Heat shock proteins (HSPs) are a family of proteins that protect against different forms of cellular stress, including those contributing to the formation of atrial fibrillation (AF), and are associated with a decreased risk of postoperative AF.1–4 Recent studies indicated that HSP27, one of the HSP proteins, may play a particularly important role in AF pathogenesis. Atrial HSP27 levels are inversely correlated with paroxysmal and persistent AF duration and myolysis extent in humans.5 In experimental AF, atrial HSP27 may protect against tachypacing-induced AF, including AF duration by burst pacing and AF vulnerability.6 Although atrial HSP27 may prevent AF occurrence and progression, the clinical correlation between serum HSP27 (HSP27S) and AF remains poorly understood. Moreover, the relationship of HSP27S with clinical electroanatomical characteristics remained unclear.

The HSP27S levels were correlated with LAD, left atrial voltage, and fractionated intervals, and predicted AF recurrence after catheter ablation. The mechanisms could be related to inflammation. (Circ Arrhythm Electrophysiol. 2012;5:334-340.)

Key Words: heat shock protein ▪ atrial fibrillation ▪ catheter ablation

On the other hand, HSPs are important mediators of the inflammatory process,7 which plays a key role in AF pathogenesis. The HSP27 interferes with the tumor necrosis factor (TNF)-α signaling pathway, a proinflammatory pathway, by protecting against oxidative stress and modulating transforming growth factor-β–activated kinase-1 activity.9,10 The HSP27 also increases interleukin (IL) 10, an anti-inflammatory cytokine, by activating p38 signaling.11 Bal-

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From the Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital (Y.-F.H., C.-T.T., Y.-J.L., S.-L.C., L.-W.L., T.-C.T., K.S., C.-H.L., T.-F.C., S.-A.C.); the Institute of Clinical Medicine and Cardiovascular Research Center, National Yang-Ming University (Y.-F.H., H.-M.T., C.-T.T., Y.-J.L., S.-L.C., L.-W.L., T.-C.T., C.-H.L., T.-F.C., S.-A.C.); the Department of Internal Medicine, Mackay Memorial Hospital, Mackay Medical College (H.-I.Y.); and the Graduate Institute of Medical Science, Taipei Medical University (H.-I.Y.), Taipei, Taiwan.

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Correspondence to Hung-I Yeh, MD, PhD, or Shih-Ann Chen, MD, FHRS, Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Mackay Medical College, 201 Section 2, Shih-Pai Road, No. 46, Section 3, Jhong-Jheng Road, Taipei, Taiwan. E-mail hiyeh@ms1.mmh.org.tw or epsachen@ms41.hinet.net

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ance between anti-inflammatory and inflammatory cytokines, such as IL-10 and TNF-α, has also been associated with AF. The information suggested that HSP27 may modulate inflammatory pathways and, therefore, prevent AF-related remodeling.

To clarify these issues, the present study had the following aims: (1) compare HSP27S levels in AF and control patients; (2) investigate the correlations among HSP27S, clinical/electrophysiological characteristics, and catheter ablation outcomes; and (3) investigate the role of HSP27S-associated inflammatory pathways in AF development.

**Methods**

**Participants**

This prospective study involved 214 consecutive patients (114 patients with AF who were referred for catheter ablation and 100 controls from the cardiology clinic who were free of AF by at least 2 ECG reports and without AF-related symptoms). Patients with hematologic, renal, or hepatic impairment, inflammation, neoplastic disorders, recent (<3 months) myocardial infarction or stroke, acute AF precipitated by thyrotoxicosis, or any acute infection were excluded. Ethical approval was granted by the Institutional Review Board of the Veterans General Hospital, Taipei, Taiwan. All subjects provided written informed consent.

**Blood Sampling**

Before catheter ablation, fresh peripheral blood samples were collected for ELISA in patients with AF. In control patients, fresh peripheral blood samples were collected in the cold fasting state before any procedure. In 16 patients, blood samples drawn from the left atrium were collected after the transseptal procedure and before catheter ablation. Total serum HSP27 and HSP70 levels were determined by ELISA (Stressgen), as were TNF-α and IL-10 levels (Bender Medsystems). High-sensitivity C-reactive protein was determined using particle-enhanced immunoturbidimetry with latex microparticles sensitized with duck anti-CRP immunoglobulin Y (Good Biotech Corp; Taichung, Taiwan).

