceptives or any drug therapy for at least 3 months before starting the study. Women in the control group were recruited from the same geographic area as the PCOS patients. All the subjects in this study were Chinese women.

Institutional review board approval was obtained for this study, and informed consent was obtained from all women before inclusion.

Serum sex hormone analysis and metabolic examinations

After undergoing a history and physical examination, including measurement of abdominal and hip circumferences, blood sampling in the fasting state of all subjects was performed on Day 2–4 of menstrual cycle or during amenorrhea, after excluding pregnancy by appropriate hormone. Fasting venous blood samples were collected between 08.00 AM and 10.00 AM after a 12-h overnight fast. Serum sex hormones, including FSH, LH, prolactin (PRL), total testosterone and estradiol (E2) were detected by chemiluminescence immunization (Beckman Access Health Co., USA). All inter- and intra-assay coefficients of variation are <5% and 8%, respectively.

In PCOS patients, blood samples also were obtained at baseline and at 30-min intervals for 2 h during a 75-g oral glucose tolerance test (OGTT) to measure glucose and insulin. Serum glucose was measured by the glucose oxidase method on an automatic biochemistry analyzer (AU640, Olympus Co., Japan) and the level of insulin was detected by chemiluminescence immunization (Beckman Access Health Co., USA). Total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL) concentrations were measured enzymatically on an automated biochemistry analyzer (AU640, Olympus Co.).

Genetic analysis

Blood samples for molecular genetic studies were collected in tubes containing EDTA as anticoagulant and stored at −20 °C.

Genomic DNA was extracted from human leukocyte nuclei isolated from whole blood and target DNA was amplified by PCR. Restriction fragment length polymorphism (RFLP) assays were designed for polymorphisms identified.

Two SNPs, Arg51-Gln(r34911341) and Leu72-Met(rs696217) in exon 2 of ghrelin gene were studied. Details of reported SNPs may be found at the dbSNP web-site (http://www.ncbi.nlm.nih.gov/SNP/) under their respective accession numbers.

PCR primers for the fragment containing these two SNPs were forward 5'-TCCAGCCTGCCACCTTAGC-3' and reverse 5'-GGACCCCTGTTCACTGCCAC-3'. Cycling parameters were denaturation at 95 °C for 10 min,
5 cycles with 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, 5 cycles with 95°C for 30 s, 61°C for 30 s, 72°C for 30 s and 25 cycles with 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and then, 72°C for 7 min. This procedure generated a 373 bp fragment that was digested with ScaI (Arg51 allele: fragments of 210 and 163 bp) and BsrI (Leu72 allele: fragments of 272 and 101 bp). Restriction patterns were visualized on 2% agarose gels stained with ethidium bromide.

In all 567 samples, the success rate of the first genotyping was 94.2%. The test was continued until all the samples were successfully genotyped, and all the samples were double genotyped in a blinded fashion with concordant results.

**Statistical analysis**

Statistical analysis was performed using the Statistical Package for the Social Sciences software, version 13 (SPSS, Chicago, IL, USA). Results are reported as mean ± SD. Normal distribution and homoscedasticity of continuous variables were tested by means of the Kolmogorov–Smirnov test. Variables that did not fulfill these tests were log transformed before analysis. The differences among PCOS patients and controls in clinical and biochemical variables were evaluated by independent samples t-test. Categorical data were expressed as frequencies and percentages. Genotypic and allelic distributions were compared using the Pearson’s χ²-test. Multivariate general linear model analyses were used to evaluate the effects of the polymorphisms on quantitative variables introducing age and BMI as a covariate. P-values of <0.05 were considered statistically significant.

**Results**

In our study, 141 patients (52.0%) had all symptoms of the revised diagnostic criteria for PCOS according to the ASRM/ESHRE Rotterdam consensus, including oligomenorrhea or amenorrhea, hyperandrogenism and PCO, 101 patients (37.3%) in the PCOS group had oligomenorrhea or amenorrhea and PCO, 20 patients (7.4%) had oligomenorrhea or amenorrhea and hyperandrogenism and 9 patients (3.3%) in the PCOS group had hyperandrogenism and PCO. However, no subjects in the control group had the symptoms mentioned above.

