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Altered Circulating Inflammatory Cytokines Are Associated with Anovulatory Polycystic Ovary Syndrome (PCOS) Women Resistant to Clomiphene Citrate Treatment

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Background: Polycystic ovary syndrome (PCOS) is a common gynecological disease characterized by chronic oligoanovulation, clinical/biochemical hyperandrogenism, polycystic ovaries, and insulin resistance. Accumulating evidence has shown that PCOS-related ovarian dysfunction is the main cause of anovulatory infertility. Clomiphene citrate (CC) is the first-line therapy for PCOS patients; however, approximately 15–40% PCOS patients are resistant to CC treatment. It has been demonstrated that PCOS is a chronic pro-inflammatory state, as some pro-inflammatory cytokines were elevated in the peripheral circulation of PCOS patients, but whether altered inflammatory cytokines expression in PCOS patients is associated with blunted response to CC remains unknown.

Material/Methods: We recruited 44 CC-resistant PCOS patients, along with 55 age and body mass index (BMI)-matched CC-sensitive PCOS patients. Ovulation was induced by administrating 50–100 mg/day CC on days 5 to 9 of each menstrual cycle. The cytokine profiles were detected by cytokine antibody microarrays and further validated by ELISAs.

Results: CC-resistant patients had higher levels of high-sensitivity C-reactive protein (hsCRP) than the CC-sensitive individuals. A growth factor, angiopoietin-2, was significantly reduced [1.64 (0.93–1.95) *vs.* 1.08 (0.85–1.34), $p<0.05$], while a chemokine CXCL-16 was significantly increased (9.10±2.35 *vs.* 10.41±2.82, $p<0.05$) in CC-resistant patients compared to the CC-sensitive subjects. CXCL-16 was positively correlated with hsCRP ($r=0.33$, $p<0.01$). Logistic regression analysis showed that angiopoietin-2 and CXCL-16 are associated with CC resistance.

Conclusions: Circulating cytokines are disturbed in CC-resistant PCOS patients. Altered angiopoietin-2 and CXCL-16 levels might compromise the responsiveness of the ovary to CC through up-regulating angiogenesis and inflammation.

MeSH Keywords: Cytokines • Inflammation • Polycystic Ovary Syndrome

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Background

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized by chronic oligoanovulation, clinical/biochemical hyperandrogenism, polycystic ovaries, and insulin resistance [1]. The PCOS-related ovarian dysfunction has emerged as the main cause of anovulatory infertility [2]. Although several approaches are available to induce ovulation in anovulatory PCOS women [3], clomiphene citrate (CC) is still considered the first-line therapy, with lower risk of adverse effects and lower cost [4]. However, evidence has demonstrated that approximately 15–40% of PCOS patients do not respond to CC treatment [5,6], which is termed CC resistance. It has been reported that PCOS women with obesity, hyperandrogenism, and insulin resistance are less likely to respond to CC, suggesting these factors could be prominent causes of CC resistance [7–9]. Given the multifaceted pathogenesis of PCOS and the great heterogeneity in the studied populations, the aforementioned factors cannot fully explain the origin of CC resistance. On the other hand, current strategies of augmenting therapy with insulin-sensitizing agents such as metformin are not proving to be sufficient [10,11].

Recently, a growing body of evidence has demonstrated that PCOS is a low-grade pro-inflammatory state. Some of the pro-inflammatory cytokines were found to be elevated in the peripheral circulation of PCOS women [12–14]. Cytokines have been shown to be directly involved in maintaining the delicate balance of the hypothalamo-pituitary-ovarian axis and in the maintenance of normal ovarian and menstrual cycles [15]. We thus hypothesized that the dysregulation of cytokines, a main feature of chronic inflammation in PCOS, may be also associated with the blunted ovarian response to CC therapy. The aim of this cross-sectional study was to identify the circulating cytokines that are expressed differently in CC-resistant and CC-sensitive PCOS women.

Material and Methods

Patients

At the Department of Reproduction Health and Infertility of the First Affiliated Hospital of Chongqing Medical University, between February 2013 and December 2014, we recruited 44 anovulatory PCOS women determined to no have response to ovulation induction by CC treatment and assigned them to the CC-resistant group. We recruited 55 age- and body mass index (BMI)-matched anovulatory PCOS women who had ovulated after CC treatment and assigned them to the CC-sensitive group as control. All the patients recruited met the following inclusion criteria:

1. Age 20–35 years;
2. Diagnosis of PCOS (with at least both oligo-amenorrhea and polycystic ovaries on ultrasonography) based on the 2003 Rotterdam consensus [16].
3. Fasting blood glucose levels <7.0 mmol/L and a 2-h glucose levels <11.1 mmol/L following a 75-g oral glucose tolerance test (OGTT);
4. Follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) levels within normal ranges;
5. No intake of the hormone medications (including oral contraceptives) within the past 2 months or of medicines that affect insulin sensitivity (e.g., metformin or thiazolidinediones) within the past 3 months;
6. No male-related infertility or tubal causes of infertility.

