Prospective Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
48(3) 1–10

DOI: 10.1177/0300060519884500
journals.sagepub.com/home/imr
SAGE

NOD-like receptor protein 3 and high mobility group box-1 are associated with prognosis of patients with congenital heart disease

Jiajie Fan[1], Yunxiang Qiu[1], Zhijie Zheng[1], Luyan Yu[2], Shanshan Shi[1] and Xiujing Wu[1]

Abstract

Objective: To investigate the association between plasma levels of nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3 (NLRP3) and high mobility group box-1 (HMGB1) and their prognostic significance in neonatal patients with congenital heart disease (CHD).

Methods: This study enrolled neonatal patients with CHD and collected their demographic and clinical data. Plasma concentrations of NLRP3 and HMGB1 were measured using enzyme-linked immunosorbent assays. Spearman's analysis was used to determine the correlation between NLRP3 and HMGB1 levels. The association between NLRP3 and HMGB1 levels and 2-year survival and mortality were evaluated using Kaplan–Meier curve and logistic regression analyses.

Results: A total of 84 neonatal patients with CHD were included in the study. Plasma NLRP3 and HMGB1 levels were significantly higher in deceased patients compared with those that survived. There was a positive correlation between NLRP3 and HMGB1 levels in neonatal patients with CHD. Patients with elevated levels of NLRP3 and HMGB1 showed significantly lower 2-year survival and higher mortality rates compared with those with lower NLRP3 and HMGB1 levels.

Conclusion: Neonatal patients with CHD and a poor prognosis had higher NLRP3 and HMGB1 levels, which suggests that these might be potential biomarkers of CHD prognosis.

[1]Cardiac Intensive Care Unit, the Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China
[2]Department of Burns and Plastic Surgery, The Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China

Corresponding author:
Luyan Yu, Department of Burns and Plastic Surgery, The Children's Hospital of Zhejiang University School of Medicine, 3333 BinSheng Road, Hangzhou, 310031, Zhejiang Province, China.
Email: lunyanyu12@126.com

Keywords
Congenital heart disease, NOD-like receptor protein 3, high mobility group box-1, children

Date received: 20 June 2019; accepted: 3 October 2019

Introduction

Congenital heart disease (CHD) is one of the most common congenital diseases with high morbidity and mortality, particularly in developing countries.[1] The prevalence of CHD has been reported to be approximately 1% in newborns worldwide.[2] It is generally considered that traditional cardiac surgery to treat cyanotic CHD is associated with risks and has a poor prognosis.[3] Accumulative evidence has demonstrated that multiple biomarkers, including cytokines, tissue factors and metabolic factors are involved in the occurrence and progression of CHD.[4,5] Measuring serum biomarker levels facilitates the diagnosis and prognosis of CHD. For example, serum homocysteine levels are known to be a significant biomarker of cardiovascular damage during the metabolism of sulphur-containing amino acids.[6]

It is well known that the innate immune response is the host's first line of defence against invasion by foreign pathogens. When the body is attacked by pathogens, one or more pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are non-specifically identified by pattern-recognition receptors (PRRs), which in turn activate a downstream signal transduction pathway that triggers cytophagy and an inflammatory reaction, thereby producing an adaptive immune response.[7] The PRRs identified to date mainly include cytomembrane-located Toll-like receptors, C-type lectin receptors, cytoplasm-located nucleotide-binding oligomerization domain-like (NOD)-like receptors and retinoic acid-inducible gene-I-like receptors.[8] Inflammasomes are intracellular multiprotein cytoplasmic complexes that play a central role in inflammatory diseases.[9] The NOD-like receptor protein 3 (NLRP3) inflammasome is a multiprotein complex expressed in macrophages and dendritic cells,[10] which can be activated by PAMPs from bacteria, fungi, viruses and DAMPs to trigger the assembly of the NLRP3/apoptosis-associated speck-like protein containing a CARD (ASC)/pro-cysteinyl aspartate-specific protease-1 (pro-caspase-1) inflammasome complex.[11]

High-mobility group box 1 (HMGB1) is an abundant protein that has been shown to be involved in the pathogenesis of vascular inflammation.[12] Recently, several studies have strongly suggested that endogenous HMGB1 released by necrotic cells can serve as a DAMP, leading to the activation of the canonical NLRP3 inflammasomes in several inflammatory diseases.[13,14] In addition, a previous study has shown that serum HMGB1 levels were prominently elevated in patients with pulmonary arterial hypertension (PHA) secondary to CHD.[15] However, the association between NLRP3 and HMGB1 in the prognosis CHD has not been reported.