The clinical and biochemical characteristics of women with PCOS and controls are given in Table I. There was no significant difference in age between the PCOS patients and the controls. As expected, the level of testosterone and LH, and the BMI and waist-hip-ratio (WHR) were significantly higher in the PCOS group than the control group.

For the RFLP analysis, the rare ghrelin variant Arg51Gln was not detected in the cohorts studied. All the Chinese people in this study were Arg51Arg. The genotype and allele frequencies of SNP Leu72-Met in both the patient and control groups followed Hardy–Weinberg equilibrium. No significant differences in genotype distributions and allele frequencies of Leu72-Met polymorphism were observed when the PCOS and the control groups were compared (Table II).

To verify whether polymorphisms could influence the PCOS phenotype, we compared the clinical characteristics of the patients with PCOS according to the presence of the ghrelin precursor polymorphism of the Met72 allele.

There was no significant difference in age, WHR and the level of FSH, LH, E₂, testosterone and PRL in different genotype PCOS patients. The level of fasting insulin in PCOS women with the Met72 allele was slightly higher than that of PCOS women with the Leu72 allele homozygotes, but the differences did not reach significance (P = 0.06). No significant differences between the groups existed for the other characters (Table III).

**Discussion**

PCOS is associated with menstrual dysfunction, hyperandrogenism and anovulation in conjunction with elevated pre-antral follicle number and arrested follicular maturation. It is related to obesity and to major metabolic alterations including both IR and beta-cell dysfunction (Franks, 1995; Dunaf and Finegood, 1996). Ghrelin is known to play a role in regulating glucose metabolism and energy balance (Saad et al., 2002). Polymorphisms in preproghrelin and ghrelin gene could be responsible for obesity, IR and low ghrelin levels observed in some individuals. Moreover, several studies reported lower level of plasma ghrelin in PCOS patients. Therefore, ghrelin gene may be involved in the pathogenesis of PCOS.

Among identified polymorphisms, Arg51Gln and Leu72Met are most often described and change the amino acid sequence of ghrelin (Arg51Gln) and preproghrelin (Leu72Met). The Arg51Gln modifies the amino acid sequence of the mature peptide and may affect its function. Several studies showed the Arg51Gln variant was associated with lower plasma ghrelin levels (Ukkola et al., 2002; Poykko et al., 2003a,b) and obesity (Ukkola et al., 2001). The most recent study of Krzyzanowska-Swiäńska et al. (2005) demonstrated that 51Gln allele carriers had higher prevalence of T2DM and hypertension than non-carriers. However, in the present study, we failed to detect the rare polymorphism Arg51Gln in the Chinese people: all the Chinese people in this study were Arg51Arg. The polymorphism was underrepresented, but it was consistent with other two studies in Chinese population (Xu et al., 2008; Zou et al., 2008). Similarly, Ukkola et al. (2002) found that Arg51Gln allele was not present among blacks in their study. Comparing the results of other studies (0.6–3%), these results suggest ethnic differences, and imply that there are much lower frequencies of the Arg51Gln in some populations.

At any rate, the functional significance of the Met72 polymorphism remains uncertain: it lies outside the region where the mature ghrelin product is encoded (Kojima et al., 1999), but leucine at position 72 is

| Table I Clinical and biochemical features of control group and PCOS group of Chinese women |
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| Variable          | Control group     | PCOS group        | P-value           |
|                   | (n = 290)         | (n = 271)         |                   |
| Age (years)       | 29.28 ± 3.70      | 28.84 ± 3.40      | 0.12              |
| BMI (kg/m²)       | 22.19 ± 3.03      | 24.98 ± 4.06      | <0.01             |
| Waist-hip ratio   | 0.80 ± 0.06       | 0.86 ± 0.07       | <0.01             |
| FSH (IU/l)        | 6.68 ± 1.89       | 6.96 ± 2.06       | 0.07              |
| LH (IU/l)         | 4.20 ± 4.07       | 9.78 ± 6.15       | <0.01             |
| Testosterone (nmol/l) | 1.26 ± 0.45   | 2.39 ± 0.81       | <0.01             |
| Prolactin (µg/l)  | 18.56 ± 9.38      | 17.70 ± 9.13      | 0.23              |
| Estradiol (pmol/l)| 52.47 ± 28.28     | 55.18 ± 41.86     | 0.36              |

