Acta Cardiol Sin 2015;31:1–7
Original Article
doi: 10.6515/ACS20140526A

EPS and Arrythmia

Decreased Cardiac Expression of Heat Shock Protein 27 is Associated with Atrial Fibrillation in Patients with Rheumatic Heart Disease

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Background: The objective of this study was to compare the expression of heat shock proteins (HSPs) between rheumatic heart disease (RHD) patients with atrial fibrillation (AF) and RHD patients without AF, and its efficacy in predicting the occurrence of AF in RHD patients.

Methods: Ninety-five patients were enrolled in our study, including 60 RHD patients with AF, and 35 RHD patients without AF. The baseline characteristics of the patients such as gender, age, AF duration, left atrial diameter and left ventricular ejection fraction were collected, and erythrocyte sedimentation rate and high-sensitivity C-reaction protein were measured from all patients. Tissue samples were obtained from the right atrial appendage during open-heart surgery and then detected using immunohistochemical methods and Western blot with HSP27, HSP60, HSP70 and HSP90 antibodies.

Results: Compared with RHD patients without AF, the density of HSP27 positive protein in RHD patients with AF was significantly lower. The density of HSP60, HSP70 or HSP90 antibodies did not indicate significant difference between the two groups. Use of the Western blot experiment showed consistent results with immunohistochemical staining. In RHD patients with AF, the expression level of HSP27 protein was negatively associated with AF duration and left atrial diameter. Left atrial enlargement and low expression of HSP27 were the independent predictors of AF.

Conclusions: The decreased expression level of HSP27 is associated with AF in RHD patients.

Key Words: Atrial fibrillation • Heat shock protein • Rheumatic heart disease

INTRODUCTION

As molecular chaperones, heat shock proteins (HSPs) play an important role in the biosynthesis process of a variety of proteins, and are active in protein folding, trafficking and cell signaling to protect cells from acute or chronic stress injury.[1] In recent years, there has been increasing interest about the relationship between HSPs and atrial fibrillation (AF). Some studies[2-6] suggested that the down-regulation of HSPs plays a certain role in the occurrence of AF after surgery, but the conclusions that were reached regarding the types and changes of HSPs in various studies were significantly different. It is of great importance to investigate the expression of HSPs in AF patients for elucidating the mechanisms of AF and also predicting the occurrence and prognosis of AF. In the present study, valuable tissues were collected from rheumatic heart disease (RHD) patients, and various expressions of HSPs that are widely studied were

Received: April 21, 2013 Accepted: May 26, 2014
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compared between RHD patients with and without AF to further clarify the relationship between the expression of HSPs and AF.

MATERIALS AND METHODS

Patient population

This investigation was approved by the institutional ethics committee in the university hospital. The patient population enrolled in this study consisted of 95 consecutive patients. The enrollment criteria included: (1) rheumatic valvular disease; (2) referred for open-heart surgery in Enshi Autonomous Prefecture Central Hospital of Wuhan University, China; (3) without coronary heart disease, renal or liver impairment, malignancy or infectious disease before the operation. Exclusion criteria included atrial flutter, fever and receiving treatment for other diseases. After written informed consent was obtained from each patient, they were divided into two groups: RHD patients with AF (Group A, N = 60) and RHD patients without AF (Group B, N = 35). According to their symptoms, the surface electrocardiogram (ECG) or 24-hour dynamic ECG was performed on all patients to determine whether they had AF. Routine preoperative echocardiography was performed to evaluate cardiac chamber size and cardiac function.

Serological testing

Blood samples were drawn from the antecubital vein in the fasting state. Serum high-sensitivity C-reaction protein (hs-CRP) and erythrocyte sedimentation rate (ESR) were measured with standard laboratory techniques on a Hitachi 912 Analyzer (Roche Diagnostics, Germany).[7]

Atrial sample collection and immunohistochemical staining

All patients underwent cardiopulmonary bypass with moderate hypothermia and antegrade crystalloid cardioplegic arrest during the open-heart surgery. Two to three millimeters of atrial tissue was obtained from the right atrial appendage for immunohistochemical and Western blot studies. During the surgery, the right atrial appendage was cannulated for extracorporeal circulation. The tissue from the tip of the right atrial appendage was collected when the appendage was sutured after the surgery. All the excised specimens together were consistent with the entire thickness of the atrial wall. All myocardial specimens were quickly frozen in liquid nitrogen and then embedded into paraffin blocks. Tissues were vertically sectioned from epicardium to endocardium, and multiple 5-μm thick serial sections were used.

