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Serum biomarker analysis in patients with premature ovarian insufficiency

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ABSTRACT

Premature ovarian insufficiency (POI) is a primary ovarian defect characterized by premature depletion of ovarian follicles before 40 years of age. The disorder has been attributed to various causes, but the study of altered proteins in serum levels as the cause is rare. Additionally, identifying novel biomarkers can contribute to more accurate diagnosis or prognosis of POI. In the present study, a solid-phase antibody array simultaneously detecting multiple proteins was used to analyze POI serum with menopausal and healthy fertile subjects as control groups. As a result, compared to the menopause and healthy fertile groups, eleven proteins, including Neurturin, Frizzled-5, Serpin D1, MMP-7, ICAM-3, IL-17F, IFN-gamma R1, IL-29, IL-17R, IL-17C and Soggy-1, were uniquely down-regulated, and Afamin was particularly up-regulated in POI serum. More importantly, all of these factors were firstly found to be associated with POI in this study, suggesting that these proteins may participate in the pathogenesis of POI and may be novel serum biomarkers for POI.

1. Introduction

Premature ovarian insufficiency (POI) is a defect of ovarian development or function and a major cause of infertility in women younger than 40 years of age. POI is characterized by primary or secondary amenorrhea, symptoms of hypoestrogenism, sex steroid deficiency, and elevated serum gonadotropin concentrations long before the natural age of menopause. POI affects approximately 1% of women [1], seriously interferes with fertility and family planning [2,3], and presents a higher risk of developing osteoporosis, cardiovascular diseases and neurodegenerative disorders [4].

The etiology of POI is heterogeneous, including chromosomal, autoimmune, iatrogenic, viral infection, and metabolic causes. Disorders of the X chromosome are considered the most common genetic causes of POI, including chromosomal numerical defects, translocations, gene mutations and isochromosomes [5]. For example, approximately 16–26% of fragile X mental retardation 1 (FMR1) gene premutation carriers will develop POI [6,7]. In addition, some autosomal genes are associated with POI, such as GDF9, FSHR, LHR and NR5A1 [8]. Nevertheless, the cause of POI remains unknown in many cases. In most cases, low-level sex chromosome mosaicism does not seem to cause any phenotypic effects on ovarian function, making diagnosis difficult [9]. Therefore, identifying novel diagnostic or prognostic biomarkers for POI is of great importance. In the present study, an antibody array technology detecting the levels of multiple proteins in a high throughput format was used to identify novel serum cytokine biomarkers for POI.

2. Materials and methods

2.1. Subjects

Fourteen POI patients who had a cessation of menses for 6 months, and serum FSH (follicle-stimulating hormone) levels > 25 IU/l before or at the age of 40 years, were recruited. Twelve menopausal women with 45–55 years of age and with serum FSH levels > 40 IU/l, and fourteen healthy fertile women under 40 years of age having serum FSH levels < 10 IU/l were included in this study as controls. All participants were from The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, and underwent a complete clinical assessment. Those who had gynecological history, an ovariectomy, hormone therapy, or cancers such as hysteromyoma, cervical carcinoma, or breast cancer, were excluded. Signed, informed consents were obtained from all subjects prior to their inclusion in this study. The study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine.

Abbreviations: POI, premature ovarian insufficiency; FMR1, fragile X mental retardation 1; FSH, follicle-stimulating hormone; ELISA, enzyme-linked immuno sorbent assay; GO, gene ontology

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Serum from each subject was collected for antibody array analysis.

2.2. Estradiol assay

The levels of estradiol in three groups were measured using the Electrochemical luminescence analyzer (MODULARE170, Roche, Germany) according to the manufacturer's instructions.

2.3. Antibody array analysis

Serum samples were separately analyzed using a high-density antibody array (AAH-BLG-1000; RayBiotech, Inc, Norcross, GA, USA) that utilizes a biotin label-based technology, simultaneously detects 1000 human proteins and is comprised of AAH-BLG-1 (detects 507 proteins) and AAH-BLG-2 (detects 493 proteins). Briefly, after dialysis, the serum was biotinylated. The biotin-labeled proteins were incubated with glass slides pre-printed with 1000 capture antibodies at room temperature for 2 h. A streptavidin-conjugated fluorescent dye (Cy3) was used to detect captured proteins. The signals were visualized using a Scan Array laser (GenePix v. 5.0, GenePix 4000B scanner, Molecular Devices, San Jose, CA, USA).

