

Placental protein 14 as a potential biomarker for diagnosis of preterm premature rupture of membranes

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Abstract. Premature rupture of membranes (PROM) is a common pregnancy complication that frequently results in maternal and perinatal morbidity. The present methods for diagnosing PROM do not satisfy clinical requirements. The present study aimed to examine the proteome profile of amniotic fluid (AF) and maternal plasma, screen unique proteins in AF, and evaluate their diagnostic value for diagnosing PROM. The proteome profiles of AF and maternal plasma were examined via liquid chromatography coupled with tandem mass spectrometry-based proteomic techniques. The protein expression levels of diagnostic candidates in AF, maternal plasma and vaginal fluid were determined by ELISA analysis and Magnetic Luminex[®] screening assays. The diagnostic value of potential biomarkers was evaluated using receiver operating characteristic curves. A lateral flow assay was developed based on colloidal gold immunochromatography

technology. The present study identified 540 unique proteins in AF, 12 of which were chosen for further detection. The present results demonstrated that expression levels of pulmonary surfactant-associated protein B, BPI fold-containing family A member 1, zymogen granule protein 16 homolog B, EGF-containing fibulin-like extracellular matrix protein 1, keratin, type II cytoskeletal 4, keratin, type I cytoskeletal 19, placental protein 14 (PP14), insulin-like growth factor-binding protein 2, mesothelin and serpin family B member 3 were significantly higher in AF compared with in maternal plasma ($P < 0.01$). Furthermore, PP14 was observed to have excellent diagnostic accuracy for preterm PROM (PPROM), with a respective sensitivity and specificity of 100 and 87.5% when the cutoff value was 0.008 $\mu\text{g/ml}$. The PP14-based lateral flow assay demonstrated a visual detection threshold of 0.008 $\mu\text{g/ml}$. The results from the present study suggested that PP14 may be a novel potential biomarker for PPRM, and may be developed into a lateral flow assay for bedside application to rapidly diagnose PPRM.

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Introduction

Premature rupture of membranes (PROM) is a common complication during pregnancy (1). According to a previous study, PROM complicates 24.3% of all pregnancies in Beijing, China (2). PROM, particularly preterm PROM (PPROM), frequently results in maternal and perinatal morbidity. The complications of PROM include premature labor, chorioamnionitis, respiratory distress syndrome, cord compression, placental abruption and antepartum fetal mortality (3). Pregnant women with vaginitis, a history of premature labor or twin pregnancies, have a higher risk of developing PROM (4). The etiology of PROM is unknown and its occurrence is unpredictable (5). Therefore, accurate and timely diagnosis may improve maternal and perinatal outcomes.

In the past 80 years, several techniques to clinically diagnose PROM have been developed, including microscopic fetal cell identification, vaginal fluid pH determination, examination

of amniotic fluid (AF) crystallization, intra-amniotic dye injection and protein marker detection in the vaginal fluid. The most accurate method to diagnose PROM is with an intra-amniotic dye injection, using a dye such as indigo carmine. However, this method is invasive and may cause infection or abortion (5). Fetal cell identification or AF crystallization examination with a microscope are time-consuming processes and false positive rates are high. Therefore, vaginal pH tests and a number of specific PROM marker-based bedside test products are more popular at present due to their safety, efficiency and convenience (3,6-9). However, during clinical practice, patients with suspected PROM frequently experience vaginal bleeding and excessive blood in the cervical-vaginal fluid (VF) that may interfere with rapid tests and result in false positive outcomes, which is concerning for clinicians.

In order to improve the diagnostic accuracy of PROM, the present focused on screening for novel PROM biomarkers. As PROM occurs, AF disseminates into the vagina, and the detection of unique fetal proteins in the VF may aid in the diagnosis of PROM. Therefore, in the present study, the proteome profiles of AF and maternal plasma of pregnant women was assessed by liquid chromatography coupled with a tandem liquid chromatography-mass spectrometry (LC-MS/MS)-based proteomic technique. Unique proteins in AF were screened and preliminarily evaluated for their potential diagnostic ability in PROM by using ELISA and a Magnetic Luminex[®] screening assay to determine a potential biomarker for PPRM.

Materials and methods

Subjects. The present study was approved by the Ethics Committees of West China Second University Hospital of Sichuan University (Sichuan, China) and Shuangliu District Maternal and Child Health Hospital (Sichuan, China). All women (≥ 18 years old) enrolled in the study signed consent forms. In the PPRM/PROM group, the inclusion criteria were defined as follows: Leaking of AF observed prior to labor; pH test positive; AF crystallization test positive and sICAM-1 strip test positive. For intact membrane group, women who met the following criteria were recruited in the study: No leaking of AF observed prior to labor; pH test negative; AF crystallization test negative and sICAM-1 strip test negative (9,10). Women who had been administered drugs vaginally in the last 72 h were excluded from both groups. A total of 133 maternal plasma, 133 AF and 133 VF samples were collected from January 2015 to October 2016. Among them, 100 maternal plasma and 100 AF samples were collected separately from pregnant women in their third trimester, and 33 maternal plasma and 33 AF samples were provided by women who selected amniocentesis in their second trimester. All 133 women were without any complex pregnancy-associated diseases. A total of 71 VF samples were obtained from 14 patients with PPRM (< 37 weeks; five patients with vaginal bleeding) and 57 patients with PROM (≥ 37 weeks; 11 patients with vaginal bleeding). The remaining 62 VF samples were collected from pregnant women at ≥ 37 weeks (53 women, five of which experienced vaginal bleeding) or < 37 weeks (nine women, one of whom experienced vaginal bleeding) with intact membranes.

