Inflammasome components and ADAMTS4 in premature rupture of membranes

JINMING ZHU[1], CHUNLING MA[2], XIAOMEI LUAN[1], JUAN LI[1], FENGYUN PENG[1] and LEI HUANG[1]

[1]Department of Obstetrics, Xuzhou Maternity and Child Health Care Hospital Affiliated to Xuzhou Medical University, Xuzhou, Jiangsu 221009; [2]Graduate School, Xuzhou Medical University, Xuzhou, Jiangsu 221004, P.R. China

Received May 28, 2020; Accepted November 9, 2020

DOI: 10.3892/mmr.2020.11740

Abstract. Inflammation may be responsible for the development of premature rupture of membranes (PROM) including preterm PROM (PPROM) and mature PROM (MPROM). A total of four classic receptor proteins have been confirmed to assemble inflammasomes: NLR family pyrin domain containing (NLRP)1, NLRP3 and NLR family CARD-domain containing 4 (NLRC4) and absent in melanoma 2 (AIM2). The activation and expression of these receptor-modulated inflammasomes in placenta and fetal membrane of PROM pregnancies requires investigation. In addition, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) is a risk factor for PROM, but whether its expression is associated with inflammasome activation remains to be elucidated. In the present study, the placenta and fetal membrane tissues of patients who had suffered PPROM and MPROM and healthy pregnancies were investigated. Reverse transcription-quantitative PCR was used to determine the mRNA expression of inflammasomes and ADAMTS4. Western blotting, immunohistochemistry and ELISA were used to investigate the protein expression levels of inflammasomes and ADAMTS4. The results demonstrated that all four inflammasomes were elevated in placenta and fetal membrane of PPROMs as were mRNA and protein expression levels of IL-18 and IL-1β (compared with controls). A further increase of inflammasomes and interleukins was observed in MPROMs compared with controls. Similar results were also observed in ADAMTS4 expression in PPROM and MPROM groups. However, immunohistochemistry results revealed no significant difference of inflammasome receptor expression in PPROMs compared with controls. Finally, a general positive correlation between ADAMTS4 and all four inflammasome receptors in placenta and fetal membrane of PPROMs and MPROMs was observed. The present study revealed that NLRP1, NLRP3, AIM2 and NLRC4 inflammasome activation in PROM was increased. Promoted ADAMTS4 level was further observed in PROM group and was significantly correlated with inflammasome expression. Inhibition of inflammasome activation may provide a therapeutic target for clinical PROM treatment.

Correspondence to: Professor Jinming Zhu, Department of Obstetrics, Xuzhou Maternity and Child Health Care Hospital Affiliated to Xuzhou Medical University, 46 Heping Road, Xuzhou, Jiangsu 221009, P.R. China
E-mail: zhujinmingxz@163.com

Key words: premature rupture of membranes, inflammasome, a disintegrin and metalloproteinase with thrombospondin motifs 4, correction

Introduction

Premature rupture of membranes (PROM) refers to the spontaneous rupture of membranes surrounding the amniotic cavity in pregnancies before labor (1). It is reported that the incidence rate of PROM worldwide is 5-15% (2), it can be observed at any gestational age (3) and occurs in ~10% of pregnancies and in ~40% of preterm deliveries (4). Preterm PROM (PPROM) is the rupture of fetal membranes in pregnancies between 20 and 37 weeks of gestation and the incidence rate is 2.0-3.5% worldwide (1). Mature PROM (MPROM) occurs after 37 weeks of gestation with a 10% incidence rate (1). PROM without proper treatment can result in endometritis, chorioamnionitis, placental abruption, premature labor, fetal infection and fetal distress, which threaten maternal and fetal health and life. Recent studies have demonstrated that reproductive system infection is one of the important causes of PROM, however, the specific mechanism is unknown (5-7).

Inflammasomes have been recognized for their crucial effects in host defense against pathogens; dysfunction of inflammasome activation is associated with the development of tumorigenesis and metabolic, neurodegenerative, autoimmune and inflammatory diseases (8). To date, four receptor proteins have been determined to assemble inflammasomes, including the NLR family pyrin domain containing (NLRP)1, NLRP3, NLR family CARD-domain containing 4 (NLRC4) and absent in melanoma 2 (AIM2) (5). Recognition of the inflammatory ligand by these receptors results in sensor activation, oligomerization and the recruitment of an adaptor protein known as apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC serves as a bridge, connecting the sensor to the downstream effector caspase1 (Casp1) (9). Proximity-induced autoprocessing results in the formation of the catalytical active protease Casp1 p20 (10), which initiates

downstream responses, including the release and conversion of IL-18 and IL-1β from proIL-18 and proIL-1β (11) and induces pyroptosis, which is a lytic form of cell death. Indeed, the expression levels of Casp1 p20 is considered an indicator of inflammasome activity (12,13). Although the activation of inflammasomes is linked to inflammatory response to some extent, there is no report that it is related to PROM progress, to the best of the authors' knowledge.

A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) is a member of ADAMTS protease family that serves an important role in many processes such as organogenesis, angiogenesis, ovulation, cervical dilation and parturition (14). ADAMTS4 is upregulated in PROM and affects the development of PROM through Ras-mediated activation of NADPH oxidase (15,16). However, the ADAMTS4 expression in placenta and fetal membrane tissues of PPROM and MPROM patients and the relationship between ADAMTS4 and inflammasome components remain to be elucidated.

The aims of the present study were to explore the activation levels of several classical inflammasomes, NLRP1, NLRP3, AIM2 and NLRC4, in PPROM, MPROM and control patients. Furthermore, ADAMTS4 expression and the correlation analysis between ADAMTS4 and inflammasome components in PROM patients were also investigated.

Materials and methods

Study design. The present study was conducted at Xuzhou Maternity and Child Health Care Hospital Affiliated to Xuzhou Medical University between April 2017 and December 2019. The protocol of the present study was approved by the Medical Ethics Committee of Xuzhou Maternity and Child Health Care Hospital Affiliated to Xuzhou Medical University (approval no. 201502) and all the patients included in the study provided signed informed consent. A total of 60 patients aged 23-41 years with a singleton pregnancy who were admitted to the Department of Obstetrics were recruited, including 20 cases of PPROM patients (gestational week <37 weeks), 20 cases of MPROM patients (gestational week ≥37 weeks) and 20 cases of healthy pregnancies as control (gestational week ≥37 weeks) groups. Gestational age was determined based on the first trimester ultrasound scan for all pregnancies.