Each subject of the present study provided signed consent. The study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Ovulation induction

All participants were induced to ovulate by giving 50 mg/day CC on day 5 to 9 of a spontaneous or a progestin-induced menstrual cycle. Ovulation was observed by vaginal B-ultrasound (GE Voluson E8, Zipf, Austria) on days 11 to 14 of the menstrual cycle, and progesterone level was measured on day 21 of the menstrual cycle. If there was no dominant follicle (size <10 mm in diameter) observed and the progesterone level was <5 ng/ml after 1 cycle of CC treatment, 100 mg/day CC was used on the same days of subsequent cycles. The maximum dose of CC used in our hospital was 100 mg/day due to the nature of the Asian population [9,17]. The duration of follow-up for all patients was at least 3 treatment cycles. The CC-resistant group was defined as those who failed to ovulate in response to CC treatment after 3 cycles. The patients who ovulated after at least 1 of 3 cycles of CC treatment were defined as CC-sensitive.

Clinical and Biochemical measurements

BMI was calculated as the ratio between weight in kilograms and the square of height in meters (kg/m^2). BMI ≥28 kg/m^2 was referred to as obesity [18]. A blood sample was collected from each patient on day 3 of the first menstrual cycle (before the initiation of CC treatment), following overnight fasting. An OGTT was performed after overnight fasting on all patients. Plasma glucose was measured by the Glucose Oxidase method (Mindray, Shenzhen, China). Fasting plasma insulin, total testosterone, FSH, and TSH were determined by chemiluminescence (Beckman, CA, USA). The plasma levels of high-sensitivity C-reactive protein (hsCRP) were measured by immunoturbidimetry on the autoanalyzer (HITACHI 7180, Ichige, Japan). Insulin resistance (IR) was estimated by Homeostasis Model Assessment of IR (HOMA-IR), which was calculated using the

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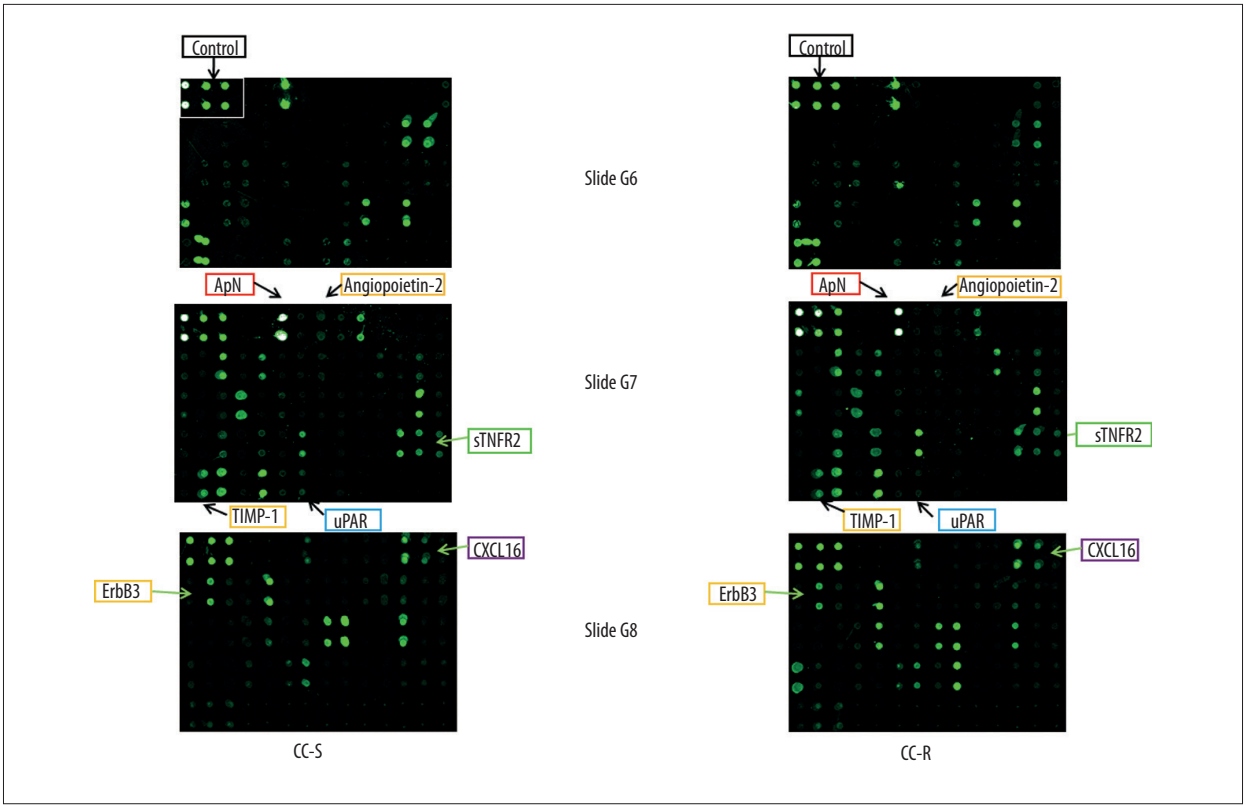

