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GENETICS

Anti-Müllerian hormone gene polymorphism is associated with androgen levels in Chinese polycystic ovary syndrome patients with insulin resistance

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Abstract
Purpose The objective of the study was to investigate whether genetic polymorphisms of the anti-Müllerian hormone (AMH) and its specific receptor anti-Müllerian hormone type II receptor (AMHRII) were associated with the hormone disorder and phenotype of polycystic ovary syndrome (PCOS).
Methods This case-control study included 141 PCOS patients and 123 normal women. Two polymorphisms of AMH and AMHRII and the clinical characteristics of participants such as body mass index (BMI), serum luteinizing hormone (LH), estradiol levels (E_2), total testosterone levels (T), and homeostasis model assessment of insulin resistance (HOMA-IR) were analyzed with the case-control sample. Gene–gene interactions of AMH and AMHRII genes were analyzed based multifactor-dimensionality reduction method.
Results A significant difference of AMH gene polymorphisms were observed in IR-PCOS women and controls. The AMH and AMHRII gene polymorphisms were not found a significant difference in non-IR-PCOS and normal groups. To IR-PCOS women, genotypes of AMH were closely related to the serum levels of LH ($P = 0.000$), testosterone ($P = 0.000$) and HOMA-IR ($P = 0.038$), while in the non-IR-PCOS and normal groups, no relationship was found. No impact of AMH and AMHRII gene–gene interactions was demonstrated.
Conclusions Our research suggests that the diversity of AMH genotypes in the AMH signal pathway may be connected with the susceptibility and phenotype of PCOS with insulin resistance.

Keywords Anti-Müllerian hormone (AMH) · Anti-Müllerian hormone type II receptor (AMHRII) · Polymorphisms · Polycystic ovary syndrome (PCOS) · Insulin resistance (IR) · Androgen

Capsule AMH genetic polymorphism in the AMH signal pathway is related to serum androgen levels in Chinese PCOS with insulin resistance.

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Introduction

Polycystic ovary syndrome (PCOS) is the major cause of anovulatory infertility and a common genetically complex endocrinopathy with a variety of clinical symptoms in women of reproductive age. There are mainly three sets of diagnostic criteria [1], and all combine three characteristic features: ovulatory dysfunction, polycystic ovarian morphology, and hyperandrogenism. Environmental and genetic factors both contribute to the development of PCOS [2]; however, the underlying etiology and pathogenesis of PCOS remain poorly understood, and there is no effective method of prevention of this disease.

Anti-Müllerian hormone (AMH), also referred to as Müllerian-inhibiting substance (MIS), may play an important role in the pathophysiology of PCOS by regulating folliculogenesis. As a member of the transforming growth factor-β (TGF-β) family, AMH expressed by granulosa cells (GCs) of early developing follicles in the ovary during the reproductive years. AMH is considered a reliable marker of ovarian reserve in human and strongly correlate with the number of antral follicles [3–5]. Studies in female mice revealed that AMH regulate the initial and cyclic recruitment of ovarian follicles by decreasing the sensitivity to follicle-stimulating hormone (FSH) [6–8]. Furthermore, AMHRII-deficient mice are a phenocopy of AMH-deficient (AMHKO) female mice [9]. Some studies found the serum AMH levels increased in PCOS patients [10, 11], and the high AMH concentrations have positive correlation with individual features of PCOS [12], supposing that the elevated AMH levels may reflect the AMH signaling pathway to be abnormal in women with PCOS.

Functional candidate genes of the biochemical pathways in PCOS affect gonadotropin secretion [13, 14], regulate androgen production and secretion, and influence insulin secretion and obesity. Kevenaar ME with his colleagues found that AMH gene (rs10407022) polymorphism contributes to the complex etiology of PCOS [15]. Previously studies have found that anti-Müllerian hormone receptor II gene (AMHRII; rs2272002) is related to androgen levels in premature ovarian failure (POF) patients [16], suggesting that AMHRII polymorphism is associated with the hormonal disorder and PCOS phenotype.