2.4. ELISA performance

To validate the results of the antibody array, ELISA was performed according to the manufacturer's instructions (RayBiotech, Norcross GA, USA) for the detections of Neurturin (Measurement ranges: 1.23–300 pg/ml, Sensitivity: 1.23 pg/ml), Serpin D1 (Measurement ranges: 1.23–300 ng/ml, Sensitivity: 1.23 ng/ml), MMP-7 (Measurement ranges: 0.15–100 ng/ml, Sensitivity: 0.15 ng/ml), ICAM-3 (Measurement ranges: 65.5–16000 pg/ml, Sensitivity: 65.5 pg/ml), IL-17F (Measurement ranges: 20–10000 pg/ml, Sensitivity: 20 pg/ml), IFN-gamma R1 (Measurement ranges: 24–6000 pg/ml, Sensitivity: 24 pg/ml), IL-29 (Measurement ranges: 20–10000 pg/ml, Sensitivity: 20 pg/ml), IL-17R (Measurement ranges: 10–3000 pg/ml, Sensitivity: 10 pg/ml), IL-17C (Measurement ranges: 125–8000 pg/ml, Sensitivity: 125 pg/ml), Soggy-1 (Measurement ranges: 20–10000 pg/ml, Sensitivity: 20 pg/ml) and Afamin (Measurement ranges: 8.23–6000 pg/ml, Sensitivity: 8.23 pg/ml). 20 cases for each group were recruited basing on the inclusion criteria used in the antibody array experiment. Briefly, after being diluted at different factors for different serum biomarkers, serum samples were incubated in the plates overnight at 4 °C. Biotin-conjugated antibody was added into the plate wells for 2 h incubation and HRP-conjugated streptavidin was added to catalyze the TMB reagent. The catalytic reaction was stopped by the addition of sulfuric acid, and the OD_{450} was measured using a microplate reader (ELx800NB, Biotek, Winooski, CT, USA).

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc, Chicago, IL, USA). Comparisons between groups were performed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test, and P values < 0.05 were considered statistically significant. All data were shown as mean ± SD. In addition, fold change (FC) was calculated, and the values were given to indicate the relative expression levels of cytokines. Proteins with average signal value more than 150 in each group were considered positively expressed. Venn Diagram analysis was performed by a website (http://bioinformatics.psb.ugent.be/webtools/Venn/). An unsupervised-hierarchical cluster was performed by Cluster 3.0 software.

2.6. Bioinformatics analysis

In order to elucidate the potential functions of some proteins screened as specific POI biomarkers in the pathogenesis of POI, gene ontology (GO) and pathway analysis were performed to reveal the biological processes, cellular components and molecular functions of these proteins using KOBAS3.0 database (http://kobas.cbi.pku.edu.cn/) using the gene IDs. The corrected p-values of enrichments < 0.05 were considered significant.

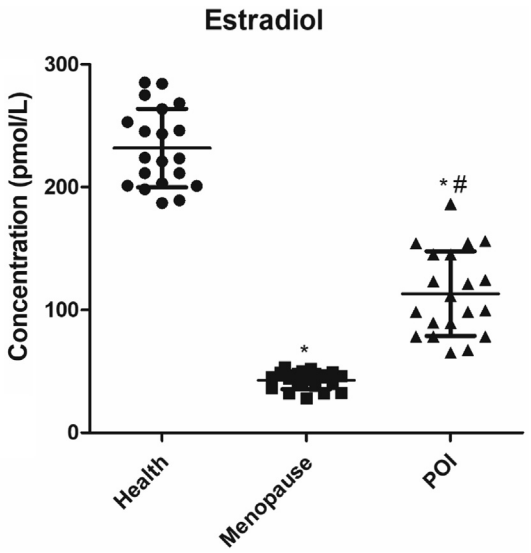

Fig. 1. Estradiol level. The estradiol level among the three groups were shown with scatter diagram. * means $p < 0.05$ vs healthy fertile group. # means $p < 0.05$ vs menopausal group.