The diagnosis of PROM was performed using a speculum examination by visualizing the characteristic pooling of amniotic fluid in the vagina, together with a positive test for the presence of insulin-like growth factor-binding protein (ACTIM PROM test; Oy Medix Biochemica Ab) in vaginal fluid. The exclusion criteria of all pregnancies in the present study were: Structural or chromosomal abnormalities of the fetus, signs of fetal hypoxia, signs of intrauterine fetal growth restriction, vaginal bleeding, or any medical complication such as diabetes mellitus hypertension, preeclampsia, or thyroid disease.

Samples. Placenta specimens were obtained from the chorion frondosum of the maternal surface of placenta during the cesarean section. Fetal membrane specimens were acquired near the rupture of fetal membrane. A part of the specimens was stored at -80˚C for total RNA and protein extraction, the other parts were immersed into 4% paraformaldehyde at room temperature for 24 h for immunohistochemical staining.

Immunohistochemistry. Placenta and fetal membrane specimen sections (4 μm) were deparaffinized, rehydrated and incubated with 3% hydrogen peroxide at room temperature for 30 min for endogenous peroxidase activity blockage. Following blocking with 5% bovine serum albumin (cat. no. A3858; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, sections were incubated with primary antibodies against human NLRP1 (cat. no. ab3683; 1:100 dilution; Abcam), human NLRRP3 (cat. no. ab214185; 1:100 dilution; Abcam), human AIM2 (cat. no. 93015; 1:100 dilution; Abcam) and human NLRC4 (cat. no. 99860; 1:100 dilution; Abcam) at 4˚C overnight. Tissue sections were then rinsed with phosphate-buffered saline and treated with streptavidin-peroxidase conjugated IgG secondary antibody (cat. no. SP-9001; OriGene Technologies, Inc.) at 37˚C for 30 min. The sections were then stained a brown color under a light microscope with diamino-3,3'-benzidine tetrahydrochloride (DAB; OriGene Technologies, Inc.) incubated at room temperature for 5 min. Slides were counterstained with hematoxylin at room temperature for 5 min, dehydrated and mounted. Protein levels were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

ELISA. The protein concentration of ADAMTS4 was determined using human ADAMTS4 ELISA kit (cat. no. CSB-E11848h; Cusabio Technology LLC) according to the manufacturer's instructions. The absorbance was determined using a microplate reader and all samples were tested in duplicate. The standard curves were drawn by plotting absorbance values against the gradient standard concentrations and the linear regression was then analyzed. The ADAMTS4 concentration in cell supernatants of placenta and fetal membrane was calculated according to the curve linear equation.

Protein preparation and western blot analysis. Placenta and fetal membranes were homogenized and the cells were lysed. In brief, ~100 mg placenta or fetal membrane tissue was homogenized at 4˚C with a homogenizer. Total protein was extracted using Tissue or Cell Total Protein Extraction kit (cat. no. C510003; Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. The concentration of proteins was established with the bicinchoninic acid (BCA) method with BCA Protein Concentration Assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology). Tissue homogenates were analyzed through standard immunoblotting analysis. Briefly, samples were boiled at 100˚C for 5 min and 40 μg protein with equal volumes (20 μl) of each sample was loaded onto 12% sodium dodecyl sulfate-acrylamide gels. Protein was separated at a constant voltage of 120 V and transferred onto nitrocellulose filter membrane. Following blocking with 5% skimmed milk at room temperature for 2 h, membranes were incubated with antibodies against human NLRP1 (cat. no. ab3683; 1:1,000 dilution; Abcam), NLRP3 (cat. no. ab214185; 1:1,000 dilution; Abcam), AIM2 (cat. no. ab93015; 1:1,000 dilution; Abcam), NLRC4 (cat. no. ab99860; 1:1,000 dilution; Abcam), ASC (cat. no. ab155970; 1:1,000 dilution; Abcam), Casp1 (cat. no. 3866; 1:1,000 dilution; Cell Signaling Technology, Inc.), Casp1 p20 (cat. no. 4199; 1:1,000 dilution; Cell Signaling Technology, Inc.), proIL-18 (cat. no. 10663-1-AP; 1:1,000 dilution; ProteinTech Group, Inc.), IL-18 (cat. no. sc-7954;

1:200 dilution; Santa Cruz Biotechnology, Inc.), proIL-1β (cat. no. ab156791; 1:1,000 dilution; Abcam), IL-1β (cat. no. 83186; 1:1,000 dilution; Cell Signaling Technology, Inc.) and ADAMTS4 (cat. no. ab185722; 1:1,000 dilution; Abcam) at 4˚C overnight. Anti-GAPDH (cat. no. ab9485; 1:1,000 dilution; Abcam) was applied as loading control. Membranes were then washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 at room temperature for 5 min three times and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (cat. no. 7076; 1:10,000 dilution; Cell Signaling Technology, Inc.) or HRP-conjugated anti-rabbit IgG (cat. no. 7074; 1:10,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 2 h. The blots were visualized with an enhanced chemiluminescence solution (cat. no. P0018; Beyotime Institute of Biotechnology), then exposed to film and quantified with ImageJ v2.0 software (National Institutes of Health). The expression level of target protein was calculated by dividing the intensity of the target protein by the intensity of GAPDH protein.

Reverse transcription-quantitative (RT-q) PCR. The frozen placenta and fetal membrane tissues were thawed and RNA was extracted using RNAiso Plus Total RNA extraction reagent (cat. no. 9109; Takara Bio, Inc.), followed by DNase I treatment. RNA concentration was analyzed using NanoDrop (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and RNA quality was quantified by the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) to produce an electrophoresis trace from which the RNA integrity number and DV200 index were calculated using the 2100 Expert Software (version no. B.02.10; Agilent Technologies, Inc.). RNA was then reverse-transcribed to cDNA on a Veriti 96-well Thermal Cycler (Applied Biosystems) with PrimeScript RT reagent kit (Takara Bio, Inc.) with thermocycling conditions of 42˚C for 15 min and 85˚C for 5 min. Primers were synthesized by Takara Bio, Inc. (sequences in Table I). PCR amplifications were performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH) with SYBR-Green PCR Master mix (Applied Biosystems) with thermocycling conditions of 95˚C for 30 sec and 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. All procedures including RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (17) following normalization to GAPDH expression.

