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ORIGINAL PAPER

Lack of association between interleukin-1a gene (*IL-1a*) C (-889) T variant and polycystic ovary syndrome in chinese women

Binbin Wang · Sirui Zhou · Jing Wang · Jingjing Liu · Feng Ni · Chunmei Liu · Jinting Yan · Yuan Mu · Yunxia Cao · Xu Ma

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Abstract Polycystic ovary syndrome (PCOS) is associated with the spontaneous development of an aberrant heterogeneous hormonal environment that thought to be related to multiple genetic or environmental factors remain undefined. Previous studies indicated that *IL-1a* gene C (-889) T, a polymorphism of the gene encoding a protein which involved in various immune responses, inflammatory processes and hematopoiesis, and is associated with PCOS. A total of 205 PCOS patients and 177 healthy controls were included in this study that used PCR-RFLP to detect C (-889) T variant of *IL-1a* gene. Clinical measures determined previously were included in the SPSS analysis. The results showed that the presence of *IL-1a* gene C (-889) T polymorphism in PCOS and healthy controls in Chinese women was not significantly different when studying genotype and allele frequencies. (Genotype: $\chi^2 = 4.62$, df = 2, $P = 0.10$; allele: $\chi^2 = 1.35$, df = 1, $P = 0.25$). Furthermore, no association was found between metabolic parameters observed and *IL-1a* genotypes in PCOS patients in this study. In conclusion, *IL-1a* gene C (-889) T polymorphism does not appear to be risk factor for PCOS in this population of Chinese women.

Keywords Polycystic ovary syndrome · *IL-1a* · C (-889) T · Polymorphism

B. Wang, S. Zhou authors contributed equally to the work.

B. Wang · S. Zhou · J. Wang · C. Liu · J. Yan · Y. Mu · X. Ma (✉)
Center for Genetics, National Research Institute for Family Planning, 12, Dahuisi Road, Haidian, Beijing 100081, China
e-mail: genetic@263.net

J. Liu · F. Ni · Y. Cao (✉)
Reproductive Medicine Center, The First Affiliated Hospital, Anhui Medical University, Hefei, China
e-mail: caoyunxia6@126.com

B. Wang · S. Zhou · J. Wang · C. Liu · J. Yan · Y. Mu · X. Ma
Peking Union Medical College, Beijing, China

X. Ma
World Health Organization Collaborating Centre for Research in Human Reproduction, Beijing, China

Introduction

Polycystic Ovary Syndrome (PCOS) affects from 4 to 12% of women in reproductive age and has been associated with a heterogeneous aberrant peripheral hormonal environment. Since the 1990 National Institutes of Health-sponsored conference on PCOS, it has been acknowledged that the syndrome encompasses a broad spectrum of signs and symptoms of ovarian dysfunction. The 2003 Rotterdam consensus workshop [1] concluded that PCOS is a syndrome of ovarian dysfunction along with the cardinal features hyperandrogenism and polycystic ovary (PCO) morphology which is diagnosed when two out of three criteria are met by a patient, including oligomenorrhea or amenorrhea, clinical or biochemical hyperandrogenism and ultrasonographic polycystic ovarian morphology [2].

PCOS patients have reproductive disorders along with anovulation, hyperandrogenism, polycystic ovaries, and obesity [3], and frequently display metabolic disturbances with a higher risk of type II diabetes. Contemporary researches tend to focus on relationships of insulin resistance, obesity, type II diabetes with PCOS [4–6], as well as the abnormal androgen production, and activity of PCOS is thought to lead to changes in the control of follicle development and maturation [7].

Although the inheritance mechanism of PCOS is still uncertain, multiple genetic factors including mutations and polymorphisms to several genes have been discovered a possible association with the risk of PCOS [4, 8], such as the insulin receptor substrate family genes which have been investigated within all the subjects of PCOS [9–11].

