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Original Article

Expression and predictive value of NLRP3 in patients with atrial fibrillation and stroke

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Abstract: Objective: To investigate the expression and prognostic value of NLRP3 in non-valvular atrial fibrillation (NVAF) patients with ischemic stroke (IS). Methods: A retrospective analysis from January 2019 to December 2021 was conducted in 105 patients with NVAF who were treated in our hospital and were divided into the simple NVAF group (simple group) and combined IS group (consolidation group) according to the occurrence of IS. The relative expression of NLRP3 in serum was tested via qRT-PCR. The serum TNF-α, IL-6, and CRP levels were measured by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), and the correlation between the expression of NLRP3, TNF-α, IL-6, CRP and non-valvular atrial fibrillation stroke risk score (CHA2DS2-VASc score) was analyzed by Pearson method. The independent predictors of NVAF combined with IS were analyzed by regression equation. Meanwhile, the predictive value of the factors was assessed by receiver operating characteristic (ROC) curve. Results: The scores of NLRP3, TNF-α, IL-6, CRP and CHA2DS2-VASc in the consolidation group were obviously higher than those in the simple group. Pearson analysis revealed that the NLRP3, TNF-α, IL-6, and CRP levels in IS patients were positively correlated with CHA2DS2-VASc score. Logistic analysis revealed that NLRP3, IL-6, CRP and CHA2DS2-VASc could be used as potential factors to predict the merging of NVAF with IS. ROC showed that combined detection of NLRP3, IL-6, CRP and CHA2DS2-VASc in predicting NVAF complicated with IS exhibited an area under the curve of more than 0.9. Conclusion: NLRP3 is highly expressed in peripheral blood of patients with NVAF complicated with IS, which is a potential indicator for predicting NVAF complicated with IS.

Keywords: NLRP3, atrial fibrillation, ischemic stroke, prediction

Introduction

Atrial Fibrillation (AF) is the most common tachyarrhythmia seen in clinical practice [1]. Patients with AF have lost regular and orderly atrial activities, and the AF disorder caused by atrial dominant reentrant circuits is the most serious disturbance of atrial activities [2]. In the 2018 epidemiological survey, the weighted prevalence rate of AF in the Chinese population aged 35 and above was 0.71%. Its prevalence increases dramatically with age, of which 34% are AF patients diagnosed for the first time [3]. Non-valvular atrial fibrillation (NVAF) is a kind of AF [4]. Recent studies have found that with the aggravation of aging, the morbidity of NVAF has increased annually, seriously affecting the quality of life of patients [5, 6]. Moreover, research has shown that NVAF can increase the risk of ischemic stroke (IS), and its morbidity is 400-500% of non-AF patients, with an annual morbidity of 1.92%. Asian AF patients are more likely to develop IS than non-Asian patients, and receiving anticoagulant therapy is another predisposing factor [7]. Therefore, how to effectively prevent AF complicated with IS has been a clinical focus.

Recently, with the continuous improvement in medicine and the deepening of clinical research, it has been found that hypercoagulability, oxidative stress and inflammatory factors play an essential role in the pathogenesis of AF complicated with IS [8]. The NLRP3 inflammasome, mainly containing NLRP3, apoptosis associated speck like protein containing (CARD) and procaspase-1 [9], is a complex of intracellular protein interactions, which can recognize injury-related molecular pattern (DAMPs) and promote the maturation of inflammatory factors, thus producing inflammation [10]. It has been found that the components of NLRP3

inflammasome are expressed in cardiomyocytes and fibroblasts, and NLRP3 is the most abundant in cardiac fibroblasts. Research has shown that NLRP3 is involved in the occurrence of AF [11]. It is discovered that NLRP3 inflammasome may be the key mediator of cell injury and inflammation after stroke. Inhibiting NLRP3 inflammasome can reduce the degree of neurological impairment and alleviate cerebral ischemia-reperfusion injury under the condition of ischemia *in vivo* and *in vitro* [12]. However, it has not been determined whether NLRP3 can be used as a prognostic indicator in patients with NVAF and IS.

