Original Article

Expression of NOD1 and downstream factors in placenta, fetal membrane and plasma from pregnancies with premature rupture of membranes and their significance

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Abstract: Objective: To investigate the expression and significance of NOD1 (nucleotide oligomerization domain 1) and its downstream factors in placenta, fetal membrane and plasma of pregnancies with premature rupture of membranes (PROM). Methods: 60 cases of PROM pregnancies were recruited, including 30 cases of preterm premature rupture of membranes (< 37 weeks) and 30 cases of mature premature rupture of membranes (≥ 37 weeks); 30 healthy pregnancies in the same period were selected as a control group (gestational weeks ≥ 37 weeks). Western blot was used to detect the expression of NOD1, receptor interacting protein 2 (RIP2) and nuclear factor-κB (NF-κB) in placenta and fetal membrane tissues of the three groups. Immunohistochemistry staining was used to investigate the protein levels of NOD1 in the placenta and fetal membrane of three groups of pregnancies. Reverse transcription quantitative PCR (RT-qPCR) demonstrated the expression of NOD1, RIP2, and NF-κB mRNA in placenta, fetal membrane, and plasma of the three groups. The content of NOD1 in plasma was detected by ELISA. Results: Western blot analysis showed that the expressions of NOD1, RIP2 and NF-κB in the placenta and fetal membrane of the preterm PROM group were significantly higher than those in the control group (P < 0.01). Furthermore, the increased levels of NOD1, RIP2 and NF-κB protein in placenta and fetal membrane of mature PROM group were more dramatic than those in the preterm PROM group (P < 0.05). The immunohistochemical staining showed that the staining intensity of NOD1 in placenta and fetal membranes of mature PROM group was stronger than those in the control group. RT-qPCR detection showed that the mRNA expressions of NOD1, RIP2 and NF-κB in placenta, fetal membrane and plasma were significantly higher than in the control group (P < 0.01). In addition, NOD1, RIP2 and NF-κB mRNA levels in the placenta, fetal membranes and plasma of mature PROM group were further increased compared with preterm PROM group (P < 0.01); ELISA assay revealed that the levels of NOD1 in plasma of the mature PROM group, preterm PROM group and control group were (8.34±0.16), (6.82±0.11) and (0.92±0.08) ng/mL, respectively. In the mature PROM group, the content of NOD1 in plasma was increased compared with preterm PROM group (P < 0.05), and plasma NOD1 in both the mature PROM group and preterm PROM group were significantly higher than those in the control group (P < 0.01). Conclusions: Premature rupture of membranes leads to increased mRNA and protein levels of NOD1, RIP2 and NF-κB in placenta, fetal membrane, and peripheral blood, which triggers an inflammatory response and increases the severity of PROM.

Keywords: Premature rupture of membranes, NOD1, placenta, fetal membranes, plasma

Introduction

Premature rupture of membranes (PROM) means the rupture of membranes caused by genital tract infections, etc. in pregnancies before labor. It is reported that the incidence rate in China is 2.7%~17%, and the incidence rate abroad is 5%~15% [1]. Mature PROM refers to the occurrence of membrane rupture in pregnancies after 37 weeks of gestation, the incidence rate is about 10%. Preterm PROM is the rupture of fetal membranes in pregnancies between 20 weeks and 37 weeks of gestation, and the incidence rate is about 2.0%~3.5%. It is
well established that PROM with improper treatment can be complicated by premature labor, placental abruption, endometritis, chorioamnionitis, neonatal infection, and fetal distress, which seriously threaten maternal and child health and life safety, and is a serious complications during perinatal period. According to relevant research, the causes of PROM include trauma, reproductive system infection, mycoplasma infection, cervical cervix relaxation and increased amniotic pressure. Among them, reproductive system infection is one of the most important factors [2]; however, the specific mechanism remains unclear.

The nucleotide-binding oligomerization domain like receptors (NLR) family is a major member of the innate immune response pattern recognition receptor, and nucleotide oligomerization domain 1 (NOD1) is one of the important members of the NLR family. When NOD1 is activated by pathogens, it can cause its own oligomerization to activate downstream receptor interacting protein 2 (RIP2), which in turn activates nuclear factor-kB (NF-kB) or mitogen-activated protein kinase (MAPK) pathway to induce the expression of cytokines and chemokines, causing inflammation [3]. Recent reports by Hoang et al. have shown that the levels of various inflammatory factors such as NOD1, NOD2, NLRP1 and NLRP3 in the isolated fetal membranes with microbial infection were increased, suggesting that microbial infection may mediate the development of fetal membrane inflammation by increasing the levels of inflammatory factors [4]. However, whether the NOD1 signaling pathway is involved in the development of fetal membrane inflammation in PROM is still unknown.