Figure 1. Cytokine antibody microarray slides used to detect cytokines secreted by CC-sensitive (CC-S) and CC-resistant (CC-R) women with PCOS. A set of 3 human cytokine antibody microarray slides, which tests up to 174 cytokines, was used and probed with fasting plasma. This figure illustrates the representative microarrays of CC-S and CC-R patients. Highlighted cytokines are differentially secreted between the 2 groups.

formula FPG (mmol/L)× fasting insulin (μIU/ml)/22.5. Patients with HOMA-IR >2.0 were considered as having IR [17].

Cytokine antibody microarray

Screening for circulating cytokines secreted by CC-sensitive or CC-resistant patients was performed by hybridizing plasma with antibody-coated glass slides according to the protocol supplied by the manufacturer [RayBio Human Cytokine Antibody Array G Series 2000, a kit combining glass slides of Array G6, G7, and G8 and allowing the simultaneous detection of 174 cytokines (cat. no. AAH-CYT-2000), for details please refer to *http://www.raybiotech.com/manual/Antibody%20Array/AAH-CYT-2000.pdf* (RayBiotech, Inc, Norcross GA, USA)]. Spots fluorescent signal intensities on slides were quantified by using a laser scanner [GenePix 4000B (Molecular Devices, LLC, CA, USA)]. Signals were normalized to internal positive controls present on each slide (Figure 1), and then expressed as intensity units in each group. The experiments on both groups were always performed simultaneously.

ELISA

The differentially secreted cytokines between the 2 groups as identified by cytokine antibody microarrays ($p<0.05$) were further validated by ELISA. The assessment of circulating angiopoietin-2, urokinase receptor (uPAR), tissue inhibitor of metalloproteinase-1 (TIMP-1), soluble tumor necrosis factor receptor-2 (sTNF-R2), adiponectin, CXCL-16, and human epidermal growth factor receptor 3 (ErbB3/Her3) were performed by using ELISA kits from RayBiotech according to the manufacturer's instructions.

Presentation of the results and statistical analysis

Statistical analysis was performed using SAS 9.13 (SAS Institute, Cary, NC). Variables are presented as means ±SD or median (interquartile ranges) or number (percentage). Means of continuous variables were compared using the unpaired *t* test or the Wilcoxon 2-sample test. The percentage differences between groups were compared using χ^2 tests. Univariate logistic regression analysis was performed to evaluate the associations with response to CC. The differences between groups were considered statistically significant at $p<0.05$. For logistic

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Table 1. Clinical and biochemical characteristics of CC-sensitive (CC-S) and CC-resistant (CC-R) women with PCOS.