In this research, we analyzed the NLRP3 expression in serum of NVAF patients with/without IS, evaluated the prognostic value, and provided potential prognostic indicators for treatment.

Methods and data

Clinical data

Retrospective analysis from January 2019 to December 2021, in 105 patients with NVAF were treated in our hospital who were divided into the simple NVAF group (n=45, simple group) and combined IS group (n=60, consolidation group, All patients met the is diagnostic criteria in the Chinese guidelines for the diagnosis and treatment of acute ischemic stroke (2018) according to the occurrence of IS. This study was approved by the Medical Ethics Committee of our hospital. All the patients included knew about this research and signed the informed consent form. Approval No.: SH1901005.

Inclusion and exclusion criteria

Inclusion criteria: 1. All patients who meet the diagnostic criteria of NVAF in the 2016 European guidelines for the Management of Atrial Fibrillation [13]; 2. Patients with paroxysmal or persistent atrial fibrillation confirmed by 12 lead routine ECG or 24-h ambulatory ECG and NVAF confirmed by transthoracic echocardiography; 3. Patients with complete clinical data.

Exclusion criteria: 1. Patients complicated with other heart diseases; 2. Patients with serious dysfunction of liver, lung, kidney and other important organs; 3. Patients with acute and chronic infectious diseases; 4. Patients complicated with malignancy; 5. Those complicated with cerebrovascular disease, or previous history of cerebrovascular disease.

Sample detection

qRT-PCR detection: Fasting peripheral blood was collected from patients in the morning and centrifuged at room temperature for 30 min to obtain serum. The collected serum was stored in the refrigerator at -80°C. The serum total RNA was extracted by EasyPure miRNA Kit, and its concentration and integrity were detected by ultraviolet spectrophotometer and agarose gel electrophoresis. The RNA was reverse-transcribed to obtain cDNA and amplified by PCR (TransScript®Two-Step RT-PCR SuperMix kit). The amplification system was configured according to the kit instructions: a pre-denaturation of 94°C for 3 min, and 40 cycles of amplification (94°C for 30 s, 60°C, 60°C for 30 s and 72°C for 30 s). GAPDH was used as the internal reference. The NLRP3 expression was calculated using $2^{-\Delta\Delta CT}$ [14]. NLRP3, Forward: 5'-CCATCGGCAAGACCAAGA-3', Reverse: 5'-ACAGGCTCAGAATGCTCATC-3'. GAPDH Forward: 5'-TGACTTCAACAGCGACACCCA-3', Reverse: 5'-CACCCTGTTGCTGTAGGCCAAA-3'.

Elisa test: The expression of inflammatory cytokines (TNF-α, ml077385; IL-6, ml058097; CRP, ml057570; All kits were from China, Shanghai, mlbio.) in the serum of patients was detected by ELISA kit. Five mL of venous blood was collected from each patient, centrifuged at 1509.3 g for 10 min, and serum was collected for subsequent experiments. The collected serum was added to 50 μL of standard solutions of different concentrations in blank microwells; 50 μL of distilled water was added to blank control wells and 50 μL of antibody was added; 40 μL of sample was added to the remaining microwells, and then 10 μL of biotin-labeled antibody was added. Then the plate was sealed and incubated at 37°C for 30 min. When washing the plate, we ensured that each well of the washing solution was full without overflowing and it was let stand for 30 s and discard, and patted dry for a total of 5 times. Fifty L enzyme labeled solution was added to each well, and the plate was incubated at 37°C for 60 min again and washed again for 5 times. At last, the plate was dried thoroughly with absorbent paper and added with horseradish peroxidase label 100 μL/well and sealed. It

was incubated 15 min without light at 37°C, added by 100 μL/well chromogenic substrate TMB, then cultured 20 min at room temperature, and finally added with 50 μL terminating solution per well. The maximum absorption wavelength at 450 nm was determined by microplate reader within 15 min. Three groups of wells were set, and the experiment was repeated 3 times.

Clinical data collection

Clinical data of patients, such as age, gender, uric acid, left atrial diameter (LAD), atrial fibrillation stroke risk score (CHA2DS2-VASc), serum total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LD-C) were collected.

