Toll-Like Receptors 2 and 4 Predict New-Onset Atrial Fibrillation in Acute Myocardial Infarction Patients

Ping Zhang,^{1*} MD, Liang Shao,^{2*} MD and Jun Ma,² MD

Summary

Myocardial infarction (MI) can cause new-onset atrial fibrillation (AF) due to cardiac remodeling. As a recent study has shown, inflammatory factors are closely tied to cell death and survival in myocardial ischemia injury. Toll-like receptors (TLRs) have been shown to participate in the process of myocardial infarction as innate immune factors.

The subjects were divided into 3 groups: healthy controls ($n = 82$), MI patients ($n = 84$), and AFMI (new-onset atrial fibrillation after myocardial infarction) patients ($n = 85$). Peripheral blood mononuclear cell (PBMC) TLR mRNA expression was detected by rt-PCR. Western blot was used to analyze PBMC TLRs and their downstream signal protein expression. PBMCs were presented as TLR2 expression or TLR4 expression using flow cytometry.

From mRNA to protein detection, PBMC TLR2 and TLR4 were significantly higher in the AFMI group than in the control group and MI group. A similar tendency was also observed in the expression of downstream signaling proteins. When further analyzed with TLR2 and TLR4 antibodies by flow cytometry, PBMC levels also appeared to be higher in AFMI patients than those in MI patients and the healthy control group.

In our study, PBMC TLRs and their downstream signaling proteins were significantly higher in the acute myocardial infarction patients with new-onset atrial fibrillation compared with healthy people and acute myocardial infarction patients without new-onset atrial fibrillation. They have the potential to be novel biomarkers for new-onset atrial fibrillation after acute myocardial infarction.

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Key words: Innate immune

As one component of the global burden of cardiac arrhythmia disease, atrial fibrillation (AF) is generally divided into valvular and non-valvular AF. Myocardial infarction (MI) is an independent risk factor for new-onset AF which occurs in 6% to 21% of acute MI patients due to post myocardial infarction remodeling.¹⁻⁵⁾ Unfortunately, AF is highly associated with substantial morbidity and mortality increases after myocardial infarction.⁶⁻⁸⁾

while TLR4 deficiency has been shown to have a protective effect against myocardial ischemic injury.^{15,16)} Furthermore, there is some evidence for TLR4 in ventricular remodeling after acute myocardial infarction.^{17,18)} However, the effect of TLR2 on heart disease remains controversial. In TLR2 knock-out mice, there was no obvious difference in infarct size compared to wild type mice, although a negative effect on fibrosis in the non-infarcted area and a positive effect on left ventricular remodeling have been reported.^{19,20)} However, TLR2 also has been found to trigger atrial fibrillation and fatal arrhythmias in ischemia reperfusion.²²⁻²⁴⁾

In this study, we investigated TLR2 and TLR4 expression and found they are a potential predictor of new-onset atrial fibrillation after myocardial infarction.

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Innate immune factors play important roles in most cardiac diseases.⁹⁻¹¹⁾ As data has shown in recent research, toll-like receptors increase in either plasma or tissues as innate immune factors in ischemia heart diseases and arrhythmias diseases.¹²⁻¹⁴⁾ Notably, TLR4 could directly stimulate macrophages and induce atheroma formation,

From the ¹Department of Geriatrics & Neurology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China and ²Department of Cardiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China.

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Address for correspondence: Ma Jun, MD, Department of Cardiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, No. 109 Xueyuanwest Road, Wenzhou, Zhejiang 326000, China. E-mail: henrymuch@163.com or Shao Liang, MD, Department of Cardiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, No. 109 Xueyuanwest Road, Wenzhou, Zhejiang, 326000, China. E-mail: shaoliang021224@hotmail.com

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Methods

Population characteristics: All volunteers were recruited from the Department of Cardiovascular Medicine, First Affiliated Hospital of Medical College of Xi'an Jiaotong University and the Department of Cardiology, The Second Affiliated Hospital of Wenzhou Medical University. The study was approved by the ethics committee of Xi'an Jiaotong University and conformed to the principles of the Declaration of Helsinki. Informed consent was obtained from each patient.