3. Result

3.1. Estradiol level

In order to better distinguish the three groups, serum estradiol was measured in all subjects. As a result, the tendency of estradiol level among the three groups was significantly healthy fertile group > POI group > menopausal group, consistent with clinical data, as shown in Fig. 1, which suggested these subjects were representative in corresponding group.

3.2. Specific POI biomarker screening

In order to look for POI specific biomarkers, both menopausal samples and healthy fertile samples were used as controls. By statistically analyzing the differentially expressed proteins between any two groups among POI, menopausal and healthy fertile groups, it was found that 188 proteins were significantly differential between POI and healthy fertile groups, 257 proteins were markedly differential between menopausal and healthy fertile groups, and 190 proteins were obviously differential between POI and menopausal groups. Specific POI biomarkers were defined as the proteins simultaneously differentially expressed in POI compared with both menopausal group and healthy fertile group, but not differential between menopausal and healthy fertile groups. After Venn diagram analysis, we found twelve specific POI biomarkers (Fig. 2), including Neurturin, Frizzled-5, Serpin D1, MMP-7, ICAM-3, IL-17F, IL-17R, IL-17C, IFN-gamma R1, IL-29, Soggy-1 and Afamin. Their expression levels were further analyzed by histogram display using the signal values. As shown in Fig. 3, when compared with both menopausal group and healthy fertile group, the levels of Neurturin, Frizzled-5, Serpin D1, MMP-7, ICAM-3, IL-17F, IFN-gamma R1, IL-29, IL-17R, Soggy-1, and IL-17C were decreased, while the expression of Afamin was increased, in POI serum. Furthermore, the array profiles were shown to reveal the differences of these twelve proteins among the three groups (Fig. 4), and the unsupervised-hierarchical

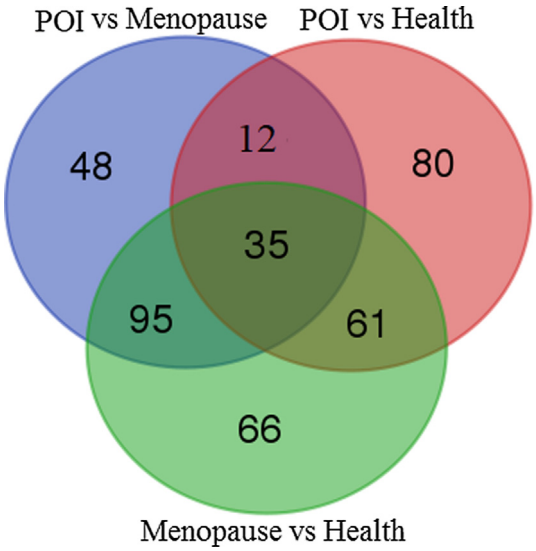

Fig. 2. Venn diagram analysis. The differentially expressed proteins among the healthy fertile vs menopausal groups, healthy fertile vs POI groups, and menopausal vs POI groups were analyzed by Venn diagram to find POI-specific biomarkers that were simultaneously differentially expressed in POI vs menopausal and POI vs healthy fertile groups, but not differentially expressed between POI and healthy fertile groups.

cluster gathered all the POI samples into the same group (Fig. 5). The mean ± SD and fold change of differential proteins were showed in Table 1.

3.3. ELISA results

To validate POI-specific biomarkers identified by antibody array, eleven biomarkers were validated by ELISA except Frizzled-5 due to the unavailable commercial kit. The expression levels of these cytokines obtained from ELISA were consistent with those obtained from the antibody array, further confirming that these cytokines were differentially expressed in POI patients (Fig. 6).

3.4. Bioinformatics analysis

GO analysis showed that the specific POI proteins mainly participated in the categories including response to stress, response to stimulus, cellular response to stimulus, single-organism process, regulation of cellular process, regulation of biological process, binding, biological regulation, cellular process, and single-organism cellular process (Fig. 7A). Pathway analysis showed the most enriched terms were interleukin-17 signaling, cytokine signaling in immune system, immune system, signaling by interleukins, cytokine-cytokine receptor interaction, and extracellular matrix organization (Fig. 7B).