CHA2DS2-VAS scores

CHA2DS2-VAS is a score for stroke risk assessment in AF patients, with a total score of 9. C: heart failure, 1 point; H: hypertension, 1 point; A2: age ≥75, 2 points; D: diabetes, 1 point; S: thromboembolism, stroke or transient ischemic attack, 2 points; V: vascular disease (myocardial infarction, peripheral arterial vascular disease or aortic valve disease), 1 point; A: aged 65-74, 1 point; S: gender, female, 1 point. For patients with a score of 0, no anticoagulation or only aspirin is required. For those with a score of 1, oral anticoagulant or aspirin replacement therapy is recommended as a priority. For those with scores ≥2, oral anticoagulant therapy is recommended.

Outcome measures

Main outcome measures: The NLRP3 expression in the two groups was analyzed. The TNF-α, IL-6 and CRP levels of patients were compared, and the changes of CHA2DS2-VASc scores were evaluated. Pearson test was conducted to assess the correlation between NLRP3, TNF-α, IL-6, CRP and CHA2DS2-VASc score.

Secondary outcome measures: The clinical indicators of both groups of patients were compared. Logistic regression was used to investigate the predictors of NVAF complicated with IS. Its predictive value was tested through ROC curve.

Statistical analysis

The collected data were statistically analyzed via the SPSS 20.0 software package, and the image rendering was done with GraphPad 7. The comparison of measurement data between groups were compared via independent sample t-test, and the paired t-test was used for those within the group. The comparison of counting data (%) was conducted by chi-square test and expressed by χ^2. Pearson test was conducted to assess the correlation between CHA2DS2-VASc score and various indexes. The risk factors for NVAF complicated with IS were analyzed by Logistic regression analysis, and the predictive value of risk factors was analzyed through ROC curve.

Results

Comparison of baseline data between two groups of patients

It was found that there was no remarkable difference in age, gender, BMI, course of disease, history of smoking and alcoholism, past medical history, systolic pressure, diastolic pressure, TG, and TC between two groups (all P>0.05) (**Table 1**).

Comparison of NLRP3 between the two groups of patients

We compared the NLRP3 level between both groups and found the level in the simple group was statistical lower than that in consolidation group (P<0.001) (**Figure 1**).

Comparison of inflammatory factors between both groups of patients

The TNF-α, IL-6, and CRP levels were detected by ELISA. The results revealed that the levels in the simple group were statistically lower than that in consolidation group (all P<0.001) (**Figure 2A-C**).

Comparison of CHA2DS2-VASc between both groups of patients

The patients were evaluated with the CHA2-DS2-VASc score. It revealed that the CHA2-DS2-VASc of the simple group was statistically lower than that of the consolidation group (P<0.001, **Figure 3**).

Table 1. Baseline data

Factor	Simple group (n=60)	Consolidation group (n=45)	*P* value
Age			0.734
>70	34	24	
≤70	26	21	
Gender			0.535
Male	33	22	
Female	27	23	
BMI			0.573
>24 kg/m^2	30	20	
≤24 kg/m^2	30	25	
Course of disease			0.777
>3 years	27	19	
≤3 years	33	26	
History of smoking			0.776
Yes	35	25	
No	25	20	
History of alcoholism			0.893
Yes	14	10	
No	46	35	
Past medical history			
Hypertension	27	22	0.693
Hyperlipidemia	11	7	0.709
Diabetes	20	16	0.558
Chronic obstructive pulmonary disease	9	6	0.809
Systolic pressure (mmHg)	131.50±12.75	136.00±15.81	0.109
Diastolic pressure (mmHg)	77.05±14.92	79.75±11.73	0.317
TG	1.51±0.40	1.49±0.40	0.798
TC	4.64±0.96	4.58±0.1.10	0.735

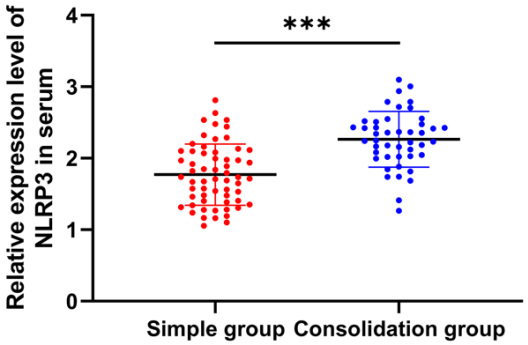

Figure 1. Expression of NLRP3 in patients with NVAF and IS. ***P<0.001.