	CC-S (n=55)	CC-R (n=44)	P value	Normal laboratory value
Age (year)	26.35±3.13	26.82±3.47	0.48	
BMI (kg/m^2)	23.02±3.72	23.30±3.23	0.70	
Subjects with BMI ≥28, n (%)	6 (10.91)	2 (4.55)	0.25	
Total testosterone (ng/ml)	0.58±0.15	0.59±0.17	0.87	<0.75
Subjects with testosterone ≥0.75, n (%)	4 (7.27)	7 (15.91)	0.17	
FSH (mIU/ml)	6.40±1.82	6.10±1.52	0.43	3.80–8.70 (follicular phase)
TSH (μIU/ml)	2.58 (1.63–3.13)	2.62 (1.69–3.22)	0.89	0.35–3.50
hsCRP (mg/L)	1.17 (0.22–1.53)	1.80 (0.50–2.38)	**0.048**	0.00–3.00
OGTT				
Fasting glucose (mmol/L)	5.24±0.37	5.24±0.89	0.99	3.90–6.00
30-min glucose (mmol/L)	9.18±1.46	9.06±2.23	0.75	
60-min glucose (mmol/L)	8.82±2.38	9.15±2.93	0.55	
120-min glucose (mmol/L)	7.31±1.99	7.89±2.74	0.24	
Fasting insulin (μIU/ml)	8.38 (4.34–11.66)	9.05 (5.07–12.49)	0.57	1.90–23.00
30-min insulin (μIU/ml)	76.82 (36.54–91.74)	76.23 (42.05–91.64)	0.79	
60-min insulin (μIU/ml)	78.95 (44.00–99.05)	92.70 (50.52–118.08)	0.19	
120-min insulin (μIU/ml)	69.75 (38.66–89.10)	77.04 (38.05–97.35)	0.63	
HOMA-IR	1.95 (0.98–2.67)	2.19 (1.13–2.95)	0.67	
Subjects with HOMA-IR >2.0, n (%)	20 (36.36)	17 (38.64)	0.82	

Data are means ±SD or median (interquartile ranges) or number (percentage) for indicated number of subjects in each group. P values for comparisons between two groups are based on Unpaired t test or Wilcoxon two-sample test or χ^2 test.
BMI – body mass index; FSH – follicle-stimulating hormone; TSH – thyroid-stimulating hormone; hsCRP – high-sensitivity C-reactive protein; OGTT – oral glucose tolerance test; HOMA-IR – homeostasis model assessment of insulin resistance.

regression analysis, the statistical significance was set as p<0.1 and the 95% Wald confidence intervals (CI) did not include 1.0.

Results

Characteristics of subjects

As shown in Table 1, the 2 groups of participants were well matched in terms of age and BMI. There were no significant differences between the 2 groups in terms of hormonal and metabolic parameters, as well the proportions of obesity, hyperandrogenism, and insulin resistance. Compared to the CC-sensitive patients, the CC-resistant patients displayed higher levels of hsCRP, a pro-inflammatory marker.

Cytokine secretion in CC-sensitive and CC-resistant PCOS patients

Fasting plasma from CC-sensitive and CC-resistant PCOS patients were subjected to cytokine antibody microarray for profiling 174 human cytokines (Figure 1). The secretory profile of plasma demonstrated that 7 cytokines – angiopoietin-2, uPAR, TIMP-1, sTNF-R2, adiponectin, CXCL-16, and ErbB3 – were differentially secreted between the 2 groups (Table 2, p<0.05).

These cytokines were further validated in 55 CC-sensitive and 44 CC-resistant individuals by ELISA. The ELISA results confirmed that 2 of 7 candidate cytokines were altered in CC-resistant individuals. Compared to the CC-sensitive group, the secretion of angiopoietin-2 was significantly reduced in the CC-resistant group [1.64 (0.93–1.95) *vs.* 1.08 (0.85–1.34),

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Table 2. Screening secretion of cytokines of CC-sensitive (CC-S) and CC-resistant (CC-R) women with PCOS.

Cytokines	Fluorescence intensity		P-value
	CC-S (n=4)	CC-R (n=4)	
Angiopoietin-2	299.03±109.37	160.35±12.56	0.05
uPAR	501.69±27.76	372.13±93.69	0.04
TIMP-1	2846.80±349.61	2143.04±295.11	0.02
sTNFR2	2214.21±203.90	1696.79±254.46	0.02
Acrp30	68648.05±1721.00	65635.77±1280.93	0.03
CXCL-16	1107.60±130.55	1549.45±194.84	0.01
ErbB3/Her3	3459.52±329.81	2693.26±266.28	0.01

Fasting plasma of each subject was incubated with the sets of cytokine antibody microarrays like those shown in Figure 1. Cytokine levels were measured by laser scanner (GenePix 4000B Microarray Scanner), normalized to internal positive controls and expressed as fluorescence intensity. Only the cytokines, which differed between the two groups of subjects, are shown in the table. Seven cytokines were differentially secreted between CC-S and CC-R women. Values are presented as means ±SD for 4 subjects per group.
uPAR – urokinase receptor; TIMP-1 – tissue inhibitor of metalloproteinase-1; sTNF-R2 – soluble tumor necrosis factor receptor-2; CXCL-16 – chemokine (C-X-C motif) ligand 16; ErbB3/Her3 – human epidermal growth factor receptor 3.

Table 3. Univariate logistic regression analysis of hsCRP and the two cytokines in 99 patients for the associations with CC-resistance.