4. Discussion

It is known that most causes of POI are idiopathic, and are likely associated with genetic aberrations in its pathogenesis. However, the detection of genetic aberrations for early diagnosis of POI is complicated and expensive. Therefore, the present study aimed to identify POI-specific serum biomarkers to simplify early diagnosis of POI. As a result, twelve proteins, including Neurturin, Frizzled-5, Serpin D1, MMP-7, ICAM-3, IL-17F, IL-17R, IL-17C, IFN-gamma R1, IL-29, Soggy-1, and Afamin, were found to be specific for POI using menopausal and healthy fertile participants as control groups.

Menopause is a biological process that occurs as natural part of aging in women, typically after 40 years of age. It is characterized by the cessation of menstruation, the loss of primordial ovarian follicles, a decline in estradiol production and the concomitant increase in FSH, which are similar to the symptoms of POI. In addition, some risk factors, such as smoking and exposure to arsenic and dioxin, advance the onset of menopause. When menopause occurs prior to 40 years of age, it is called POI. Therefore, there will be some similar pathological processes between natural menopause and POI, and some specific pathomechanisms stimulating the onset of POI. Therefore, in the study of screening POI-specific biomarkers, it is necessary to add natural menopausal samples in addition to healthy fertile samples.

The pathway analysis of the twelve biomarkers shows that ICAM-3, IL-17F, IL-17R, IL-17C, IFN-gamma R1, and IL-29 participate in immune system. Increasing evidences have shown that the human ovary is one target of an autoimmune response, leading to ovarian dysfunction, and autoimmune response is an important etiology of POI [10–12]. ICAM-3 has been identified as the third leukocyte-function associated-1(LFA-1) ligand and plays an important role in the initiation of immune responses [13]. However, there have been no reports on the relationship between ICAM-3 and ovarian function. We inferred that decreased ICAM-3 might reduce immunity of ovarian to cause POI, which needs further validation. IL-17F, IL-17C and IL-17R are from IL-17 signaling and are important in cancers and immune system. IL-17F and IL-17C appear to interact with IL-17R and contribute to the pathology of

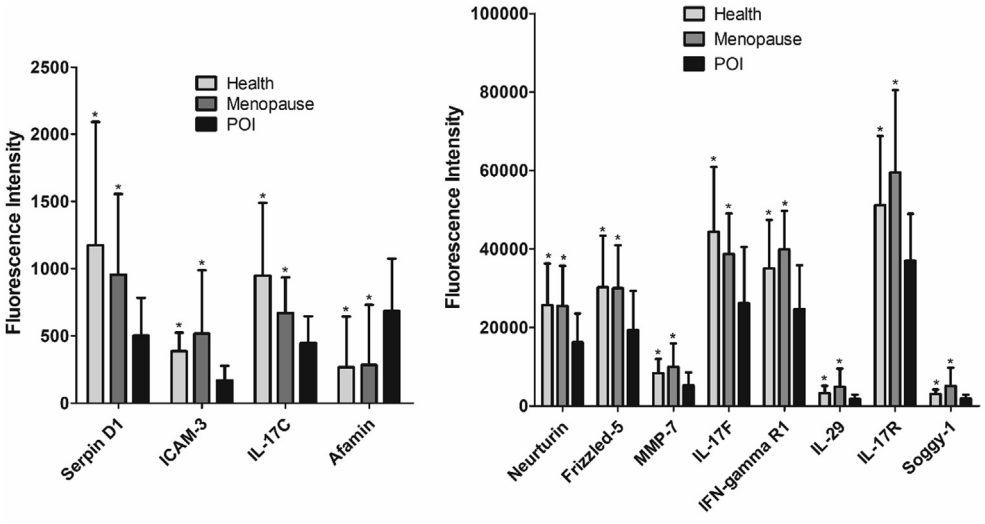

Fig. 3. Histogram analysis. Histogram shows levels of POI-specific proteins in the three groups. * means $p < 0.05$ when compared to the POI group.