Statistical analysis. Data are expressed as mean ± SEM and statistical analyses were performed using SPSS 23.0 software (IBM Corp.). Comparison between groups was carried out by unpaired Student's t-test, chi-square test, or one-way ANOVA followed by Bonferroni's post hoc test. Bivariate correlation analysis was performed using Pearson or Spearman rank correlation. All calculated P-values were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequences
NLRP1	F: 5'-CCACAACCCTCTGTCTACATTAC-3'
	R: 5'-GCCCCATCTAACCCATGCTTC-3'
NLRP3	F: 5'-GATCTTCGCTGCGATCAACA-3'
	R: 5'-GGGATTCGAAACACGTGCATTA-3'
AIM2	F: 5'-CTGCAGTGATGAAGACCATTCGTA-3'
	R: 5'-GGTGCAGCACGTTGCTTTG-3'
NLRC4	F: 5'-CCAGTCCCCTCACCATAGAAG-3'
	R: 5'-ACCCAAGCTGTCAGTCAGACC-3'
ASC	F: 5'-AACCCAAGCAAGATGCGGAAG-3'
	R: 5'-TTAGGGCCTGGAGGAGCAAG-3'
Caspase-1	F: 5'-GCCTGTTCCTGTGATGTGGAG-3'
	R: 5'-TGCCCACAGACATTCATACAGTTTC-3'
IL-18	F: 5'-CTGCCACCTGCTGCAGTCTA-3'
	R: 5'-TCTACTGGTTCAGCAGCCATCTTTA-3'
IL-1β	F: 5'-CCAGGGACAGGATATGGAGCA-3'
	R: 5'-TTCAACACGCAGGACAGGTACAG-3'
ADAMTS4	F: 5'-GAGGGAGGCACCCCTAACT-3'
	R: 5'-CCTTGACGTTGCACATGGGA-3
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'
	R: 5'-TGGTGAAGACGCCAGTGGA-3'

NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4; ASC, the apoptosis-associated speck-like protein that contains a caspase recruitment domain; ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs 4; F, forward; R, reverse.

Results

Elevated expression of inflammasome-related receptor proteins in placenta of PROM pregnancies. The mRNA and protein expression of inflammasome-related receptors in placenta tissues were identified. As shown in Fig. 1A, the mRNA expression of NLRP1, NLRP3, AIM2 and NLRC4 in PPROM groups were all significantly increased compared with controls. In addition, mRNA expression of these receptors was further upregulated in MPROM groups compared with controls. As common components of several inflammasomes, the expression of ASC and Casp1 mRNA were also promoted in PPROM and MPROM groups when compared with control group. The protein levels of these inflammasome components in PPROM and MPROM groups changed in accordance with the changes in mRNA levels (Fig. 1B-D). These results suggested that the mRNA and protein expression of all inflammasome components, NLRP1, NLRP3, AIM2, NLRC4, ASC and Casp1, were upregulated in placenta tissue of PPROM patients. Notably, these trends of increasing inflammasome components expression were more pronounced in MPROM groups.

Increased expression of inflammasomes in fetal membrane of PROM patients. The mRNA levels of inflammasomes in fetal membrane of PPROM were notably enhanced compared with controls except for AIM2 expression (Fig. 2A). In addition to the mRNA levels, protein expression of these inflammasomes in PPROM groups was also increased compared with the

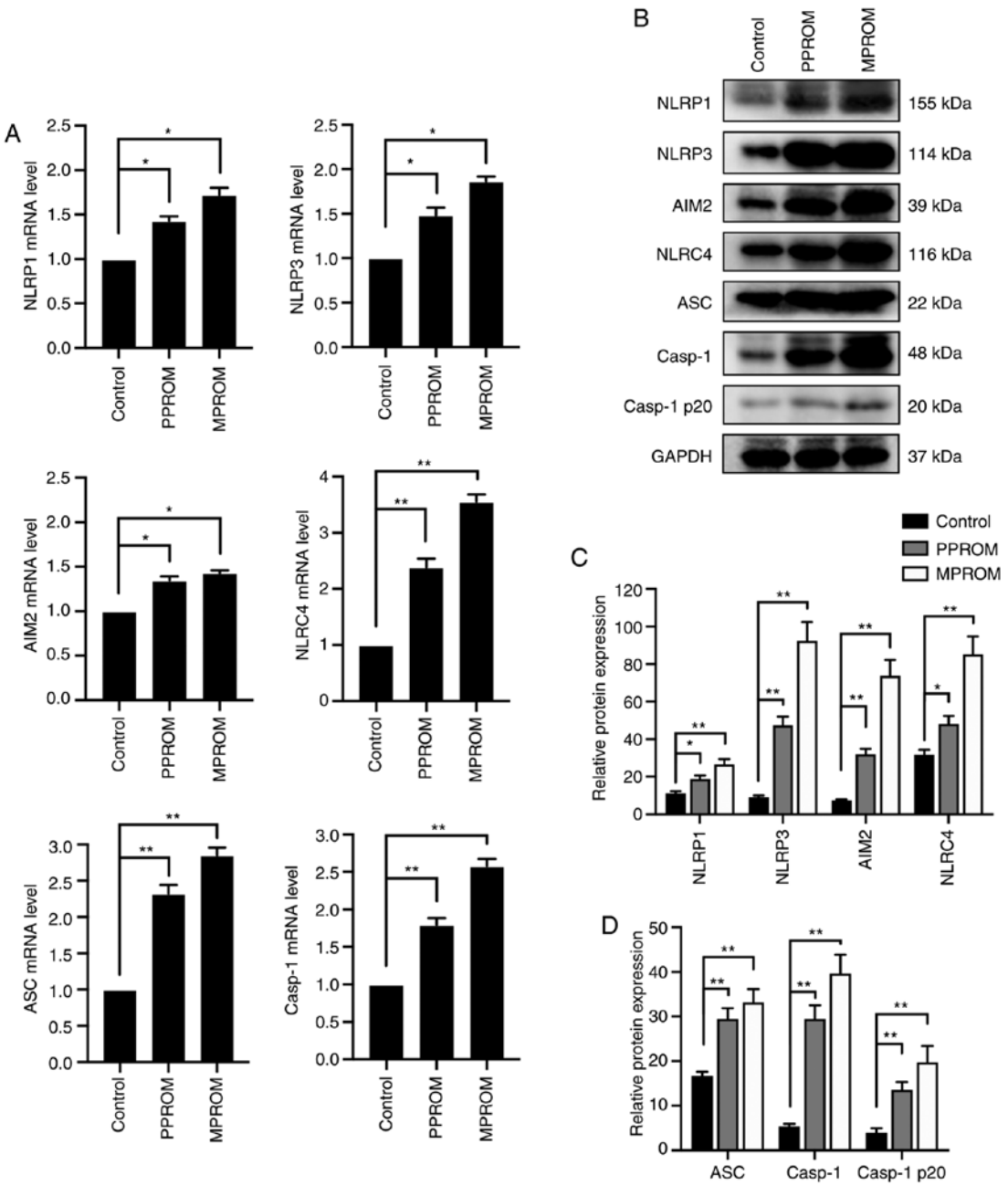

Figure 1. Enhanced mRNA and protein levels of inflammasome components in placenta tissues from PROM patients. (A) The mRNA expression of NLRP1, NLRP3, AIM2, NLRC4, ASC and caspase-1 in control, PPROM and MPROM placenta tissues was determined by reverse transcription-quantitative PCR (n=20 in each group). (B) Representative blots of NLRP1, NLRP3, AIM2, NLRC4, ASC, Casp1 and Casp1 p20 in control, PPROM and MPROM placenta tissues. Corresponding quantitative analysis of (C) NLRP1, NLRP3, AIM2 and NLRC4 and (D) ASC, Casp1 and Casp1 p20 (n=20 in each group). *P<0.05, **P<0.01 vs. indicated groups. PROM, premature rupture of membranes; NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4; ASC, the apoptosis-associated speck-like protein that contains a caspase recruitment domain; Casp1, caspase-1; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes.