The present study measured the plasma NLRP3 and HMGB1 levels in neonatal patients with CHD in order to investigate the difference in clinical parameters and prognosis in patients stratified according to their levels of plasma NLRP3 and HMGB1.

Patients and methods

Participants and data collection

This prospective study enrolled consecutive neonatal patients with a diagnosis of CHD according to the clinical classification criteria of the European Society of Cardiology,[16] who were admitted to the Cardiac Intensive Care Unit, the Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China for cardiac surgery between July 2011 and December 2012. The exclusion criteria were as follows: (i) neonates without a diagnosis of CHD; (ii) neonates aged >3 years; (iii) neonates that died during admission; (iv) those with a neurological event post-surgery or those in whom cardiac surgery was not performed; (v) patients complicated by other congenital malformations, coronary atherosclerotic heart disease, inherited metabolic diseases, infection and rheumatoid connective tissue diseases. All patients were stratified according to their outcome into two groups: deceased group and survived group. Clinical and demographic characteristics (e.g. age, sex, weight) were recorded for each patient.

All experimental protocols were approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, China. Written informed consent was obtained from the parents or legal guardians of the study participants.

Measurement of plasma NLRP3 and HMGB1

Blood samples were collected from all participants at hospital admission. Briefly, 5-ml blood samples from each patient were collected in ethylenediaminetetraacetic acid-containing tubes and then centrifuged at 1000 *g* for 10 min at 4°C (TDL-40B centrifuge; Shanghai Anting Scientific Instrument Factory, Shanghai, China). Plasma samples were stored at −70°C. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to determine the plasma levels of the following antigens: NLRP3 (minimum detectable concentration 0.781 ng/ml, sensitivity 0.469 ng/ml; Uscn Life Science Inc., Wuhan, China); HMGB1 (minimum detectable concentration 2.5 ng/ml, sensitivity 1 ng/ml; Shino-Test Corporation, Kanagawa, Tokyo, Japan); N-terminal pro-brain natriuretic peptide (NT-pro-BNP; minimum detectable concentration 30.9 ng/l, sensitivity 12.2 ng/l; LifeSpan BioSciences, Seattle, WA, USA); high-sensitivity troponin T (hsTnT; minimum detectable concentration 0.156 ng/ml, sensitivity 0.039 ng/ml; Life Science Inc.); and C-reactive protein (CRP; minimum detectable concentration 1 μg/ml, sensitivity 1 μg/ml; Life Science Inc.) on an Elecsys 300 analyser (Roche Diagnostic Systems, Branchburg, NJ, USA) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation for all ELISAs were <8% and <6%, respectively.

Follow-up

All patients were followed up for long-term major cardiovascular events (MACE) including cardiac death, hospitalization, new onset of arrhythmias and surgical or percutaneous intervention. For survival analysis, all-cause death for all patients was recorded and the follow-up lasted for 2 years via clinical visits or telephone interviews.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 18.0 (SPSS Inc., Chicago, IL, USA)

for Windows®. Continuous data are expressed as mean ± SD if normally distributed, or as median (min–max) if otherwise, and they were compared using Student's *t*-test or Mann–Whitney *U*-test. Categorical data were compared using χ^2-test. Spearman's analysis was used to determine the correlation between the plasma levels of NLRP3 and HMGB1. Kaplan–Meier curve analysis was undertaken to compare survival and the prediction of MACE between groups. The relationship between the plasma levels of NLRP3 and HMGB1 and 2-year mortality was analysed using a logistic regression model. A *P*-value <0.05 was considered to be statistically significant.

Results

This prospective study enrolled 84 neonatal patients with CHD, which were divided into a deceased group ($n = 11$) and a survived group ($n = 73$). The clinical and demographic characteristics of the patients are summarized in Table 1. Compared with the patients that survived, the deceased patients showed significantly higher plasma levels of NLRP3, HMGB1 and NT-pro-BNP ($P < 0.01$ for all comparisons), while there were no significant differences among the other parameters. Spearman's correlation analysis demonstrated that the plasma HMGB1 level was positively correlated with the plasma NLRP3 level in neonatal patients with CHD ($r = 0.615$; $P < 0.001$) (Figure 1).

To further determine the underlying roles of NLRP3 and HMGB1 in CHD, neonatal patients with CHD were stratified into high and low groups based on the median plasma levels of NLRP3 (23 ng/ml) and HMGB1 (25 ng/ml) (Table 2). Neonatal patients with elevated plasma levels of NLRP3 or HMGB1 had significantly higher rates of 2-year MACE including cardiac death, hospitalization, new onset of arrhythmias and surgical or percutaneous intervention than patients with lower plasma NLRP3 or HMGB1 levels ($P < 0.05$ for all comparisons).