Details of the staining techniques were the same as previously described.[4] The paraffin-embedded sections were dewaxed, dehydrated, and incubated with 3% peroxidase for 10 min at room temperature. These sections were rinsed with distilled water and saturated in phosphate buffered saline (PBS) for 5 min. Then the sections were incubated overnight at 4 °C with a 1:100 dilution of mouse monoclonal anti-HSP27, anti-HSP60, anti-HSP70 and anti-HSP90 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated with a polymer helper reagent for 20 min at 37 °C and rinsed with PBS. Afterwards, the samples were incubated with poly peroxidase-anti-mouse IgG for 20 min at room temperature. After PBS washing, the sections were stained with diaminobenzidine solution, counterstained with hematoxylin, routinely dehydrated, and then mounted. The tissues from each group's patients were stained in the same session.

Detection of atrial positive expression density

The densities of HSP27, HSP60, HSP70 and HSP90 were determined using computerized image analysis (Image Pro-Plus 3.0, Media Cybernetics, Rockville, MD, USA).[8] Under the microscope, three visual fields with the highest positive expression density were selected from each slice to be determined. The computer automatically detected the positive expression in the slices and calculated the occupied area. The positive expression density is the positive expression area divided by the total detection area ($\mu m^2/mm^2$). The positive expression density of each slice is the average of the measured values of three visual fields.

Western blot

Five patients from each group were randomly chosen for Western blot experiments. Proteins from formalin-fixed, paraffin-embedded tissue sections were extracted as previously described.[9] The proteins were separated

by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk and then probed with horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-HSP27, anti-HSP60, anti-HSP70 and anti-HSP90 antibodies (Santa Cruz Biotechnology, USA). The working dilutions were 1:1000 for all the primary antibodies. The resulting reaction was visualized using HRP-conjugated anti-mouse IgG secondary antibody (Santa-Cruz Biotechnology, USA), followed by incubation with enhanced chemiluminescence (ECL) Western Blot Detection Kit (Amersham, the Netherlands) for 1 min. These experiments were repeated five times and the mean was scored.

Data acquisition

Based on medical records and medical history, the patients' age, gender, body mass index (BMI), AF type and AF duration were collected. The size of each chamber was measured by conventional echocardiography, and the densities of HSP27, HSP60, HSP70 and HSP90 were analyzed by immunohistochemical staining method. The result of the Western blot analysis was expressed as the ratio to the levels of GAPDH. The differences found among these indicators were compared between the two groups of patients.

Statistical analysis

The data are expressed as mean ± standard deviation. We used the independent t test for continuous variables analysis, and the x^2 or the Fisher's exact test for discrete variables analysis. A stepwise logistic regression analysis was used for multivariate analysis. Statistical analysis was carried out using the SPSS 16.0 (SPSS Inc, Chicago, IL, USA). When $p < 0.05$, that indicated a significant difference.

RESULTS

General information

Among the 95 patients selected for this study, 49 were male and 46 were female, with an average age of 44 ± 7 years. Group A included 22 cases of paroxysmal AF (mean duration, 24 ± 13 hours) and 38 cases of non-paroxysmal AF (including 31 persistent AF and 7 permanent AF; the mean duration was 12 ± 5 days). The incidences of structural diseases including mitral stenosis/insufficiency, aortic stenosis/insufficiency and/or tricuspid insufficiency were similar between Group A and Group B. The proportion of isolated mitral stenosis was 48.3% (29/60) in Group A and 45.7% (16/35) in Group B. The orally administered drugs were comparable in the two groups except for warfarin. The detailed information of patients in Group A and Group B are listed in Table 1. The age, gender, systolic blood pressure, BMI, left ventricular ejection fraction, right atrial diameter, right ventricular diameter and left ventricular end-diastolic diameter were not significantly different between the two groups of patients. The left atrial diameter of Group A patients was significantly larger than that of Group B patients ($p < 0.01$) (Table 1).

Serological test results

There were no significant differences in the ESR and hs-CRP levels of the two groups of patients, as shown in Table 1.

HSP positive density

The expression of HSPs was mainly observed in myocardial cells. Compared with Group B, the positive density of HSP27 in Group A patients was significantly lower (Figure 1, Table 2), while there was no significant difference in positive density of HSP60, HSP70 or HSP90 between the two groups (Table 2). In Group A, the HSP27 positive density showed a significant negative correlation with AF duration and left atrial size, whereas there were no significant correlations of HSP27 expression with gender, age, BMI, left ventricular ejection fraction, left ventricular end-diastolic diameter, left atrial diameter, ESR or hs-CRP (Table 3). In Group A, the HSP27 positive density of patients with non-paroxysmal AF was significantly lower than that of patients with paroxysmal AF ($255 \pm 94\ \mu m^2/mm^2$ vs. $289 \pm 102\ \mu m^2/mm^2$, $p < 0.05$). In Group B, HSP27 was not associated with any factor.