The clinical characteristics of all subjects are presented in Table I.

Sample collection. Maternal plasma and AF samples were obtained from the women during their third trimester by cesarean section. Samples from women in their second trimester were collected during amniocentesis. A total of 3 ml EDTA-anticoagulated peripheral whole blood and 10 ml uncontaminated AF was collected from each woman. VF samples were collected from the posterior fornix using vaginal swabs. The swabs were inserted in 5 ml microcentrifuge tubes containing 1 ml sterile 0.01 M PBS, and rotated for 30 sec. All samples were centrifuged at 400 x g for 10 min at 4°C, and the supernatant was removed, aliquoted and stored at -80°C.

LC-MS/MS analysis. A total of 10 maternal plasma and 10 AF samples which originated from five second trimester women and 10 third trimester women were separately mixed into a plasma sample and an AF sample pool. Protein identification in the two samples was performed using the LC-MS/MS technique. The whole procedure may be defined as follows. Firstly, the proteins in the two samples were separately enriched and extracted using ProteoMiner[™] kits (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for LC-MS/MS analysis, according to the manufacturer's protocol. Secondly, 100 μ g total protein/sample was digested into peptides using trypsin with a protein/enzyme ratio of 30:1 (w/w) at 37°C for 16 h. Following digestion, the mixture was acidified by the addition of 10 μ l formic acid. Thirdly, the peptides were fractionated using strong cation exchange chromatography with a LC-20AB high performance liquid chromatography pump system (Shimadzu Corporation, Kyoto, Japan). Peptides were eluted at a flow rate of 1 ml/min with a gradient of buffer A for 10 min, 5-60% buffer B (25 mM NaH₂PO₄, 1 M KCl in 25% CAN; pH 2.7) for 27 min and 60-100% buffer B for 1 min at 25°C. The system was subsequently maintained at 100% buffer B for 1 min before equilibration with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex, Inc., Tianjin, China) and vacuum dried. Each fraction was subsequently resuspended in buffer C (5% ACN, 0.1% FA) and centrifuged at 20,000 x g for 10 min at 4°C; the final concentration of peptide was approximately 0.5 μ g/ μ l. Supernatant (10 μ l) was loaded on a LC-20AD nanoHPLC (Shimadzu Corporation) by the autosampler onto a 2 cm C18 trap column. Peptides were eluted onto a 10 cm analytical C18 column (inner diameter, 75 μ m) packed in-house. The samples were loaded at 8 μ l/min for 4 min. The gradient was then run at 300 nl/min for 35 min, starting from 2 to 35% buffer D (95% ACN, 0.1% FA), followed by a 5 min linear gradient to 60% and a 2 min linear gradient to 80%/Buffer D was maintained at 80% for 4 min, and finally returned to 5% in 1 min. Data acquisition was performed with a TripleTOF 5600 System (Shanghai AB SCIEX Analytical Instrument Trading Co., Shanghai, China) fitted with a Nanospray III source (Shanghai AB SCIEX Analytical Instrument Trading Co.) and a pulled quartz tip as the emitter (New Objective, Inc., Woburn, MA, USA). Data were acquired using a positive-ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas

Table I. Clinical characteristics of the subjects from whom the AF, maternal plasma and VF samples were collected.

	AF		Plasma		VF of PROM		VF of healthy control	
	<37 weeks	≥37 weeks	<37 weeks	≥37 weeks	<37 weeks	≥37 weeks	<37 weeks	≥37 weeks
Maternal age, y	25.9±3.6	27.4±3.8	25.9±3.6	27.8±4.8	26.7±5.6	25.7±3.3	25.4±3.6	27.2±4.8
Mean ± SD (range)	(19-33)	(22-34)	(19-33)	(19-39)	(19-38)	(20-35)	(20-31)	(20-38)
Gestational age at sample collection, d	145.8±10.4	276.7±6.2 ^a	145.8±10.4	276.9±6.4 ^a	221.3±34.9	274.8±6.7 ^a	227.6±39.3	275.8±8.4 ^a
Mean ± SD (range)	(127-163)	(260-286)	(127-163)	(260-286)	(174-258)	(265-286)	(147-258)	(260-288)
Gravidity, n	2.0±1.0	2.3±1.3	2.0±1.0	2.9±1.6	2.9±2.0	2.2±1.3	1.9±1.4	1.8±0.7
Mean ± SD (range)	(1-4)	(1-5)	(1-4)	(1-6)	(1-7)	(1-5)	(1-5)	(1-3)
Parity, n	0.2±0.4	0.4±0.5	0.2±0.4	0.6±0.7	0.6±0.5	0.3±0.5	0.1±0.3	0.4±0.5
Mean ± SD (range)	(0-1)	(0-1)	(0-1)	(0-2)	(0-1)	(0-1)	(0-1)	(0-1)

^aP<0.05, <37 weeks vs. ≥37 weeks pregnant women. AF, amniotic fluid; VF, vaginal fluid; PROM, premature rupture of membranes; SD, standard deviation.

of 15 psi and an interface heater temperature of 150°C. MS analysis was performed in a high sensitivity scan mode. The MS scan range was 350-1,500 m/z. The top 30 precursor ions were selected into the MS² scan, and the MS/MS scan range was 350-1,250 m/z. The data were analyzed using the Mascot search engine (Matrix Science, Ltd., London, UK; version 2.3.02) for protein identification. Protein function methods were described by the Gene Ontology (GO) system and the Cluster of Orthologous Groups of Proteins (COGs) database.