control group except for the Casp1 expression (Fig. 2B-D). Consistently, mRNA and protein levels of inflammasomes in fetal membrane tissues of MPROM groups were further elevated compared with controls.

Increased maturation and expression of inflammasome downstream effectors in PROM patients. The mRNA expression of IL-18 and IL-1β in placenta and fetal membrane of PROM pregnancies was also investigated. As shown in Fig. 3A, IL-18 and IL-1β mRNA levels in PPROM groups in both placenta and fetal membrane were increased significantly compared with controls. A further increase of IL-18 and IL-1β mRNA levels in MPROM groups was observed compared with controls. Furthermore, the protein expression of proIL-18, IL-18, proIL-1β and IL-1β in both placenta and fetal membrane of PPROM patients were markedly elevated compared with controls (Fig. 3B-D). A consistent trend of an increase in protein expression of proIL-18, IL-18, proIL-1β and IL-1β was observed in MPROM group, as well as increased mRNA expression.

Analysis of NLRP1, NLRP3, AIM2 and NLRC4 protein levels in placenta and fetal membrane by immunohistochemistry.

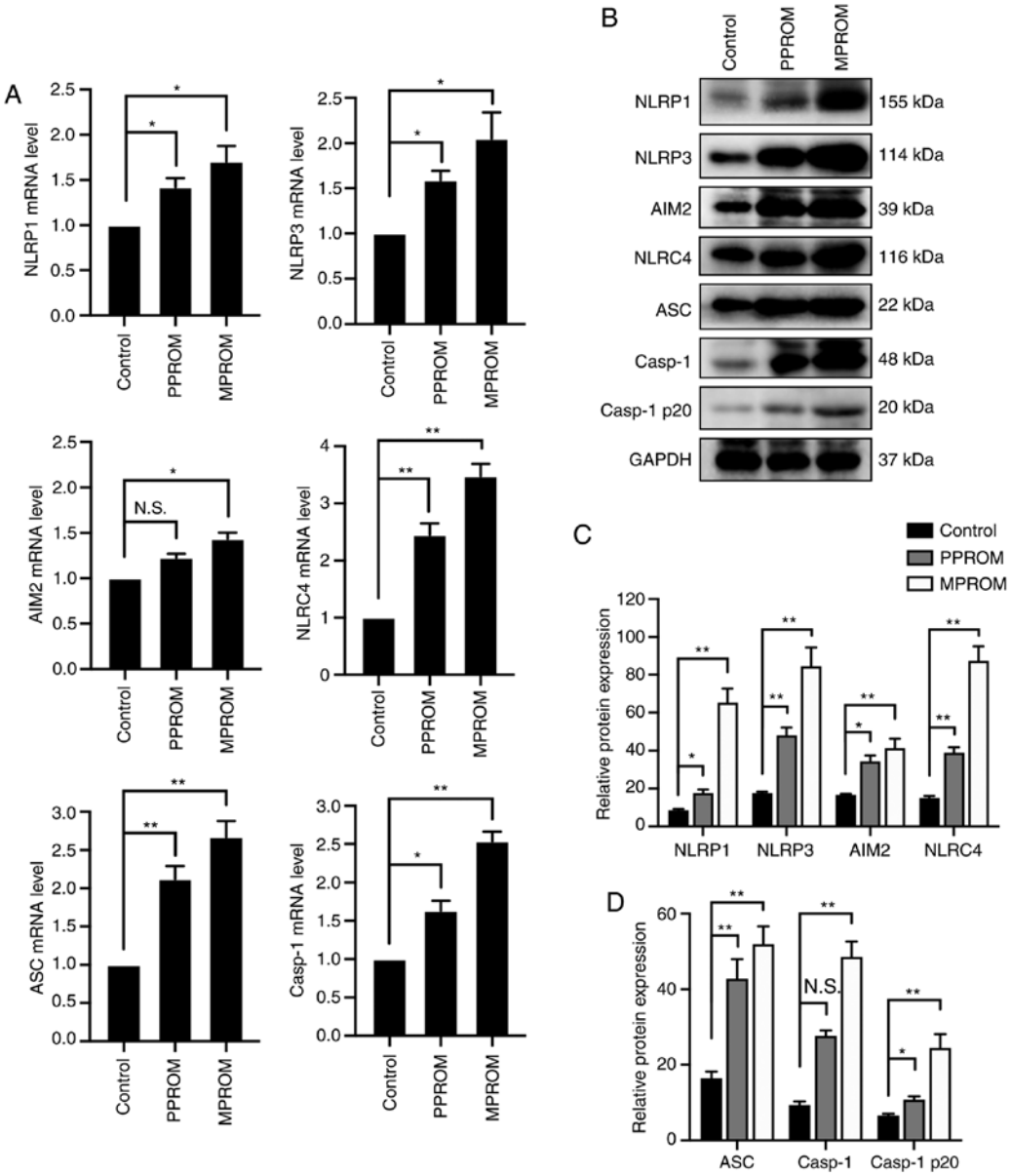

Figure 2. Elevation of inflammasome expression was observed in fetal membrane tissues of PROM pregnancies. (A) The mRNA levels of NLRP1, NLRP3, AIM2, NLRC4, ASC and Casp1 in control, PPROM and MPROM fetal membrane tissues (n=20 in each group). (B) Representative blots of NLRP1, NLRP3, AIM2, NLRC4, ASC, Casp1 and Casp1 p20 in control, PPROM and MPROM fetal membrane tissues. Corresponding quantitative analysis of (C) NLRP1, NLRP3, AIM2 and NLRC4 and (D) ASC, Casp1 and Casp1 p20 (n=20 in each group). *P<0.05, **P<0.01 vs. indicated groups. PROM, premature rupture of membranes; NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4; ASC, the apoptosis-associated speck-like protein that contains a caspase recruitment domain; Casp1, caspase-1; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes; N.S., no significance.