Previous studies suggested a chronic low-level inflammation might usually associate with PCOS [12]. One of the most prominent mediators of inflammation is the interleukin-1 (*IL-1*) family. The *IL-1* gene cluster on chromosome 2q12-q13 contains three related genes *IL-1a, IL-1b*, and *IL-1RN,* encoding the pro-inflammatory cytokines *IL-1a, IL-1b*, and their endogenous receptor antagonists. They are the potent pro-inflammatory cytokines and play a central role in many inflammatory cascades [13].

The protein encoded by Interleukin-1 alpha (*IL-1a*) gene is a pleiotropic cytokine involved in various immune responses, inflammatory processes, and hematopoiesis, is produced by monocytes, macrophages, neutrophils, and epithelial cells, and affects nearly every cell type [14]. The *IL1*-mediated inflammatory processes have been proposed to influence the processes of ovulation, fertilization, and implantation. All these parameters may be affected in women with PCOS [15].

The *IL-1a* gene has a polymorphism present at position −889 of the promoter region in a 430 kb sequence on chromosome 2q14.2 with a C–T base change [16]. This polymorphism lies upstream of the sequence that suppresses transcription of the *IL-1a* gene and corresponds with altered *IL-1a* protein expression in ovarian tissue [17]. Research supporting this has shown that a polymorphism in the *IL-1a* gene correlates with the occurrence of PCOS [18]. Furthermore, the serum level of FSH and subsequent LH/FSH ratio correlated with the polymorphism of *IL-1a* within the PCOS group [19]. Although it is already known that *IL-1a* gene represents a risk factor of many diseases [20, 21], there appears to be no association between this polymorphism and ovarian cancer [21].

Based on the previous observations, we investigated the relative occurrence of the *IL-1a* gene C (-889) T polymorphism in women with PCOS and normal women to determine whether it could be used as a prognostic marker in the pathogenesis of PCOS.

Results

Clinical and laboratory variables

The clinical characteristics of PCOS patients and controls enrolled in the study are listed in Table 1. The differences between PCOS and controls of the following serum levels are determined after blood samples were drawn in the hospital. No statistically significant differences were observed between PCOS and control groups for serum levels of FSH, prolactin, and total testosterone levels. However, E2 and LH levels in PCOS group were significantly higher than those in controls. Furthermore, comparing with controls, PCOS women presented a significantly younger mean age and a higher BMI value.

Genotype and allele frequencies with clinical and biochemical parameters

Genotype frequencies and the frequency concerning the appearance of the mutated *IL-1a* allele versus the respective wild-type alleles were determined for women with PCOS, and for healthy controls.

The genotype and allele frequencies are summarized in Table 2. We compared genotypes carrying at least one mutation (C/T and T/T, respectively) with wild type (C/C, respectively). The distribution of genotype frequencies in women with PCOS and controls were in Hardy–Weinberg equilibrium. No statistical significant difference was ascertained regarding the polymorphisms of the *IL-1a* between the patient and control group.

In addition, we also investigated the clinical and biochemical parameters among different groups of genotypes, including mean ages of menarche,BMI values, E2, FSH, LH, PRL levels, and total testosterone levels to investigate the possible association between *IL-1a* gene polymorphism and these parameters in PCOS patients. The clinical and

Table 1 Demographic and clinical characteristics of the study population

Parameter	PCOS ($n = 205$)	Control ($n = 177$)	P
Age (year)	26.84 ± 4.05	31.14 ± 4.22	NS
Menarche age (year)	14.43 ± 2.06	14.33 ± 1.38	NS
BMI (kg/m^2)	23.16 ± 4.17	21.37 ± 2.47	$P < 0.001$
E2 levels (pg/ml)	233.1 ± 208.0	176.1 ± 124.6	$P = 0.001$
FSH levels (mIU/ml)	5.46 ± 2.45	6.71 ± 2.13	NS
LH levels (mIU/ml)	13.07 ± 7.00	4.98 ± 3.12	$P < 0.001$
Prolactin levels (ng/ml)	17.57 ± 32.93	18.07 ± 19.46	NS
Total testosterone (ng/ml)	3.45 ± 8.45	2.07 ± 6.96	NS