Relationship between NLRP3, inflammatory factors and CHA2DS2-VASc score

The correlation of NLRP3, inflammatory factors and CHA2DS2-VASc score was analyzed by Pearson test. It showed that NLRP3, TNF-α, CRP and IL-6 were positively correlated with CHA2DS2-VASc (P<0.05, **Figure 4A-D**).

Predictive value of NLRP3, TNF-α, CRP and IL-6 in NVAF complicated with IS

The factors with statistical difference between the two groups of patients were collected for assignment (**Table 2**). Multivariate analysis manifested that NLRP3, CRP, IL-6, and CHA2-DS2-VASc were independently related to NVAF complicated with IS (**Figure 5**, P<0.05). To clarify the predictive value of the risk factors in NVAF complicated with IS, we drew the ROC curve. It was found that the areas under the curve of NLRP3, CRP, IL-6 and CHA2DS2-VASc were all greater than 0.8, and that of NLRP3 for predicting NVAF complicated with IS was the largest (**Figure 6A**). Then, we predicted the joint ROC curve according to logistics regression. Through further analysis, we found that

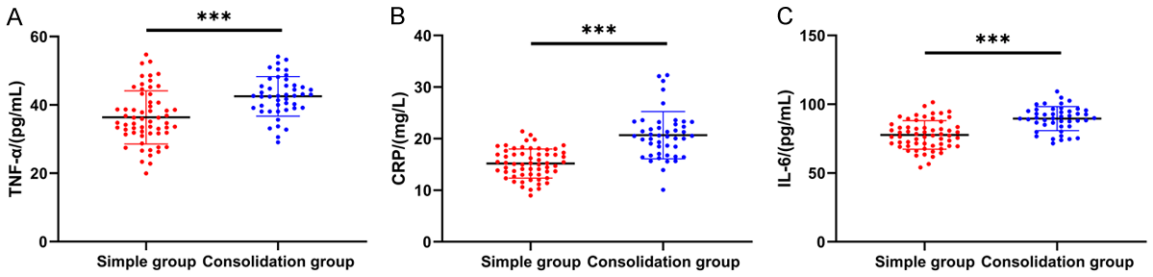

Figure 2. Expression of TNF-α, CRP, and IL-6 in serum of patients with NVAF and IS. A. Detection of serum TNF-α level in simple group and consolidation group by ELISA; B. Detection of serum CRP level in simple group and consolidation group by ELISA; C. Detection of serum IL-6 level in simple group and consolidation group by ELISA. ***P<0.001.

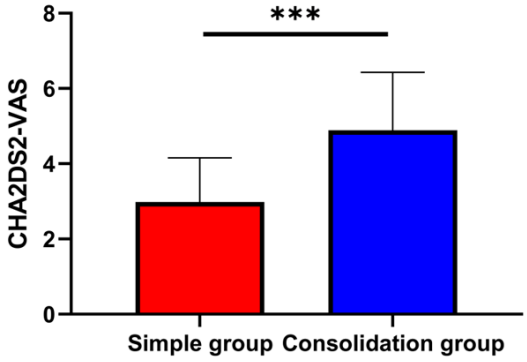

Figure 3. Comparison of CHA2DS2-VASc in patients with NVAF and IS. ***P<0.001.

the area under the joint curve was 0.996 (**Figure 6B**). This indicates that the combined detection of NLRP3, CRP, IL-6 and CHA2DS2-VASc can be used as a potential indicator for predicting NVAF complicated with IS.

Discussion

Ischemic stroke (IS) is a familiar clinical neurological disease. Its manifestations are mainly neurological defects, with high morbidity and mortality [15]. NVAF, an independent risk factor of IS, can easily form left atrial thrombus and increase the risk [16]. Recently, research has found [17] that inflammation and oxidative stress are involved in the process of cerebrovascular injury, so finding relevant effective markers is quite valuable for the early diagnosis and treatment of NVAF complicated with IS.