The expression of inflammasome components was then determined by immunohistochemical staining *in vivo*. As shown in Fig. 4A-C and E, NLRP1, NLRP3 and NLRC4 expression were all increased in placenta of PPROM groups compared with controls, whereas no significant difference in AIM2 expression levels was observed between PPROMs and controls (Fig. 4D). The expression of these inflammasome components were all markedly upregulated in the placenta of MPROMs compared with controls. Unexpectedly, no significant difference was observed in their expression levels in the fetal membrane of PPROMs compared with controls (Fig. 5A-E) and a promotion of inflammasome component expression was identified in the fetal membrane of MPROMs compared with controls. These results suggested that all four inflammasomes may be responsible for PROM progress.

ADAMTS4 expression and its correlation analysis with inflammasome components in placenta and fetal membrane of PROM pregnancies. The expression of ADAMTS4, one of the risk factors of PROM, was detected. As shown in Fig. 6A and B, ADAMTS4 protein levels in placenta and fetal membrane of PPROM and MPROM groups were all significantly increased compared with control groups. In addition to protein expression, upregulated mRNA expression of ADAMTS4 was observed in placenta and fetal membrane of PPROMs and MPROMs compared with controls (Fig. 6C). Furthermore, a general positive correlation between ADAMTS4 and all four inflammasome components in PPROMs (Fig. 6D) and MPROMs (Fig. 6E) was observed.

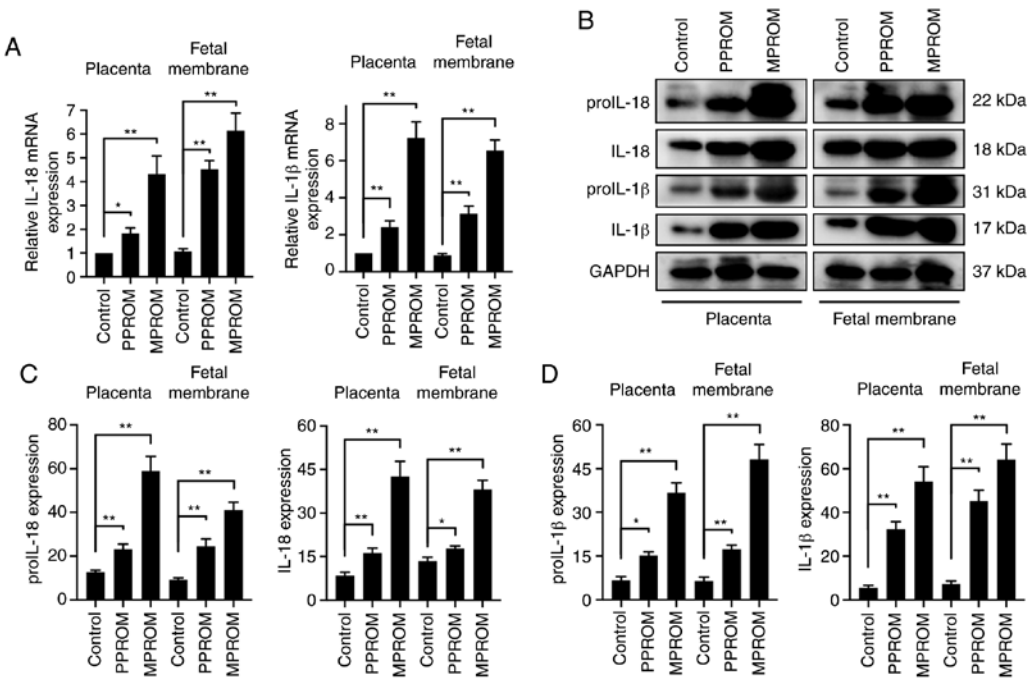

Figure 3. Increased expression and maturation of IL-18 and IL-1β in placenta and fetal membrane tissues from PROM patients. (A) The mRNA expression of IL-18 and IL-1β in placenta and fetal membrane from control, PPROM and MPROM patients (n=20 in each group). (B) Representative blots of proIL-18, IL-18, proIL-1β and IL-1β in placenta and fetal membrane tissues with control, PPROM and MPROM patients. Corresponding quantitative analysis of (C) proIL-18 and IL-18 and (D) proIL-1β and IL-1β expression (n=20 in each group). *P<0.05, **P<0.01 vs. indicated groups. PROM, premature rupture of membranes; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes.