We performed a case-control association study to investigate whether genes polymorphisms of the AMH signaling pathway are related to the hormone disorder and phenotype of PCOS.

Materials and methods

The study recruited 141 unrelated women with PCOS and 123 normal women, from January 2013 to December 2014, at the Reproductive Medicine Center of General Hospital of Ningxia Medical University in China. The diagnosis of PCOS was made based on the presence of two of three Rotterdam 2003 criteria: oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries, and exclusion of other etiologies (congenital adrenal hyperplasias, androgen-secreting tumors, Cushing's syndrome).[17]. Oligo-anovulation typically presents as oligomenorrhea (<9 cycles per year) or amenorrhea, and as a consequence, have a higher risk of infertility. Hyperandrogenism is clinically manifested by hirsutism, acne and androgenic alopecia, and/or levels of total testosterone higher than 55.07 ng/dl (1.91 nmol/l). The diagnosis of PCO is presence of 12 or more follicles in each ovary measuring 2 ± 9 mm in diameter, and/or increased ovarian volume (>10 ml). PCOS subjects who were diagnosed with congenital adrenal hyperplasia, Cushing'syndrome, and androgen-secreting tumors were not included in this study. Patients with diabetes and glucose intolerance were absolutely ruled out to exclude the PCOS patients caused by metabolic disorders. Healthy women without any endocrine dysfunctions and any other kind of disease were recruited as controls. These women were monitored for follicular growth for 3 months before the testing by transvaginal ultrasound. All members of the control population were normal women, who had regular menstrual cycles, had normal ovulation, and had no endocrine dysfunction related to PCOS, for assisted reproduction with male factor or tubal factor. All the participants had not taken hormonotherapy during the preceding 3 months before the testing.

This study followed the EQUATOR (Enhancing the QUAlity and Transparency Of health Research) network's guidelines (http://www.equator-network.org/), the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement [18], the Helsinki Declaration and the Committee on Publication Ethics (COPE) guidelines (http://publicationethics.org/). The study was performed according to the principles of the local institutional review board at General Hospital of Ningxia Medical University, and all participants gave written informed consent.

Height and weight were measured directly, and the body mass index (BMI) of individual participant was calculated by dividing weight (Kg) by squared height (m). Fasting venous blood was collected by a single venipuncture between day 3 and day 5 of a menstrual cycle. Serums obtained from the blood samples by centrifugation were stored at −80 °C. The levels of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), estradiol (E_2), total testosterone (T), insulin, and glucose were determined. Insulin resistance index calculated by homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following formula: fasting glucose (FPG, mmol/L) × fasting insulin (FINS, mIU/L) /22.5 [19]. The HOMA-IR ≥ 2.69 was considered as insulin resistance (IR) [20]. According to the WHO standard, for Asians, obesity is defined as the BMI ≥ 25 Kg/m^2.

For each patient, a blood sample was obtained for DNA analysis. Genomic DNA was extracted from whole peripheral blood samples by using the TIANamp Blood DNA Kit (Tiangen Biotech, China) according to the manufacturer's protocol. Primers were designed using Primer3 online software (http://www.ncbi.nlm.nih.gov/), and four primers were used to amplify the rs10407022 and rs2272002 with 430 bp and 190 bp amplicons (Table 1). Each PCR reaction mixture contained 2 μL genomic DNA, 1 μL 10 mmol/L forward primers, 1 μL 10 mmol/L reverse primers, 2 μL 2.5 mmol/L dNTP, 2.5 μL 10 × rTaq buffer, 0.2 μL rTaq enzymes, and 16. 3 μL sterile distilled water mix in a total volume of 25 μL. The

Table 1 The primers that were used for determination of AMH gene and AMHRII gene

Gene	SNP	Primer	Sequence (5' → 3')	Product size (bp)
AMH	rs10407022	Forward Reverse	CCTTCCACTCGGCTCATTTA CACCAGGATGTGGACCTCCT	430
AMHRII	rs2272002	Forward Reverse	CCCTTTGGAAGAGTGGTGAG GAGACGTAAGTGAGGGTGGA	190

PCR cycling conditions consisted of one 2-min cycle at 94 °C, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and one 2-min cycle of 72 °C. The PCR products were recovered and purified by using a 96-well PCR plate (MiLLipore, USA) according to the manufacturer's protocol. Genotypes were determined from the purified samples by using an ABI 3730xl DNA Analyzer (Applied Biosystems, USA).

