Protein Expression Profile in IVF Follicular Fluid and Pregnancy Outcome Analysis in Euthyroid Women with Thyroid Autoimmunity

Yuting Liu, Yijia Wu, Mingyuan Tian, Wenwen Luo, Chanyu Zhang, Yongjian Liu, Ke Li, Wei Cheng, and Dongfang Liu*

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ABSTRACT: The objective of this study is to investigate the influence of the thyroid autoantibodies on the protein expression in follicular fluid and the clinical outcome of assisted reproductive technology. A total of 602 patients treated for infertility were screened; 49 euthyroid women who were positive for thyroid autoantibodies and 63 negative controls were recruited. Follicular fluid samples were analyzed using proteomics. Validation of target proteins in follicular fluid was performed by using parallel reaction monitoring. Differentially expressed proteins in follicular fluid, clinical pregnancy rate, abortion rate, and live-birth rate were analyzed. Clinical pregnancy rates and take-home baby rates in the thyroid autoimmunity (TAI) group were less than in the control group, but abortion rates in the TAI group were higher than in the control group (all \( p < 0.005 \)). A total of 49 proteins were differentially expressed in the TAI-positive group. In Gene Ontology secondary annotations of all the proteins identified, five types of proteins were associated with the reproductive process. Among 11 proteins quantitatively identified by parallel reaction monitoring, angiotensinogen and fetuin-B were associated with reproduction. These differentially expressed proteins identified in this study involved multiple pathways according to the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis. Our study provides evidence that some differentially expressed proteins between TAI-positive women and controls were associated with the reproductive process and closely related to important physiologic effects, which could partially explain the underlying mechanism link between TAI and the adverse outcomes of assisted reproductive technology.

INTRODUCTION

Thyroid autoimmunity (TAI), which is mainly characterized by the abnormally elevated antithyroperoxidase antibody (antiTPO) and antithyroglobulin antibody (antiTG), is one of the most common causes of thyroid dysfunction among women of reproductive age. Thyroid hormones not only can affect reproductive health, but also stimulate fetal nerve and brain development. Thus, the influence of maternal thyroid function on pregnancy outcome is a topic worthy of attention.1,2

The incidence rate of TAI among childbearing women was reported as 8−14% worldwide.3 A number of studies indicated that women with positive antiTPO and antiTG bodies were more likely to be infertile,4 and had a higher risk of adverse pregnancy outcome. There are some hypotheses that, on the one hand, TAI causes hypothyroidism or subclinical hypothyroidism, which may impair the development of the fetal brain. On the other hand, positive thyroid autoantibody makes the organism autoimmune, which leads to a direct interference on normal placental function, and ultimately resulted in the increase of spontaneous miscarriage.5,6 However, the specific mechanism of thyroid autoantibodies on pregnancy outcome has still needed further exploration.

In vitro fertilization (IVF) is currently considered as one of the most effective treatment strategies in female infertility.7 Previous studies have shown that euthyroid women with positive antiTPO and/or antiTG antibodies were more prone to have fertility problems.3,8 Antithyroid antibodies, as an independent factor for the failure of IVF, may be associated with recurrent implantation failure.3,7 Follicular fluid contains a wide variety of biologically active materials, such as proteins that can provide the microenvironment for oocytes development and modulate ovulation. Besides, the products of thecal
and granulose cell metabolism and blood serum composition that passes through the blood-follicle barrier can be found in the follicular fluid; therefore, such biologic fluid provides a unique window to search the factors affecting follicle maturation. The specific ingredients in the follicular fluid can help us better explore the signaling within the follicles and reveal the possible influence of the thyroid autoantibodies on pregnancy outcomes, particularly for women undergoing assisted reproductive IVF treatment. Proteomics has been applied to different research topics. Based on the proteins dictating biological function to a heavy extent, these novel methodologies can be used for recognition of biomarkers for noninvasive diagnosis, as well as predictive risk factors for the assisted reproduction techniques in infertile patients. Available data pertaining to proteomics of the follicular fluid in euthyroid women with autoimmune thyroid diseases undergoing IVF are still scarce. Therefore, we conducted this study to compare protein expression difference of the follicular fluid and IVF clinical outcome between euthyroid women with positive thyroid autoantibodies and controls, which could find the important relationship between TAI and adverse outcomes of pregnancy.