ELISA and Magnetic Luminex[®] screening assay. A total of 14 proteins were detected. Sandwich ELISA kits purchased from RayBiotech, Inc. (Norcross, GA, USA) and Cloud-Clone Corp. (Houston, TX, USA) were used to analyze AF and maternal plasma samples for the concentrations of EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1; cat. no. SEF422Hu), keratin type II cytoskeletal 4 (KRT4; cat. no. SEA489Hu), keratin type II cytoskeletal 6A (KRT6A; cat. no. SED234Hu), keratin type II cytoskeletal 8 (KRT8; cat. no. SEC025Hu), keratin type I cytoskeletal 15 (KRT15; cat. no. SEA517Hu), keratin type I cytoskeletal 17 (KRT17; cat. no. SEB822Hu), keratin type I cytoskeletal 19 (KRT19; cat. no. ELH-CYT19-1), BPI fold-containing family A member 1 (BPIFA1 or PLUNC; cat. no. ELH-PLUNC-1), pulmonary surfactant-associated protein B (SFTPB; cat. no. SEB622Hu) and zymogen granule protein 16 homolog B (ZG16B; cat. no. SES158Hu). The assays were performed according to the manufacturers' protocols. Dilutions (100 μl each) of standard, blank and diluted samples was added into a 96 well plate in duplicate. Plates were incubated for 2.5 h at room temperature (RT) followed with gentle for 2 h at 37°C Following washing, biotinylated antibodies were incubated for 1 h at RT with gentle shaking at 37°C. Horseradish peroxidase-streptavidin solution was incubated for 45 min at RT followed by gentle shaking for 30 min at 37°C. Tetramethylbenzidine dihydrochloride substrates were added into each well for 30 min in the dark. The reactions were terminated by adding 0.2 M sulfuric acid. The absorbance of each well was recorded at a wavelength of 450 nm.

The concentrations of insulin-like growth factor-binding protein 2 (IGFBP2), mesothelin, placental protein 14 (PP14), and serpin family B member 3 (serpin B3) in AF, maternal plasma and VF samples were determined by Magnetic Luminex[®] Screening Assay multiplex kits (R&D Systems, Inc., Minneapolis, MN, USA). The kits were used according to the manufacturer's protocol. The microparticle cocktail (50 μl) was added into a 96 well plate, followed by 50 μl standard and diluted samples. The microplates were incubated for 2 h at RT with gentle shaking. A volume of 50 μl diluted biotin antibody cocktail and diluted streptavidin-phycoerythrin were added and incubated at RT with shaking. The microparticles were re-suspended with 100 μl wash buffer. Following incubation for 2 min, the microplates were read using the Luminex[®] Liquid Chip.

Lateral flow assay development. A lateral flow assay based on colloidal gold immunochromatography technology was developed to qualitatively detect PP14. A concentration of 1 mg/ml mouse monoclonal to anti-pp14 antibody (cat. no. ab17247; Abcam, Cambridge, UK) was conjugated with 40 nm colloidal gold particles, and the antibody-gold conjugate was atomized into a glass fiber pad with an AirJet dispenser (BioDot, Inc., Irvine, CA, USA). A concentration of 1 mg/ml rabbit anti-pp14 polyclonal antibody (cat. no. abs124712; Absin, Shanghai, China) and 0.5 mg/ml goat-anti-mouse immunoglobulin G (cat. no. TA130072; OriGene Technologies, Inc., Beijing, China) were atomized as a test line and control line (BioDot, Inc.), respectively. Recombinant human PP14 protein (Abcam) was diluted to 0.04, 0.02, 0.01, 0.008, 0.005 and 0.004 μg/ml with 0.01 M PBS. Diluted samples (80 μl) were dropped into the sample wells of the lateral flow strip, and the results were subsequently observed within 10 min. A positive result was judged when the test line and the control line appeared. A negative result was determined when only the control line was visible.

Statistical analysis. The normality of distribution of continuous variables was tested by a one-sample Kolmogorov-Smirnov

test in SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables with a normal distribution are presented as the mean \pm standard deviation; non-normal variables were reported as median (interquartile range). The means of two and three or more groups of continuous normally distributed variables were compared by independent sample Student's t-test or one-way analysis of variance (ANOVA), respectively. If the ANOVA was significant, Holm-Sidak's post-hoc test was used to analyze the difference between two groups. The Mann-Whitney U-test or Kruskal-Wallis test was used to compare the means of two and three or more groups of variables not normally distributed, and Dunn's post hoc test was performed to determine the difference between two groups if the P-value of the Kruskal-Wallis test was <0.05 . The diagnostic values of each detected protein were evaluated via Receiver Operating Characteristic (ROC) curve and the area under the curve (AUC). Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Protein identification by LC-MS/MS. Two pooled samples were analyzed by LC-MS/MS. A total of 648,074 identified spectra [1.09% false discovery rate (FDR)] and 625,162 identified spectra (0.89% FDR) were obtained in AF and maternal plasma samples, respectively. 4,343 peptides and 896 proteins were identified in AF samples, and 3,788 peptides and 681 proteins were identified in maternal plasma samples.