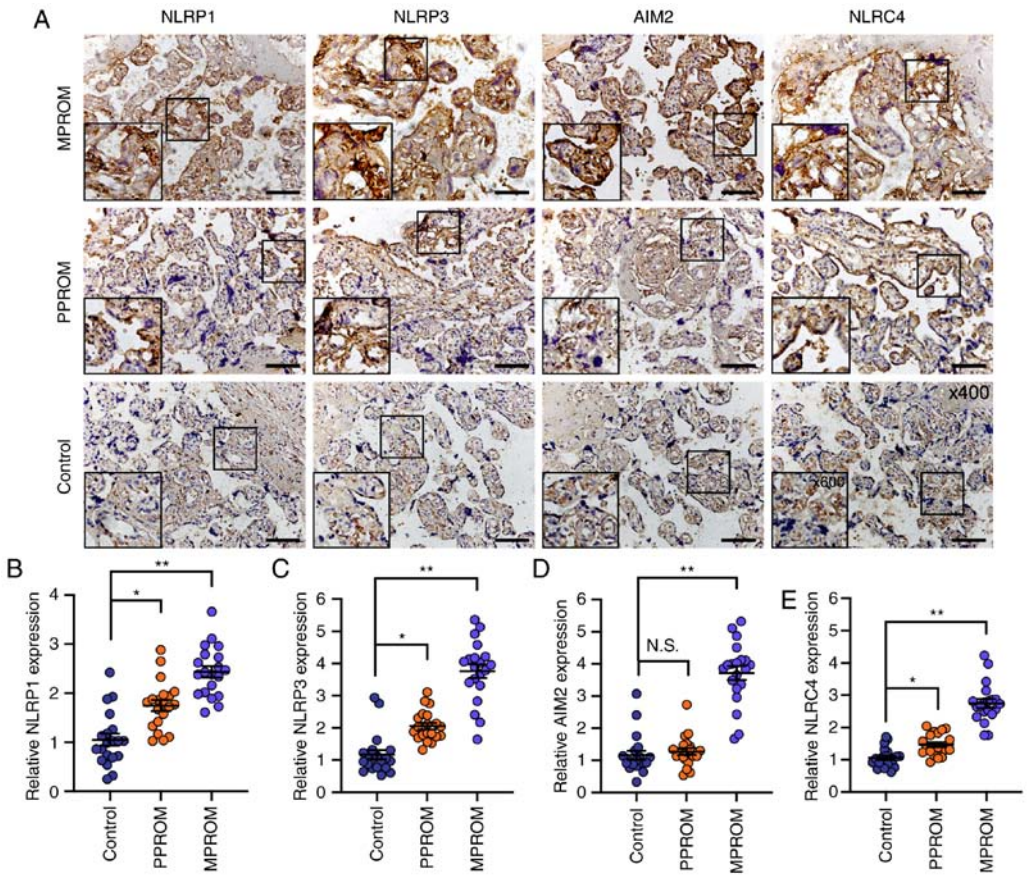

Figure 4. Evaluation of NLRP1, NLRP3, AIM2 and NLRC4 expression in placenta tissues. (A) Representative immunohistochemical staining images of NLRP1, NLRP3, AIM2 and NLRC4 in placenta tissue sections from control, PPROM and MPROM patients (magnification of main image, x400; Scale bar, 250 μm; box indicates area enlarged on lower left). Quantified expression levels of (B) NLRP1, (C) NLRP3, (D) AIM2 and (E) NLRC4 in control, PPROM and MPROM tissues (n=20 in each group). *P<0.05, **P<0.01 vs. indicated groups. N.S. indicates no significance. NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes.

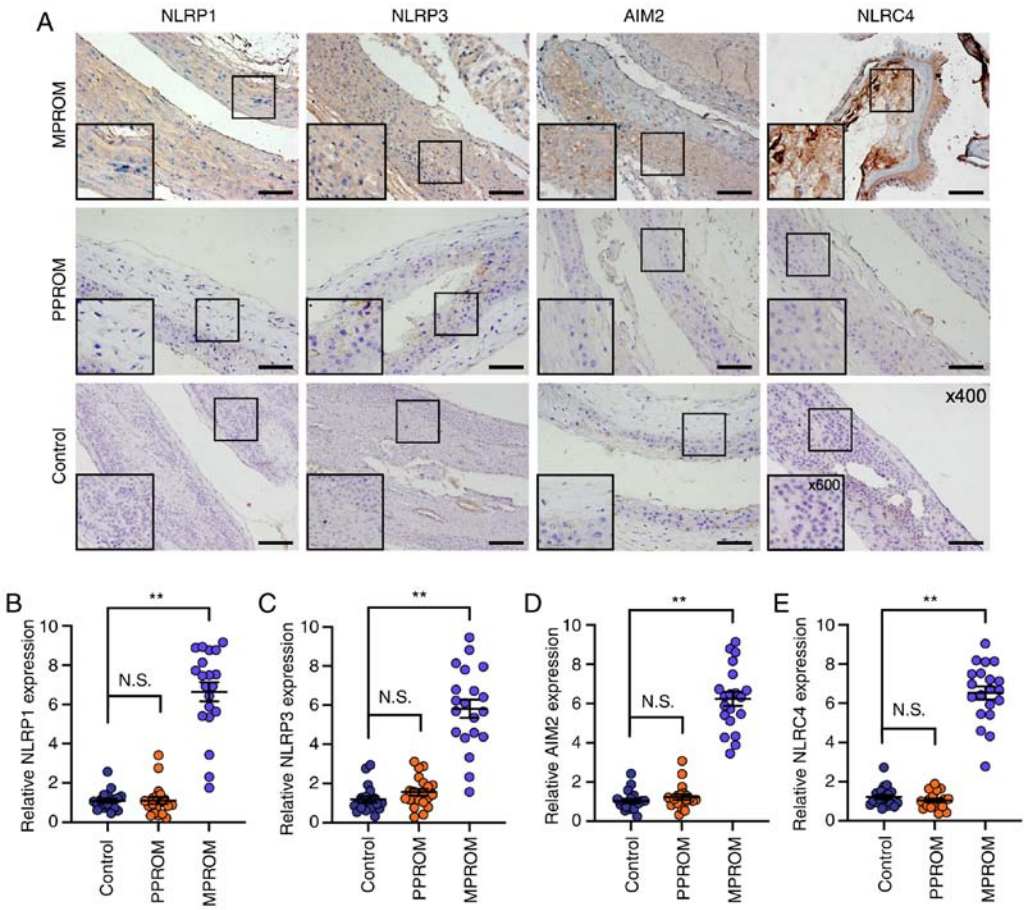

Figure 5. Increased inflammasome components levels in fetal membrane of PROM patients. (A) Representative immunohistochemical staining images of NLRP1, NLRP3, AIM2 and NLRC4 in fetal membrane sections from control, PPROM and MPROM groups (magnification of main image, x400; Scale bar, 250 μm; box indicates area enlarged on lower left). Quantified expression levels of (B) NLRP1, (C) NLRP3, (D) AIM2 and (E) NLRC4 in control, PPROM and MPROM tissues (n=20 in each group). **P<0.01 vs. indicated groups. PROM, premature rupture of membranes; NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes.

Discussion

PROM is defined as rupture of the fetal membrane before (PPROM) or after (MPROM) 37 weeks of completed gestation. At present, PROM is often ignored and regarded as an adverse outcome of pregnancy (18). Despite advanced progress in prenatal care over the past three decades, rates of PROM and subsequent miscarriage remain high (1). The etiology of PROM is diverse and complicated, including cervix relaxation, reproductive system infection, abnormal amniotic cavity pressure and lack of trace elements, and infection is considered as a crucial cause of PROM (3,19,20).

The inflammasome was named by Martinon *et al* (21) in 2002 to describe a high-molecular-weight complex in the cytoplasm of stimulated immune cells which modulates the activation of inflammatory caspases. Since then, this research field has expanded substantially and multiple major inflammasomes have been identified, with the assembly of them being directed by a unique pattern-recognition receptor in response to pathogen-associated molecular patterns or endogenous danger signals in the cytoplasm of the host cells (22). The four classic inflammasome receptors NLRP1, NLRP3, AIM2 and NLRC4 are crucial for inflammasome activation and host defense against pathogens (8). However, the relationship between inflammasomes and PROM remains to be elucidated.

ADAMTS4 is also known as aggrecanase-1 and possesses a role in aggrecan cleavage and collagen degradation, which result in extracellular matrix (ECM) remodeling (23), the vital pathogenesis of PROM. ADAMTS4 is abundantly expressed in brain, lungs and myocardium tissues and a small amount is expressed in placenta and skeletal muscle cells (23). In addition, its aggrecanase activity and mRNA expression are linked with IL-1 and TNF-α accumulation (24). The combination of oxidative mediators with inflammation, infection and proteases affect the structural and functional changes of ECM and form a vicious cycle in ECM degradation (15). Inflammation and oxidative stress also lead to proteoglycan degradation in ECM, collagen collapse and degradation of cell connections via released proteases such as ADAMTS4 (25). Decreased tensile forces and thinning of the fetal membrane result in rupture of membranes in the early weeks of pregnancy ultimately (15). As one of the risk factors of PROM, whether ADAMTS4 is associated with inflammasome expression need to be determined.