**RESULTS**

**Laboratory and Clinical Findings.** The laboratory findings, clinical features, and proportion of reasons for infertility in women with positive (TAI-positive group) and negative (TAI-negative group) titers of antithyroid antibodies are described in Table 1. The baseline characteristics were not statistically significant between the two groups except for their serum antiTPO antibody and antiTG antibody levels.

**IVF Outcome.** IVF results are illustrated in Table 2. The differences of the number of oocytes retrieved and of embryos transferred, fertilization rate, implantation rate, and clinical pregnancy rate between the two groups were not statistically significant. The abortion rate, the take-home baby rate were 61 and 39%, respectively, in women with TAI, whereas they was 25 and 75% in negative controls (*P*<0.05).

**Proteomics Profiling of Follicular Fluid Samples.** A total of 727 proteins were identified in follicular fluid samples, of which 656 were quantified. Besides, 49 proteins were differentially expressed in the TAI-positive group, including 31 upregulated and 18 downregulated genes (Table 3). In Gene Ontology (GO) secondary annotations of all the proteins identified, five types of proteins [T-complex protein 1 subunit α, corticotropin-releasing factor-binding protein, angiotensinogen (AGT), apolipoprotein B-100, fetuin-B (FETUB)] were associated with the reproductive process. Furthermore, the identified 49 proteins were associated with the metabolic process, developmental process, cellular component organization or biogenesis, immune system process, growth, and so on. Figure 2 shows the statistical evaluation for enrichment of GO categories, indicating the cellular component, molecular function, and biological process in differentially expressed proteins. The results has revealed an upregulation of the biological process with the following aspects: cellular response to gonadotropin-releasing hormone and immune response-regulating cell surface receptor signaling pathway. Meanwhile, a downregulation of the following aspects has also been revealed: fertilization, skin development, and regulation of tissue remodeling, and so forth. The aspects mentioned above play a significant role in physiological and pathological processes of the autoimmune thyroid.

| Table 1. Comparison of the Basic Characteristics between the Positive Titer of the Antithyroid Antibodies Group (TAI Positive) and the Negative Control Group (TAI-Negative) |
|---|
| **item** | **TAI-positive group** | **TAI-negative group** | **P Values** |
| number | 49 | 63 | |
| age (year) | 32.06 ± 4.96 | 32.94 ± 5.23 | 0.399 |
| BMI (kg/m²) | 22.01 ± 2.91 | 21.82 ± 2.51 | 0.803 |
| TSH (μIU/mL) | 2.60 (1.90, 3.06) | 2.15 (1.66, 2.86) | 0.077 |
| Free T3 (pmol/L) | 4.6 (4.3, 5) | 4.7 (4.5, 5.3) | 0.21 |
| Free T4 (pmol/L) | 16.5 (15.6, 18.7) | 16.8 (14.9, 17.9) | 0.3346 |
| TPOAb (IU/mL) | 180.8 (66.6, 238.8) | 10.8 (7, 15.2) | <0.0001 |
| TgAb (IU/ml) | 268.9 (139.6, 451.1) | 38 (28.6, 42.8) | <0.0001 |
| TRAb (IU/L) | 0.3 (0.3, 0.58) | 0.3 (0.3, 0.52) | 0.4856 |
| FSH (IU/L) | 7.25 (6.47, 8.37) | 7.69 (6.4, 9.47) | 0.2125 |
| LH (IU/L) | 4.33 (2.85, 6.16) | 3.92 (2.99, 5.23) | 0.1562 |
| LH/FSH | 0.57 (0.41, 0.87) | 0.51 (0.36, 0.66) | 0.0968 |
| estradiol (pmol/L) | 43.12 (28.75, 59.59) | 45.47 (29.27, 70.8) | 0.2544 |
| progesterone (nmol/L) | 0.71 (0.52, 0.93) | 0.69 (0.48, 0.97) | 0.4767 |
| prolactin (mIU/L) | 12.27 (9.31, 16.16) | 12.94 (9.45, 18.75) | 0.1071 |
| duration of infertility (year) | 4 (3, 6) | 5 (3, 8) | 0.2866 |
| causes (%) | | | |
| tubal factor | 73.47 (36/49) | 65.08 (41/63) | 0.342 |
| male factor | 20.41 (10/49) | 28.57 (18/63) | 0.322 |
| tubal + male factor | 6.12 (3/49) | 6.35 (4/63) | 0.342 |
| proportion (%) | | | |
| abortion rate (%) | 61.11 (11/18) | 25.0 (9/36) | 0.010 |
| clinical pregnancy rate (%) | 36.73 (18/49) | 57.14 (36/63) | 0.032 |
| implantation rate (%) | 24.62 (33/130) | 38.82 (66/170) | 0.079 |
| BMI (kg/m²) | 22.01 | 22.20 | 0.156 |
| prolactin (mIU/L) | 12.20 (9.31, 16.16) | 12.94 (9.45, 18.75) | 0.1071 |
| estradiol (nmol/L) | 49.88 (29.27, 70.8) | 51.65 (36.63, 72.6) | 0.1071 |
| progesterone (nmol/L) | 0.71 (0.52, 0.93) | 0.69 (0.48, 0.97) | 0.4767 |
| prolactin (mIU/L) | 12.27 (9.31, 16.16) | 12.94 (9.45, 18.75) | 0.1071 |
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According to The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, hasa04921 oxytocin signaling pathway, hasa04512 extracellular matrix (ECM)–receptor interaction, and hasa04611 platelet activation were revealed in upregulated proteins, whereas hasa04940 type I diabetes mellitus was revealed in downregulated proteins. The KEGG enrichment pathway visually illustrating the differentially expressed proteins is shown in Figure 3.