**Real-Time Polymerase Chain Reaction**

Before catheter ablation, fresh anticoagulated peripheral blood samples were collected. Total mRNA was prepared with a DNA-free RNA Blood Mini-Kit (Invitrogen) from the buffy coat. The mRNA concentration was estimated by absorbance at 260 nm. Reverse transcription was performed with the First Strand cDNA Synthesis Kit (Ambion; Austin, TX) using equal amounts of total RNA. The real-time polymerase chain reaction was performed using iQ SYBR Green Supermix and an iCycler iQ thermal cycler (Bio-Rad) following the manufacturer’s protocols. Gene expression levels were normalized relative to α-actin references. At least 2 independent experiments were conducted. The oligonucleotide primers were as follows: β-actin (sense, 5'-TCCCTGTCGATCCACGAAAACCT-3'; antisense, 5'-GAAGACATTTCGCGTCAGGAT-3'), TNF-α (sense, 5'-CTCTTCGACCCCCAGTGA-3'; antisense, 5'-GGAAGCTGCCCCTCAGCTT-3'), IL-10 (sense, 5'-CCAGACATCAAGGCGCAGC-3'; antisense, 5'-CCTAGATGTCATAGGACTCGG-3'), and HSP27 (sense, 5'-GTCCCGTGATGCTGACCACCTT-3'; antisense, 5'-CAGTCTCAGGATTTTGCACC-3'). Relative mRNA expression changes were assessed by comparative analysis of quantitative real-time polymerase chain reaction using the ΔΔCt method. The Ct is the fractional cycle number at which the fluorescence signal of normalizedized samples passes a fixed threshold higher than baseline. The ΔΔCt values of the sample were determined by subtracting the average Ct value of the test gene from the average Ct value of the β-actin gene.

**Catheter Ablation of AF**

The electrophysiological study, the contact electroanatomical mapping, signal analysis, identification of pulmonary vein and non-pulmonary vein ectopic beats, catheter ablation of AF, and the follow-up of recurrences were performed as described previously. Those techniques have been described as detailed in the online-only Data Supplement.

According to the 2006 American College of Cardiology/American Heart Association guideline, recurrent AF is defined as paroxysmal if the arrhythmia terminates spontaneously. When sustained beyond 7 days, AF is defined as persistent. The category of persistent AF also includes cases of long-standing AF (eg, >1 year), usually leading to permanent AF, in which cardioversion has failed or has not been attempted. In the present study, persistent and permanent AF were classified as nonparoxysmal AF. The duration of AF history in those with nonparoxysmal AF was 66.1±46.5 months.

**Follow-Up of AF Recurrences**

After discharge, the patients underwent follow-up (2 weeks after the catheter ablation and then every 1–3 months thereafter, with a
follow-up of 1 year) at our cardiology clinic or with the referring physicians, during which either 24-hour Holter monitoring or cardiac event recording with a recording duration of 1 week was performed; antiarrhythmic drugs were prescribed for 8 weeks to prevent any early recurrence of AF. An AF recurrence was defined as an episode lasting >1 minute, and was confirmed by ECGs 3 months after the ablation (blanking period). The end point for the follow-up was the clinically documented recurrence of atrial arrhythmias or repeat ablation procedures.

Definition of Subgroups
A receiver operating characteristic curve was used to find the optimal cut point of HSP27S for classifying the recurrence after catheter ablation. At the cut point of 3.85 ng/mL, identified by the receiver operating characteristic curve (sensitivity, 0.5; specificity, 0.75; area under the curve, 0.64; 95% CI, 0.52–0.76; \( P = 0.03 \); online-only Data Supplement Figure 1), the patients were classified as having either a low HSP27S level (≤3.85 ng/mL) or a high HSP27S level (>3.85 ng/mL).

Statistical Analysis
The 2-sample \( t \) test and the Wilcoxon rank-sum test were used as appropriate. Categorical data were compared using a \( \chi^2 \) test with Yates correction or the Fisher exact test. Binary logistic regression was used for the comparison of AF with control. Freedom from AF (including recurrences or repeat procedures) was determined and compared using a Kaplan-Meier analysis and log-rank test. A Cox regression analysis was used to identify the independent factors associated with AF recurrences. The correlations were analyzed by different models of regression, and the best-fit model with the highest \( r^2 \) value was chosen and presented. Statistical significance was established at \( P < 0.05 \). PSAW SPSS 18.0 was used for statistical analysis.