	OR (95% CI)	P value
hsCRP	1.28 (0.94–1.75)	0.12
Angiopoietin-2	0.29 (0.12–0.70)	<0.01
CXCL16	1.23 (1.03–1.46)	0.02

p<0.05], while CXCL-16 was over-secreted by the CC-resistant group (9.10±2.35 *vs.* 10.41±2.82, p<0.05).

Correlation analysis

Pearson correlation analysis showed that CXCL-16 was positively correlated with hsCRP (r=0.33, p<0.01). Univariate logistic regression analysis further showed that decreased angiopoietin-2 levels and increased CXCL-16 levels were significantly associated with the presence of CC resistance (Table 3).

Discussion

Some studies have suggested that serum androgen levels, BMI, blood glucose, and insulin resistance are associated with CC resistance. Other researchers have reported that FSH receptor polymorphism at position 680 and basal FSH levels predict the ovarian response to CC [19]. However, in the present study, the CC-sensitive and CC-resistant groups had similar BMI, and showed no significant differences in blood glucose, insulin levels, total testosterone, or FSH levels before CC treatment. However, the CC-resistant group displayed a higher pro-inflammatory state, as shown by an increase in hsCRP levels. This indicates that inflammation might be associated with the blunted CC response. Therefore, we then focused on investigating the correlation between circulating cytokines and CC resistance without the interference of obvious hormonal and metabolic confounding factors.

The regulation of cytokines in the ovary has been described as promoting processes of follicular growth, steroidogenesis, recruitment, and activation of leukocytes necessary for ovulation and tissue remodelling during ovulation, luteinization, and luteolysis [20]. Ovulation is regarded as an inflammatory event that is followed by anti-inflammatory reactions; these processes need the coordination of both pro-inflammatory and anti-inflammatory cytokines [21]. Moreover, cytokines are involved in both the inhibition and stimulation of follicular responsiveness to gonadotrophins [22]. However, the involvement of cytokines in the ovulatory response to CC medication has not been thoroughly investigated. Serum levels of IL-6 have been reported to be decreased in anovulatory PCOS women resistant to CC when compared to ovulatory women, suggesting that low serum IL-6 could be a marker of CC resistance [23]. Another study found that circulating levels of leptin, an adipokine, were significantly higher in CC non-responders and could be used to predict CC resistance [8]. Using cytokine antibody microarray technology, we investigated 174 human cytokines, including the pro-inflammatory factors, growth factors, chemokines, anti-inflammatory factors, extracellular matrix

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factors, and adipokines. By subsequently performing ELISA, we ultimately identified 2 cytokines that had different circulating levels between the CC-sensitive and CC-resistant patients. Angiopoietin-2, a growth factor, is decreased in CC-resistant women, and is also reversely correlated with the presence of CC resistance as determined by logistic regression analysis. The angiopoietins, including angiopoietin-1 and angiopoietin-2, are key regulators of angiogenesis. It is known that angiopoietin-1 is necessary for the recruitment of peri-vascular cells that lead to the maturation and stabilization of newly developed capillaries, while angiopoietin-2 acts as a natural antagonist of angiopoietin-1, resulting in loosening of the supporting cell matrix and destabilization of existing vessels [24]. In the physiological condition, angiopoietin-2 protein level in the ovary increases during follicular enlargement and decreases during maturation of follicles, suggesting that angiopoietin-2 may be critical for follicular development [25,26]. In contrast, in the pathological PCOS condition, angiopoietin-2 level was decreased in the ovaries of PCOS rats compared to the normal controls, resulting in higher ovarian vascularity and vessel stabilization [27]. The compromised angiopoietin-2 expression in CC-resistant PCOS women suggest that defects of follicular development and ovulation may result from excessive ovary angiogenesis.

We also found that CXCL-16 was increased in CC-resistant women and was positively correlated with CC resistance. Although the function of CXCL-16 in the ovaries or hypothalamo-pituitary is still poorly documented, it is known that CXCL-16 plays an important role in initiating and shaping immune-inflammatory responses by attracting inflammatory mononuclear cells [28]. Consistently, we have found a significant positive correlation between CXCL-16 and hsCRP, indicating that CXCL-16-mediated pro-inflammation might also be involved in regulating CC sensitivity in PCOS patients.

Conclusions

Circulating cytokines are disturbed in CC-resistant PCOS patients. Altered angiopoietin-2 and CXCL-16 levels might impair the responsiveness of the ovaries to CC through up-regulating angiogenesis and inflammation.

Conflict of interest

The authors declare that they have no competing interests.

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