**Verification Results of Target Proteins.** The parallel reaction monitoring (PRM) for targeted quantitative proteo-
mics by mass spectrometry was used to quantitatively identify target proteins. Because of the limited properties of some proteins and the abundance of their expression, 11 proteins were quantitatively identified. Among them, AGT and FETUB were associated with reproduction, lactotransferrin (LTF) and apolipoprotein D (APOD) were associated with growth, and serum paraoxonase/arylesterase 1 (PON1) was related to oxidative stress. All the results are shown in Table 4.

**DISCUSSION**

There is a common consensus that thyroid autoantibodies are associated with adverse clinical pregnancy outcomes in women with positive thyroid antibodies.\(^{13,14}\) The infertility ratio in

| swiss-prot | gene name | protein description | score | matched peptide | MW kDa | test/control | p value | regulated type |
|------------|-----------|---------------------|-------|-----------------|-------|--------------|---------|----------------|
| A0JNW5     | UHRF1BP1L | UHRF1-binding protein 1-like | ~2    | 1               | 164.2 | 0.0238       | up      |                |
| P01624     | IGKV3-15  | immunoglobulin kappa variable 3-15 | 42.557 | 1               | 12.496 | 0.0452       | up      |                |
| P01703     | IGLV1-40  | immunoglobulin lambda variable 1-40 | 6.9764 | 1               | 12.301 | 0.00684      | up      |                |
| P01742     | IGHV1-69  | immunoglobulin heavy variable 1-69 | 2.0791 | 2               | 12.659 | 0.0161       | up      |                |
| P02788     | LTF       | lactotransferrin       | 239.11 | 25              | 78.181 | 0.000344     | up      |                |
| P05060     | CHGB      | secretogranin-1        | 164.19 | 15              | 78.275 | 0.000109     | up      |                |
| P05090     | APOD      | apolipoprotein D       | 173.05 | 9               | 21.275 | 0.0344       | up      |                |
| P07359     | GP1BA     | platelet glycoprotein Ib alpha chain | 5.0133 | 1               | 71.539 | 0.0337       | up      |                |
| P12107     | COL11A1   | collagen alpha-1(IX) chain | 14.492 | 7               | 181.06 | 0.00285      | up      |                |
| P13639     | EEF2      | elongation factor 2    | 9.3068 | 1               | 60.343 | 0.0201       | up      |                |
| P17987     | TC2P1     | T-complex protein 1 subunitz | 36.304 | 3               | 23.602 | 0.0395       | up      |                |
| P19652     | ORM2      | alpha-1-acid glycoprotein 2 | 323.31 | 97              | 468.83 | 0.00954      | up      |                |
| Q12907     | LMAN2     | vesicular integral-membrane protein VIP36 | 2.3936 | 1               | 40.228 | 0.018        | up      |                |
| Q13509     | TUBB3     | tubulin beta-3 chain   | 10.234 | 3               | 50.432 | 0.000118     | up      |                |
| Q16787     | LAM3      | lamin subunit alpha-3   | 2.3989 | 2               | 366.65 | 0.0486       | up      |                |
| Q18958     | ST13P4    | putative protein FAM10A4 | 11.095 | 3               | 27.406 | 0.0486       | up      |                |
| Q87DB4     | MGARP     | protein MGARP          | 1.2233 | 1               | 25.389 | 0.018        | up      |                |