MI was diagnosed according the guidelines for Chinese coronary artery disease therapy (2015 edition) based on a rise in cardiac troponin levels, typical symptoms (chest pain), and a typical electrocardiogram (pathological Q waves, ST elevation or depression).

The subjects were divided into the following groups: healthy control group (n=82); myocardial infarction group without arrhythmia (MI group, n=84); and new-onset atrial fibrillation (in 1 month after reperfusion) after myocardial infarction group (AFMI group, n=85). Blood samples were collected from a radial artery before any reperfusion therapy within 2 hours after MI onset.

Exclusion criteria included fast arrhythmia disease history, heart valve disease, neoplastic disease, advanced liver or renal disease, and infection or autoimmune or immunodeficiency disease.

TLR2 and TLR4 mRNA detection: Blood samples were mixed with Ficoll-Paqueplus reagent (Sigma, China) and then centrifuged for 20 minutes at 3000 g. Peripheral blood mononuclear cells (PBMCs) were extracted from the intermediate mixture, and washed with phosphate buffer saline (PBS). TRIzol (Invitrogen Corp, Carlsbad, CA, USA) was used to extract RNA from PBMCs for storage at -80°C. The total RNA (400 ng) was reverse transcribed using a First Strand cDNA Synthesis Kit (Fermentas MBI, CA). TLR2, TLR4, and β -actin mRNA expression was detected according to a two-step real-time RT-PCR method. The TLR2 forward and reverse sequences were 5'-TTG TGC CCA TTG CTC TTT TCA C-3' and 5'-TGG CAT TGT CCA GTG CTT CA-3'. The forward and reverse sequences of TLR4 were 5'-TGC GGG TTC TAC ATC AAA-3' and 5'-CCA TCC GAA ATT ATA AGA AA AGT C-3'. In β -actin, they were 5'-AGC CTC GCC TTT GCC GA-3' and 5'-CTG GTG CCT GGG GCG-3'. Thermal cycling consisted of 95°C for 5 minutes and 40 cycles of 95°C for 30 s, 56°C for 20 s, 56°C for 20 s, and 72°C for 30 s. PCR was performed using an IQ5 Multicolor Real-Time PCR Detection System (BIO-RAD, USA). Results are presented as TLR2 mRNA level/ β -actin mRNA level and TLR4 mRNA level/ β -actin mRNA level after $2^{-\Delta\Delta CT}$ method analysis.

Western blotting: Blood samples were mixed with Ficoll-Paqueplus reagent (Sigma, China) and then centrifuged for 20 minutes at 3000 g. Peripheral blood mononuclear cells (PBMCs) were extracted from the intermediate mixture and washed with phosphate buffer saline (PBS). TRIzol (Invitrogen Corp, Carlsbad, CA, USA) was used to extract protein from PBMCs for storage at -80°C. Proteins in the samples were purified and quantified with a BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai,

China). Pre-cast any-kd SDS-PAGE gels were used to separate sample protein which was homogenized in a loading buffer. Next, the immunoblots were transferred to a polyvinylidene difluoride membrane and blocked in 5 % skim milk. Anti-human TLR2 antibody (ab1655, Abcam, USA) diluted 1:1000, anti-human TLR4 antibody (bs3489, Bioworld Technology, Inc., St. Louis Park, MN, USA) diluted 1:1000, anti-human myeloid differentiation factor 88 (MyD88, bs3521, Bioworld Technology, Inc., USA) antibody diluted 1:1000, anti-human TIR domain-containing protein- β antibody (TRIF- β , ab13810, Abcam, USA) diluted 1:1000, and anti-human interferon regulatory factor 3 antibody (IRF-3, ab21680, Abcam, USA) diluted 1:1000 were used as primary antibodies in a 4°C room overnight. Corresponding secondary antibody was used as appropriate before exposure. Immunoreactive bands were visualized in the dark with an enhanced chemiluminescence kit (Amersham, Sweden). A densitometer analysis system (Quantity One V4.62) was used to measure band intensities. Equal protein loading was confirmed by staining the gel with Ponceau S and probing with β -actin antibody (Abcam). The results are shown as the grey scale ratio of target protein to β -actin.