Kaplan–Meier survival curves revealed that neonatal patients with higher NLRP3 or HMGB1 levels showed a lower survival rate than those with lower levels of NLRP3 ($P < 0.001$) or HMGB1 ($P < 0.001$) based

Table 1. Baseline clinical and demographic characteristics of neonatal patients ($n = 84$) with congenital heart disease stratified according to their outcome.

Characteristic	All patients $n = 84$	Deceased $n = 11$	Survived $n = 73$	Statistical significance[a]
Gestational age, weeks	39.0 ± 1.2	38.4 ± 1.3	39.1 ± 1.3	NS
Male:female	51:33	7:4	44:29	NS
Weight, kg	3.5 ± 0.4	3.4 ± 0.6	3.3 ± 0.5	NS
NLRP3, ng/ml	23 (12–58)	38 (26–58)	20 (12–29)	$P < 0.001$
HMGB1, ng/ml	25 (14–65)	47.5 (29–65)	21 (14–32)	$P < 0.001$
NT-pro-BNP, ng/l	511 (124–1532)	993 (701–1532)	386 (124–594)	$P = 0.005$
hsTnT, ng/ml	0.412 (0.193–0.615)	0.562 (0.347–0.615)	0.463 (0.193–0.12)	NS
CRP, mg/l	7 (3–16)	10 (5–16)	6 (3–11)	NS

Data presented as mean ± SD, *n* of patients or median (min–max).

[a]Between-group comparison: continuous data were compared using Student's *t*-test or Mann–Whitney *U*-test; and categorical data were compared using χ^2-test.

NLRP3, nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3; HMGB1, high-mobility group box 1; NT-pro-BNP, N-Terminal Pro-Brain Natriuretic Peptide; hsTnT, high-sensitivity troponin T; CRP, C-reactive protein; NS, no significant between-group difference ($P \geq 0.05$).

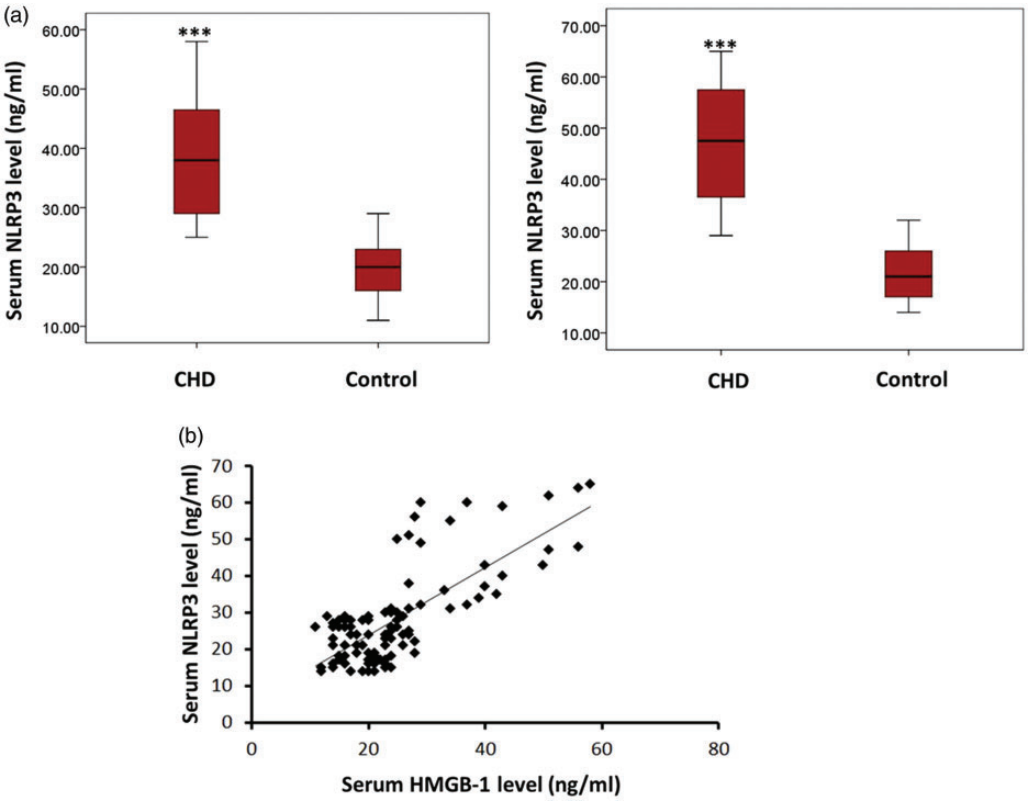

Figure 1. Spearman's correlation analysis between plasma levels of nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3 (NLRP3) and high-mobility group box 1 (HMGB1) in neonatal patients with congenital heart disease.