BMI, body mass index; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone

Table 2 Genotype distribution and relative allele frequencies of C (-889) T polymorphism of *IL-1a* gene in Chinese with PCOS ($n = 205$) and controls ($n = 177$)

Group	No.	Genotype frequency (%)			Allele frequency (%)	
		C/C	C/T	T/T	C	T
PCOS	205	171(83.4)	32(15.6)	2(1.0)	374(91.2)	36(8.8)
Controls	177	137(77.4)	40(22.6)	0(0)	314(88.7)	40(11.3)
		$\chi^2 = 4.62$, df = 2, $P = 0.10$			$\chi^2 = 1.35$, df = 1, $P = 0.25$	

Table 3 Biochemical profile (Mean ± SD) of Chinese PCOS women with regard to genotypes for C (-889) T polymorphism of *IL-1a* gene

Parameter	Group		
	C/T ($n = 34$; 16.6%)	C/C ($n = 171$; 83.4%)	*P*
Menarche age	14.22 ± 1.74	14.48 ± 2.12	NS
BMI	22.74 ± 4.22	23.25 ± 4.17	NS
E2 levels (pg/ml)	248.6 ± 300.6	230.0 ± 185.3	NS
FSH levels (mIU/ml)	4.63 ± 2.05	5.62 ± 2.49	NS
LH levels (mIU/ml)	12.23 ± 6.98	13.24 ± 7.02	NS
Prolactin levels (ng/ml)	32.07 ± 76.65	14.69 ± 10.52	NS
Total testosterone (ng/ml)	2.13 ± 0.75	3.71 ± 9.23	NS
LH/FSH	4.11 ± 6.95	2.61 ± 1.66	NS

biochemical profiles (Mean ± SD) of PCOS patients are shown in Table 3 with regards to genotypes C/T (including individuals with the T/T genotype) and the C/C variant of the *IL-1a* gene. Although the C/T genotype in *IL-1a* gene recorded a higher mean of E2 and PRL levels and with a lower mean BMI, FSH, LH, and total testosterone levels, no statistical differences were observed with regard to clinical and biochemical parameters as well as these genotypes ($P > 0.05$).

Genotype and allele frequencies in obesity and non-obesity

In order to investigate whether there was a simultaneous effect between *IL-1a* and insulin resistance on the risk for PCOS, we divided PCOS and controls into obese and non-obese groups and performed an analysis, using BMI > 24 as criteria. After stratifing all samples by obese status, the genotypic and allelic distributions of *IL-1a* in PCOS subjects and controls of subgroups are shown in Table 4. We found no statistical significance between PCOS and controls in either the obesity or the non-obesity group, which suggested that there was no interaction between *IL-1a* polymorphism and obesity on the risk of PCOS.

Discussion

Polycystic ovary syndrome (PCOS) is the single most common endocrine abnormality in reproductive aged women, and also a leading cause of infertility. Its clinical manifestations may include: menstrual irregularities, signs of androgen excess, and obesity according to the Rotterdam consensus on diagnostic criteria for PCOS [1].

There are theories showing that PCOS is a complex genetic disorder with multiple susceptibility genes [22],

Table 4 Genotype and allele distributions of *IL-1a* gene C (-889) T polymorphism in obese and non-obese women

Group	No.	Genotype frequency (%)		Allele frequency (%)	
		C/C	C/T	C	T
Non-obese					
PCOS	130	108(83.1)	23(16.9)	238(91.5)	24(8.5)
Controls	151	117(77.5)	34(22.5)	268(88.7)	34(11.3)
	$\chi^2 = 1.07$, df = 1, $P = 0.30$			$\chi^2 = 0.67$, df = 1, $P = 0.41$	
Obese					
PCOS	74	63(85.1)	11(14.9)	136(91.9)	12(8.1)
Controls	26	20(76.9)	6(23.1)	46(88.5)	6(11.5)
	$\chi^2 = 0.92$, df = 1, $P = 0.34$			$\chi^2 = 0.55$, df = 1, $P = 0.45$	