Data were analyzed using the Statistical Package for the Social Sciences software (SPSS 20.0). Descriptive statistics are expressed as means, standard deviations, percentage distributions, median, and 95 % confidence. One-sample Kolmogorov–Smirnoff test was used to test the normality of distribution for continuous variables. One-way analysis of (co) variance was used to determine differences between groups. Non-normally distributed hormone levels data were reciprocal-transformed prior to ANOVA analysis. Date was adjusted for age and BMI. Post hoc Tukey or multiple comparison tests were performed if a significant overall difference was found. Genotype distributions of AMH and AMHRII polymorphisms were assessed by using goodness-of-fit chi-squared test for Hardy–Weinberg equilibrium. Gene–gene interactions of AMH and AMHRII genes were analyzed based multifactor-dimensionality reduction method by using MDR software (version3.0). Values of $P<0.05$ or 0.01 were considered to be significant.

Results

In the early statistics, data among different PCOS classifications including insulin resistance, hyperandrogenism, anovulation, and BMI were analyzed. Only the group of insulin resistance made a positive result. So, insulin resistance was taken as entry point for follow-up study. In PCOS patients, insulin resistance was found in 66.7 % (94/141) and non-IR was 33.3 % (47/141). In the total PCOS group, BMI, LH, LH/FSH ratio, T, AMH, and HOMA-IR were observed to be significantly higher when compared with the control group (all values $P<0.05$, data not shown). Clinical characteristics of PCOS cases and controls are shown in Table 2. No statistically significant difference of age was found between the groups. However, some clinical characteristics in the pairwise comparisons found statistically significant differences ($P<0.01$). Ninety-four PCOS patients with insulin resistance and 123 normal women were selected for further analysis.

Significant difference in the distribution of AMH genotypes was observed between insulin-resistant PCOS and normal women ($P=0.013$), although no difference was found in the distribution of AMHRII genotypes in cases and controls ($P=0.728$) (Table 3). Allele frequencies of AMH and AMHRII in PCOS women with insulin resistance did not differ from frequencies in the controls ($P=0.353$, $P=0.387$). No statistically significant difference of AMH and AMHRII gene polymorphism was found between the non-IR-PCOS

Table 2 Clinical characteristics of PCOS cases and controls

	IR-PCOS ($n=94$)	Non-IR-PCOS ($N=47$)	Control ($n=123$)
Age (years) (range)	27.8 ± 4.1 (20–39)	27.4 ± 4.7 (20–38)	27.9 ± 4.3 (20–41)
BMI (Kg/m^2)	$23.8 \pm 4.5^{a,b}$	21.9 ± 2.9^{a}	21.7 ± 2.6^{b}
LH (IU/L)	13.3 ± 7.6^{a}	12.7 ± 8.1^{b}	$4.4 \pm 2.0^{a,b}$
FSH (IU/L)	5.5 ± 1.8	5.6 ± 2.1	5.7 ± 1.8
LH/FSH	2.4 ± 0.5^{a}	2.3 ± 0.6^{b}	$0.8 \pm 0.4^{a,b}$
PRL (ng/mL)	11.6 ± 8.5	12.1 ± 7.6	11.7 ± 4.6
E_2(pg/mL)	53.9 ± 36.8	52.7 ± 37.9	46.9 ± 40.1
T (pg/mL)	57.1 ± 33.0^{a}	56.9 ± 34.1^{b}	$44.0 \pm 12.2^{a,b}$
AMH (ng/ml)	13.3 ± 5.2^{a}	12.1 ± 5.6^{b}	$3.7 \pm 1.4^{a,b}$
HOMA-IR	$2.9\ (2.7, 3.8)^{a,b}$	$1.9\ (1.4, 2.6)^{b}$	$1.8\ (1.2, 2.6)^{a}$