Results
Correlation Between HSP27S and AF
Both groups’ clinical, biochemical, and echocardiographic characteristics are summarized in Table 1. Between the AF and control patients, there were no significant differences in age, sex, underlying diseases, smoking, alcohol use, medications, renal function, blood glucose, or lipid profiles. The patients with AF had received at least one antiarrhythmic drug and, compared with controls, had a larger left atrial diameter (LAD; 40.2 ± 7.0 versus 33.8 ± 6.7 mm; \( P < 0.001 \)), waist circumference (87.3 ± 8.9 versus 83.8 ± 11.1 cm; \( P = 0.03 \)), body mass index (25.6 ± 4.3 versus 24.2 ± 3.8 kg/m\(^2\); \( P = 0.01 \)), and lower HSP27S levels (5.73 ± 0.52 ng/mL; \( P = 0.02 \)). The left ventricular ejection fraction did not differ; HSP70 (0.22 ± 0.15 versus 0.22 ± 0.15 ng/mL; \( P = 0.87 \)), TNF-\( \alpha \) (0.22 ± 0.07 versus 0.22 ± 0.10 pg/mL), and IL-10 (0.10 ± 0.10 versus 0.12 ± 0.20 pg/mL) did not differ.

By using age, sex, diabetes mellitus, hypertension, hyperlipidemia, smoking, LAD, waist circumference, body mass index, and HSP27S levels as variables for logistic multivariate analysis, the HSP27S level was still independently correlated with AF (odds ratio, 0.92 per ng/mL; 95% CI, 0.85–0.99; \( P = 0.03 \)).

In the patients with AF, the HSP27S levels of the left atrial blood were similar to those of peripheral venous blood (3.58 ± 0.65 versus 3.53 ± 0.75 ng/mL; \( n = 16 \); \( P = 0.95 \)). There were 83 patients with paroxysmal AF and 31 patients with nonparoxysmal AF. However, HSP27S levels were lower in those with nonparoxysmal AF than in those with paroxysmal AF (4.70 ± 0.35 ng/mL [\( n = 31 \)] versus 6.13 ± 0.45 ng/mL [\( n = 83 \)]; \( P = 0.02 \); Figure 1).
HSP27S and Left Atrial Size
In the control patients, HSP27S levels did not correlate with high LAD. However, in the patients with paroxysmal AF, LAD/4 cm was associated with lower HSP27S levels (4.77 ± 0.43 ng/mL [n = 28] versus 6.25 ± 0.54 ng/mL [n = 52]; P = 0.03, Figure 2), compared with LAD <4 cm. In patients with nonparoxysmal AF (n = 31), only 5 patients had LAD (≤4 cm), which was not representative. Therefore, the statistics were not performed. The HSP70, TNF-α, and IL-10 levels have no correlation (B).

HSP27S and AF-Related Electrophysiological Characteristics
There were 63 patients with paroxysmal AF with left atrial voltage recordings. Low HSP27S (<3.85 ng/mL) was associated with low atrial voltage (1.78 ± 0.16 mV [n = 19] versus 2.19 ± 0.08 mV [n = 44]; P = 0.01; Figure 3A). Among the patients with paroxysmal AF receiving catheter ablation, low HSP27S was associated with more nonpulmonary vein ectopies, including those from the ligament of Marshall, left atrial free wall or septum, and coronary sinus (16.7% [n = 24] versus 0% [n = 54]; P = 0.01). Frequency analysis recordings were available for 17 patients with nonparoxysmal AF. The mean fractionated interval correlated well with the HSP27S level (r=0.72, P<0.001, Figure 3B). The HSP70, TNF-α, and IL-10 levels had no correlation with left atrial voltage, the incidence of nonpulmonary vein ectopies, and mean fractionated interval.

HSP27S and Catheter Ablation Outcome
A total of 105 patients with AF (paroxysmal and nonparoxysmal) were included for the Kaplan-Meier and Cox regression analysis (mean follow-up, 301.6 days). There were 30 patients who had AF recurrence after catheter ablation. At a cut point of 3.85 ng/mL, Kaplan-Meier analysis showed that the maintenance rate of sinus rhythm within 1 year after catheter ablation was lower in patients with HSP27S levels ≤3.85 ng/mL, compared with those with levels >3.85 ng/mL (55.9% [n = 34] versus 78.9% [n = 71]; P = 0.015; Figure 4). Seven patients who did not receive catheter ablation because of severe coronary stenosis or atrial thrombus and 2 patients lost to follow-up within 3 months after the ablation were not included in the analysis; however, 7 patients lost to follow-up 3 months after catheter ablation were included. The analysis was further performed for the patients with paroxysmal AF.
In the patients with paroxysmal AF, a high HSP27S level (3.85 ng/mL, n=54) was the only factor that was independently associated with sinus rhythm maintenance after catheter ablation (hazard ratio, 3.07; 95% CI, 1.06–8.85; P=0.039), adjusted for age, sex, waist circumference, and LAD (Figure 4).

