The Potential Value of Plasma Receptor Interacting Protein 3 for Neonatal Sepsis Diagnosis

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Research Article

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Running title: Value of Plasma Receptor Interacting Protein 3 in Neonatal Sepsis

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Abstract

Background: Neonatal sepsis is a systemic inflammatory response syndrome in neonates. The molecular mechanism of neonatal sepsis remains incompletely clarified. The purpose of this study was to explore the potential value of receptor interacting protein 3 (RIP3) in neonatal sepsis.

Methods: 93 neonates with sepsis and 93 neonates without infectious diseases were enrolled in this study from September 2019 to March 2021. Plasma RIP3 was detected by enzyme-linked immunosorbent assay (ELISA) and assessed along with whole blood hypersensitive C-reactive protein (hs-CRP) and platelet count (PLT). Differences of RIP3, hs-CRP and PLT between the two groups were compared. Changes of the three indicators in sepsis were also observed after treatment. The diagnostic value of indicators for neonatal sepsis was evaluated by receiver operating characteristic (ROC) curve.

Results: In sepsis group, RIP3 and hs-CRP levels were significantly higher than those
in control group (RIP3, $p < 0.0001$; hs-CRP, $p < 0.0001$), and PLT level was significantly lower than that in control group ($p < 0.0001$). After treatment, RIP3 and hs-CRP levels among septic survivors had a significant decrease ($p < 0.0001$) and PLT level had a significant improvement ($p = 0.0028$). With RIP3 $> 15464.72$ pg/mL, CRP$> 3.24$ mg/L, PLT$< 205.00 \times 10^9$/L as the positive criteria, the sensitivity of the three indicators in the diagnosis of neonatal sepsis was 68.8%, 64.5%, 59.1%, and the specificity was 91.4%, 82.8%, 79.6%, respectively. The combination of RIP3, CRP and PLT showed 76.3% sensitivity and 94.6% sensitivity.

**Conclusions:** RIP3 may attribute to the early diagnosis and understanding therapeutic effect of neonatal sepsis. The combined detection of RIP3, CRP and PLT may be more effective than individual ones in the diagnosis of neonatal sepsis.

**Keywords:** neonate; sepsis; receptor interacting protein 3

**Background**

Neonatal sepsis is the cause of morbidity and mortality in neonatal populations[1-3]. The early diagnosis of neonatal sepsis is challenging because its clinical features are highly variable and may be confused with non-infectious disorders[4, 5]. Missed identification and delayed treatment can rapidly lead to multiple organ dysfunctions[6-8]. Moreover, some survivors of neonatal sepsis may have behavioral and neurocognitive dysfunction, mood disorders, and a low quality of life, which causes a huge burden to the medical system and society[2, 9]. Prompt diagnosis of neonatal sepsis is crucial to improve outcomes and prognosis. However, the early diagnosis of neonatal sepsis remains a serious global problem despite major efforts.

Isolation of causative microorganisms by blood culture is still the gold diagnosis
standard of neonatal sepsis \cite{10}, but it has many limits in practical application for long time-consuming and low sensitivity. In addition, some traditional non-specific indicators, such as white blood cell count (WBC), platelet count (PLT), C-reactive protein (CRP) and procalcitonin (PCT) could not offer sufficient sensitivity or specificity for early identification. Over the past few years, distinct biomarkers have been evaluated, including cell surface antigens, bacterial surface antigens and genetic biomarkers are being investigated. Protein biomarkers, cytokines and chemokines are getting much interest for identification of neonatal sepsis \cite{11, 12}. However, till date there is no single ideal biomarker that has high sensitivity and specificity \cite{1, 11}.