Values are means ± SD, median (95 % confidence)

PCOS polycystic ovary syndrome, *BMI* body mass index, *LH* luteinizing hormone, *FSH* follicle stimulating hormone, *PRL* prolactin, E_2 estradiol, *T* testosterone

[a,b] Similar superscripts indicate a statistically significant difference ($P<0.01$)

Table 3 The distribution of the AMH and AMHRII gene polymorphism in IR-PCOS cases and controls

Groups	*N*	Genotypes			*P*	Allele frequency		*P*
AMH		T/T	T/G	G/G		T	G	
IR-PCOS	94	20 (21.3)	48 (51.1)	26 (27.7)	0.013	97 (51.6)	91 (48.4)	0.353
Control	123	34 (36.2)	29 (30.9)	31 (33.0)		88 (46.8)	100 (53.2)	
AMHRII		A/A	A/T	T/T		A	T	
IR-PCOS	94	80 (85.1)	12 (12.8)	2 (2.1)	0.728	172 (91.5)	16 (8.5)	0.387
Control	123	76 (80.9)	15 (16.0)	3 (3.2)		167 (88.8)	21 (11.2)	

Values are *n* (%)

AMH anti-Müllerian hormone, *AMHRII* anti-Müllerian hormone type II receptor, *PCOS* polycystic ovary syndrome

and normal women ($P > 0.05$, data not shown). Also, no difference was found in the distribution of allele frequencies of AMH and AMHRII in PCOS women and controls ($P > 0.05$, data not shown). Odds ratios (ORs) of AMH and AMHRII allele frequency that associated with PCOS risk are also performed, and there was no statistically significant found (data not shown). The genotype distributions of AMH and AMHRII polymorphisms were assessed Hardy–Weinberg equilibrium through a goodness-of-fit chi-squared test in both PCOS cases and control groups. The Hardy–Weinberg equilibrium value *P* of AMH genotype distribution in insulin resistant PCOS population was 0.001 ($P < 0.05$), though distributions of the other three genotype groups were consistent with the principle (all values $P > 0.05$). Gene–gene interactions of AMH and AMHRII were analyzed, but the result showed that two polymorphisms of genotypes had no interactive effect (Table 4).

Clinical characteristics of insulin resistant PCOS women within diverse genotypes are shown in Table 5. As shown in the table, genotypes of the AMH polymorphisms were associated with the serum levels of LH ($P = 0.000$) and testosterone ($P = 0.000$), and other general characteristics were observed no significant differences (all values $P < 0.05$). No difference was found in the distribution of AMH and AMHRII genotypes in non-IR-PCOS and normal cohort (results not shown).

Discussion

Despite the gradually increasing recognition of the clinical importance of PCOS, few convincing results are produced on the researches of the polymorphism or combination of causative genes. The current study was designed to investigate the association between the gene polymorphisms of AMH and AMHR2 with PCOS. To accomplish this, polymorphisms in two genes of the AMH signal transduction pathway, AMH and its specific receptor AMHR2, were identified.

Table 4 Gene-gene interactions of AMH and AMHRII polymorphisms in IR-PCOS and normal groups

Model	Bal. Acc. CV testing	CV consistency	*P*
rs10407022	0.6011	10/10	0.3729
rs10407022, rs2272002	0.5957	10/10	0.3995

This research found that AMH (rs10407022) genetic polymorphisms might affect the ovarian function. The results of our study showed that only one significant difference in the genotype distribution of the AMH polymorphisms in PCOS women with insulin resistance compared with the normal controls. It suggested that the AMH genotype polymorphisms may be connected with the susceptibility to IR-PCOS. Kevenaar et al. discovered that genotype and allele frequencies of the AMH (rs10407022) polymorphism in the PCOS women did not differ from frequencies in the normal control. The various results which were significant rates of AMH genotype and allele frequencies between Caucasians and Chinese may be due to the different races. The phenotype of PCOS we selected in this study may also contribute to the different results.