Therefore, the aim of this study was to determine the role of NOD1 signaling pathway in PROM by measuring the expression of NOD1, RIP2, and NF-kB in placenta, fetal membrane and plasma of pregnancies with PROM, providing an experimental and theoretical basis for early prediction and clinical diagnosis and treatment of PROM.

### Materials and methods

#### Study design

The Institutional Review Board at Affiliated Maternal and Child Health Hospital of Xuzhou Medical University approved the study and written informed consent was obtained from all the participants. Between November 2016 and June 2018, a prospective cohort study was conducted. 60 cases of PROM pregnancies aged ≥ 18 years with a singleton pregnancy who were admitted to the Department of Obstetrics, Affiliated Maternal and Child Health Hospital of Xuzhou Medical University were recruited, including 30 cases of preterm PROM pregnancies (< 37 weeks) and 30 cases of mature PROM pregnancies (≥ 37 weeks); 30 healthy pregnancies in the same period were selected as the control group (gestational weeks ≥ 37 weeks). Exclusion criteria were: vaginal bleeding, structural or chromosomal abnormalities of the fetus, signs of fetal hypoxia, signs of intrauterine fetal growth restriction, or any medical complication such as hypertension, preeclampsia, diabetes mellitus or thyroid disease. Gestational age was evaluated based on the first trimester ultrasound scan for all pregnancies. All three groups of pregnancies were singleton pregnancies, and all were delivered by cesarean section. The average age, gravidity, parity, time of membrane rupture and gestational age at delivery were compared between the three groups (Table 1). The average age, gravidity and parity were not statistically significantly different (P > 0.05). The mature PROM group was different from the control group in terms of rupture time (P < 0.05), and the preterm PROM group had further

### Table 1. Maternal characteristics of PROM pregnancies (means ± SEM)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Numbers</th>
<th>Average age (years)</th>
<th>Gravidity (times)</th>
<th>Parity (times)</th>
<th>Rupture time (hours)</th>
<th>GAD (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature PROM</td>
<td>30</td>
<td>30.1±3.0</td>
<td>2.3±0.6</td>
<td>1.1±0.2</td>
<td>2.3±0.1</td>
<td>38.1±1.1</td>
</tr>
<tr>
<td>Preterm PROM</td>
<td>30</td>
<td>29.4±4.1</td>
<td>2.1±0.7</td>
<td>1.2±0.2</td>
<td>3.8±0.2**</td>
<td>35.3±2.1**</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>30.6±3.3</td>
<td>2.1±0.9</td>
<td>1.1±0.3</td>
<td>1.1±0.2</td>
<td>37.2±0.8</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs. control group, ***P < 0.01 vs. mature PROM group. Data were compared using two-tailed Student’s t test. PROM: premature rupture of membranes. GAD: gestational age at delivery.
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increases compared with the mature PROM group (P < 0.01). The gestational age at delivery in preterm PROM group was significantly lower than in the control group (P < 0.01). In addition, there was no significant difference in the mature PROM group compared with control group in terms of gestational age at delivery (P > 0.05).

PROM was defined as leakage of amniotic fluid for at least two hours prior to labor. The diagnosis of PPROM was made using a sterile 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; Medix Biochemica, Kauniainen, Finland) in vaginal fluid.

Biopsies collection and preparation

Placenta and fetal membrane tissues: All specimens in this study were taken from the central area of the maternal surface of the placenta immediately after removal of the placenta during cesarean section. The decidua basalis was removed, and 3 pieces of chorion frondosum tissues were taken away from the calcified necrotic area (1.0 cm×1.0 cm×0.5 cm). Furthermore, 3 pieces of fetal membrane tissues were taken within 2 cm near the rupture zone of fetal membranes (1.0 cm×1.0 cm). Both of the placenta and fetal membrane tissues were washed with diethylpyrocarbonate (DEPC) water, placed in liquid nitrogen for 10 min, and then transferred to a -80°C refrigerator for extraction of total RNA for reverse transcription quantitative PCR (RT-qPCR) testing. In addition, 6 pieces of placental (1.0 cm×1.0 cm×0.5 cm) and fetal membrane tissues (1.0 cm×1.0 cm) were taken, 3 of them were washed with physiological saline at 4°C, fixed in 4% paraformaldehyde for 24 h, processed through a graded alcohol series and embedded in paraffin, sectioned 4 μm for immunohistochemistry. Another 3 placental and fetal membrane tissues were used for western blot analysis. Plasma sample: Patients from the three groups of pregnancies underwent sampling of 4 ml of elbow venous blood under fasting conditions on the morning after admission. Blood was settled for 30 min, centrifuged at 3000 r/min for 15 min, stored the plasma, and the plasma sample was stored in a -80°C refrigerator for RT-qPCR and ELISA.