| Q910J4     | QRIC2H2   | glutamine-rich protein 2 | ~2    | 1               | 180.83 | 0.0438       | up      |                |
| Q918L6     | MMRN2     | multimerin-2            | 22.904 | 3               | 104.41 | 0.0126       | up      |                |
| Q94UBF2    | COPG2     | costomer subunit gamma-2 | 1.5956 | 1               | 97.621 | 0.0115       | up      |                |
| Q9UGH3     | PCYOX1    | prenylcytochrome oxidase 1 | 133.95 | 10              | 56.639 | 0.00375      | up      |                |
| Q00300     | TNFRSF11B | tumor necrosis factor receptor superfamily member 11B | 3.3657 | 4               | 46.026 | 0.0462       | down    |                |
| O75339     | CILP      | cartilage intermediate layer protein 1 | 37.162 | 14              | 132.56 | 0.0426       | down    |                |
| P01019     | AGT       | angiotensinogen         | 33.13  | 4               | 53.154 | 0.00561      | down    |                |
| P01594     | IGKV1-33  | immunoglobulin kappa variable 1-33 | 179.25 | 1               | 12.848 | 0.025       | down    |                |
| P01861     | IGHG4     | Ig gamma chain C region | 31.477 | 12              | 35.94  | 0.018       | down    |                |
| P02452     | COL1A1    | collagen alpha-1(I) chain | 18.687 | 3               | 138.94 | 0.00767      | down    |                |
| P04114     | APOB      | apolipoprotein B-100  | 171.07 | 42              | 515.6  | 0.000527     | down    |                |
| P10321     | HLA-C     | HLA class I histocompatibility antigen, Cw-7 alpha chain | 6.1809 | 8               | 40.648 | 0.000134     | down    |                |
| P16870     | CPE       | carboxypeptidase E      | 6.8274 | 4               | 53.15  | 0.0142       | down    |                |
| P22692     | IGFBP4    | insulin-like growth factor-binding protein 4 | 28.999 | 3               | 27.934 | 0.0343       | down    |                |
| P31151     | SI00A7    | protein SI00A7          | 2.3387 | 1               | 11.471 | 0.00899      | down    |                |
| Q08830     | FGL1      | fibrinogen-like protein 1 | 88.167 | 5               | 36.379 | 0.00862      | down    |                |
| Q31303     | SFP2      | secreted phosphoprotein 24 | 41.647 | 4               | 24.337 | 0.0158       | down    |                |
| Q35157     | CDKN1    | corneodesmosin          | 1.4127 | 1               | 51.522 | 0.0299       | down    |                |
| Q92626     | PXDN      | peroxidasin homolog     | 119.1  | 12              | 165.27 | 0.0266       | down    |                |
| Q9B2Z6     | WD1R1     | WD repeat-containing protein 11 | 1.391  | 1               | 136.68 | 0.0475       | down    |                |
| Q9HNR5     | DOLMIL    | olfactomedin-like protein 3 | 27.574 | 6               | 46.031 | 0.0113       | down    |                |
| Q9UUGM5    | FETUB     | fetuin-B               | 25.354 | 3               | 42.054 | 0.0493       | down    |                |
women with thyroid autoantibodies becomes higher; the relationship between thyroid autoantibodies and IVF has been a topic of focus during recent years. We here report, for the first time to our knowledge, that the protein expression profile in follicular fluid of TAI women was highly different from controls, which may be related to failure of pregnancy in positive thyroid autoantibodies in women undergoing IVF/ICSI.