COGs analysis illustrated 21 classifications of proteins in AF and maternal plasma samples. For AF samples, proteins focused on the functional class O (post-translation modification, protein turnover and chaperones), class R (general function prediction) and class G (carbohydrate transport and metabolism). However, for maternal plasma samples, a greater number of proteins focused on Class O, Class R and Class Z (cytoskeleton) (data not shown).

GO analysis demonstrated that there was similar functional grouping and protein localization for proteins in AF and maternal plasma. However, the proteins in AF have a unique molecular function called protein tag, compared with proteins in maternal plasma (data not shown).

Unique proteins in AF sample. Subsequent to eliminating proteins identified in AF and maternal plasma, 540 unique proteins were observed for AF (data not shown). COG functional classification revealed that these proteins were involved in post-translational modification, protein turnover, chaperones, general function prediction, carbohydrate transport and metabolism. The GO system analysis demonstrated that these proteins were involved in 'development', 'reproduction, single-organism processes', 'organelle' and 'binding' (data not shown). Proteins EFEMP1, KRT4, KRT6A, KRT8, KRT15, KRT17, KRT19, PLUNC, SFTPB, ZG16B, IGFBP2, mesothelin, PP14 and serpin B3 were selected for further evaluation. Criteria for their selection is presented in Fig. 1.

Expression levels of 14 proteins in AF and maternal plasma. Expression levels of the 14 proteins in small panel samples

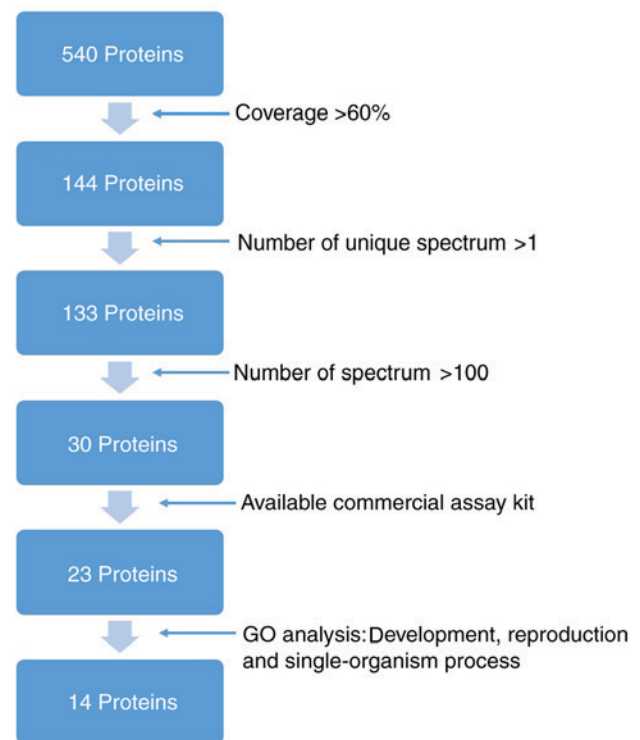


Figure 1. Criteria of selecting proteins for further detection.

were detected initially. The results demonstrated that the expression levels of KRT6A, KRT8, KRT15 and KRT17 in maternal plasma samples were higher compared with in AF samples, and thus these proteins were excluded for further analysis (data not shown). The expression levels of SFTPB, PLUNC, ZG16B, EFEMP1, KRT4 and KRT19 were detected by ELISA (Fig. 2). Additionally, the expression levels of PP14, IGFBP2, mesothelin and serpin B3 were determined (Fig. 2) using a magnetic Luminex[®] screening assay for the AF and maternal plasma samples. The expression levels of SFTPB and PLUNC in AF significantly decreased, and PP14 expression levels significantly increased during the second trimester, compared with those of the third trimester ($P < 0.05$). In maternal plasma samples, there was no detection of KRT19 via ELISA. The ELISA kit detected SFTPB in 28 maternal plasma samples (8 from the second trimester and 20 from the third trimester). For EFEMP1, the expression levels of 18 maternal plasma samples from the second trimester and 95 maternal plasma samples from the third trimester were undetected. As the number of samples that were detected was insufficient for the purpose of the statistical analysis, the undetected samples were assigned a value with the minimum corresponding detectable levels stated by the ELISA kit. The results revealed that no significant differences were observed between the second and third trimester groups in the expression levels of all proteins in the maternal plasma samples ($P > 0.05$). For SFTPB, the expression levels in AF during the second trimester were not statistically significant different from the maternal plasma of the second trimester ($P > 0.05$).

Expression levels of KRT19, PP14, IGFBP2, mesothelin and serpin B3 in VF samples. As the expression level of SFTPB in AF of the second trimester was not significantly different