Flow-cytometer analysis: Blood samples were mixed with Ficoll-Paqueplus reagent (Sigma, China) and then centrifuged for 20 minutes at 3000 g. Peripheral blood mononuclear cells were extracted from the intermediate mixture, washed with phosphate buffer saline (PBS), and then frozen at -80°C. Anti-human TLR2 antibody (ab9101, Abcam, USA) diluted 1:50 and anti-human TLR4 antibody (ab30667, Abcam, USA) diluted 1:50 were used as primary antibodies and then reacted with the corresponding fluorescent secondary antibody. Side scatter was set for the TLR2 level and forward scatter was set for the TLR4 level.

Statistical analysis: SPSS 13.0 software was used to analyze the data, which are presented as the mean $\pm$ SD or as the median and interquartile range. The one-way ANOVA method followed by Scheffe's test was performed to compare continuous data among the groups. A P value < 0.05 was regarded as a statistically significant difference.

Results

Basic population characteristics: The basic population characteristics are presented in the Table. There were no significant differences in age, gender, smoking, drinking, or echocardiographic findings ($P > 0.05$), while there were significant differences in b-LDH, CK-MB, cTnI, and hs-CRP among the 3 groups ($P < 0.05$). Other than routine medical treatment, there was no further treatment in the control group. Blood samples were obtained before reperfusion therapy, and with aspirin 300 mg, plavix 300 mg, and statin administration in the MI group and AFMI group.

TLRs mRNA expression: RT-PCR analysis (Figure 1) revealed TLR2 and TLR4 mRNA expressions were lower in the control group than in the MI group (TLR2, $P = 0.027$; TLR4, $P = 0.032$) and AFMI group (TLR2, $P = 0.017$; TLR4, $P = 0.021$). Furthermore, the TLR2 mRNA level

Table. Basic Clinical Characteristics of the Study Subjects

	Control (n = 42)	MI (n = 44)	AFMI (n = 45)	P
Age	52 ± 3	53 ± 4	52 ± 3	0.873
Gender (M/F)	21:21	22:22	22:23	0.891
Smoking	22 (52%)	22 (50%)	22 (48%)	0.724
Drinking	28 (66%)	29 (65%)	29 (64%)	0.717
Hypertension	26 (64%)	28 (64%)	29 (64%)	0.914
Diabetes mellitus	15 (36%)	16 (37%)	19 (42%)	0.673
BMI (kg/m ²)	22.74 ± 1.16	21.89 ± 1.22	23.08 ± 1.07	0.542
WHR	0.82 ± 0.11	0.79 ± 0.09	0.81 ± 0.12	0.662
TC (mmol/L)	3.33 ± 0.41	3.19 ± 0.28	3.26 ± 0.57	0.718
TG (mmol/L)	1.09 ± 0.14	1.16 ± 0.13	1.22 ± 0.21	0.426
HDL-cholesterol (μmol/L)	1.47 ± 0.28	1.52 ± 0.22	1.36 ± 0.31	0.477
LDL-cholesterol (μmol/L)	2.08 ± 0.17	2.12 ± 0.22	1.97 ± 0.21	0.536
BUN (mmol/L)	4.92 ± 1.35	5.07 ± 1.23	5.11 ± 1.42	0.491
MDRD-eGFR (mL/minute/1.73 m ²)	65.35 ± 5.41	64.13 ± 5.33	66.52 ± 5.62	0.824
b-LDH (IU/L)	132.31 ± 27.68	241.14 ± 34.79	236.58 ± 39.22	0.014
CK-MB (IU/L)	7.27 (4.52, 9.43) Max 11.47	10.38 (6.14, 14.37) Max 16.53	11.42 (7.71, 13.12) Max 17.32	0.018
cTnI (ng/mL)	0.93 (0.47, 1.25)	2.54 (2.17, 2.98)	2.62 (2.25, 3.01)	0.011
hs-CRP (mg/L)	4.27 (2.03, 6.18)	9.42 (4.87, 14.62)	9.38 (4.94, 15.33)	0.009
WBC (×10 ⁹ /L)	7.45 ± 1.33	6.82 ± 1.31	8.01 ± 1.61	0.467
NT-proBNP (ng/L)	321.56 ± 43.75	353.12 ± 61.33	349.91 ± 39.01	0.322
UCG index				
LVEF (%)	65.12 ± 8.63	60.44 ± 4.13	62.57 ± 7.31	0.332
E/A ratio	1.32 ± 0.26	1.41 ± 0.21	1.38 ± 0.25	0.457
LVDd (mm)	51.42 ± 4.16	52.11 ± 3.87	50.96 ± 3.67	0.526
LVFS (%)	27.15 ± 5.44	28.66 ± 5.17	27.63 ± 5.19	0.713