Conflicting results have been reported on the correlation between thyroid autoantibodies and IVF. One study showed that the existence of thyroid autoantibodies would have adverse effects on IVF outcomes. However, a meta-analysis revealed that thyroid autoantibody positivity did not affect fertility, implantation, or clinical pregnancy, but increased miscarriage rates and decreased the live births. Our experiment

Figure 1. Flow of Participants.

Figure 2. GO enrichment results of upregulated (A) and downregulated (B) proteins.

Figure 3. KEGG enrichment results of upregulated (A) and downregulated (B) proteins.
Table 4. Verification Results of Target Proteins

| protein name                        | TEST/control ratio | TEST/control P-value | TMT result |
|-------------------------------------|--------------------|----------------------|------------|
| APOD                                | 1.74               | 1.17 × 10⁻²          | 1.32       |
| basement membrane-specific          | 1.81               | 6.90 × 10⁻²          | 1.36       |
| serum                               | 1.90               | 1.27 × 10⁻¹          | 1.21       |
| paraoxonase/arylesterase 1          | 1.81               | 1.66 × 10⁻²          | 1.42       |
| LTF                                 | 0.90               | 7.37 × 10⁻¹          | 0.61       |
| insulin-like growth                 | 0.85               | 1.29 × 10⁻¹          | 0.80       |
| factor-binding protein 4            | 1.02               | 9.14 × 10⁻¹          | 0.83       |
| olfactomedin-like protein 3         | 1.89               | 1.11 × 10⁻¹          | 1.46       |
| α-1-acid glycoprotein 2             | 0.77               | 7.56 × 10⁻¹          | 0.74       |
| secreted phosphoprotein 24          | 1.76               | 3.71 × 10⁻¹          | 1.33       |
| prenylcysteine oxidase 1            | 0.79               | 1.41 × 10⁻¹          | 0.79       |

demonstrated that the clinical pregnancy rates and infantile rates in women with positive thyroid antibodies were significantly decreased than those with negative antibodies, which suggested that thyroid autoantibodies may have a detrimental influence on the course of a pregnancy.

Proteomics has been widely used to explore the pathogenesis and discover follicular fluid biomarkers from women undergoing IVF/ICSI treatment with endocrine diseases.11,16 Currently, the follicular fluid protein spectrum was examined in positive thyroid autoantibodies in women undergoing IVF/ICSI treatment by application of proteomics in order to explore the mechanistic effects of thyroid autoantibodies on the IVF/ICSI outcome.

In the present study, we found 31 upregulated and 18 downregulated proteins. Among them, five proteins were associated with reproductive function. 17 proteins were involved in the immune response, and differentially expressed proteins were associated with various biological functions, such as embryonic development, free radical scavenging, and organismal injury. Our findings showed that the cellular responses to gonadotropin-releasing hormone and immune response-regulating cell surface receptor signaling pathway were upregulated. The proper amount of gonadotropin secreted by the pituitary gland is essential for reproduction. The MAPK family of second messengers is strongly induced in gonadotropes upon GnRH stimulation. Reactive oxygen species (ROS) can fulfill its role in the GnRH receptor signaling through activation of MAPK signaling cascades, control of negative feedback, and participation in the secretory process. Oxidative stress may lead to biologic macromolecule oxidative damage. Research has shown that ROS in follicular fluid was negatively associated with embryo quality. Therefore, the upregulation of the signaling pathway may affect pregnancy outcomes through abnormal activation of MAPK to induce oxidative stress response.17,18 Furthermore, biological processes, including fertilization and skin development were downregulated. The fore-mentioned changes may lead to negative influences on the outcomes of assisted reproductive technology. Through KEGG enrichment analysis, differentially expressed proteins identified in this study involved multiple pathways. Oxytocin inflammation was considered as an endogenous signal to molecules to stimulate uterus and to promote the inflammatory pathways in cell membranes at the grass-roots level. Hence, abnormal activation of oxytocin signaling pathways (hsa04921) may be associated with the aggravation of inflammatory changes in pregnant women and affect embryonic development. ECM has a significant influence on cell proliferation, morphology, and other biological processes. Disorder of the ECM−receptor (hsa04512) pathway plays a vital role in the occurrence of spontaneous preterm birth. Normal physiological changes, such as coagulation and activation of the inflammatory system, may occur during pregnancy, whereas their excessive activation may cause pregnancy complications, such as gestational vascular diseases. Abnormal platelet activation (hsa04611) may also cause excessive coagulation and inflammatory reaction, leading to aseptic inflammation of the placenta, which results in adverse clinical pregnancy outcomes. All the fore-mentioned pathways were activated in women with positive thyroid autoantibody in this study, which explained the possible mechanism of the negative effect of thyroid autoantibody on the pregnancy outcomes.19