Receptor interacting protein (RIP) 3 belongs to the RIP kinase family \cite{13}. Recent evidence suggests that RIP3-dependent necroptosis is activated in a variety of diseases \cite{14-16}. Stimulated by biological or physicochemical factors, tumor necrosis factor receptor binds to ligand and recruits Fas-associated protein with death domain (FADD), RIP1 and caspase-8 to form complex I, which promotes apoptosis \cite{16}. When caspase-8 activity is inhibited, RIP1 binds to RIP3 and recruits FADD and caspase-8 to form complex II, which suppresses apoptosis and turns into programmed necrosis pathway, leading to cell swelling and rupture \cite{17}. A large number of damage associated molecular patterns (DAMPs) are released, triggering a strong inflammatory response. So far, studies on the predictive value of RIP3 in sepsis are mainly in adult patients. However, the role of RIP3 has not been comprehensively reported in neonatal sepsis.

In this pilot study, we detected the plasma level of RIP3 in children with neonatal sepsis, and compared it with hypersensitive C-reactive protein (hs-CRP) and PLT, in
order to explore its potential value for neonatal sepsis diagnosis.

Methods

Patients and study design

This study recruited patients from September 2019 to March 2021 in the Department of Neonatology of Suzhou Municipal Hospital. Infants who were diagnosed with neonatal sepsis were included. The diagnosis was based on the criteria published by the International Pediatric Sepsis Consensus (Sepsis-2)\(^7\). In brief, the definition of neonatal sepsis includes the confirmed sepsis supported by blood culture and the clinical sepsis with negative blood culture. Other inclusive criteria were as follows: (a) age $< 28$ days; (b) blood routine examination, hs-CRP and blood culture were detected before the use of antibiotics. In addition, neonates admitted for non-infectious diseases were included as controls, including premature infants whose mothers have severe eclampsia or mental disorders, hypoglycemia and breast feeding related jaundice. The baseline data of maternal and perinatal period in the control group were well balanced with those in the sepsis group. The exclusive criteria included congenital malformation, inherited metabolic disease and immunodeficiency. Besides, infants who had incomplete information or with discontinued therapy due to their guardians signed a request to give up treatment were also excluded.

This study was approved by the Medical Ethics Committee of Suzhou Municipal Hospital. All cases were conducted with informed consent by the legal guardians.

Sample collection and data recording
General information including gender, birth order, gestational age, delivery mode, APGAR score, amniotic fluid, birth weight, age of mother, pregnancy status and age at admission were collected.

Before antibiotic treatment, 3 mL of peripheral blood samples were collected from sepsis infants. 2 mL of each sample was stored in the culture bottles (Becton, Dickinson and Company [BD] BACTEC Peds Plus/F and BD BACTEC Lytic/10 Anaerobic/F) and sent to the Bacteria Laboratory for blood culture using BACTEC fx200 blood culture system (BD, Franklin Lakes, USA). The other 1 mL of each sample was detected in the Clinical Laboratory for hs-CRP and blood routine by immune turbidimetry and XE-2100 blood cell analyzer (Sysmex Corporation, Kobe, Japan) separately, and then centrifuged with 1 610 × g for 10 min. The supernatant was collected and stored at -80°C for RIP3 detection. After treatment (about 7 days of effective antibiotics treatment), 1 mL of blood samples were collected for hs-CRP and routine blood test. In the control group, 1 mL of blood samples were taken to detect hs-CRP and blood routine. Plasma was separated and stored by the same method.

The level of RIP3 in plasma was detected using Enzyme linked immunosorbent assay (ELISA) kits (CUSABIO Technology LLC, Wuhan, China), following the manufacturer’s instructions.

**Statistical analysis**

The quantitative data were firstly treated for normal distribution by kolmooorov-smirnov test. Consistent with normal distribution, the data were described as mean ± standard deviation and compared between groups using t test. Meet
non-normal distribution of quantitative data were expressed as median with interquartile range, and compared between groups using non-parametric Mann-Whitney $U$ test. The categorical data were expressed as number (percentage), and compared between groups using the chi-square test or Fisher’s exact test. Data collected from the research were analyzed by SPSS version 22.0 (SPSS, Chicago, IL, USA). GraphPad Prism version 8.3.0 (Graph-Pad, San Diego, CA, USA) was used for preparation of graphs. Receiver operating characteristic (ROC) curve was drawn by MedCalc version 18.2.1 (MedCalc, Ostend, Belgium) to determine the best cut-off value of plasma RIP3 and assess the efficacy of a single index and combined indexes for prediction of neonatal sepsis. The difference with a $p$ value of less than 0.05 was statistically significant.