Values are means ± SD.
highly conserved among different mammalian species investigated so far (Kaiya et al., 2001) and may play a significant role in post-translational processing. Carriers of the Met72 allele of the preproghrelin gene may be associated with metabolic co-morbidities in the general population and in obese populations as well (Steinle et al., 2002). Studies on the association between the Leu72Met polymorphism of the ghrelin gene and obesity generated controversial results. The Met72 allele has been reported to be protective against fat accumulation and associated metabolic co-morbidities in one study (Ukkola et al., 2002), but it was shown to associate with obesity in other studies (Ukkola et al., 2001; Miraglia et al., 2004; Kuzu et al., 2006). In our study, we also estimated the potential impact of the Leu72Met polymorphism on BMI in PCOS patients. Our data showed that the Met72 allele was not associated with BMI in Chinese PCOS patients. Our findings were consistent with previous observations by Larsen et al. (2005) that the Leu72Met variant was not associated with BMI among Danish Caucasians, and also in agreement with a study of ghrelin variants among 215 extremely obese children, and 93 normal weight students in the German population (Hinney et al., 2002).

A number of previous studies had assessed the associations between Leu72Met polymorphism and plasma lipids, glucose and insulin levels, but the results were inconsistent. Steinle et al. (2005) found evidence that the Leu72Met variant was associated with increased prevalence of metabolic syndrome as well as higher fasting glucose, lower high-density lipoprotein and higher TG levels in 856 adult order Amish. Korbonits et al. (2002) reported a reduced first-phase insulin secretion in OGTT in tall, obese children carrying the Met72 allele, which may suggest a defect in insulin secretion in OGTT in tall, obese children carrying the Met72 allele, which may suggest a defect in insulin secretion.
those subjects. However, Kim et al. (2006) reported no association between the Leu72Met polymorphism and T2DM in 206 Korean T2DM patients. Similarly, no difference in genotype distribution for Leu72Met polymorphism was found between the metabolic syndrome group and subjects classified as not having the metabolic syndrome from Denmark by Bing et al. (2005). Additionally, there were no significant differences across the three groups of genotypes with respect to fasting serum lipids, plasma glucose and serum insulin in their study. We also investigated the associations of the Leu72Met polymorphism with the TC, TG, LDL, plasma glucose and insulin levels among subjects with PCOS, but none were found. Although the level of fasting insulin in PCOS women with Met72 allele was higher than that of PCOS women with AA genotype, the differences were not significant.

Although the mechanisms of action of ghrelin and its potential interplay with other known regulators of the reproductive system remained largely unexplored, the growing evidence suggested that ghrelin might be involved in steroid synthesis and/or action (Parid et al., 2005). Concerning gonadotrophin secretion, ghrelin was shown to suppress LH secretion in vivo (Furuta et al., 2001), and to decrease LH responsiveness to GnRH in vitro, whereas FSH secretion was not affected (Fernandez-Fernandez et al., 2004). In addition, ghrelin was able to inhibit stimulated testicular testosterone secretion (Tena-Sempere et al., 2002), whereas androgens have been proven independent modulators of circulating ghrelin levels (Pagotto et al., 2002, 2003; Gambineri et al., 2003). So there might be a very close relationship between ghrelin gene and the endocrine disorder of PCOS women. In present study, our data showed that LH and testosterone levels increased significantly in the PCOS group than the control group, but no significant difference in genotypes distribution among different populations. The frequencies of Met72 allele of ghrelin gene was not associated with the metabolic syndrome and to delineate the molecular and physiological mechanisms of their effects.

To the best of our knowledge, our study covering a relatively large series of Chinese women with PCOS represents the first one providing data concerning ghrelin gene and PCOS. Though we have not conducted a comprehensive analysis of all variants in and around the ghrelin gene and cannot exclude the role of other variants outside this region, on the available evidence we conclude that Arg51Gln and Leu72Met polymorphisms of ghrelin gene were not associated with PCOS in Chinese population. However, our study was conducted only in a Chinese population and these data should be extrapolated to other ethnic groups with caution.

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