There were several proteins increasing in positive thyroid autoantibodies in women undergoing IVF/ICSI. APOD was associated with cholesterol homeostasis and steroid production, acting as a pivotal part in maintaining the microenvironment of oocyte maturation. APOD increased in positive thyroid autoantibodies in women undergoing IVF/ICSI currently. A previous study by Kushnir et al. 20 performed with proteomics found that potential biomarkers including APOD and other differential proteins, which were used to predict IVF outcomes, were upregulated under various stress stimulations and different pathological conditions, indicating that APOD was highly related to adverse pregnancy outcomes due partly to unbalanced microenvironment of oocyte maturation. Heparan sulfate proteoglycan (HSPG) is a potential biomarker for oocyte maturation in human follicular fluid (HFF). In addition, HSPG, which played a key role in controlling inflammation by binding and activating antithrombin III during folliculogenesis, was upregulated currently in the HFF of fertilized oocytes. It has been suggested that abnormal expression of this protein may lead to subsequent failure of oocyte maturation, fertilization, and IVF treatment.21 Oxidative stress has also been implicated as an important cause of several pregnancy complications, and paraoxonase/arylesterase 1 (PON1) can hydrolyze the activated phospholipids and lipid peroxide products. Thus, the changes in paraoxonase/arylesterase activities may have an impact on pregnancy outcomes by increased susceptibility to lipid peroxidation in pregnant women with positive thyroid autoantibodies.22 In the present study, we observed that the PON1 level was increased in women with positive thyroid autoantibodies in women with IVF/ICSI, a finding which, with respect to raised oxidative stress, was consistent with previous studies. Transferrin, which is a vital β-globulin, can combine with and carry iron to various tissues and organs, promoting cell development. Accumulating evidence showed that iron-promoted generation of ROS could lead to tissue injury and organ failure, such as accelerated follicle aging and ovarian tissue injury.23,24 High transferrin levels are responsible for formation of non-transferrin-bound iron, a toxic form of iron with a propensity to induce oxidative stress.25 Previous studies have shown that the transferrin level in FF was significantly increased compared to non-PCOS samples.26 There is ample evidence that PCOS, which is a common cause of female infertility, has been associated with increased production of ROS.27 Furthermore, Saller et al. found that ROS levels in PCOS-derived follicular fluid were dramatically increased compared to non-PCOS samples.
cells. Our study showed that the levels of transferrin in the positive thyroid autoantibodies group were substantially higher than that in the control group, which indicated that abnormalities of increased transfer iron protein in FF may damage the oocytes by increased ROS. AGT exists in two forms with oxidized sulfhydryl-bridge form as well as the reduced unbridged free thiol form in plasma. The decrease of free thiol AGT was highly associated with hypertension in pre-eclampsia patients, accompanied by generation of an excess load of free radicals as well as ROS and less antioxidants in the maternal systemic circulation. The free thiol AGT was also found downregulated currently in follicular fluid in women with positive thyroid autoantibodies, which suggested that AGT was related to adverse pregnancy outcome through imbalance between production of ROS and antioxidant defense system in maternal circulation. FETUB, which is expected to act as a potent ovastatin inhibitor, prevents zona pellucida hardening (ZPH) before fertilization and accordingly maintains oocytes in a fertilizable condition that is vital for IVF success. Animal experiments have confirmed that a deficiency of FETUB may lead to premature zona pellucida hardening mediated by zona protease ovastatin, which was associated with female infertility. Furthermore, a human study performed by Julia Floehr et al. showed that serum FETUB, which could be used as a substitute for follicular fluid FETUB, had increased during successful IVF cycles. The down-regulation of FETUB in follicular fluid of thyroid autoantibody-positive women was found in the current study. These findings collectively suggest that FETUB could be useful in predicting the fertilization failure in IVF.