Western blot

The results of western blot were generally consis-

Table 1. Baseline characteristics of patient population

	Group A (N = 60)	Group B (N = 35)	p
Clinical characteristics			
Age (y)	45 ± 7	43 ± 9	0.77
Male/female	33/27	16/19	0.76
SBP (mmHg)	118 ± 12	120 ± 14	0.51
Body mass index (kg/m^2)	23 ± 7	25 ± 6	0.39
LVEF (%)	36 ± 9	39 ± 11	0.48
Left atrial diameter (mm)	52 ± 12	47 ± 9	0.009
LVEDD (mm)	58 ± 13	56 ± 11	0.22
RA (mm)	48 ± 11	46 ± 7	0.18
RV (mm)	34 ± 9	32 ± 9	0.37
ESR (mm/h)	8.3 ± 2.2	8.2 ± 2.9	0.71
hs-CRP (mg/L)	4.2 ± 1.9	4.4 ± 1.5	0.20
Associated heart diseases			
Mitral stenosis	47 (orifice area, 0.9 ± 0.3 cm^2)	29 (orifice area, 0.9 ± 0.2 cm^2)	0.19
Mitral insufficiency	23 (mild, 8; moderate, 12; severe 3)	11 (mild, 4; moderate, 4; severe 3)	0.14
Aortic stenosis	17	9	0.18
Aortic insufficiency	13	6	0.19
Tricuspid insufficiency	29	13	0.10
Treatments			
Beta-blockers	33	15	0.09
ACE-inhibitors/ARBs	56	31	0.21
Statins	21	8	0.09
Warfarin	59	11	< 0.001

ACE, angiotensin converter enzyme; ARBs, angiotensin receptor blockers; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reaction protein; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; RA, right atrium; RV, right ventricle; SBP, systolic blood pressure.

tent with the results of immunohistochemistry (Figure 2). The expression of HSP27 in Group A patients was significantly lower than that in Group B patients. The expression of HSP60, HSP70 or HSP90 was similar in the two groups.

Predictors of AF

We then combined the data from both Group A and Group B incorporating the ten clinical variables of gender, age, left ventricular ejection fraction, left atrial diameter, left ventricular end-diastolic diameter, systolic blood pressure, BMI, ESR, hs-CRP and HSP27. Multivariate analysis revealed that the left atrial diameter [odds ratio (OR) 1.57, 95% confidence interval (CI) 1.12-2.03, p = 0.01] and HSP27 (OR 0.81, 95% CI 0.34- 0.90, p = 0.03) were the independent predictors of AF.

DISCUSSION

Main findings

Compared with RHD patients without AF, the positive density of HSP27 protein in RHD patients with AF was significantly lower. The expression of HSP27 in non-paroxysmal AF was even further lower than the paroxysmal type of AF. The positive densities of HSP60, HSP70 and HSP90 protein did not indicate a significant difference between the two groups. Left atrial enlargement and low expression of HSP27 were the independent predictors of AF. These results suggest that the expression level of HSP27 is associated with AF in RHD patients.

HSPs and AF

HSPs, also known as stress proteins, are divided into the following four families according to the differences