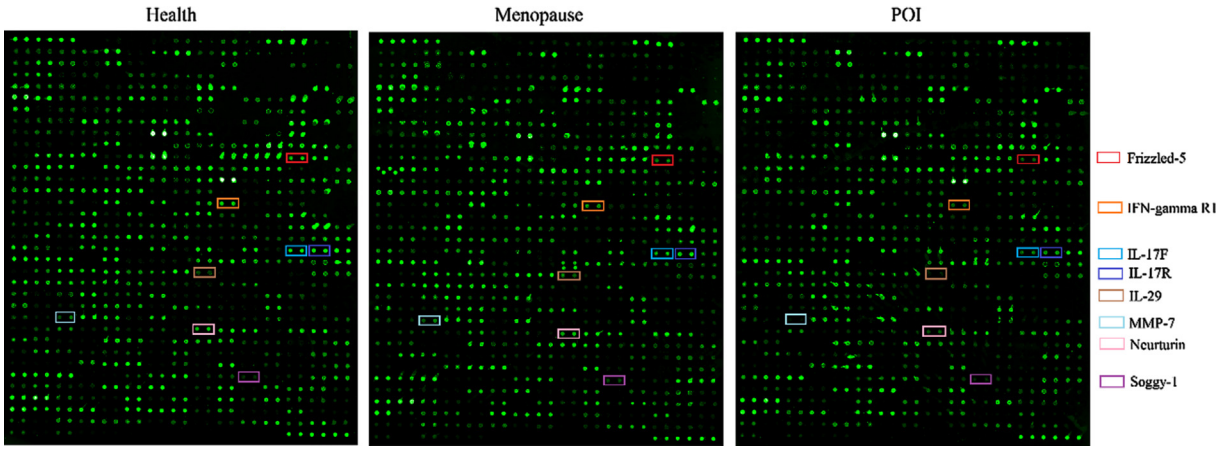

Fig. 4. The specific POI protein profiles. The locations of some specific POI proteins from AAH-BLG-1 antibody array are noted in colored boxes and the levels of cytokines are proportional to their fluorescence intensity. In these arrays, each antibody was printed in duplicate.

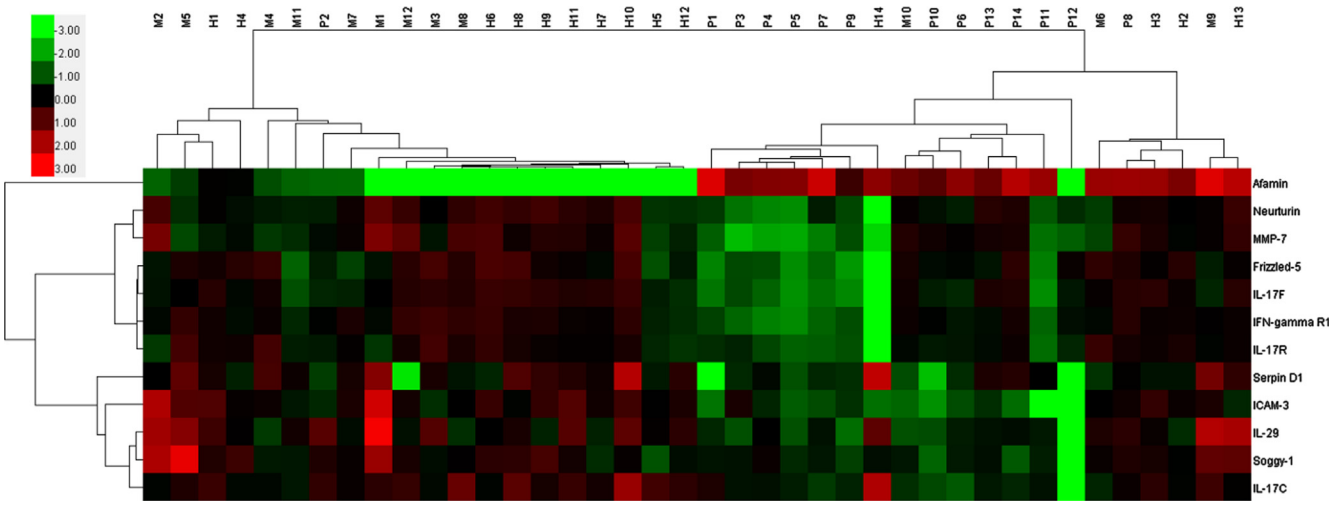

Fig. 5. Unsupervised-hierarchical cluster analysis. Green indicates low levels of the proteins, black for median levels, and red for high levels. M: menopausal group; H: healthy fertile group; P: POI group.