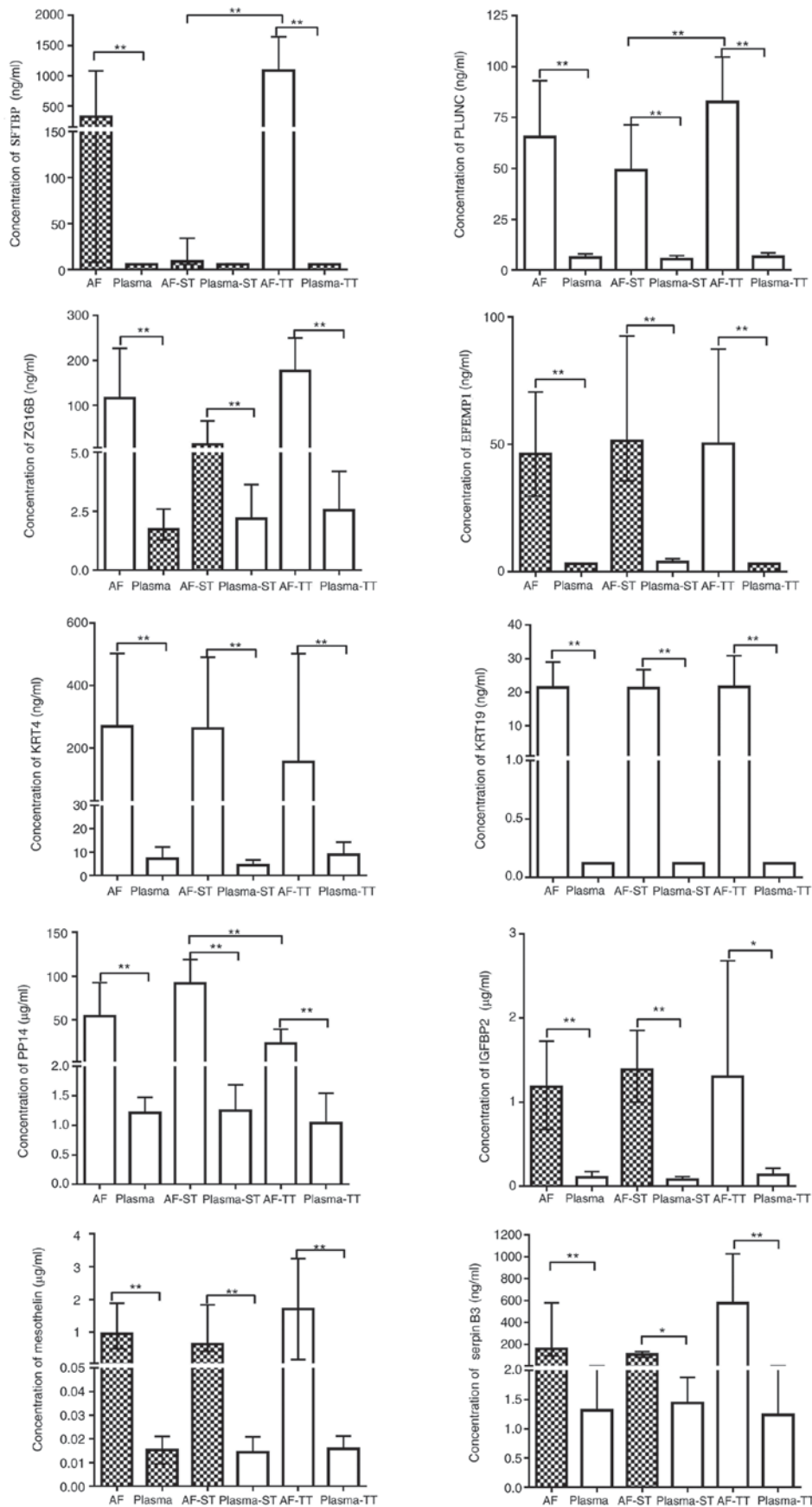


Figure 2. Concentrations of SFTPB, PLUNC, ZG16B, EFEMP1, KRT4, KRT19, PP14, IGFBP2, mesothelin and serpin B3 in AF and maternal plasma. Blank bars mean continuous variables are normally distributed. Filled bars mean continuous variables are non-normally distributed. The normal variables are presented as the mean ± standard deviation (above error bar); the non-normal variable error bars are presented as the median (interquartile range). *P<0.05, **P<0.001. SFTPB, pulmonary surfactant-associated protein B; PLUNC, BPI fold-containing family A member 1; ZG16B, zymogen granule protein 16 homolog B; EFEMP1, EGF-containing fibulin-like extracellular matrix protein 1; KRT4, keratin, type II cytoskeletal 4; KRT19, keratin, type I cytoskeletal 19; PP14, placental protein 14; IGFBP2, insulin-like growth factor-binding protein 2; serpin B3, serpin family B member 3; AF, amniotic fluid; ST, second trimester; TT, third trimester.