The present study demonstrated that the mRNA and protein expression of all four inflammasomes plus ASC and Casp1 were upregulated in placenta and fetal membrane tissues of PPROM patients compared with control groups (except for the AIM2 expression in fetal membrane; Fig. 2A). The expression of these inflammasomes was further elevated in MPROM groups compared with controls. Additionally, the expression levels of

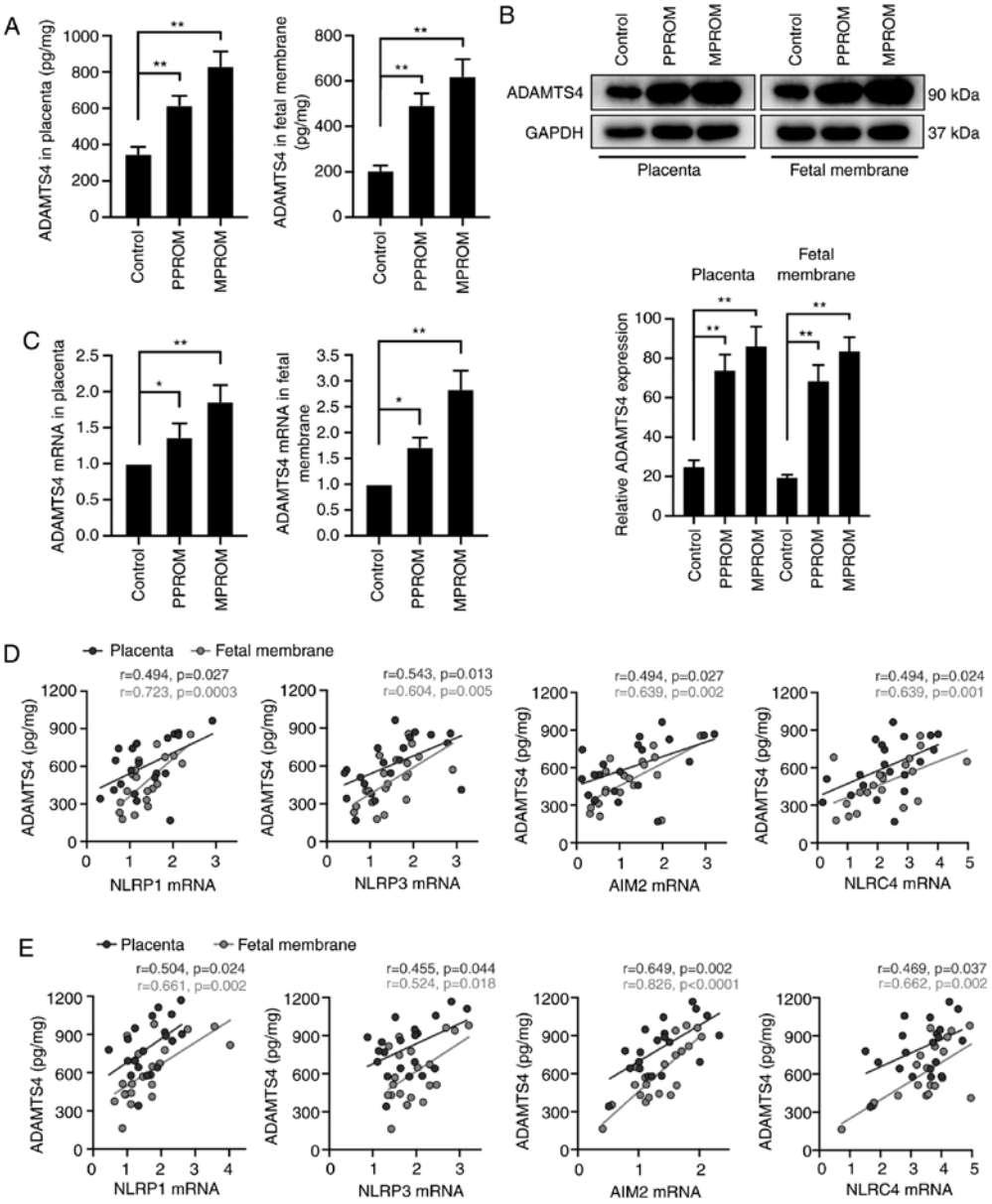

Figure 6. Correlation between inflammasome mRNA expression and ADAMTS4 concentrations in placenta and fetal membrane tissues of PROM patients. (A) ADAMTS4 expression levels were investigated in placenta and fetal membrane tissues by ELISA analysis (n=20 in each group). (B) Western blot and quantified expression levels of ADAMTS4 in placenta and fetal membrane of control, PPROM and MPROM patients (n=20 in each group). (C) The mRNA levels of ADAMTS4 in placenta and fetal membrane (n=20 in each group). *P<0.05, **P<0.01 vs. indicated groups. Correlation between ADAMTS4 protein levels and mRNA expression of inflammasome components (NLRP1, NLRP3, AIM2 and NLRC4) in placenta and fetal membrane of (D) PPROM or (E) MPROM patients (n=20 in each group). A bivariate correlation analysis was performed using the Spearman rank test. R indicates the Spearman correlation coefficient. ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs 4; PROM, premature rupture of membranes; PPROM, preterm premature rupture of membranes; MPROM, mature premature rupture of membranes; NLRP, NLR family pyrin domain containing; AIM2, absent in melanoma 2; NLRC4, CARD-domain containing 4.

downstream proIL-18, IL-18, proIL-1β and IL-1β demonstrated a consistent trend with the inflammasome expression in PPROM and MPROM groups. These results indicated that the activation of inflammasomes and their downstream effectors are more pronounced in MPROM groups compared with the PPROM groups. Notably, results from immunohistochemistry suggested that no significant difference of inflammasome receptor expression was observed in fetal membrane of PPROMs compared with controls (Fig. 5B-E). These results, which were inconsistent with the western blotting results, suggested that the inflammasomes may not be significantly activated in fetal membrane tissue of PPROM patients. Finally, the mRNA and protein levels of ADAMTS4 were investigated. Increased mRNA and protein expression of ADAMTS4 were observed in placenta and fetal membrane of PPROM pregnancies. As expected, ADAMTS4 expression was further promoted in MPROMs compared with controls. A general positive correlation between ADAMTS4 and all four inflammasome receptors in placenta and fetal membrane of PPROMs and MPROMs was observed, indicating that the activation of these inflammasomes may be potential factors influencing the expression of ADAMTS4 which triggers the development of PROM subsequently.