The genotype C/T including individuals with the T/T genotype

and pervious research has suggested an association between *IL-1a* and ovarian function in both human and rat [23, 24]. The human protein *IL-1a* acts as an inflammatory control and defensive host response resulting in vasorelaxation and increased adherence of lymphocytes, neutrophils to endothelial cells. The *IL-1a* gene encoding this protein is known to be critically involved in ovarian carcinogenesis and influences ovarian functions such as the processes of ovulation, fertilization, and implantation. The expression quantity of *IL-1a* was found and varied in human granulose and cumulus cells, which also suggested *IL-1a* might influence the process of ovulation. Furthermore, PCOS is associated with hyperandrogenism which would be in line with previous in vitro studies and studies in animal models demonstrating that *IL-1a* possibly influences steroidogenesis [14, 15, 25].

In this study, however, which contains 205 PCOS patients and 177 healthy controls, the differences of genotype and allele distributions observed did not establish any statistical significance. Therefore, there appears to be no association between genotype frequencies in *IL-1a* C (-889) T polymorphism among Chinese population.

Similarly, there was no difference associated with clinical and biochemical parameters (BMI, E2, LH, FSH, PRL levels, mean total testosterone levels) and genotype. Thus, our results suggested that this polymorphism was not associated with PCOS in Chinese women. As for the reasons why normal subjects were significantly older than the women recruited for the PCOS group, it may be because the normally cycling younger and unmarried women are less likely to seek annual check-ups that might make them available to become the healthy controls of our study. We note that E2 and LH levels in PCOS patients remain significantly higher than controls, which supported the view that LH hypothesis and granulosa cells can increase an estradiol production in the patients [18]. Steroids are known to regulate fat mass, adipose deposition and differentiation, and adipocyte metabolism which would link estrogen value to obesity and PCOS [26].

Furthermore, the stratified sample analysis provides a result that the genotype and allele frequencies between cases and controls in neither obese nor non-obese women are statistically significant. This indicates that the portion of patients who are obese does not matter crucially with regard to the negative result of gene *IL-1a* C (-889) T polymorphism associated with PCOS in Chinese woman.

In contrast, Kolbus discovered an association between genotype frequencies of the *IL-1a* promoter, but not between those of the IL-1b promoter or IL-1b exon 5 in a cohort of Austrian [15]. Further, there was a correlation between FSH serum levels and the *IL-1a* polymorphism among the patients with PCOS [15].

The reason that *IL-1a* C (-889) T polymorphism in not being associated with PCOS among Chinese as we demonstrated in this study might be because there are other less recognized polymorphisms in this gene, which also influence the protein production. Furthermore, other genes contained in the *IL-1* gene clusters such as *IL-1b* might play an important role in ovarian function of PCOS especially among Chinese population [15]. Further investigation of the *IL-1* family is required to determine whether or not this concept is correct.

The reason for the conflicting results with Kolbus might mainly lay in the following context.

First, the number of studied subjects in our research is limited. Second, the patients of our study (Asian women) are not the same ethnic population as those in the Kolbus' study (Caucasian women). Furthermore, there may be certain genetic variant to in interaction with other variants and different local environmental condition may exist. Such considerations suggest the possibility of false positive or negative conclusions in either situation. And we have to note that the criteria and the clinical and biochemical characteristics used in Kolbus' study probably were not exactly the same as ours [27].

Therefore, using different criteria (such as the times uncomplicated pregnancy and subsequent delivery of patients and controls) may provide different results. So the applied criteria that influence the samples and controls had to be taken into consideration.

In conclusion, since the frequencies have not reached statistical significance in Chinese PCOS patients, the *IL-1a* gene does not appear to be a potential candidate gene associated with the etiology of this syndrome.

