Austin Journal of Obstetrics and Gynecology

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Austin Publishing Group

Research Article

Proteomic Analysis of Peripheral Blood in Pregnant Women with Preterm Premature Rupture of Membranes

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Received: October 27, 2021; **Accepted:** November 18, 2021; **Published:** November 25, 2021

Abstract

Background: Preterm Premature Rupture of Membranes (PPROM) is an important obstetric complication faced by modern maternal and foetal medicine and accounts for 30% of all preterm births. At present, it has been reported that there are no clear biomarkers that can accurately predict PPROM. Therefore, there is an urgent need to identify new biomarkers revealing this condition.

Objective and Methods: This study aimed to identify differentially expressed proteins in peripheral blood between women with PPROM (n=5) and women with term delivery (n=5). Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) combined with label-free proteomics methods was used to distinguish differential expression between the pooled groups. Go analysis, KEGG pathway analysis and PPI interaction analysis were used to identify key proteins and their potential functions and mechanisms in PPROM.

Results: Proteins that were significantly increased with PPROM included the following: retinoic acid receptor reactive protein 2, complement factor D, extracellular matrix protein 1, and von Willebrand factor. In contrast, adhesion G protein coupled receptor G6 (fragment), fibrinogen 2, fibrinogen 3, C3/C5 invertase and pregnancy-specific glycoprotein 6 were significantly reduced. These proteins have known biological functions in the reproduction, vesicle mediated transport, embryo development and aging promoting membrane rupture, which may play important roles in the physiological and pathological processes of preterm premature rupture of membranes and provide a theoretical basis for predicting candidate biomarkers of preterm premature rupture of membranes.

Keywords: Preterm premature rupture of membranes; Differentially expressed proteins; Label-free; Bioinformatics analysis

Abbreviations

PPROM: Preterm Premature Rupture of Membranes; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; Deps: Differentially Expressed Proteins; GO: Gene Ontology; KEGG: The Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-Protein Interaction; RARRP2: Retinoic Acid Receptor Reactive Protein 2; CFD: Complement Factor D; ECM1: Extracellular Matrix Protein 1; Vwf: Von Willebrand Factor; TIG2: Tazarotene Induction Gene 2

Introduction

Preterm Premature Rupture of Membrane (PPROM) refers to rupture of the foetal membranes before parturiency at >20 weeks and <37 weeks of gestation. The incidence of PPROM is 1%-2% in all pregnancies. Among pregnant women with PPROM, only 7.7% to 9.7% of the rupture of membranes heals naturally, while for pregnant women with continuous vaginal discharge, despite active foetal protection, approximately 50% of pregnant women deliver within 1 week of rupture of membranes, accounting for approximately 1/3 of the causes of preterm delivery [1]. The incidence of singleton PPROM is 2%-4%, and the incidence of twin PPROM can be as high as 7%-20%. The amniotic cavity of PPROM is in a state of communication with the outside world. Continuous amniotic fluid leakage can not only lead to oligohydramnios, uterine infection, foetal distress, umbilical cord prolapse, and foetal lung dysplasia but also significantly increase maternal and foetal infection rates and perinatal mortality. Fifteen percent to 25% are complicated with clinically symptomatic chorioamnionitis, 15% to 20% have postpartum infection, and the earlier the gestational week of rupture of the membrane, the higher the incidence of infection, and 2% to 5% of cases result in placental abruption [2]. PPROM is seriously harmful to maternal and foetal health. To date, there is no clear biomarker that can accurately predict PPROM.