All data are presented as mean ± SD or *n* (%) or median and interquartile range. BMI indicates body mass index; WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; b-LDH, blood lactate dehydrogenase; BUN, blood urea nitrogen; SCr, serum creatinine; MDRD-eGFR, modification of diet in renal disease estimated glomerular filtration rate; cTnI, cardiac troponin I; CK-MB, creatine kinase isoenzyme MB; hs-CRP, high-sensitivity C-reactive protein; UCG, ultrasound echocardiogram; LVEF, left ventricular ejection fraction; E/A ratio, ratio of early (E) to late (A) ventricular filling velocities; LVDd, left ventricular end-diastolic diameter; and LVFS, left ventricular fractional shortening. A *P* value < 0.05 was considered statistically significant.

Figure 1. mRNA levels of TLR2 and TLR4. **P* < 0.05 MI group compared with control group; #*P* < 0.05 AFMI group compared with control group and MI group. *n* = 42 in control group, *n* = 44 in MI group, and *n* = 45 in AFMI group. A *P* value < 0.05 was considered statistically significant.

Figure 2. TLRs and downstream pathway protein expression in PBMCs. TLR indicates toll-like receptor; MyD88, myeloid differentiation factor 88; TRIF-β, TIR domain-containing protein-β; and IRF-3, interferon regulatory factor 3. **P* < 0.05 compared with control group; #*P* < 0.05 compared with control group and MI group. *n* = 42 in control group, *n* = 44 in MI group, and *n* = 45 in AFMI group. A *P* value < 0.05 was considered statistically significant.

was significantly higher in the AFMI group than in the MI group (*P* = 0.018), as was the TLR4 mRNA level (*P* = 0.022).

TLRs and signaling pathway protein expression: Protein expression is shown in Figure 2. TLR2 and TLR4 expression levels were higher in the AFMI group compared with the MI group (TLR2, *P* = 0.013; TLR4, *P* = 0.022)

and control group (TLR2, *P* = 0.009; TLR4, *P* = 0.006). TLR2 and TLR4 expression levels were significantly higher in the MI group than in the control group (TLR2, *P* = 0.014; TLR4, *P* = 0.011).

Furthermore, we detected the expressions of MyD88, TRIF-β, and IRF-3, which are downstream pathway proteins of TLRs, and they were similar to TLR2 and TLR4

Figure 3. TLR2 and TLR4 levels by flow cytometry. **A, B** and **C**: 1×10^4 cells per sample test. Side scatter was set for TLR2 level and forward scatter was set for TLR4 level. **D**: $n = 42$ in control group, $n = 44$ in MI group, and $n = 45$ in AFMI group. A P value < 0.05 was considered statistically significant.

in PBMCs. The expression levels of MyD88, TRIF- β , and IRF-3 in the control group were the lowest among the 3 groups ($P < 0.05$). In contrast, the AFMI group had the highest levels of MyD88, TRIF- β , and IRF-3 among the 3 groups ($P < 0.05$).

TLRs level detection by flow cytometry: PBMCs with TLR2 and TLR4 expression were analyzed by flow cytometry. As shown in Figure 3, the control group had lower levels of TLR2 and TLR4 than that of the AFMI group and MI group ($P < 0.05$), and TLR2 and TLR4 in the AFMI group exhibited stronger fluorescence than that of the MI group ($P < 0.05$).