Our study implied that the AMH and AMHR2 allele variations may not contribute to the risk of PCOS. This is because no difference in the distribution of AMH and AMHR2 (rs2272002) allele polymorphisms was observed between PCOS patients and controls in our research. Sproul et al. found no differences in the frequency of the AMHR2 polymorphism (rs2002555) between the PCOS groups and the general population [21]. Similar findings among different SNPs of the same gene further suggested that AMHR2 gene polymorphisms may not be related to the pathogenesis of PCOS. Moreover, rs2272002 is located in the intron region of AMHR2. This is preferable to explain the AMHR2 genetic polymorphism may be independent of the pathogenesis of PCOS. The consequences of gene-gene interactions of AMH and AMHR2 polymorphisms between IR-PCOS cases and controls indicated that two polymorphisms had no synergy and antagonism to the susceptibility to IR-PCOS.

According to our research, serum androgen levels are significantly higher in patients with PCOS, especially the

Table 5 Clinical characteristics of IR-PCOS patients by AMH and AMHRII polymorphisms

IR-PCOS	AMH ($n=94$)			P	AMHRII ($n=94$)			P
	T/T	T/G	G/G		A/A	A/T	T/T	
Age (years)	27.6 ± 4.4	28.1 ± 4.0	27.8 ± 3.9	0.852	27.9 ± 4.1	27.8 ± 4.6	28.0 ± 4.2	0.935
BMI (Kg/m2)	23.6 ± 3.3	23.4 ± 3.5	23.8 ± 2.9	0.732	23.5 ± 3.9	23.3 ± 4.2	23.9 ± 2.6	0.872
LH (IU/L)[a]	10.6 ± 3.9	18.1 ± 10.2	11.8 ± 5.4	0.000	13.4 ± 8.1	12.9 ± 3.9	13.1 ± 2.1	0.934
FSH (IU/L)[a]	5.1 ± 1.6	5.7 ± 1.9	5.7 ± 1.9	0.368	5.5 ± 1.8	5.7 ± 2.3	5.8 ± 1.6	0.778
PRL (ng/mL)[a]	10.0 ± 5.3	12.1 ± 5.7	12.9 ± 12.6	0.349	11.9 ± 9.0	9.6 ± 4.3	13.9 ± 7.6	0.647
E_2 (pg/mL)[a]	45.0 ± 28.6	61.2 ± 38.2	56.8 ± 42.4	0.194	53.4 ± 35.8	53.4 ± 47.1	61.2 ± 15.7	0.933
T (pg/mL)[a]	40.7 ± 25.6	89.2 ± 28.5	45.3 ± 21.0	0.000	56.1 ± 33.5	63.9 ± 32.6	47.0 ± 8.4	0.739
AMH (ng/ml)	11.4 ± 4.6	18.6. ± 5.6	11.0 ± 4.9	0.000	12.9 ± 5.2	13.1 ± 4.8	13.3 ± 5.3	0.673
HOMA-IR	2.8 (2.7, 3.8)	3.1 (2.8, 4.2)	2.9 (2.7,4.0)	0.038	2.9 (2.7, 3.9)	3.3 (2.7, 3.9)	2.9 (2.8, 3.8)	0.432

Values are means ± SD, Values are means ± SD, median (95 % confidence)

PCOS polycystic ovary syndrome, *AMH* anti-Müllerian hormone, *AMHRII* anti-Müllerian hormone type II receptor