Table 2. The occurrence of 2-year major cardiovascular events (MACE) in neonatal patients ($n = 84$) with congenital heart disease stratified according to the plasma levels of nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3 (NLRP3) and high-mobility group box 1 (HMGB1).

MACE	Low NLRP3 group $n = 48$	High NLRP3 group $n = 36$	Low HMGB1 group $n = 41$	High HMGB1 group $n = 43$
Cardiac death	2 (4.2)	9 (25.0)*	3 (7.3)	8 (18.6)#
Hospitalization	14 (29.2)	22 (61.1)*	11 (26.8)	25 (58.1)#
New onset of arrhythmias	24 (50.0)	27 (75.0)*	15 (36.6)	36 (83.7)#
Surgical or percutaneous intervention	16 (33.3)	24 (66.7)*	13 (31.7)	27 (62.8)#

Data presented as *n* of patients (%).
*$P < 0.05$ compared with the lower NLRP3 group; #$P < 0.05$ compared with the lower HMGB1 group; categorical data were compared using χ^2-test.

on log rank test (Figure 2). Multivariate analysis demonstrated that plasma NLRP3 (odds ratio [OR] 2.159, 95% confidence interval [CI] 1.309, 3.561; $P = 0.003$) and HMGB1 (OR 2.726, 95% CI 1.221, 6.038; $P = 0.014$) were both independent risk factors for 2-year mortality (Table 3).

Discussion

Congenital heart disease is the most common birth defect, which is characterized by structural and functional abnormalities of the blood vessels caused by genetic and environmental factors.[17,18] Children with CHD have aberrant haemodynamics, including cyanosis, PHA and abnormalities of the vertebral artery, which in turn lead to serious complications such as arrhythmias, cardiac dysfunction or heart failure.[19,20] Several blood biomarkers have been proposed for the diagnosis and prognosis of cardiovascular disease (CVD). For example, creatine kinase isoenzyme is a crucial biomarker for the early diagnosis and prognosis of acute myocardial infarction.[21] Moreover, serum asymmetric dimethylarginine concentration is associated with the severity of early-onset coronary artery disease.[22] Nevertheless, little is known about cardiac biomarkers in children with CHD.

N-terminal pro-brain natriuretic peptide is a novel biomarker of cardiac dysfunction.[23] Under normal physiological conditions, serum NT-pro-BNP levels diminish with increasing age during childhood.[24]

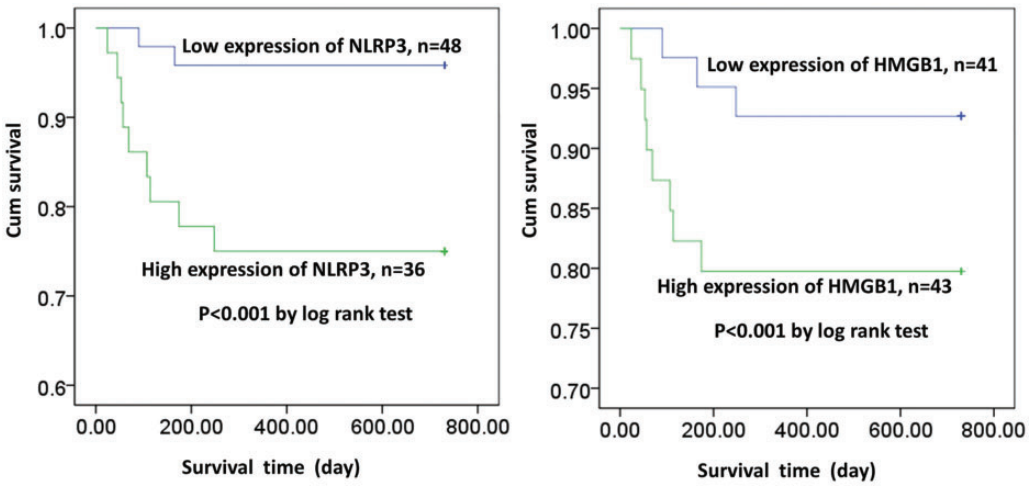

Figure 2. Kaplan–Meier survival curves of neonatal patients with congenital heart disease stratified according to the plasma levels of nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3 (NLRP3) and high-mobility group box 1 (HMGB1).