Multivariate logistic regression analysis: Scatter plots of TLR levels over CRP and TLR over CK-MB are shown in Figure 4. In the control group, the expression of TLR levels over CRP was significantly lower than that of the AFMI group and MI group ($P < 0.05$), and that of TLR over CK-MB were clearly lower than that of the AFMI group and MI group ($P < 0.05$). However, there were no differences between the AFMI group and MI group ($P > 0.05$).

Finally, predictors significantly associated with new-onset atrial fibrillation after myocardial infarction from univariate analysis ($P < 0.05$) were included in a multi-

Figure 4. Scatter plots of TLRs levels against CRP and CK-MB values. $n = 42$ in control group, $n = 44$ in MI group, and $n = 45$ in AFMI group. A P value < 0.05 was considered statistically significant.

variate logistic regression analysis, and backward selection techniques were used to determine the baseline risk factors. When CK-MB, cTNI, hs-CRP, and b-LDH were variable parameters in multivariate analysis, it was revealed that TLR2 and TLR4 levels were independent predictors of new-onset atrial fibrillation after myocardial infarction (TLR2: OR 1.08, 95% CI = 5504 sites per 10^4 cells, $P = 0.0015$; TLR4: OR 1.02, 95% CI = 5672 sites per 10^4 cells, $P = 0.0014$). There was no significant relationship between other variable parameters (CK-MB, cTNI, hs-CRP or b-LDH) and new-onset atrial fibrillation after myocardial infarction ($P > 0.05$).

Discussion

Toll-like receptors (TLRs), as innate immunological factors, participate in the pathophysiological progression of coronary disease. Recently, we have also reported that TLR expression is closely correlated with coronary atherosclerotic disease and its complications.^{25,26)}

In the TLR family, TLR2 and TLR4 are mostly expressed in myocardial infarctions and atrial fibrillation based on the results of animal studies.^{14,17)} In a TLR2^{-/-} mice study, TLR2 deficiency had the benefits of restoring myocardial blood flow after ischemia-reperfusion, reduc-

ing infarction size, and subsequently preventing lethal arrhythmias. Furthermore, TLR2 is obviously higher in non-valvular AF patients than in control patients with sinus rhythm.^{14,22)} TLR4 has also been shown to promote atrial fibrillation via MyD88-independent and IRF3-dependent pathways. This is closely attributed to myocardial infarction and ventricular remodeling.^{17,27)} However, there is a lack of data on TLR2 and TLR4 expression in acute myocardial infarction patients with or without new-onset atrial fibrillation.

Our initial aim was to investigate the TLR2 and TLR4 expression from mRNA to protein in acute myocardial infarction patients before reperfusion. An interesting finding was that TLR2 and TLR4 had higher expression levels in the new-onset atrial fibrillation group than in the health control group and myocardial infarction patients without atrial fibrillation. Common downstream signaling proteins (MyD88, TRIF- β and IRF-3) of TLR2 and TLR4 were also significantly higher in the AFMI group than in the control group and MI group. PBMCs with TLR2 and TLR4 exhibited higher levels in the AFMI group than in the MI group. Furthermore, there were no differences between TLR2 and TLR4 expressions in these patients from mRNA level to protein expression ($P < 0.05$). We speculate that TLR2 and TLR4 have the same signal (MyD88-

dependent) pathway in new-onset atrial fibrillation patients after acute myocardial infarction.^{28,29)}

As past studies have indicated, new-onset AF after MI is most dependent on infarction size, and AF also could cause harmful cardiovascular events and high mortality. A blood sample is collected before reperfusion therapy and patients only received aspirin, plavix, and statins. There are no published results on the impacts of aspirin or plavix on TLR pathway signaling. Only statins can lower TLR expression and downstream signaling protein levels.^{30,31)}

In our study, TLR2, TLR4, and their downstream signaling proteins were significantly higher in new-onset atrial fibrillation after acute myocardial infarction. They have the potential to be novel biomarkers for new-onset atrial fibrillation after acute myocardial infarction.

Disclosures

Conflicts of interest: The authors have no conflicts of interest to declare.

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