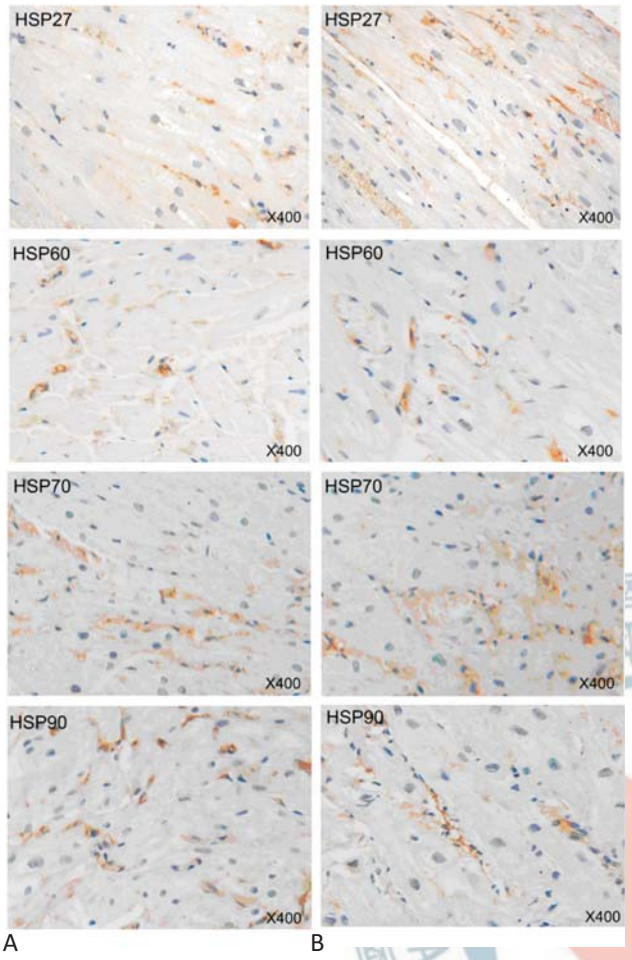

Figure 1. *Representative examples of distribution of different HSP in the two groups. As shown above, Group A represents RHD patients with atrial fibrillation; Group B represents RHD patients without atrial fibrillation. Brown areas show positive protein expression. As seen, the positive expression of HSP27 for Group A was significantly lower than that in Group B patients, whereas the positive expressions of other HSPs were similar between the two groups. HSP, heat shock protein; RHD, rheumatic heart disease.*

Table 2. HSP densities in the two groups ($\mu m^2/mm^2$)

	HSP27	HSP60	HSP70	HSP90
Group A (N = 60)	279 ± 108*	424 ± 113	554 ± 187	436 ± 110
Group B (N = 35)	558 ± 133	510 ± 167	549 ± 192	465 ± 132

* $p < 0.05$ compared with Group B. HSP, heat shock protein.

in their molecular size and amino acid sequence homology: the HSP90 family, the small molecular weight smHSP family (as represented by HSP27), the HSP60 family and the HSP70 family.[1,2,10] In recent years, several studies have suggested that the incidence of AF and HSP family may be correlated.[1-7] In this study, human specimens were obtained from clinical patients who had undergone rheumatic heart surgery. The expressions of HSPs of the atrial appendage were detected. Compared to RHD patients without AF in the control group, the right atrial appendage of RHD patients with AF showed a significant decrease in HSP27 distribution density, whereas the distribution densities of HSP60, HSP70 and HSP90 were not significantly different between the two groups. It was further established that HSP27 distribution density was negatively correlated with AF duration and left atrial diameter, but was unrelated to gender, age, BMI, left ventricular ejection fraction, left ventricular end-diastolic diameter, left atrial diameter, ESR or hs-CRP. This conclusion is consistent with that of recently published research by Hu et al.[11] The study found that the concentration of HSP27 in the serum of patients with AF was significantly lower than that in the control group patients without AF. The HSP27 content in patients with paroxysmal AF was higher than that of pa-

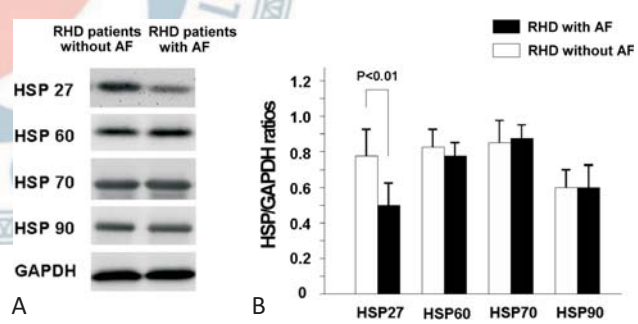

Figure 2. *Expression of HSP27, HSP 60, HSP70 and HSP90 in RHD patients without and with AF. (A) Representative Western immunoblots of HSP27, HSP 60, HSP70, HSP90 and GAPDH. (B) Values of Western blot analysis are expressed as mean ± SEM, N = 5. AF, atrial fibrillation; HSP, heat shock protein; RHD, rheumatic heart disease.*

Table 3. Analysis of related factors of HSP27 in Group A patients

	Age	Gender	AF duration	SBP	BMI	LVEF	LAD	LVEDD	ESR	hs-CRP
Correlation coefficient (r)	-0.24	0.11	-0.59	0.29	0.89	-0.18	-0.55	-0.21	-0.65	-0.17
p value	0.57	0.79	0.03	0.55	0.21	0.15	0.005	0.10	0.60	0.12