Table 1
The data of antibody array.

	Menopausal group	POI group	Healthy group	Menopausal/healthy		POI/Healthy		POI/Menopausal	
Target names	mean ± SD	mean ± SD	mean ± SD	p value	Fold change	p value	Fold change	p value	Fold change
Serpin D1	955.0 ± 601.3	503.3 ± 279.3	1175.3 ± 916.9	0.484	0.813	0.019	0.428	0.030	0.527
ICAM-3	516.0 ± 471.5	169.9 ± 106.8	386.1 ± 136.1	0.374	1.336	0.000	0.440	0.029	0.329
IL-17C	670.4 ± 264.1	447.9 ± 197.6	948.9 ± 541.6	0.105	0.707	0.005	0.472	0.022	0.668
IL-29	4883.5 ± 4646.2	1790.0 ± 1050.2	3277.8 ± 1892.0	0.281	1.490	0.018	0.546	0.043	0.367
IL-17F	38775.3 ± 10284.9	26163.8 ± 14327.7	44354.2 ± 16541.7	0.322	0.874	0.004	0.590	0.018	0.675
Neurturin	25521.2 ± 10212.4	16212.1 ± 7341.	25661.6 ± 10650.5	0.973	0.995	0.011	0.632	0.013	0.635
MMP-7	9982.3 ± 5972.8	5290.1 ± 3300.0	8370.5 ± 3611.5	0.406	1.193	0.026	0.632	0.027	0.530
Frizzled-5	30014.0 ± 10931.1	19264.2 ± 10119.6	30255.4 ± 13130.1	0.960	0.992	0.020	0.637	0.016	0.642
Soggy-1	5093.3 ± 4647.6	1963.0 ± 855.8	3078.5 ± 1097.2	0.168	1.654	0.006	0.638	0.041	0.385
IFN-gamma R1	39855.7 ± 9885.7	24660.0 ± 11237.9	35050.7 ± 12395.3	0.291	1.137	0.028	0.704	0.001	0.619
IL-17R	59547.1 ± 20933.4	36970.3 ± 11998.0	51190.5 ± 17626.4	0.280	1.163	0.019	0.722	0.002	0.621
Afamin	284.0 ± 447.0	685.0 ± 390.6	267.6 ± 376.2	0.920	1.061	0.008	2.560	0.022	2.412

inflammatory disorders [14,15]. It was found IL-17 expression is elevated in ovarian cancer, IL-17A level in POI was increased [16,17]. However, high IL-17 expression was correlated with improved progression-free survival in advanced ovarian cancer [16], and IL-17 members including IL-17F, IL-17C and IL-17R were decreased in POI patients in the present study, suggesting the complex roles of IL-17 members in ovarian diseases, which needs further research. IFN-gamma R1 is a receptor of IFN-gamma and is critical in the immune response [18]. The loss of IFN-gamma R1 was an independent prognostic factor in ovarian cancer, overexpression of a dominant-negative mutant of IFN-gamma R1 could enhance tumorogenicity and reduce immunogenicity in a mouse model [19,20], which suggested low level of IFN-gamma R1 might be an important factor of POI development by reducing immunogenicity. IL-29 is a member of the interferon (IFN)