The NLRP3 inflammasome plays a vital role in various cardiovascular diseases, including myocardial infarction, atherosclerosis, hypertension, and dilated cardiomyopathy [18, 19]. It is reported that NLRP3 is involved in AF development and progression. For example, Yao et al. [20] found that NLRP3, ASC and Casp1-p20, the components of the NLRP3 inflammasome, were expressed in atrial myocytes from AF patients and normal people. Among them, the NLRP3 protein in patients with persistent AF was much higher than that in normal people. As the most common acute and severe disease worldwide, IS has a high risk of death and disability, and data shows that it accounts for 87% of the total number of strokes [21]. It has been reported that AF increases the morbidity of IS [22]. Hua et al. [23] discovered that IS injury can be improved by inhibiting the activation of NLRP3 inflammasome, which indicates that NLRP3 is also involved in the occurrence of IS. Nowadays, there are few clinical observation indicators about NVAF complicated with IS. Based on the above studies, we speculate that NLRP3 may be involved in NVAF complicated with IS.

It was found that the NLRP3 level in serum of patients with NVAF and IS was higher than that of those with pure NVAF, indicating that the level changes were related to IS. Furthermore, we also analyzed the TNF-α, CRP and IL-6 levels in patients. As a classic inflammatory factor, the elevated TNF-α level can further activate the inflammation and participate in the occurrence of stroke [24]. CRP aggravates vascular intimal injury and aggravates lumen stenosis symptoms through multiple pathways [25]. IL-6 is a pro-inflammatory factor that can promote the differentiation of inflammatory cells and can also boost the aggregation of those cells in the arterial intima and increase the synthesis of stress response proteins, increasing the risk of IS [26]. TNF-α, CRP and IL-6 were found to have increased expression