Results

Patient characteristics

In the initial results, we excluded eight infants who had incomplete information, and three infants whose guardians signed a request to give up treatment within seven days of onset. Eventually 186 infants were included in this study with 93 in the sepsis group and 93 in the control group. In the sepsis group, one child died the next day, and another child was transferred to the children's hospital because of intestinal obstruction. There were no statistically significant differences in maternal, demographic and clinical properties between the two groups (Supplemental Table 1).

Comparison of RIP3, hs-CRP and PLT levels between sepsis group and control group
The levels of RIP3, hs-CRP and PLT in the sepsis group before treatment were 19202(14646~24720) pg/mL, 7(1~25.4) mg/L and 191(111.5~267)×10⁹/L, and those in the control group were 11648(9219~13530) pg/mL, 1(1~3) mg/L and 278(215.5~353)×10⁹/L. Further comparative analysis showed that RIP3 and hs-CRP levels in the sepsis group were significantly higher than those in the control group (p < 0.0001, Fig. 1a and p < 0.0001, Fig. 1b). The levels of PLT were significantly lower compared with control group infants (p < 0.0001, Fig. 1c).

**Comparison of RIP3, hs-CRP and PLT levels in sepsis group before and after treatment**

In the sepsis group, the levels of RIP3, hs-CRP and PLT before treatment were 19202(14646~24720) pg/mL, 7(1~25.35) mg/L and 191(111.5~267)×10⁹/L, and those after treatment were 11411(7973~14158) pg/mL, 1(2~4) mg/L and 240(170-332)×10⁹/L. Furthermore, comparative analysis showed that after effective treatment, RIP3 and hs-CRP levels significantly decreased than those before treatment (p < 0.0001, Fig. 2a and p < 0.0001, Fig. 2b). While the levels of PLT were significantly increased than those before treatment (p =0.0028, Fig. 2c).

**ROC curve analysis of the predictive value of RIP3, hs-CRP and PLT for neonatal sepsis**

ROC curves for the ability of RIP3, hs-CRP and PLT to predict neonatal sepsis were constructed (Fig. 3). The optimal cut-off value for plasma RIP3 level was 15464.72 pg/mL with the area under the curve(AUC) of 0.872. The sensitivity of RIP3 was 68.8% and the specificity was 91.4%. Further comparative analysis showed that the AUC, sensitivity, specificity, positive and negative predictive values, positive probability
ratio and Youden index of hs-CRP and PLT were smaller than those of RIP3 (Table 1).

**ROC curves for the performance of biomarker combinations to predict neonatal sepsis**

ROC curves were plotted to examine the performance of the combinations of RIP3 + CRP, RIP3 + PLT, CRP + PLT and RIP3 + CRP + PLT, respectively (Fig. 4). The AUC, sensitivity, specificity, positive predictive value and negative predictive value of RIP3 + CRP + PLT were 0.898, 73.1%, 97.9%, 97.1% and 78.4%, respectively. These values were higher than those for a single index and other combined indexes (Table 2).

**Discussion**

In the pathological state, homeostasis is maintained by apoptosis, which is generally considered to be a non-inflammatory biological process \(^{[18]}\). Once this process is inhibited, necrosis can be activated as an alternative pathway to cell death, and participates in a series of inflammatory reactions \(^{[19]}\). At present, the mechanisms of necrosis mainly covers programmed necrosis, ferroptosis, inflammatory necrosis and other pathways \(^{[14, 20-22]}\). Previous studies have shown the critical role of programmed necrosis in the regulation of systemic inflammatory response syndrome (SIRS). Linde Duprez *et al.* showed that the deletion of RIP3 reduced the amounts of circulating DAMPs in mice and conferred complete protection against lethal SIRS \(^{[23]}\). Apostolos Polykratis *et al.* had similar results in mice pretreated with the RIP1 inhibitor Necrostatin-1 \(^{[24]}\). In view of the fact that SIRS is the essence of neonatal sepsis, we
speculate that programmed necrosis had specific roles in the pathogenesis of neonatal sepsis.