However, there are several limitations in the present study. Since it is difficult to establish a PROM model in small animals, how to determine whether deleting the expression of inflammasomes can improve PROM at the animal level is

worthy of further study. In addition, although the inflammasome expression has a significant correlation with ADAMTS4 expression, whether inflammasome regulates the expression of ADAMTS4 remains to be confirmed.

In conclusion, the present study suggested that NLRP1, NLRP3, AIM2 and NLRC4 inflammasome activation and its effector expression in PROM were upregulated. Increased expression of ADAMTS4 was also observed in PROM group and was significantly correlated with the expression of inflammasomes. Whether downregulation of inflammasome elements contribute to modulating ADAMTS4 expression and alleviating PROM progress requires further evidence. The present study on the expression and correlation of inflammatory bodies and ADAMTS4 may be instrumental in the expansion of PROM etiology and may provide a potential therapeutic target for clinical PROM treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the project of Xuzhou Science and Technology (grant no. KC20121).

Availability of data and materials

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

Authors' contributions

JZ, CM and XL conceived and designed the research. JZ, CM, JL and FP were responsible for subject recruitment and collection of specimens. JZ, CM and LH performed the experiments. XL, JL, FP and LH contributed reagents, materials, instruments and analysis tools. CM and JL analyzed the data. JZ and FP were responsible for data analysis and writing of the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Xuzhou Maternity and Child Health Care Hospital Affiliated to Xuzhou Medical University (approval no. 201502) and all the patients included in the study signed informed consents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Goldenberg RL, Culhane JF, Iams JD and Romero R: Epidemiology and causes of preterm birth. Lancet 371: 75-84, 2008.
2. Lee T and Silver H: Etiology and epidemiology of preterm premature rupture of the membranes. Clin Perinatol 28: 721-734, 2001.
3. Parry S and Strauss JF III: Premature rupture of the fetal membranes. N Engl J Med 338: 663-670, 1998.
4. Kumar D, Moore RM, Mercer BM, Mansour JM, Redline RW and Moore JJ: The physiology of fetal membrane weakening and rupture: Insights gained from the determination of physical properties revisited. Placenta 42: 59-73, 2016.
5. Kacerovsky M, Pliskova L, Bolehovska R, Musilova I, Hornychova H, Tambor V and Jacobsson B: The microbial load with genital mycoplasmas correlates with the degree of histologic chorioamnionitis in preterm PROM. Am J Obstet Gynecol 205: 213.e1-e7, 2011.
6. Hornychova H, Kacerovsky M, Musilova I, Pliskova L, Zemlickova H, Matejkova A, Vosmikova H, Rozkosova K, Cermakova P, Bolehovska R, *et al*: Cervical human papillomavirus infection in women with preterm prelabor rupture of membranes. PLoS One 13: e0207896, 2018.
7. Suzuki Y, Horie K, Yada Y, Kono Y, Hirashima C, Usui R, Matsubara S and Ohkuchi A: Vaginal ureaplasma species increase chorioamnionitis in very preterm infants with preterm premature rupture of the membranes at <28 weeks of gestation. Eur J Clin Microbiol Infect Dis 37: 2371-2380, 2018.
8. von Moltke J, Ayres JS, Kofoed EM, Chavarria-Smith J and Vance RE: Recognition of bacteria by inflammasomes. Annu Rev Immunol 31: 73-106, 2013.
9. Liu D, Zeng X, Li X, Cui C, Hou R, Guo Z, Mehta JL and Wang X: Advances in the molecular mechanisms of NLRP3 inflammasome activators and inactivators. Biochem Pharmacol 175: 113863, 2020.
10. Schroder K, Zhou R and Tschopp J: The NLRP3 inflammasome: A sensor for metabolic danger? Science 327: 296-300, 2010.
11. Slaats J, Ten Oever J, van de Veerdonk FL and Netea MG: IL-1beta/IL-6/CRP and IL-18/ferritin: Distinct inflammatory programs in infections. PLoS Pathog 12: e1005973, 2016.
12. Schroder K and Tschopp J: The inflammasomes. Cell 140: 821-832, 2010.
13. Lamkanfi M and Dixit VM: Mechanisms and functions of inflammasomes. Cell 157: 1013-1022, 2014.
14. Porter S, Clark IM, Kevorkian L and Edwards DR: The ADAMTS metalloproteinases. Biochem J 386: 15-27, 2005.
15. Ozler S, Oztas E, Guler BG, Ergin M, Uygur D, Yucel A, Erel O and Danisman N: ADAMTS4 and oxidative/antioxidative status in preterm premature rupture of membranes. Fetal Pediatr Pathol 35: 239-250, 2016.
16. Ahmad R, Sylvester J, Ahmad M and Zafarullah M: Adaptor proteins and Ras synergistically regulate IL-1-induced ADAMTS-4 expression in human chondrocytes. J Immunol 182: 5081-5087, 2009.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Menon R and Richardson LS: Preterm prelabor rupture of the membranes: A disease of the fetal membranes. Semin Perinatol 41: 409-419, 2017.
19. Murtha AP and Menon R: Regulation of fetal membrane inflammation: A critical step in reducing adverse pregnancy outcome. Am J Obstet Gynecol 213: 447-448, 2015.
20. Gomez-Lopez N, Romero R, Xu Y, Garci z-Flores V, Leng Y, Panaitescu B, Miller D, Abrahams VM and Hassan SS: Inflammasome assembly in the chorioamniotic membranes during spontaneous labor at term. Am J Reprod Immunol 77: 10.1111/aji.12648, 2017.
21. Martinon F, Burns K and Tschopp J: The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10: 417-426, 2002.
22. Broz P and Dixit VM: Inflammasomes: Mechanism of assembly, regulation and signalling. Nat Rev Immunol 16: 407-420, 2016.
23. Naito S, Shiomi T, Okada A, Kimura T, Chijiiwa M, Fujita Y, Yatabe T, Komiya K, Enomoto H, Fujikawa K and Okada Y: Expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic cartilage. Pathol Int 57: 703-711, 2007.
24. Pratta MA, Scherle PA, Yang G, Liu RQ and Newton RC: Induction of aggrecanase 1 (ADAM-TS4) by interleukin-1 occurs through activation of constitutively produced protein. Arthritis Rheum 48: 119-133, 2003.
25. Lockwood CJ and Kuczynski E: Risk stratification and pathological mechanisms in preterm delivery. Paediatr Perinat Epidemiol 15 (Suppl 2): S78-S89, 2001.