The risk factors for PPROM include reproductive tract infections (e.g., bacterial vaginosis, trichomoniasis, gonorrhoea, and Chlamydia trachomatis), behavioural factors (e.g., cigarette smoking, poor nutritional status, and coitus during pregnancy), and obstetric complications (e.g., multiple gestations, polyhydramnios, incompetent cervix, gestational bleeding, cervical surgery, and antenatal trauma). Environmental factors (e.g., stress, toxin exposure) and genetic predisposition have also been proposed. In addition, biochemical signals from the foetus, including endocrine signals that promote foetal membrane apoptosis, may initiate PPROM [3-8]. In these conditions, high-risk factors can cause inflammation in the amniotic cavity and the release of inflammatory factors; they

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Citation: Wei D, Zhang Y, Lu C, Leng J and Wang S. Proteomic Analysis of Peripheral Blood in Pregnant Women with Preterm Premature Rupture of Membranes. Austin J Obstet Gynecol. 2021; 8(8): 1196.

may also lead to excessive pressure or uneven force in the amniotic cavity, which can accelerate the adverse outcomes of foetal membrane rupture. However, the exact pathogenesis of PPROM is still unclear. To further explore the molecular mechanism, it is necessary to determine the normal physiological and pathophysiological mechanisms of PPROM and healthy full-term pregnancy.

Protein expression patterns are a diagnostic index of physiological or pathological states. Proteomics is a large-scale study of proteins that has made great contributions to the understanding of gene function in the post genome era. Proteomic methods have previously been used to analyse amniotic fluid, placental tissue, etc. The basic principle of these analyses is based on the possibility that amniotic fluid, placenta and foetal membranes are involved in physiological processes. The purpose of this study was to identify potential protein changes in patients with PPROM, to identify possible biomarkers and to provide a theoretical basis for early prediction and targeted intervention. We chose to draw the peripheral blood of patients with PPROM for analysis because peripheral blood samples are accessible and can be sampled routinely and repeatably (the peripheral blood may not be sensitive enough to other tissues, and the molecular changes released to the peripheral blood may not be detected after dilution). Although amniotic fluid may be faster to sample and more sensitive to inflammatory changes, transvaginal noninvasive sampling is at risk of contamination, thus affecting the results. Placental tissue can be obtained after delivery, but because the placenta is a complex organ that can produce environmental changes in response to hormones and other molecules and mediate the selective transfer of solute and gas between the mother and the foetus, it is impossible to determine whether the change in protein profile expression is caused by PPROM.

In this study, label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify specific changes in protein levels in patients with PPROM and healthy full-term pregnancy controls, which allowed us to better understand and more deeply study the molecular mechanism of the pathophysiology in PPROM.

Materials and Methods

Peripheral blood samples were collected in May 2018 in the Department of Obstetrics of Shandong Provincial Hospital affiliated with Shandong University as the case group (Group D, n = 5) and normal term delivery (≥40 weeks, the foetal membrane was not ruptured when uterine contraction was not initiated or the cervical was dilated) as the control group (Group C, n = 5). Relevant information is given in Table 1.

Inclusion criteria

All singleton pregnancies; no recent use of antibiotics; pregnant women without placenta previa, preeclampsia, infectious diseases, intrauterine growth retardation, foetal distress, autoimmune diseases, nephropathy, diabetes, trauma and pregnancy complications requiring induced labour.

Diagnosis of PPROM

The diagnosis of PPROM includes amniotic fluid pooling in the vagina, fern tests and positive amniotic sac staining under a microscope, test paper and amniotic injection of methylene blue.

Informed consent was obtained from all participants, and this study was approved by the Ethics Review Committee of Shandong Provincial Hospital. After admission, peripheral blood was collected within 30 min, and the blood samples were immediately frozen and stored at -80°C for later use.

Proteomic exploratory phase

For sample protein extraction and mass spectrometry detection, the sample was diluted in the appropriate amount of cleavage buffer, vortexed to mix, and protease inhibitor was added (lysate: protease inhibitor 50:1). The sample was lysed with an ultrasonic cell breaker (ultrasonic 1s, stop 1s, accumulate 10s) and was then sampled at 14,000 x g for 30min. The supernatant was then taken and packed separately; 5μl was quantified, and the rest was frozen at -80°C. The concentration of extracted protein was determined by the Bradford method [9]. Twenty micrograms of the sample was subjected to SDS-PAGE electrophoresis and then trypsin hydrolysis. The label-free algorithm in MaxQuant was used for unlabelled quantitative calculation of proteomic data. Label-free mass spectrometry analysis was completed by Thermo Q-Exactive mass spectrometry, and the original mass spectrometry documents were processed by MaxQuant software (parameter: use the UniProt Homo sapiens database). Then, bioinformatics analysis was performed on these Differentially Expressed Proteins (DEPs). Gene Ontology (GO) analysis was performed to identify the biological processes, molecular functions, and cellular component terms of the identified DEPs, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to identify key enriched pathways. Protein-Protein Interaction (PPI) networks were analysed to identify the top high-degree core proteins.