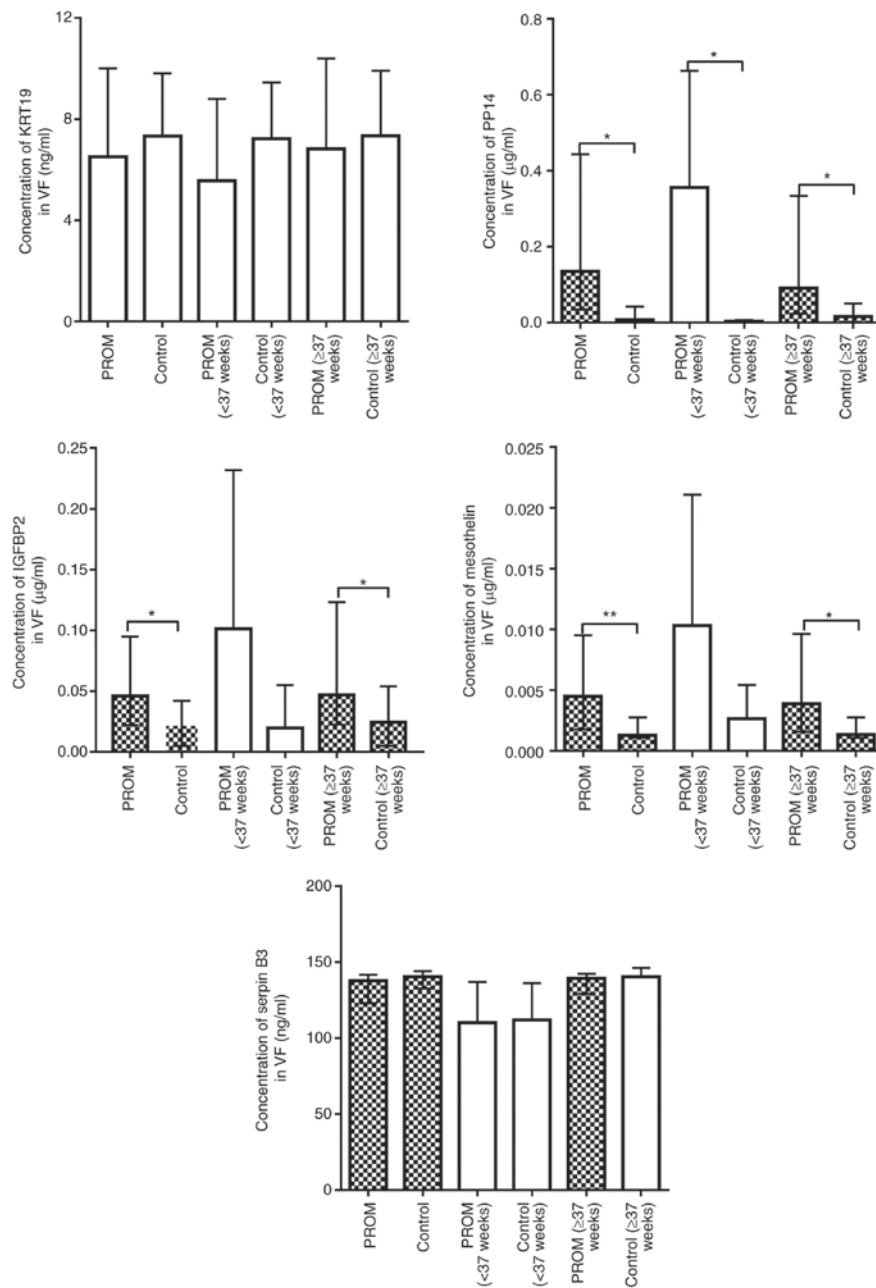


Figure 3. Concentrations of KRT19, PP14, IGFBP2, mesothelin and serpin B3 in the VF of women with overall PROM, PROM (<37 weeks) and PROM (≥37 weeks) and overall control, control (<37 weeks) and control (≥37 weeks). Blank bars mean continuous variables are normally distributed. Filled bars mean continuous variables are non-normally distributed. The normal variables are presented as the mean ± standard deviation (above error bar); the normal variables are presented as the median (interquartile range). * $P < 0.05$, ** $P < 0.001$. KRT19, keratin, type I cytoskeletal 19; PP14, placental protein 14; IGFBP2, insulin-like growth factor-binding protein 2; serpin B3, serpin family B member 3; VF, vaginal fluid; PROM, premature rupture of membranes.

compared with that in maternal plasma in the same trimester, PLUNC, ZG16B, EFEMP1, KRT4, KRT19, PP14, IGFBP2, mesothelin and serpin B3 were considered more valuable for evaluating VF samples. Unfortunately, since the expression levels of the majority of samples were below the minimum detectable limit of the ELISA kit, no valid data were obtained for the expression levels of PLUNC, ZG16B, EFEMP1 and KRT4 in VF samples. The results (Fig. 3) demonstrated that the expression levels of KRT19 and serpin B3 were not significantly different in the overall PROM groups compared with the control groups, PROM for ≥37 weeks vs. control for ≥37 weeks, or PPRM for <37 weeks vs. control for <37 weeks ($P > 0.05$). Expression levels of mesothelin and

IGFBP2 in the PROM group were significantly increased compared with those in the control group ($P < 0.05$). However, following <37 weeks and ≥37 weeks classification, the expression levels of mesothelin and IGFBP2 in the PPRM groups were not significantly different compared with those in the control for <37 weeks ($P > 0.05$), but were significantly different at ≥37 weeks compared with the control group ($P < 0.05$). Therefore, mesothelin and IGFBP2 were observed to be more suitable for PROM diagnosis, occurring at ≥37 weeks of pregnancy. As for PP14, the expression level was significantly increased in the PROM group compared with the control group at either <37 weeks or ≥37 weeks ($P < 0.05$).

To investigate whether the expression levels of mesothelin, IGFBP2 and PP14 in VF samples may have been altered due to blood contamination, analysis of the data in blood-contaminated and blood-free VF samples was conducted (Table II). The results indicated that the expression levels of mesothelin, IGFBP2 and PP14 in VF samples of the PROM (either <37 weeks or ≥37 weeks) or control (≥37 weeks) groups was not significantly different in blood-contaminated samples compared with blood-free samples ($P>0.05$). There was only one blood-contaminated VF sample in control (<37 weeks), thus the data are not presented. These results indicated that mesothelin, IGFBP2, and PP14 may be potential biomarkers for diagnosing PROM.

Diagnostic value of mesothelin, IGFBP2, and PP14 for PROM. The ROC curve was used to evaluate the diagnostic values of mesothelin, IGFBP2 and PP14 for PROM (Fig. 4). PP14 was observed to have an excellent diagnostic accuracy for PPRM, with a respective sensitivity and specificity of 100 and 87.5% for a cutoff value of 0.008 $\mu\text{g/ml}$ (AUC of 0.99 ± 0.02 , $P<0.001$).