Materials and methods

Subjects

A total of 205 PCOS patients and 177 unrelated healthy controls were recruited from the First Affiliated Hospital, Anhui Medical University, China. Women with PCOS were diagnosed following the criteria of Rotterdam Revised 2003 (two out of three) diagnosis: oligomenorrhea or amenorrhea for at least 6 months; clinical and/or biochemical signs of hyperandrogenism; polycystic ovaries (presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increased ovarian volume(10 ml)). Congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumor, hyperprolactinemia, and thyroid dysfunction were excluded [2]. Controls were individuals of proven fertility, with normal menstrual cycles, and ovarian morphology, without a history of subfertility treatment. They were recruited from the Anhui Medical University.

The study protocol was approved by the Ethics Committee of the National Research Institute for Family Planning and informed consent was obtained from all participants.

Biochemical and hormonal measures

We determined the blood sample's plasma concentrations through the laboratory analysis of total testosterone (t), progesterone (P4), follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2) of each woman. Body mass index (BMI) was calculated as weight/(height)^2 (kg/m^2) to assess obesity.

To analyze hormone content, blood samples were drawn on the 3rd day of menstrual onset in the early follicular phase of the cycle after an overnight fast. Radioimmunity systems were used for the determination of testosterone, LH, FSH, estradiol, and progesterone. In patients with amenorrhea after excluding pregnancy by a commercially available pregnancy test, blood is taken from the antecubital vein on average day after an overnight fast [15, 28].

DNA analysis

Blood samples from PCOS patients and controls were collected and stored at −20°C. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp genomic DNA kits under the brief protocol listed below.

Add 20 µl Proteinase K and 200 µl Buffer AL to the 200 µl blood sample into a 1.5 ml microcentrifuge tube, mix by pulse-vortexing for 15 s and incubate at 56°C for 10 min or more. Add 200 µl 100% ethanol to the sample, and mix again for 15 s. Then apply the mixture to the QIAamp Spin Column (in a clean 2 ml collection tube) and centrifuge at 8,000 rpm for 1 min. Then add 500 µl Buffer AW1 and centrifuge at 8,000 rpm for 1 min and add 500 µl Buffer AW2 and centrifuge at 14,000 rpm for 3 min. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube and add 200 µl Buffer AE. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 8,000 rpm for 1 min [29].

The genotyping of *IL-1a* gene C [-889] T polymorphism of PCOS patients and controls was amplified by polymerase chain reaction (PCR) using the following primer set according to previous study [30]:

5′-GGG GGC TTC ACT ATG TTG CCC ACA CTG GAC TAA-3′
5′-GAA GGC ATG GAT TTT TAC ATA TGA CCT TCC ATG-3′

The PCR was started with a denaturating step at 94°C for 5 min and the amplification by 40 cycles at 94°C for 30 s, 61°C for 40 s, 72°C for 30 s, and a final extension at 72°C for 10 min. ddH_2O (2-µl each) is used as a replacement for the 2 µl templates of every 96 samples (20 µl each mix) for PCR, serving as PCR controls.

The polymorphic allele T lacked an Nco1 restriction site which allows differentiation from the wild type allele through generation of 271 and 29 bp bands upon Nco1 restriction of the PCR product. The 300 bp PCR product and restriction fragments were resolved on a 4% agarose gel with ethidium bromide.

Three different genotypes were defined for the individual polymorphisms: the homozygous wild type C/C,the heterozygous variant C/T, and the homozygous variant T/T.

Statistical analysis

The allelic and genotypic distributions of *IL-1a* C (-889) T polymorphisms were estimated by allele counting and compared in the PCOS and control groups by χ^2 test.

Differences between noncontiguous variables, genotype distribution and allele frequencies were tested by chi-square analysis. The *t*-test was used to compare data of the clinical parameters such as age at menarche, E2, FSH, LH levels, prolactin levels, and total testosterone levels between different genotypes.

All these statistical analyses were carried out using the Statistical Package for Social Sciences version 10.0 (SPSS 10.0). The criterion for significance was set at $P < 0.05$.

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