Table 3. Multivariate analysis of the relationship between plasma levels of nucleotide-binding oligomerization domain-like (NOD)-like receptor protein 3 (NLRP3) and high-mobility group box 1 (HMGB1) and 2-year mortality in neonatal patients with congenital heart disease ($n = 84$).

	Wald	Odds ratio	95% confidence interval	Statistical significance
NLRP3	9.078	2.159	1.309, 3.561	$P = 0.003$
HMGB1	5.992	2.726	1.221, 6.038	$P = 0.014$

However, NT-pro-BNP can be secreted by cardiac atrium or ventricular myocytes in response to cardiac overload or myocardial ischaemia.[25] A previous study reported that high levels of NT-pro-BNP in the cord blood of neonates with CHD were associated with 1-year survival.[26] The serum levels of NT-pro-BNP in survivors were significantly lower than those of deceased patients.[27] In addition, patients with a higher level of NT-pro-BNP had a lower long-term survival rate.[27] In this present study, the plasma NT-pro-BNP level was downregulated in neonatal patients with CHD that survived compared with deceased patients, suggesting that NT-pro-BNP is a cardiac biomarker in children with CHD. In addition, cardiac hsTnT is a specific marker of myocardial injury.[28] There is growing evidence of the importance of hsTnT in CHD progression. For example, serum hsTnT levels were positively correlated with brain natriuretic peptide in adult patients with CHD.[29] Patients with CHD-PAH with elevated levels of hsTnT showed a significantly higher mortality rate compared with those with normal hsTnT levels.[30] In the present study, there was no significant difference in the hsTnT level between those patients that survived and those that died, suggesting that the hsTnT level might not be associated with the prognosis of neonatal patients with CHD.

Inflammasomes, composed of intracellular PRRs, ASC and caspase-1, are molecular platforms built around several proteins, which mainly serve as the cellular machinery triggering the maturation of proinflammatory cytokines to participate in innate immune defences.[31] Among them, NLRP3 is the most intensively studied inflammasome in the pathogenesis of CVD.[32] A previous study demonstrated that NLRP3 and downstream cytokines were highly expressed in atherosclerotic lesions in patients with atherosclerosis.[33] A study in NLRP-knockout mice observed an increased infarct size following ischaemia/reperfusion.[34] This current study found that plasma NLRP3 levels were significantly higher in deceased patients compared with patients that survived, which suggests that elevated plasma NLRP3 levels might be a prognostic marker in neonatal patients with CHD.

High mobility group box-1 is identified as a non-chromosomal nuclear protein that is highly conserved among most mammals.[35] A growing body of evidence suggests that plasma HMGB1 functions as a mediator in inflammatory responses that result in worsening cardiac dysfunction and remodelling. For example, elevated HMGB1 levels were observed in patients with cerebral and myocardial ischaemia and patients with CHD-PAH.[15,36] In this current study, HMGB1 levels were significantly increased in deceased patients with CHD compared with those patients that survived and the levels were positively correlated with the plasma NLRP3 levels. Patients with higher plasma levels of NLRP3 and HMGB1 were more likely to experience MACE including cardiac death, hospitalization, new onset arrhythmias and surgical or percutaneous intervention. More importantly, the plasma levels of NLRP3 and HMGB1 were strongly associated with the risk of death.

This current study had several limitations. First, although this study had a relatively large sample size compared with other related studies in the literature, the sample size was small for the stratified analyses. Secondly, the baseline evaluation and classification of the patient groups were made according to clinical assessment and echocardiographic findings. Thirdly, longer follow-up information on the patients would have improved the study. Therefore, a larger multicentre study using a longer follow-up period is required to confirm these current findings.

In conclusion, this current study demonstrated significantly higher plasma NLRP3 and HMGB1 levels in deceased patients with CHD than in those patients that survived. A significant inverse association was observed between plasma NLRP3 and HMGB1 levels and survival. Therefore, insight into the function of NLRP3 and HMGB1 might provide a novel preventive and therapeutic approach for patients with CHD.

Author contributions

Jiajie Fan conducted most of the experiments and wrote the manuscript; Yunxiang Qiu and Zhijie Zheng conducted the experiments and analysed the data; Luyan Yu designed the study and revised the manuscript; and Shanshan Shi and Xiujing Wu reviewed and revised the manuscript.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ORCID iD

Jiajie Fan https://orcid.org/0000-0003-0258-3628

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