[a] Corrected for age and BMI

genotype T/G of AMH in IR-PCOS. In women, androgens are produced by the ovaries and the adrenal glands, and estrogens are synthesized from androgens, specifically testosterone and androstenedione. The biosynthesis of estrogens in the ovarian is accomplished by the theca interna cells and granulosa cells [22, 23]. Testosterone and androstenedione are converted from the cholesterol through the agency of ovary theca cells [23, 24]. The compounds cross the basal membrane into the granulosa cells around to convert into estradiols and estrones respectively [23, 25]. The process is catalyzed by the aromatase enzyme which encoded by the gene CYP19 [23, 25], and AMH suppress the expression of aromatase [26, 27]. In our research, the patients with PCOS hold a high serum level of testosterone than controls. It is possible that the low expression of aromatase affected by the high serum level of AMH results in downregulation aromatization of androgens into estrogens. We suspect that the elevated serum androgen level is associated with AMH gene polymorphisms.

In insulin-resistant PCOS women, the serum LH levels are significantly higher, especially in the genotype T/G of AMH. The pulse amplitude and frequency of LH are regulated by the hypothalamus–pituitary ovary axis. Abnormal release of gonadotrophin-releasing hormone (GnRH) leading to increased LH levels with relative decrease glucose impairment in FSH. LH stimulates the theca cells in the ovaries to provide androgens precursors. The genotype T/G of AMH may contribute to the high serum levels of LH and the increased LH levels enhanced the production of androgenic precursors, and then testosterone, thereby contribute to hyperandrogenism in PCOS women. Also, the genotype T/G of AMH may contribute to the elevated serum androgen levels directly. The intrinsic potential relationship between the AMH polymorphisms and hormone abnormalities needs further study.

It is now widely agreed that the basic lesion of PCOS lies in the ovaries themselves and that the hypersecretion of androgens by the ovaries is the basal endocrinological disturbance. Associated extra-ovarian factors such as insulin resistance, the consequent hyperinsulinaemia, and elevated concentrations of LH, all take a part in exacerbating the disease and intertwined into an 'androgen circle'[28]. The research show that AMH polymorphisms connected with the serum levels of AMH, LH, and T in IR PCOS population. In IR PCOS women, insulin resistance, and hyperinsulinemia may increase the T levels [29] and induce production of LH [30], then the ovulation that stimulated by the hormones. Meanwhile, IR and hyperinsulinemia may suppress the expression of androgen in theca cells with LH [31].

Our research shown that the polymorphisms of AMH was connected with clinical characteristics of IR-PCOS. Indirect evidence to strengthen this correlation is provided by the fact that insulin sensitizers, metformin, can reduce the serum AMH level and increase ovulation after 2 month treatment to PCOS [32]. Therefore, we suspect that AMH gene polymorphism is associated with the etiology of IR-PCOS.

Genotype distribution of AMH polymorphisms in PCOS population was not in Hardy-Weinberg equilibrium proportions. This kind of result may be due to the not large enough sample size in the research, Berkson's bias in this case–control study, or the genetic polymorphism was sufficiently relevant to the pathogenesis of PCOS. If our study can provide the data on the number of follicles by ultrasound and other metabolic/hormonal parameters towards homeostasis in PCOS patients, it should go deep into discuss the possible role of AMH polymorphisms and PCOS. Moreover, populations from different regions and a large sample size are needed for further study.

In conclusion, in our study, the AMH gene polymorphism was connected with the susceptibility and phenotype of PCOS, implying that it is one of the factors devotes to the

pathogenesis of PCOS. AMH genetic polymorphism is related to serum androgen and luteinizing hormone levels in PCOS with insulin resistance.

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Authors' contributions Meng-Xue Zheng and Yan Li participated in study design, performed the experiments, and drafted the manuscript. Rong Hu participated in study design, directed the execution of experiments, and revised the article critically for intellectual content. Fei-Miao Wang participated in sample collection and performed statistical analysis. Xiao-Mei Zhang and Bing Guan participated in mentoring and critical discussion. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. The manuscript has been reviewed and approved by all authors, and state that the manuscript has not been previously published, and is not being considered for publication by another journal.

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