Immunohistochemistry staining

Placental and fetal membrane sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 30 min, and the sections were then washed with phosphate-buffered saline (PBS; pH 7.4). Primary antibody against NOD1 (Cell Signal Technology, Danvers, USA; 1:100) were used and incubated at 4°C overnight. The slides were then washed with PBS three times and incubated with the secondary antibody from the streptavidin-peroxidase secondary antibody kit (ZSGB-BIO, Beijing, China) according to the manufacturer’s instructions. After chromogenic development with 3,3’-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-BIO, Beijing, China), the slides were then counterstained with haematoxylin, dehydrated through a graded alcohol series, and mounted with neutral gum.

Western blot

Placental and fetal membrane tissue proteins were extracted using a lysis buffer (100 mmol/L Tris-HCl, 4% SDS, 20% glycerine, 200 mmol/L DTT, phosphatase and protease inhibitors; pH 6.8). For nuclear lysates, proteins were extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. Protein concentrations were measured using a bicinchoninic acid (BCA) assay. Equivalent amount of protein was prepared and separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electro-transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany). Then probed with NOD1, RIP2, NF-κB, Lamin B1 or β-actin antibody 4°C overnight, and incubated with secondary antibody for 2 hours at room temperature. Finally, signals were detected by Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, USA). Digitized images were analyzed using Image J (NIH, Bethesda, USA). For all western blot analyses, protein levels were calculated from the ratio of corresponding protein/β-actin or Lamin B1.
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RT-qPCR

TRizol reagent (Invitrogen, Carlsbad, USA) was used to extract the total RNA from placental and fetal membrane tissues following the manufacturer's protocol. The RNA (1000 nmol) was then subjected to reverse transcription using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Afterwards, 20-μL reactions with primers (GENEWIZ Inc., South Plainfield, USA) were detected by Light Cycler 480II (Roche, Basel, Switzerland) using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, USA). Relative quantitative analysis of the change in expression levels was obtained using the ΔΔCT method and normalized to β-actin. The sequences of the primer are as follows: NOD1 forward: 5'-ACGATGAAGTGCGAGAGTT-3'; NOD1 reverse: 5'-GGCAGTTCCCTTAGCTGTGA-3'; RIP2 forward: 5'-CCTCTCGTGTTCCTTGGC-3'; RIP2 reverse: 5'-GGTCCTTGTAGGTTTGGTGCT-3'; NF-κB forward: 5'-ACGAGCAGATGGTCAAGGAG-3'; NF-κB reverse: 5'-CTTCCATGGTCAGTGCCTTT-3'; β-actin forward: 5'-CTTCATGGTCAGTGCCTTT-3'; β-actin reverse: 5'-CACGATGGAGGGCCGGACTCATC-3'.

Enzyme linked immunosorbent assay (ELISA)

The content of free NOD1 in plasma was measured by human NOD1 ELISA kit (Lifesience, Darmstadt, Germany) according to the manufacturer's instructions. Standards, plasma sample and horseradish peroxidase (HRP)-

Figure 1. NOD1, RIP2, and NF-κB protein expression in placenta. A. Immunostaining showed the expression of NOD1 protein in placenta, bar = 250 μm. B. Western blot analysis of NOD1, RIP2, and NF-κB (nucleus) protein levels in placenta. C. Bar graph of western blot results, n = 30. **P < 0.01 vs. control group, *P < 0.05 vs. preterm PROM group. Data were compared using two-tailed Student’s t test.
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labeled detection antibody were sequentially added to the pre-coated microwells, and then incubated and thoroughly washed. The substrate was developed with tetramethylbenzidine (TMB) which was converted to blue under the catalysis of HRP and finally converted to yellow. The depth of the color is positively correlated with the level of NOD1 in the sample. The absorbance was measured at 450 nm using an enzyme-labeled analyzer. The standard concentrations (0, 1.25, 2.5, 5, 10, 20 ng/mL) was used as the abscissa, and the relative absorbance was plotted as the ordinate. The linear regression curve of the standard was drawn. The NOD1 content in the plasma of the three groups was calculated according to the curve equation.