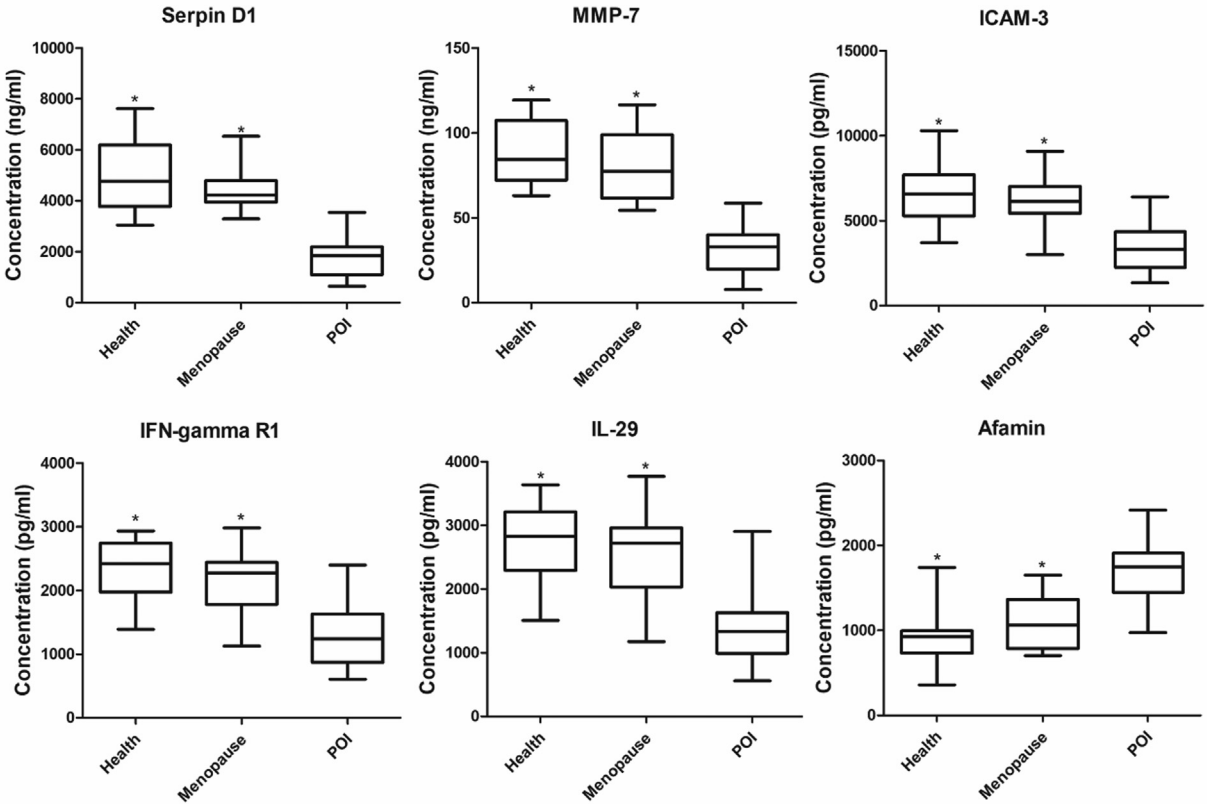

Fig. 6. ELISA validation results. The levels of validated cytokines in each group are shown in histograms with median values. * means $p < 0.05$ when compared to POI group.

family known to have beneficial role in interfering with viruses and in protective immune responses, and against tumor [21]. Although IL-29 was upregulated in polycystic ovary syndrome [22], it was found IL-29 was downregulated in POI in this study, suggesting decreased IL-29 might be beneficial to an autoimmune response to promote POI progress. Interestingly, it was found for the first time these six proteins were decreased in POI serum in the present study. The down-regulations of these proteins indicate their possibly protective roles in ovarian function, probably by the interaction of ligands and their receptors consistent with the pathway analysis, especially in the enriched cytokine-cytokine receptor interactions. These roles, however, require further research to confirm and elucidate.