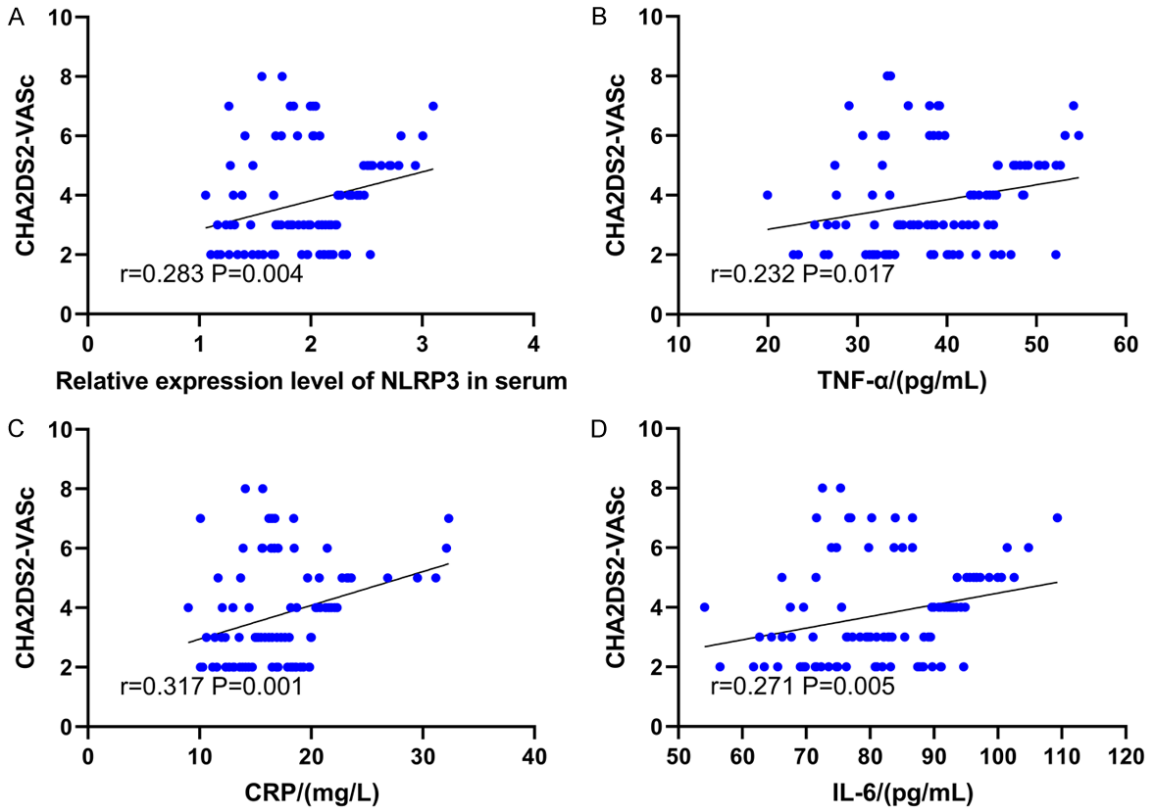

Figure 4. Correlation between NLRP3, inflammatory factors and CHA2DS2-VASc score. A. Pearson test for the correlation between NLRP3 and CHA2DS2-VASc score; B. Pearson test for the correlation between TNF-α and CHA2DS2-VASc score; C. Pearson test for the correlation between CRP and CHA2DS2-VASc score; D. Pearson test for the correlation between IL-6 and CHA2DS2-VASc score.

Table 2. Assignment table

Factor	Assignment
NLRP3	≥1.996=1, <1.996=0 (Cut-off value)
TNF-α	≥38.84=1, <38.84=0 (Cut-off value)
CRP	≥18.39=1, <18.39=0 (Cut-off value)
IL-6	≥83.58=1, <83.58=0 (Cut-off value)
CHA2DS2-VASc	1=1, 2=2, ..., 8=8
NVAF complicated with IS	Consolidation group =1; Simple group =0

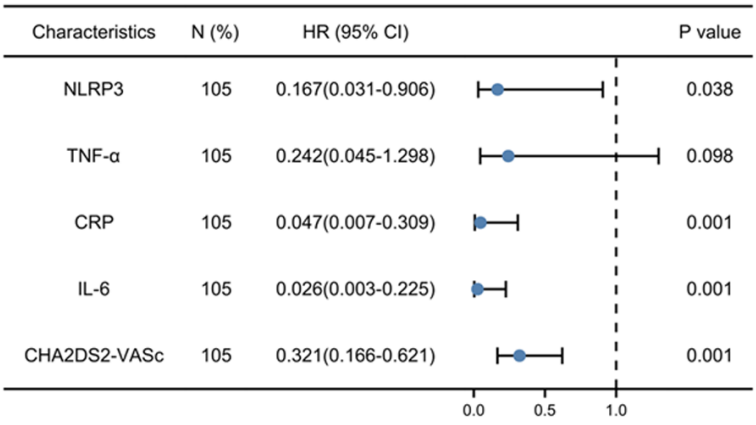

Figure 5. Multivariate analysis of NVAF complicated with IS.

in patients with NVAF complicated by IS. The CHA2DS2-VASc score is often used to assess the risk of stroke in NVAF patients and can guide clinical anticoagulation therapy. It was found that NLRP3, TNF-α, CRP and IL-6 were all positively correlated with the CHA2DS2-VASc score. This suggests that the level changes may indicate the risk of NVAF complicated by IS. Aynaci [27] and Lip [28] found that TNF-α, CRP and IL-6 were highly expressed in patients with NVAF complicated by IS, and were related to their prognosis. We also verified that TNF-α, CRP and IL-6 increased in patients with NVAF com-