**CONCLUSIONS**

In this study, we showed that the clinical pregnancy outcome is different between the women with positive or negative antibodies in those females with normal thyroid function. Proteomics was applied for the first time to identify follicular fluid proteins of euthyroid women with positive thyroid autoantibodies who underwent adjuvant reproductive therapy. This research confirmed that the proteins in the follicular fluid in women with positive thyroid autoantibodies were different from controls. Some differentially expressed proteins were associated with the reproductive process and closely related to important physiologic effects, which could partially explain the underlying mechanism link between TAI and adverse outcomes of assisted reproductive technology. Our findings will provide important clues for future studies on the mechanism of how thyroid autoantibodies affect protein expression and eventually affect outcome of IVF.

**MATERIALS AND METHODS**

**Enrolment of Study Participants.** A total of 602 infertile patients between June 2015 and January 2017 at the Center of Assisted Reproductive Technology of the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China) were screened for positive antithyroid antibodies (antiTPOAb > 34 iu/mL and/or antiTGAb > 115 iu/mL). Finally, 49 euthyroid women with positive thyroperoxidase antibody and/or thyroglobulin antibody and 63 negative controls were eventually included (Figure 1). The eligible subjects had primary infertility because of tubal factor, male factor, or both, in case of known female factor induce the study offset. Women were not eligible if they suffered from PCOS, endometriosis, or other diseases that may affect follicular fluid composition; or were diagnosed with diabetes mellitus or other endocrinologic or autoimmune disorders; or benefitted from medication-based strategies, such as a thyroid hormone, antithyroid medication, glucocorticoids, or other relevant treatments. The routine examination included laboratory assays, ultrasonography, hysteroscopy, hysterosalphingography, and a sperm test to confirm that the subjects are eligible. According to the monitoring of thyroid function during the study, once overt or subclinical thyroid diseases happened, the patients were then considered for exclusion. We refer to the article of Boudjenah, Lee et al., and calculate the correlation rates as follows: fertilization rate = number of fertilized eggs/total number of eggs obtained × 100%; implantation rate = total number of implanted embryos/total number of transferred embryos × 100%; clinical pregnancy rate = number of clinical pregnancies/transplant cycles × 100%; abortion rate = number of miscarriages/number of pregnancy × 100%; Take-home baby rate = number of deliveries with live births/number of pregnancy × 100%.

This cohort study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from each eligible participant as well.

**IVF-ET Protocols.** Subjects included in the present study used the following scheme to promote ovulation: a gonadotropin-releasing hormone agonist (1 mg/d) was injected in the middle luteal period of the patient’s previous menstrual cycle, in which color ultrasound and blood hormone were monitored from days of 3–5 of the next menstrual cycle. The human chorionic gonadotropin (250 iu) was administered when the follicle diameter was not less than 18 mm or two were not less than 17 mm. Transvaginal follicular aspiration was performed for oocyte retrieval after approximate administration for 36 h, in which intracytoplasmic sperm injection along with IVF in addition to embryo transfer were accordingly undertaken.