PP14-based lateral flow assay. The human recombinant PP14 protein was used to determine the visible threshold of the PP14 strip. Different concentrations (0.04, 0.02, 0.01, 0.008, 0.005 and 0.004 $\mu\text{g/ml}$) of PP14 diluted with 0.01 M PBS were added to the test strip, and detection was performed three times for each sample. The results demonstrated that the PP14-based strip may be effective in clinical use and had a detection threshold of 0.008 $\mu\text{g/ml}$ (Fig. 5).

Discussion

In a previous study, a biomarker [soluble intercellular adhesion molecule-1 (sICAM-1)] was identified for the diagnosis of PROM, using a cytokine antibody array (9). A rapid test strip for clinical use was developed, based on colloidal gold immunochromatography technology. The validity of the strip was 95% (10). The product was approved for access to the medical market by China's SFDA. However, a limitation of the product is that the results may be false positive if the VF is contaminated with blood. Therefore, an aim of the present study was to discover novel biomarkers that are not influenced by blood contamination using LC-MS/MS-based proteomic techniques.

As an excellent biomarker for diagnosing PROM, sICAM-1 was observed to have high expression in AF and low expression in VF with an intact fetal membrane throughout the whole pregnancy. Once PROM occurred, AF was disseminated into the vagina and diluted by VF; sICAM-1 was observed to have high detectable expression levels in VF of PROM, regardless of the existence of blood. AF contains numerous proteins; certain proteins are derived from maternal blood and others from the fetus (11-13). Proteins derived from the fetus are more suitable PROM biomarkers. Therefore, a number of proteins were selected that primarily have functions associated with development or reproduction, for further quantitative detection and evaluation of diagnostic value.

In the present study, 14 unique proteins in AF were examined according to the selection criteria. The results demonstrated that the expression levels of these 14 unique

Table II. Concentration of mesothelin and PP14 in blood-contaminated or blood-free vaginal VF samples.

	PROM ≥37 weeks; blood-contaminated	PROM ≥37 weeks; blood-free	Control ≥37 weeks; blood-contaminated	Control ≥37 weeks; blood-free	PROM <37 weeks; blood-contaminated	PROM <37 weeks; blood-free
Mesothelin, $\mu\text{g/ml}$	0.006±0.008	0.007±0.007 ^a	0.002±0.001	0.001 (0.001,0.003)	0.005±0.003	0.016±0.008
IGFBP2, $\mu\text{g/ml}$	0.044±0.059	0.047 (0.024,0.106) ^a	0.069±0.091	0.030±0.033	0.035±0.030	0.168±0.163
PP14, $\mu\text{g/ml}$	0.197±0.254	0.063 (0.015,0.239) ^a	0.115±0.095	0.015 (0.002,0.068)	0.521±0.273	0.160±0.279

^a $P<0.05$, PROM (≥37 weeks) blood-free vs. Control (≥37 weeks) blood-free VF samples. Data are presented as the mean ± standard deviation or the median (interquartile range). PP14, placental protein 14; VF, vaginal fluid; PROM, premature rupture of membranes; IGFBP2, insulin-like growth factor-binding protein 2.

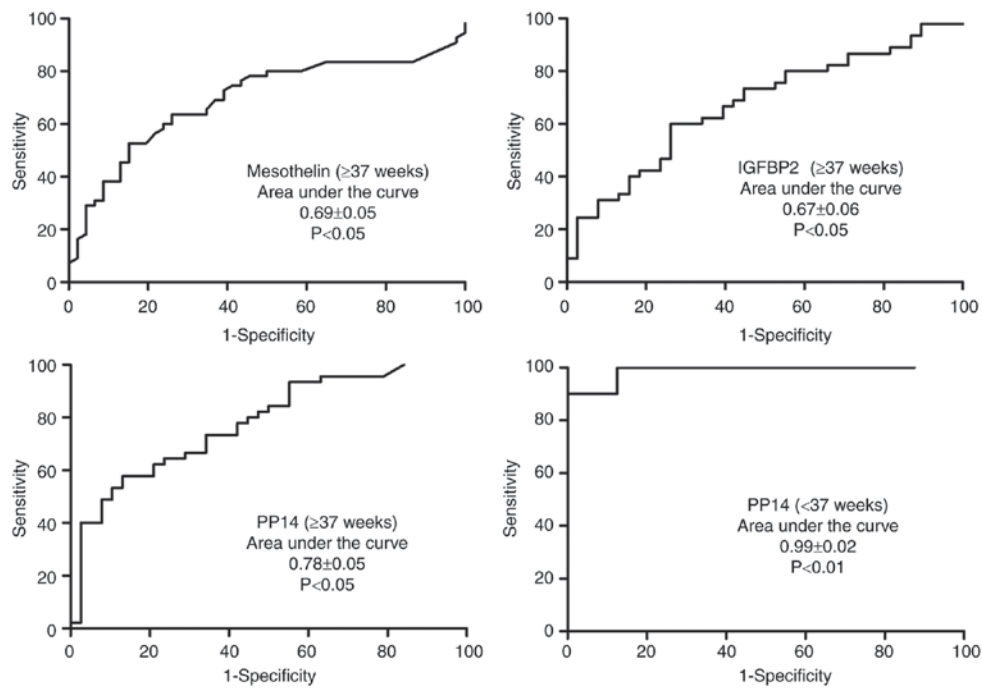


Figure 4. Receiver operating characteristic curves of mesothelin, IGFBP2 and PP14 for diagnosing PROM. IGFBP2, insulin-like growth factor-binding protein 2; PP14, placental protein 14; PROM, premature rupture of membranes.