Results

There was no significant difference in maternal age or parity; however, compared with those of the controls, the gestational age and birth weight in the PPROM group were significantly lower (Figure 1).

Through the statistical analysis of the original data, a total of 4428 peptides and 415 proteins were identified in the five groups of samples. Compared with the control group, 26 differentially expressed proteins were downregulated or upregulated in the PPROM group ($p<0.05$ and fold change > 1.2-fold were significantly different). Among these differentially expressed proteins, 21 proteins were upregulated, and the other 5 proteins were downregulated.

Bioinformatics analysis

GO analysis (Figure 2a-2c) revealed that in terms of biological processes, DEPs were mainly enriched in the reproduction, vesicle mediated transport, embryo development and aging, among which upregulated DEPs were also mainly enriched in these processes. Downregulated DEPs were mainly concentrated in the reproduction.

Table 1: Demographic and clinical characteristics of pregnancies.

	Group D	Group C	p value
Maternal age (years)	31.4±4.2	31.6±4.8	0.95
Parity	1.8 ±0.4	1.6 ±0.5	0.55
Pregnancy body mass index	25.8±3.0	27.3±1.9	0.38
Gestational age (days)	234.0±14.2	280.2±4.1	0.001
Birth weight (grams)	2204.0±640.8	3468.0±465.2	0.007