**Collection of Follicular Fluid and Blood Samples.** The follicular fluid was aspirated and processed from a single, mature, and the largest follicle (>18 mm) visualized on ultrasound before using any flushing medium to avoid being polluted by blood. A clear and uncontaminated solution was centrifuged at 2000 rpm for 15 min at room temperature, and the collected supernatant was labeled and stored at −80 °C. Thereafter, nine follicular fluid samples were obtained from those two groups, respectively, by a clinical data management randomization system, confirming that the baseline index of the sample taken is not different from the original group. Then, proteomics analysis and comparison were undertaken so that the differential expression of proteins associated with the follicular fluid was conducted among euthyroid women with and without positive thyroid autoantibodies. Blood samples were collected from all patients on the day of oocyte retrieval and were stored in the same conditions as the follicular fluid.

**Tandem Mass Tag Labeling and High-Performance Liquid Chromatography Fractionation.** After removing the high-abundance proteins in the follicle samples, the supernatant was collected, and the concentration of protein was measured with the BCA kit. Protein samples were digested and labeled with the tandem mass tag (TMT) as well. The tryptic peptides were fractionated into fractions by high-performance liquid chromatography (HPLC) with the help of...
LC–Tandem Mass Spectrometry Analysis and Database Searching. Peptides were eluted using a linear gradient of 6–80% acetonitrile over 40 min, and all increases were at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system. The peptides were subjected to a nanospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in a Thermo Scientific Q ExactiveTM Plus (Thermo Fisher Scientific, Waltham, MA, USA) coupled online to the UPLC. The full MS scans were obtained with a range of m/z 350–1800, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using the NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated one MS scan followed by 20 MS/MS scans (the fixed first mas:100 m/z). The resulted MS/MS data were processed using MaxQuant (version 1.5.2.8). Tandem mass spectra were searched at the SWISS-PROT database concatenated with the reverse decoy database. Proteins with a minimal 1.5-fold change between groups and P-value < 0.05 were statistically considered significant.

Bioinformatics Analysis. The protein ID was converted to a GO annotation using the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Unannotated proteins used the InterProScan software to annotate their GO functions. A double-tailed Fisher exact test was used to examine the enrichment of differentially expressed proteins to all identified proteins. FDP takes 1%. Also, GO with a corrected P-value < 0.05 was considered statistically significant.

Validation of Differentially Expressed Proteins Using PRM. The differential abundance of selected proteins was verified using PRM analysis. The Skyline software (MacCoss Lab, University of Washington, WA, USA) was used to process the acquired MS data. Parameters of the peptide segment were determined as follows: the protease was set at trypsin [KR/P], the maximum number of leak-off position was set at 0, the length of the peptide segment was set as 7–25 amino acid residues, and the cysteine alkylation was set as the “fixed modification”. Transition parameters were as follows: the parent ion charge was set to 2, 3, the daughter ion charge is set to 1, and the ion type was set to b, y. The fragment ion selection started from the third to the last, and the mass error tolerance of ion matching was set to 0.02 Da.

Statistical Analysis. The normal distribution results were expressed as mean ± standard deviation, and the non-normal distribution results were expressed by the median. Data analysis is conducted using SPSS 23.0 for Windows. Student’s t-test is used for data with normal distribution, and the nonparametric test is used for data with non-normal distribution, whereas a comparison of percentage values between groups was made by a chi-square test. A P value < 0.05 was considered as statistically significant.

The P-values were calculated using right-tailed Fisher’s exact tests. Bars represent significance as −log (P-value).

The P-values were calculated using right-tailed Fisher’s exact tests. Bars represent significance as −log (P-value).
**ABBREVIATIONS**

AGT, angiotensinogen; APOD, apolipoprotein D; ECM, extracellular matrix; FETUB, fetuin-B; GO, Gene Ontology; HFF, human follicular fluid; HPLC, high-performance liquid chromatography; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; IGFBP4, factor binding protein 4; KEGG, Kyoto Encyclopedia of Genes and Genomes; LTF, lactotransferrin; MMRN2, multimerin-2; MS/MS, mass spectrometry; NSI, nanoparticles ionization; OLFML3, olfactomedin-like protein 3; PCOS, polycystic ovary syndrome; PON1, paraoxonase/arylesterase 1; PRM, parallel reaction monitoring; ROS, reactive oxygen species; TAI, thyroid autoimmunity; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; TMT, tandem mass tag; ZPH, zona pellucida hardening; BMI, body mass index; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone

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