Statistical analysis

For all quantitative analyses, data are expressed as means ± SEM. Statistical analysis was performed using PASW Statistic 21 (SPSS Inc., Chicago, USA). Data between two groups were compared using two-tailed Student’s t test. P < 0.05 was considered a significant difference. Analyses were performed using GraphPad prism (La Jolla, USA).

Results

Protein expression of NOD1, RIP2 and NF-κB in placenta of pregnancies

Immunohistochemistry staining demonstrated that NOD1 protein is mainly expressed in the cell membrane and cytoplasm of syncytiotrophoblasts and vascular endothelial cells in placental and fetal membrane tissue, showing brown-yellow staining. As shown in Figure 1A, the intensity of NOD1 protein staining in the placenta of mature and preterm PROM groups were significantly higher than in the control group, and the NOD1 protein staining intensity in mature PROM group was higher than that in the preterm PROM group. Furthermore, western blot revealed the similar result that the NOD1 protein level in preterm PROM group was significantly higher than in the control group (P < 0.01), and the NOD1 protein level in the mature PROM group was dramatically increased compared with the preterm PROM group (P < 0.05) (Figure 1B, 1C). In addition, the levels of RIP2 and NF-κB protein (nucleus) in placentas of the preterm PROM group were significantly higher than those in the control group (P < 0.01); and the levels of RIP2 and NF-κB in mature PROM group were improved compared with the preterm PROM group (P < 0.05).

NOD1, RIP2 and NF-κB mRNA expression in placenta

Next we examined the expression levels of NOD1, RIP2, and NF-κB mRNA in placenta. As shown in Figure 2A-C, the mRNA levels of NOD1, RIP2, and NF-κB in the preterm PROM group were significantly higher than those in the control group (P < 0.01), and the mRNA levels of the three in the mature PROM group were dramatically increased compared with the pre-
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In the preterm PROM group (P < 0.01). In addition, in the mature PROM group, RIP2 mRNA increased more sharply than NOD1 and NF-κB (~5 folds vs. ~4 folds).

Protein expression of NOD1, RIP2 and NF-κB in fetal membrane

NOD1 protein staining intensity by immunostaining in fetal membrane of the mature and preterm PROM groups was significantly higher than that of the control group, and the NOD1 staining intensity in the mature PROM group was significantly increased compared with the preterm PROM group (Figure 3A). Figure 3B, 3C showed that the protein levels of NOD1, RIP2 and NF-κB (nucleus) in fetal membrane of preterm PROM group were significantly higher than those in the control group (P < 0.01), and the NOD1, RIP2 and NF-κB expression in mature PROM group were dramatically increased compared with preterm PROM group (P < 0.05).

Figure 3. NOD1, RIP2, and NF-κB protein expression in fetal membrane. A. Immunostaining showed the expression of NOD1 protein in fetal membrane, bar = 250 μm. B. Western blot analysis of NOD1, RIP2 and NF-κB (nucleus) protein levels in fetal membrane. C. Bar graph of western blot results, n = 30. "P < 0.01 vs. control group, "P < 0.05 vs. preterm PROM group. Data were compared using two-tailed Student’s t test.

NOD1, RIP2 and NF-κB mRNA expression in fetal membrane

Similar to the previous results in placenta, NOD1, RIP2 and NF-κB mRNA expression in fetal membrane of preterm PROM group were significantly higher than those in the control
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group (P < 0.01), and the mRNA levels of NOD1, RIP2 and NF-κB in the mature PROM group were sharply improved compared with the preterm PROM group (P < 0.01) (Figure 4A-C). In addition, in the fetal membrane of mature PROM group, the increase of RIP2 mRNA was also more dramatic than that of NOD1 and NF-κB (~5 folds vs. ~4 folds).

Expression of NOD1, RIP2 and NF-κB in plasma during pregnancies

Finally, we examined the expression of NOD1, RIP2, and NF-κB in plasma of each group. As shown in Figure 5A, the concentration of NOD1 in plasma of the mature PROM group, preterm PROM group and control group were (8.34±0.16), (6.82±0.11) and (0.92±0.08) ng/mL, respectively. The NOD1 concentration in the preterm PROM group was significantly higher than in the control group (P < 0.01), and the plasma NOD1 protein concentration in the mature PROM group was dramatically increased compared with the preterm PROM group (P < 0.05). The mRNA levels of NOD1, RIP2, and NF-κB in plasma of the mature PROM group were significantly higher than those in preterm PROM group (P < 0.01), and NOD1, RIP2, and NF-κB mRNA contents in preterm PROM group were significantly increased compared with controls (P < 0.01) (Figure 5B-D). In addition, the levels of NOD1, RIP2, and NF-κB mRNA in plasma of the mature PROM group were more moderate than those in placenta and fetal membranes (~3 folds vs. 4-5 folds).