The latest studies showed that the deficiency in the RIP3 protein attenuated inflammatory response and organ damage of newborn mice with sepsis\(^ {25}\) suggesting that RIP3 may participate in the pathogenesis of neonatal sepsis. Till now, the role of RIP3 in neonatal sepsis has not been reported in clinic. The current study evaluated the clinical value of plasma RIP3 for neonatal sepsis. We demonstrated that the levels of RIP3 in neonatal sepsis group before treatment was significantly higher than those in control group, revealing that plasma RIP3 may be an important biomarker of neonatal sepsis. Our finding is consistent with the reports about the expression of plasma RIP3 in adult patients in intensive care unit. It has been discovered that RIP3 was detectable in the plasma of adult patients with severe sepsis, and the expression of RIP3 in the plasma of the patients with sepsis death was significantly higher than that of the survivors, suggesting that the abnormal production or clearance of RIP3 may predict the poor prognosis of sepsis\(^ {26}\). Another study conducted a prospective study on 953 patients in five intensive care units. It was found that the elevated levels of plasma were associated with in-hospital mortality and organ failure\(^ {27}\), which indirectly confirmed that the high expression of RIP3 could indicate the adverse consequences of critical illness. We further observed plasma RIP3 level in neonatal sepsis group significantly decreased after the effective treatment. It is speculated that RIP3 would be helpful to judge the clinical efficacy and prognosis of neonatal sepsis.

At present, the early diagnosis of neonatal sepsis in practice mainly depends on
non-specific infection indicators such as WBC, PLT, CRP and PCT \cite{28-30}. In this study, hs-CRP and PLT levels were also investigated, and compared with RIP3 to evaluate the value of different indicators in early diagnosis of neonatal sepsis. The results showed that the sensitivity, specificity, positive and negative predictive values, positive probability ratio and Youden index of hs-CRP and PLT were lower than those of RIP3, indicating that RIP3 was a biomarker with high diagnostic efficiency. Previous studies showed that the diagnostic value can be improved by combination of different biomarkers \cite{31-33}. According to this point, further comparison of the diagnostic value of combined multiple indicators for neonatal sepsis were conducted, showing the sensitivity, specificity, positive predictive value and negative predictive value of RIP3+hs-CRP+PLT in the diagnosis of neonatal sepsis were higher than those of RIP3+hs-CRP, RIP3+PLT and hs-CRP+PLT. Meanwhile, the AUC of RIP3+hs-CRP+PLT in the diagnosis of neonatal sepsis was the highest. It is suggested that the combination of RIP3, hs-CRP and PLT was more robust diagnostic performance for neonatal sepsis.

The deficiencies of this preliminary study are as follows: Firstly, newborns are prone to iatrogenic anemia, so the amount of blood collection was relatively small, which was not enough to be detected multiple indicators. The differences of RIP3 and many other biomarkers like PCT and IL in the diagnosis of neonatal sepsis were unclear. Secondly, because all newborns once diagnosed with sepsis were actively treated with antibiotics, there was no untreated sepsis as a control study. The clinical intervention factor of antibiotic treatment had not been deeply studied. Thirdly, there
was a lack of monitoring at multiple time points to explore the dynamic expression of different indicators in neonatal sepsis. Besides, due to ethical limitations, it was difficult to obtain blood samples of newborns under physiological conditions in practice, and the sample size was small, so the comparison of RIP3 expression between different gestational age and different body weight under physiological conditions was unknown. Lastly, this is a single center preliminary study with a small sample size, our findings need to be confirmed by long-term and large-scale multicenter studies.

In conclusion, RIP3 may attribute to the early diagnosis and understanding therapeutic effect of neonatal sepsis. The combined detection of RIP3, CRP and PLT may be more effective than individual ones in the diagnosis of neonatal sepsis.

Declaration

Ethics approval and consent to participate

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration. This study has been approved by the ethics committee of Suzhou Municipal Hospital (K-2019-013-H01) and written informed consent was obtained from each patient’s legal guardians before collection of sample.