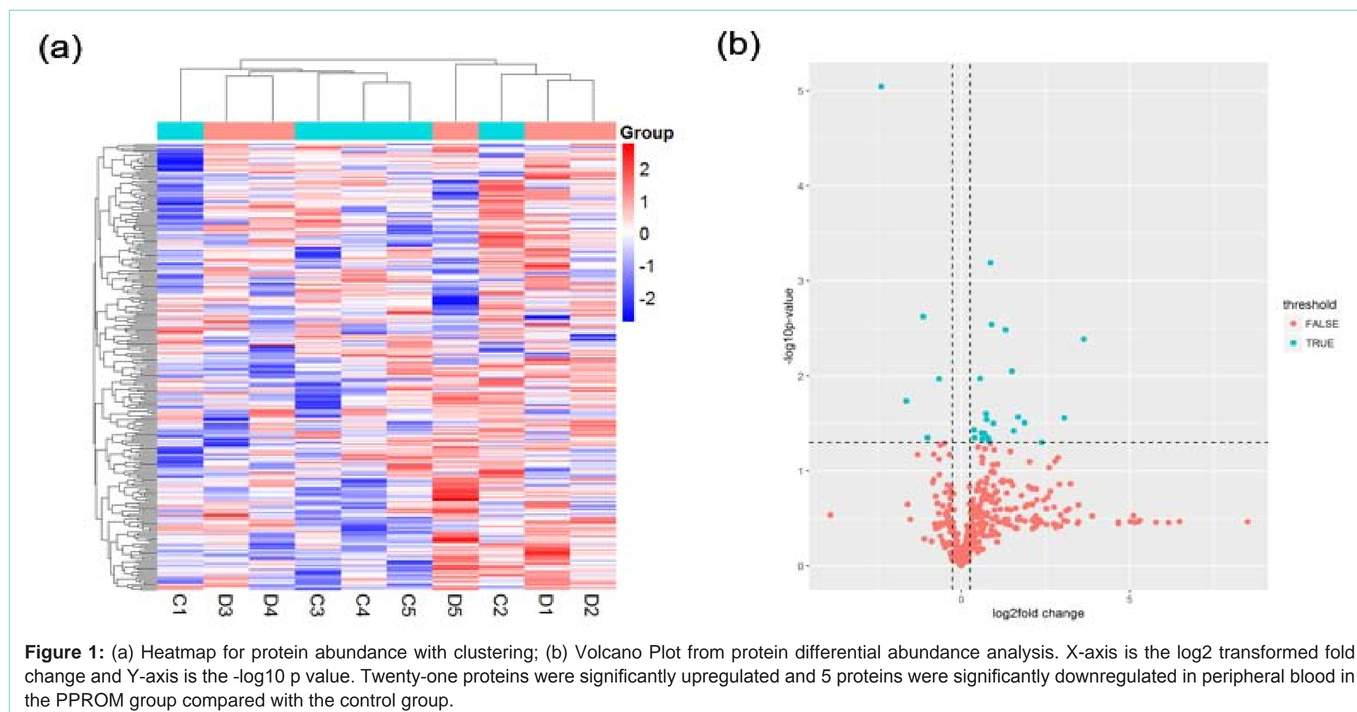

Figure 1: (a) Heatmap for protein abundance with clustering; (b) Volcano Plot from protein differential abundance analysis. X-axis is the log2 transformed fold change and Y-axis is the -log10 p value. Twenty-one proteins were significantly upregulated and 5 proteins were significantly downregulated in peripheral blood in the PPROM group compared with the control group.

In terms of intracellular components, DEPs were mainly enriched in the extracellular region, plasma membrane; in terms of molecular function, DEPs were mainly enriched in receptor binding and integrin binding among others.

KEGG pathway analysis revealed that the main pathways enriched by DEPs were the Wnt signalling pathway, complement and coagulation cascades, among others (Figure 2d). PPI interaction analysis (STRING) showed 11 nodes and 11 connections in DEPs (Figure 3). The former 4 core DEPs were Retinoic Acid Receptor Reactive Protein 2 (RARRP2), Complement Factor D (lipoprotease, CFD), Extracellular Matrix Protein 1 (ECM1) and Von Willebrand Factor (vWF). RARR2 showed the highest node degree among the 11 proteins. These core DEPs were all upregulated. PPI network analysis showed that RARRP2, CFD, ECM1, and vWF were the core DEPs in the occurrence of PPROM, which may play an important role in the pathological process.

Discussion

Retinoic acid plays important roles in vertebrate development, cell differentiation and proliferation. On the one hand, the retinoic acid signalling pathway is complex and intersects with other signalling pathways. On the other hand, retinoic acid receptors are diverse and have different spatiotemporal expression patterns during embryonic development, and their expression in adults is tissue-specific. Retinoic Acid Receptor Reactive Protein 2 (RARRP2) was identified as the product of the retinoic acid receptor responder 2 gene in 2003 [10]. The gene is located on chromosome 7 in humans and consists of five coding exons, with a full length of 1618bp. It was initially identified as a protein produced by a gene upregulated by selective antipsoriatic synthesis of retinoic acid-tazarotene, also known as Tazarotene Induction Gene 2 (TIG2) or chemerin or adipokine [11,12]. It