Discussion

For a long time, PROM has been a problem in perinatal medicine, and it is the leading identifiable cause of preterm birth. The cause of PROM has attracted widespread attention since the 1950s. The etiology is complicated and diverse. The relaxation of the cervix, the lack of trace elements, and increase or unevenness of pressure in the amniotic cavity can all lead to PROM. However, it is now widely accepted that microbial infection is the main cause of PROM [5-7]. When an infection occurs, inflammatory cells can be stimulated to produce a variety of pro-inflammatory cytokines such as interleukin-1 and interleukin-6. Furthermore, T and B lymphocytes can be stimulated by interleukin-6, and produce C-reactive protein after differentiation and maturation. C-reactive protein activates an acute inflammatory response, thereby potentiating chemokines and stimulating the production of prostaglandins [8]. Genital tract infections can occur several weeks or months before PROM. Pathogenic microbes from the genital tract cause chorioamnionitis. In addition, the proinflammatory cytokines induce matrix metalloproteinases expression which degrades the fetal membrane extracellular matrix leading to rupture [5, 9]. After rupture, microbes can further aggravate infection, in a vicious circle. Recent studies have demonstrated that infectious factors account for 30%-40% of PROM [10-12]. Shobokshi et al. [13] also confirmed through large-scale data that infection is closely related to PROM.
Host pattern recognition receptor (PRR) detects a variety of pathogen-associated molecular patterns [14], and recent researches in the PRR field have raised our understanding of the NLR family in detecting bacterial infections [15]. NOD1 is a vital member of the NLR family and is widely expressed in many cell types [14, 16]. NOD1 senses microbial pathogens by detecting the conserved structure of the Gram-negative bacterial peptidoglycan (PG) [17, 18]. After activation of PG, NOD1 signals through the caspase activation recruitment domain (CARD) and subsequently interacts with the adaptor protein RIP2 [19-21]. The interaction of NOD1-RIP2 leads to the activation of NF-κB [18] and MAPK [22], which drives the production of inflammatory cytokines such as interleukin-1 and interleukin-8.

In the present study, we found that the NOD1 signaling pathway plays an important role in PROM: immunostaining showed that levels of NOD1 protein in placenta and fetal membrane of mature and preterm PROM groups were significantly higher than in the control group, and the NOD1 level in the mature PROM group was more elevated than that in the preterm PROM group. Western blot revealed that except for NOD1, the expression of RIP2 and nuclear NF-κB protein of placenta and fetal membrane of pregnancies in mature and preterm PROM groups was significantly higher than that in the control group, and the expression of NOD1, RIP2, and NF-κB in the mature PROM group was higher than in the preterm PROM group. Furthermore, RT-qPCR demonstrated that except for the increase in protein expression, the mRNA levels of NOD1, RIP2 and NF-κB in the placenta, fetal membrane, and plasma of pregnancies in mature and preterm PROM groups were significantly higher than those in the control group. Similarly, elevated levels of NOD1, RIP2, and NF-κB mRNA in the mature PROM group were more pronounced compared to the preterm PROM group. ELISA assay showed significantly higher plasma NOD1 in PROM groups than the control group, and the NOD1 concentration in mature PROM was more increased compared with preterm PROM. These results indicate that the NOD1 signaling
pathway is involved in the development of PROM, and the activation of NOD1 signaling pathway in placenta, fetal membrane, and plasma of mature PROM is more dramatic compared with the preterm PROM group. Limitations of our study include the restriction to the NOD1 signaling pathway in PROM rather than plus the NOD2 signaling pathway. Whether inhibition of NOD1 will reduce the incidence of PROM and premature rate still needs further confirmation.

In conclusion, our study reveals for the first time that the NOD1-RIP2-NF-κB signaling pathway is involved in the development of PROM, and its activation is related to gestational age (sharp activation in mature PROM group). The important role of NOD1 signaling pathway in PROM in this study may provide a benefit theoretical basis for early prediction and treatment strategies of PROM.

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Disclosure of conflict of interest

None.

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