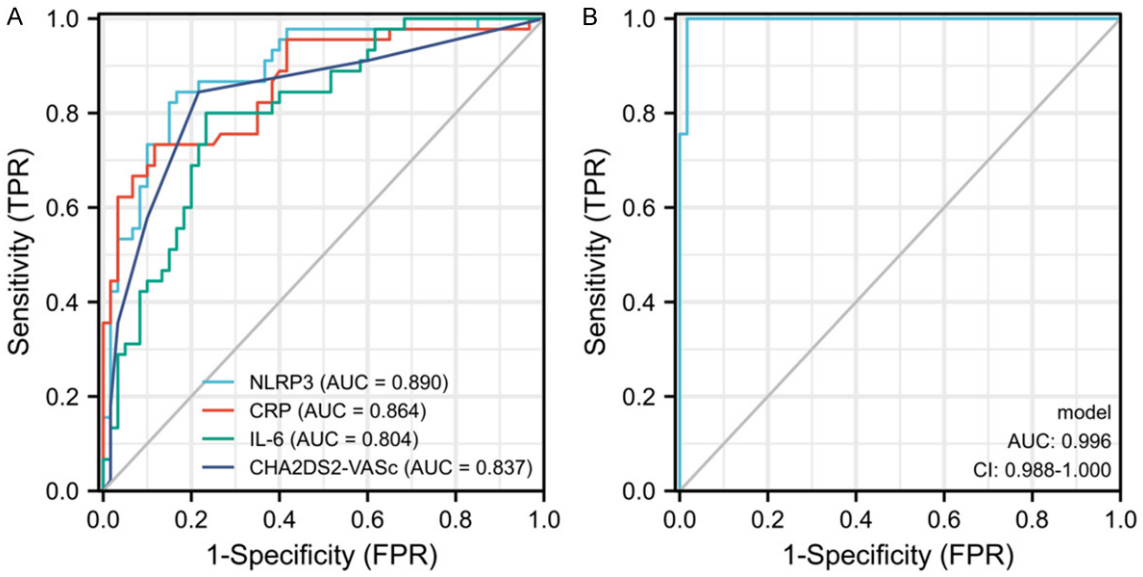

Figure 6. ROC curves of NLRP3, CRP, IL-6 and CHA2DS2-VASc predicting NVAF complicated with IS. A. NLRP3, CRP, IL-6 and CHA2DS2-VASc alone predicted the ROC curve of NVAF complicated with IS; B. NLRP3, CRP, IL-6 and CHA2DS2-VASc combined to predict the ROC curve of NVAF complicated with IS.

plicated by IS. In addition, we first discovered that the NLRP3 level in patients with NVAF complicated by IS was higher than that in those with NVAF alone. Early studies have shown [29] that cardiomyocyte NLRP3 inflammasome activation is a major factor for the overall increase in NLRP3 activity in atrial tissue from patients with paroxysmal AF. We believe that when the NLRP3 inflammasome is activated, it will cause a series of inflammatory cascade responses in the body, inducing nerve cell damage and inflammation. IS is the most serious complication of AF. When IS occurs in patients, the inflammation in the body is further aggravated, resulting in a further increase in NLRP3. Finally, we analyzed potential predictors for patients with NVAF complicated by IS. Through logistic regression, we found that NLRP3, CRP, IL-6 and CHA2DS2-VASc can be used as potential factors for predicting NVAF complicated with IS. While ROC curve analysis denoted that the area under the curve of each indicator alone was greater than 0.8, and that of NLRP3 was the largest. This suggests that NLRP3 has a high predictive value in NVAF complicated with IS.

We first verified that NLRP3 is highly expressed in NVAF complicated with IS, and it has a high predictive value. Nevertheless, the research still has some limitations. First, we did not observe whether there is a difference in NLRP3 between normal people and NVAF complicated with IS. Secondly, it is still vague whether there is a change in NLRP3 before and after treatment. What's more, no follow-up was performed in this research, and further analysis is needed to determine whether there is a link between NLRP3 and prognosis. Finally, the inflammatory bodies of NLRP3 mainly contain NLRP3, CARD and procaspase-1. In this research, we only detected NLRP3 in the serum of patients, but not the other two proteins. It is vague whether CARD and procaspase-1 can improve the predictive performance in predicting NVAF complicated with IS. Thus, in the follow-up study, we will further assess the relationship between NLRP3 and the prognosis of NVAF patients complicated with IS, to provide potential indicators for clinical treatment and efficacy evaluation.

Overall, NLRP3 is highly expressed in the peripheral blood of patients with NVAF complicated by IS, which can be used as a potential predictive indicator.

Disclosure of conflict of interest

None.

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