is highly expressed in white adipose tissue, liver and placenta but less highly expressed in brown adipose tissue, lung, skeletal muscle, kidney, ovary and heart. Pro-pre RARRP2 secretes a 163-amino acid protein encoded by this gene in an inactive form. An inactive pro-RARRP2 of 143 amino acids (18kDa) is formed after cleavage of the N-terminal signal peptide and is then activated by different proteolysis pathways to produce the active form (16kDa), which plays a functional role [13]. Recent evidence shows that RARRP2 is a natural ligand of chemerin receptors (ChemerinR), and their receptors exist all over the body and play multifunctional roles in regulating chemotaxis, autocrine/paracrine processes, adipogenic metabolism, angiogenesis and reproductive function [14-18]. The role of RARRP2 in female reproduction has been studied extensively. The expression level of RARRP2 in the peripheral blood of pregnant women was significantly higher than that of nonpregnant women, and the content of RARRP2 in the cord blood of older pregnant women was significantly higher than that of normal pregnant women. RARRP2 and CMKLR1 are expressed in placental trophoblast cells, and many kinds of cells are involved in uterine decidualization. RARRP2 is highly expressed in uterine stromal cells and extravillous trophoblast cells of pregnant women early in the pregnancy. The RARRP2/CMKLR1 signalling pathway plays an important role in maintaining early pregnancy [19]. However, with the progression of pregnancy, the level of serum RARRP2 decreases significantly [20], and there is differential regulation of RARRP2 in the process of pregnancy, which indicates that RARRP2 may play a key role in the process of pregnancy. Our study found that the level of RARRP2 in the peripheral blood of pregnant women with PPROM was significantly higher than that of full-term pregnant women. We speculate that before the occurrence of PPROM, the inflammatory response may have already started. RARRP2 may promote the recruitment, chemotaxis and retention of macrophages at the inflammatory site by stimulating the adhesion of macrophages to extracellular matrix proteins and other adhesion molecules [21]. As a chemokine of innate immune cells, RARRP2