AF, atrial fibrillation; BMI, body mass index; ESR, erythrocyte sedimentation rate; HSP, heat shock protein; hs-CRP, high-sensitivity C-reaction protein; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; LVEF, leftventricular ejection fraction; SBP, systolic blood pressure.

tients with persistent AF. After the catheter ablation of atrial fibrillation, the patients with high HSP27 content have a higher possibility of maintaining sinus rhythm. The study conducted by Hu et al. and our study suggest that HSP27 plays a protective role not only in idiopathic AF but also in patients with structural heart diseases. Furthermore, another study by Brundel et al.[12] found that HSP27 may represent the crucial HSP to protect myocardial cells from AF-induced atrial remodeling and may delay or prevent progression of AF, consistent with our result showing that the HSP-positive density in patients with non-paroxysmal AF was significantly lower than that in patients with paroxysmal AF. This result can be explained by the previous reports indicating that HSP27 protects against stress-induced disruption of F-actin and myofibril structures and/or accelerates recovery of cytoskeletal integrity after disruption.[12-14] The protective effect of HSP27 was lost and eventually led to the occurrence and progression of AF in these patients.

Previous studies[15-19] have found different changes in HSP family members including HSP25, HSP27, HSP60, HSP65, HSP70, HSP90 and other small HSP proteins in different types of AF, such as rapid pacing induced AF, rheumatic valvular AF, acute atrial ischaemia induced AF and idiopathic AF. However, the methods used to detect HSP in these studies were inconsistent; for example, they primarily found HSP content in the serum while the other detected HSP in the tissue. Therefore, the obtained findings were not consistent and the clinical significance is limited. This study investigated four representative HSPs from the four families in RHD patients, and we found that only HSP27 manifested a significant difference between patients with and without AF. The consistent result supporting the protective role of HSP27 was not only observed in rheumatic valvular AF, but also in rapid pacing inducing AF and idiopathic AF.[12-15] A similar study by Yang et al.[1] showed that HSP60 may be associated with the degree of atrial myolysis in RHD patients; however, no significant difference was found between patients with and without AF. This result may indicate that atrial myolysis may be not the most crucial factor for the occurrence of AF in this type of AF. Another study conducted by Oc et al.[6] found that a high level of serum HSP70 antibody may be a marker for subsequent development of AF. Their study involved patients who underwent coronary artery bypass surgery, where the circulating HSP70 may not exactly reflect the local expression of HSP70 in the heart.

The protective mechanisms of HSP27

Previous studies have shown that the protective mechanisms of HSP27 on myocardial cells include:[10] (1) promoting glutathione production to improve the restoration ability of cells; (2) inhibition of cytochrome C release and apoptotic protease-activating factor-1 cytochrome C (Apaf-1/CytC) complex formation; (3) inhibiting the activation of caspase precursor; and (4) stabilizing the cytoskeleton. These factors play an important role in stabilizing individual myocardial cells as well as in the electrophysiological function of the entire heart.

Clinical implications

As suggested by this study, HSP27 is possibly related to the occurrence and progression of AF in RHD patients. Therefore, determining the HSP27 content helps in predicting the occurrence and progression of AF. On the other hand, our study raised the possibility that AF may be inhibited by up-regulating the expression of HSP27 in the heart. In fact, an animal study[15] has provided the primary evidence showing that treatment with HSP27 (but not HSP70) protects against atrial tachycardia – induced remodeling, and suggests the possibility that HSP induction may be an interesting and novel approach to preventing clinical AF.

Study limitations

Firstly, the right atrial appendage was the only representative site for the immunohistochemical studies due to the ethical considerations involved when working with living patients. However, the histological and Western blot results clearly showed the decrease in expression of HSP27 in the atrial appendage in RHD patients with AF. Secondly, the HSP content of the serum was not determined since the HSP in the serum can originate in many organs, not only in the heart. Furthermore, the results of this study need to be verified in comparable studies utilizing a larger number of patients.

CONCLUSIONS

This study showed that HSP27, but not other HSPs, was the crucial HSP to protect the heart from AF in this group of specific patients, which was consistent with observations made regarding HSPs in several previous reports.

CONFLICT OF INTEREST STATEMENT

None declared.

ACKNOWLEDGEMENT

This work was supported by grants 81200389, 81100128, 81070143, 81370281 from the National Natural Science Foundation of China (WW, ZL, HJ), grant 4101024 and 121069 from the Fundamental Research Funds for the Central Universities (ZL, WW), grant 20120141120077 and 20100141120072 from the Specialized Research Fund for the Doctoral Program of Higher Education of China (WW, ZL) and Wuhan Planning Project of Science and Technology (No. 201271031429, ZL).

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