Figure 5. Results of PP14-based lateral flow assay. (A) A total of $0.005 \mu\text{g/ml}$ PP14 recombinant protein was added. The strip was negative. (B) A total of $0.008 \mu\text{g/ml}$ PP14 recombinant protein was added. The strip was positive. PP14, placental protein 14; C, control line; T, test line; S, sample well.

proteins could be detected in AF samples and maternal plasma samples, with KRT6A, KRT8, KRT15 and KRT17 exhibiting higher expression levels in maternal plasma samples compared with AF samples, which appeared to contradict the results of the LC-MS/MS analysis. This may be explained by the characteristics of ELISA. It is known that false positive signals from ELISA may be caused by heterophilic antibodies, which in the case of the present study were the human anti-mouse antibodies in the blood (14). The intensity of the signal is easily influenced by ambient temperature, as higher temperatures are able to increase the optical density value. The

quality of the ELISA kit may additionally interfere with the results.

The present results demonstrated that mesothelin, IGFBP2 and PP14 had potential value for diagnosing PROM. Mesothelin is a 40-kDa membrane-glycoprotein; it is reported to associate with a number of types of cancer, although, its biological function in normal conditions is unclear (15). IGFBP2 is a 36-kDa protein, which is primarily involved in metabolic disease and cancer (16). PP14 is a secretory glycoprotein produced by the endometrium during pregnancy and has a high expression level in AF (17). The results of the present study demonstrated that mesothelin and IGFBP2 had insufficient ability to diagnose PPRM, and PP14 was the superior biomarker for diagnosing PPRM, with a respective sensitivity and specificity of 100 and 87.5% with a cutoff value of $0.008 \mu\text{g/ml}$. The present study revealed that the expression level of PP14 in AF during the second trimester was significantly higher compared with in the third trimester. This was in agreement with previous studies that the concentration of PP14 in AF reached its peak at 18-20 weeks of gestation, and subsequently decreased (18,19). This may explain why PP14 had a higher accuracy in diagnosing PPRM compared with PROM (≥ 37 weeks). Furthermore, the concentration of PP14 in VF samples was stable in blood-contaminated VF and blood-free VF samples, which is an advantage for the future clinical use of PP14.

As the cutoff value of PP14 for diagnosis of PPRM was at the level of $\mu\text{g/ml}$, a colloid-gold lateral flow strip was developed to rapidly test PP14. The strip was revealed to have a detection threshold of $0.008 \mu\text{g/ml}$, which was in accordance with the cutoff value of PP14. This strip may become a useful and rapid tool to supply indicative information for diagnosing PPRM in hospitals, particularly in vaginal bleeding-complicated cases.

However, there were five limitations to the present study. First, no sufficient volume of VF samples for LC-MS/MS analysis was available. As 50 ml VF samples was required, it was impossible to obtain the proteome profile of VF samples. Second, a total of 540 unique proteins were identified in AF samples. However, it was impossible to examine all of them. Accordingly, only 14 proteins of interest were selected for confirmation analysis, and thus it is possible that some effective biomarkers were missed. Third, the sample size of PPROM was small. A greater number of samples are required for further verification that PP14 may be an excellent biomarker for PPROM, which is not influenced by blood contamination. Fourth, the accuracy of the PP14 strip for clinical samples of PPROM requires confirmation using large samples, and multiple-center and single-blind clinical trials. As a standard clinical trial must be approved by China SFDA, this may be a future direction. Fifth, the expression levels of STFBP, KRT19 and EFEMP1 in maternal plasma samples, and PLUNC, ZG16B, EFEMP1 and KRT4 levels in VF samples were undetectable, so accurate levels could not be determined. This may have been caused by insufficient ELISA kit sensitivity.

To the best of the authors' knowledge, the present study was the first study to provide data on the expression levels of SFTPB, PLUNC, ZG16B, EFEMP1, KRT4, KRT19, PP14, IGFBP2, mesothelin and serpin B3 in AF and maternal plasma during the second and third trimester of pregnancy. Expression levels of KRT19, PP14, IGFBP2, mesothelin and serpin B3 in PROM and non-PROM VF samples in the Chinese population may aid the understanding of physiological processes experienced by pregnant women. Finally, the present study suggested that PP14 may be a novel potential biomarker for PPROM and that the PP14-based strip may be a helpful bedside test for rapidly diagnosing PPROM.

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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.

Authors' contributions

YW made substantial contributions to conception and design of the study. HL made substantial contributions to data acquisition and was involved in drafting the manuscript. GC, BZ, LG and TW analyzed and interpreted the data. YL, JG and QY collected samples and critically revised the manuscript

for important intellectual content. YuL was accountable for all aspects of the work, in ensuring that questions related to the accuracy or integrity of the research were appropriately investigated and resolved, and analyzed the data. LZ made substantial contributions to conception and design of the study, data acquisition, revised the manuscript critically for important intellectual content, and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committees of West China Second University Hospital of Sichuan University (Sichuan, China) and Shuangliu District Maternal and Child Health Hospital (Sichuan, China). All subjects signed consent forms.

Consent for publication

All patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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