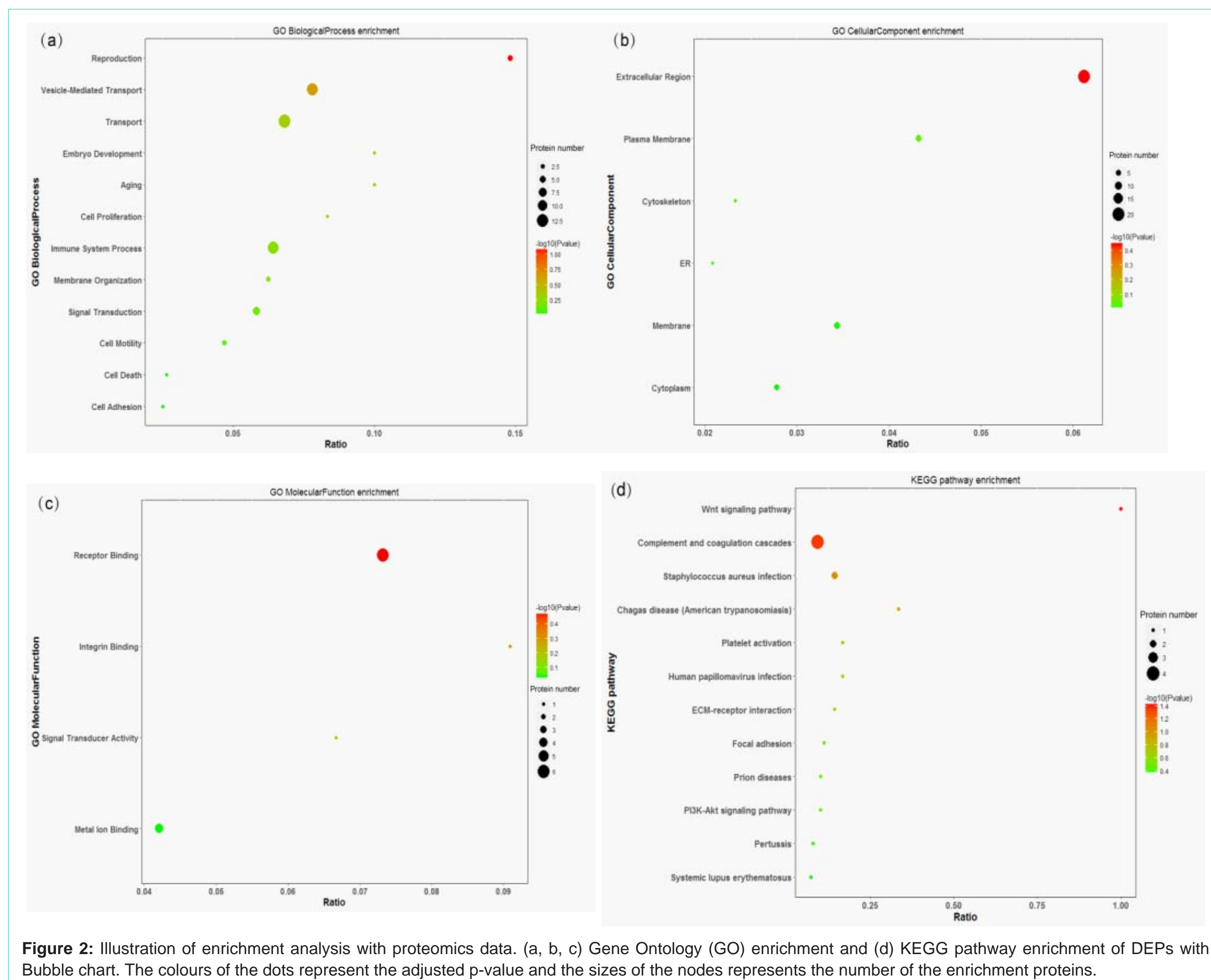

Figure 2: Illustration of enrichment analysis with proteomics data. (a, b, c) Gene Ontology (GO) enrichment and (d) KEGG pathway enrichment of DEPs with Bubble chart. The colours of the dots represent the adjusted p-value and the sizes of the nodes represents the number of the enrichment proteins.

plays a key role in initiating the immune response, thus accelerating the occurrence of PPROM. Moreover, some scholars have found that RARRP2 inhibits human chorionic gonadotropin and induces testosterone production in primary cultured rat Leydig cells [22]. In cattle, RARRP2 and its three receptors, CMKLR1, GPR1 and CCRL2, are expressed in the ovary, and recombinant RARRP2 decreases steroid synthesis and cholesterol synthesis *in vitro* after IGF-1 and/or FSH induction [23]. As an adipose cytokine, RARRP2 may reduce the production of steroids and cholesterol during pregnancy, thus reducing the synthesis or affecting the function of progesterone, resulting in enhanced uterine contraction and accelerating the occurrence of PPROM.

Extracellular matrix protein 1 (ECM1) is an 85-kDa secreted glycoprotein that contains six cysteine bimodal peaks and has a CC-(X7-10)-C pattern [24,25]. ECM1 has a wide tissue distribution. It has been reported that it can participate in endochondral bone formation as a negative regulator of bone mineralization, stimulate endothelial cell proliferation, promote angiogenesis, inhibit Matrix Metalloproteinase 9 (MMP9) proteolysis activity, and participate in extracellular matrix remodeling [26]. Some researchers believe that although the pathophysiology of PPROM has not been clarified, as pregnancy progresses, important changes take place in the morphology, biochemistry and structure of foetal membranes, and rupture of foetal membranes eventually occurs. The connective tissue layer was obviously ruptured and oedematous, the cytotrophoblast and decidual layers were significantly thinner, and the characteristics of extracellular matrix remodelling were significantly related to PPROM. Lei H [27], et al. found that mouse amniotic epithelial cells began programmed death and extracellular matrix degradation processes before delivery. Fortunato SJ [28], et al. confirmed the apoptotic DNA fragmentation of foetal membranes by polymerase chain reaction and found that the incidence of apoptosis of PPROM was higher than those of preterm and term deliveries. Kataoka S [29], et al. found that the foetal membranes in the neck of the uterus were thinner than those in other parts of the uterus, and the degree of programmed death was higher. In our proteomic study, ECM1 was found to be elevated in PPROM, indicating that extracellular matrix remodelling, such as oedema, increased fragility and collagen fibre breakage, may occur in the connective tissue layer of membranes