Consent for publication

No applicable.

Availability of data and materials

The data and materials supporting the conclusions of the study are available from the
corresponding author on reasonable request.

**Competing interests**

The authors declare no conflicts of interest.

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**Authors’ contributions**

GCC, FZT and WLX had primary responsibility for the study design, data analysis and manuscript preparation. ZXX participated in the sample collection and prepared the draft manuscript. FK helped with clinical data analysis. MSR helped in conducting the ELISA experiments. YZM helped in the data analysis. WSN and YSL contributed to the study design and provided valuable suggestions for conducting the experiments. All authors read and approved the final manuscript.

**Acknowledgments**

None.

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Figure. 1 Quantification of RIP3, hs-CRP and PLT levels in the sepsis group and control group. BT: before treatment. *** $p<0.0001$

Figure. 2 Quantification of RIP3, hs-CRP and PLT levels in the sepsis group and control group. BT: before treatment; AT: after treatment. *** $p<0.0001$, ** $p<0.01$

Figure. 3 ROC curve for the ability of RIP3, hs-CRP and PLT quantification to predict neonatal sepsis

Figure. 4 ROC curve for the ability of different combinations of RIP3, hs-CRP and PLT to predict neonatal sepsis

Table 1. Predictive value of RIP3, hs-CRP and PLT for neonatal sepsis

| Variable   | RIP3(pg/mL) | hs-CRP(mg/L) | PLT($\times 10^9$/L) |
|------------|-------------|--------------|-----------------------|
| Cut-off value | 15464.72    | 3.24         | 205.00                |
| AUC        | 0.872       | 0.765        | 0.708                 |
| Sensitivity | 68.8        | 64.5         | 59.1                  |
| Specificity | 91.4        | 82.8         | 79.6                  |
| PPV        | 88.9        | 78.9         | 74.3                  |
| NPV        | 74.6        | 70.0         | 66.1                  |
| PPR        | 8.00        | 3.75         | 2.89                  |
| NPR        | 0.34        | 0.43         | 0.51                  |
| 95%CI      | 0.815-0.917 | 0.698-0.824  | 0.637-0.772           |
| YI         | 0.602       | 0.473        | 0.387                 |

AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; PPR: positive probability ratio; NPR: negative probability ratio; YI: Youden index
Table 2. Predictive value of biomarker combinations for neonatal sepsis

| Variable       | RIP3+hs-CRP | RIP3+PLT | hs-CRP+PLT | RIP3+hs-CRP+PLT |
|----------------|-------------|----------|------------|-----------------|
| AUC            | 0.898       | 0.877    | 0.808      | 0.902           |
| Sensitivity    | 73.1        | 71.0     | 61.3       | 76.3            |
| Specificity    | 97.9        | 91.4     | 89.3       | 94.6            |
| PPV            | 97.1        | 89.2     | 85.1       | 93.4            |
| NPV            | 78.4        | 75.9     | 69.7       | 80.0            |
| PPR            | 34.00       | 8.25     | 5.70       | 14.20           |
| NPR            | 0.27        | 0.32     | 0.43       | 0.25            |
| 95%CI          | 0.854-0.942 | 0.829-0.926 | 0.745-0.871 | 0.858-0.946  |
| YI             | 0.710       | 0.624    | 0.505      | 0.710           |

AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; PPR: positive probability ratio; NPR: negative probability ratio; YI: Youden index

Supplementary file

Supplemental Table 1. Characteristics of infants in the sepsis group and control group
Figure 1

Quantification of RIP3, hs-CRP and PLT levels in the sepsis group and control group. BT: before treatment. *** p<0.0001
Figure 2

Quantification of RIP3, hs-CRP and PLT levels in the sepsis group and control group. BT: before treatment; AT: after treatment. *** p<0.0001, ** p<0.01
Figure 3

ROC curve for the ability of RIP3, hs-CRP and PLT quantification to predict neonatal sepsis
Figure 4

ROC curve for the ability of different combinations of RIP3, hs-CRP and PLT to predict neonatal sepsis

Supplementary Files

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- SupplementalTable1.docx