Neurturin, another protein differentially expressed in POI, as a neurotrophic factor, plays an important role in parasympathetic neural development. However, it was found that, in the female reproductive tract, neurturin was expressed at high levels in the epithelium and mucosa of the oviduct and at low levels in developing follicles within the ovary, suggesting neurturin may be involved in the growth or maintenance of the oviduct lining and in the process of follicle maturation or ovulation [23]. In this study, we found that neurturin was decreased in POI serum, suggesting that the ability of the follicle maturation or ovulation in POI patients was weakened due to the decrease of neurturin. Frizzled-5 is a key molecule functioning as Wnt5a receptor. Thus far, there have not been reports about the relationship between ovarian function and Frizzled-5. However, Abedini et al. [24] found that WNT5a could inhibit FSH as a key regulator of follicle development. In this research, Frizzled-5 was found to be reduced in POI serum, so we speculate that WNT5a may inhibit FSH by interacting with Frizzled-5. The down-regulation of Frizzled-5 can weaken the protective function of WNT5a on ovary via decreasing FSH expression to promote the development of POI. Serpin D1 is a serum glycoprotein and protease inhibitor and MMP7 is a proteolytic enzyme. It was discovered that Serpin D1 was over-expressed in ovarian cancer and that MMP7 played an essential role in enhancing ovarian cancer cell ability to migrate and invade [25,26]. Soggy-1, also called Dickkopf-Like1 (DKKL1), was found to play an important role in testicular development, spermatogenesis and male infertility [27], but no reports on the role in ovary. However, no previous research found that Serpin D1, MMP7 or Soggy-1 affected POI, but the present study showed Serpin D1, MMP7 and Soggy-1 were decreased in POI serum for the first time, suggesting that Serpin D1, MMP7 and Soggy-1 may promote ovarian cell growth, and decreased expressions may facilitate POI development. Afamin is a member of the albumin multigene family and is abundantly expressed not only in serum, but also in extravascular fluids such as follicular, seminal, and cerebrospinal fluid. In a previous study, Afamin was identified as a novel protein marker for ovarian cancer and polycystic ovarian syndrome with elevated serum levels [28,29]. In this study, Afamin was found, for the first time, to be increased in POI, suggesting that Afamin also may be a novel protein marker for POI. Afamin is increased in response to various conditions of oxidative stress, and POI is a status of elevated oxidative stress [30], suggesting Afamin may involve into POI through oxidative stress, and service as a novel biomarker for POI.

In conclusion, we found twelve proteins differentially expressed in POI serum, eleven of which were down-regulated and one up-regulated, compared with menopausal and healthy fertile groups. More importantly, these twelve factors were revealed to be associated with POI for the first time in the current research, suggesting these targets may be able to serve as novel serum biomarkers for POI.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

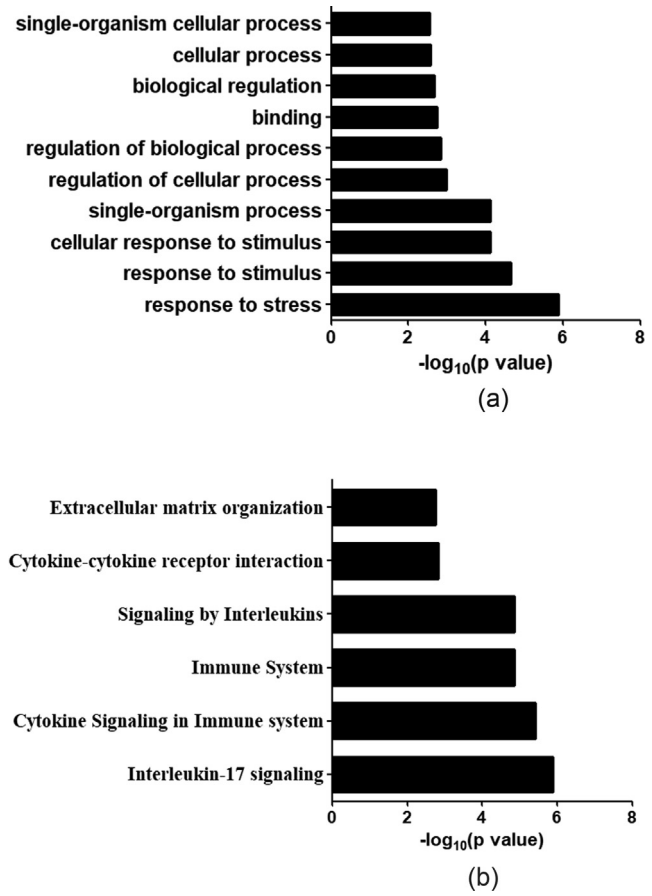

Fig. 7. Bioinformatics analysis. (A) the most enriched pathways. (B) the most enriched gene ontology terms.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Contributors

Jian Liu conceived and designed the experiments. Jian Liu, Xunchun Huang, Xiaojing Cao, Xuan Feng, Xiaoyun Wang performed the experiments. Jian Liu and Xiaoyun Wang analyzed the data. Jian Liu wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine. The ethical approval number is B2013-074-01.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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