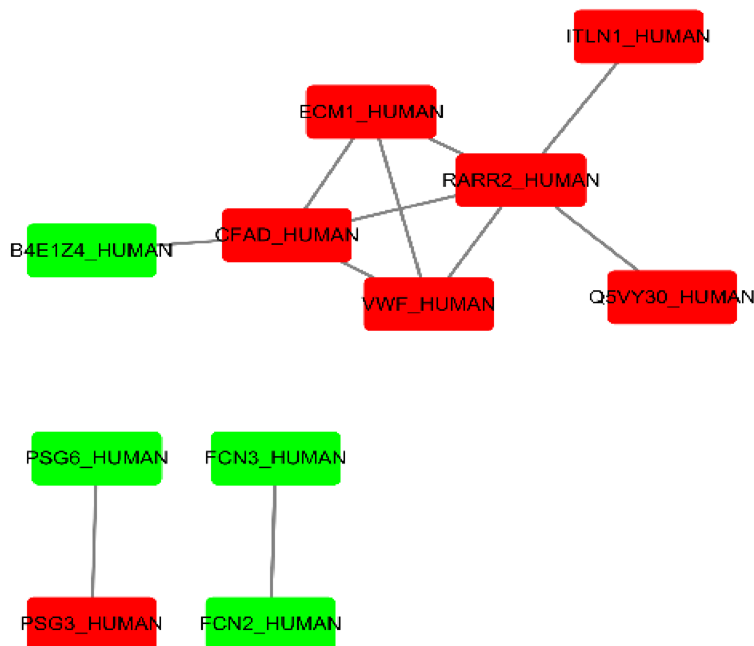

Figure 3: Protein-Protein Interaction (PPI) analysis of Differentially Expressed Proteins (DEPs). Nodes indicate proteins, whereas lines indicate interactions between proteins. Red represent the up-regulated proteins and green represent the down-regulated proteins.

before PPROM, coupled with inflammation, stress response and increased stress of foetal membranes eventually leading to PPROM. ECM1 may play an important role in this pathophysiological process.

Complement Factor D (CFD) cleaves factor B when the latter is complexed with factor C3b, activating the C3bBb complex, which then becomes the C3 convertase of the alternate pathway. Its function is homologous to that of C1s in the classical pathway. Factor D is the only enzyme in the blood that can catalyse this reaction; thus, it is essential for the activation of alternative pathways [30]. The concentration of factor D in blood is the lowest of all complement proteins, which makes factor D a restrictive enzyme in the alternative pathway activation sequence. Previous studies have shown that improper complement activation, whether too little or too much, can lead to adverse pregnancy outcomes. Specifically, over activation of complement components in the placenta can lead to placental damage, increasing the risk of preeclampsia and foetal loss. The bypass pathway can not only directly initiate complement activation but also play a feedback amplification role in the activation of the classical and lectin pathways. Its abnormal activation or regulation is involved in the occurrence and development of many diseases, such as C3 glomerulopathy, atypical haemolytic uraemic syndrome, IgA nephropathy, and rheumatoid arthritis [31]. The liver is the main synthesis site of CFD, and adipocytes, macrophages, endometrium and endothelial cells can also be synthesized [32]. Studies have shown that young fibroblasts treated with CFD can increase the expression of the MMP1 gene, while CFD knockout decreases MMP1 expression. Cell senescence can increase the secretion of CFD, decrease the expression of collagen type α-1 chain and elastin, and increase the expression of matrix metalloproteinase-1, which has a negative effect on the matrix of the dermis and promotes the degradation of nearby fibroblasts [33]. In this study, CFD was found to be elevated in the peripheral blood of PPROM, suggesting that factor D may play a role in foetal membrane cell senescence, apoptosis and matrix remodelling by affecting matrix metalloproteinases, thus accelerating the occurrence of foetal membrane rupture. In addition, some scholars found that CFD deficiency decreased the expression of inflammatory factors (TNF and CCL2) and fibrosis markers and reduced the accumulation of F4/80+ macrophages [34]. Indicating that CFD can promote the occurrence of inflammatory processes and indirectly participate in the pathophysiological process of PPROM through the inflammatory response.

Because of the convenience and availability of blood testing, the detection of related proteins in the blood of pregnant women is expected to become a new and efficient method to predict early PPROM. The occurrence of PPROM is a multifactor process, and its pathogenesis has not been fully elucidated. In this study, SDS-PAGE coupled with Q-Exactive proteomics methods identified 26 DEPs in the peripheral blood of pregnant women with PPROM and full-term delivery. Bioinformatics analysis identified the core DEPs, including RARR2, CFD, and ECM1 that may contribute to the pathogenesis of PPROM, which is expected to provide new predictors for PPROM in the future.

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