**HSP27S-Related Inflammatory Mechanisms**

The possible inflammatory mechanisms of HSP27 in the patients with AF were further studied. Circulating TNF-α, IL-10, and high-sensitivity CRP protein levels were determined, and mRNA samples of white blood cells were analyzed (n=54). There was no relationship between high-sensitivity CRP and HSP27S levels (r=0.01, P=0.94). The HSP27S protein level was correlated with IL-10 (r=0.57, P=0.01) and TNF-α protein (r=0.63, P=0.01) levels. HSP27 mRNA levels were correlated with IL-10 (r=0.55, P=0.01) and TNF-α mRNA (r=0.71, P<0.01) levels.

The correlation between different types of leukocytes, HSP27S, HSP70, TNF-α, and IL-10 level showed that only the HSP27S level was associated with lymphocyte ratio (r=0.19, P=0.04 by Pearson correlation). No correlation was found between leukocyte types and other cytokines (Table 2).

**Table 2. Association of Different Cytokines With Leukocyte Types**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>HSP27 (ng/mL)</th>
<th>HSP70 (ng/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>IL-10 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (no./mm³)</td>
<td>−0.17</td>
<td>0.02</td>
<td>−0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.19*</td>
<td>0.04</td>
<td>0.002</td>
<td>−0.04</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>−0.16</td>
<td>−0.03</td>
<td>−0.04</td>
<td>−0.03</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>0.08</td>
<td>0.03</td>
<td>0.14</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*Correlations (r values) are given. Information in parentheses indicates how these variables were measured.

HSP indicates heat shock protein; TNF, tumor necrosis factor; IL, interleukin; WBC, white blood cell.

*P<0.05.

**Discussion**

**Major Findings**

This study comprehensively examined the role of HSP27S in human AF, and the results showed that the HSP27S level is low in AF. In addition, HSP27S levels predicted the catheter ablation outcomes. Regarding the underlying mechanisms, the findings that HSP27S levels were associated with LAD, left atrial voltage, and fractionated interval imply that HSP27S contributes to the pathogenesis of AF through structural and electric remodeling.
HSP27S-Related Inflammatory Pathways

Atrial HSP27-related myolysis and electrophysiological changes were reported in the previous study. The present study further found a link between HSP27S and inflammation-related pathways, which has never been previously reported in AF. Inflammation is important in AF pathogenesis, and HSP27 is considered an anti-inflammatory, regulating TNF-α and IL-10, which are associated with AF. The IL-10 single-nucleotide polymorphism (A allele), associated with low IL-10 levels, is at risk for AF. The TNF-α levels in patients with AF are higher than in those without AF. The interactions between HSP27, TNF-α, and IL-10 are associated with mitogen-activated protein kinase-associated pathways, which are activated by cytokines, and critical in the production of cytokines. The HSP27 increases IL-10 by activating p38 mitogen-activated protein kinase signaling, through which TNF-α also regulates HSP27. Other than through mitogen-activated protein kinase-associated pathways, TNF-α also decreases HSP27 through protein kinase C activation. Beyond interactively regulating the cellular levels, HSP27 protects against TNF-α-induced oxidative stress and apoptosis. It also interferes with TNF-α down-streaming signals by modulating transforming growth factor-β-activated kinase-1 activity, which is not reported in IL-10-associated signals. The present study demonstrated a good correlation between HSP27S, IL-10, and TNF-α at each of the transcriptional and translational levels. However, HSP27S levels were not correlated with high-sensitivity CRP, suggesting that the interactive regulation between HSP27S, inflammation, and atrial remodeling was through specific inflammatory cytokines, not general inflammatory pathways, as indicated by high-sensitivity CRP level.

HSP27S and Catheter Ablation Outcome

Although several studies suggested the potential role of atrial HSP27 in AF, it has never been reported that HSP27S was correlated to catheter ablation outcome. The present study demonstrated that the HSP27S levels could predict 1-year AF-free survival, demonstrating HSP27’s importance in the secondary prevention of AF after catheter ablation. Many inflammatory cytokines were associated with AF, but few demonstrated a link to electroanatomical properties and affected clinical outcome. Endothelin-1 has been linked with atrial dilatation and fibrosis, and plasma endothelin-1 has been associated with the failure of pulmonary vein isolation. The HSP27’s potential role in electroanatomical remodeling might explain why HSP27S levels could predict long-term catheter ablation outcome.

Study Limitations

The present study only studied serum HSP27 levels. The expression level in atrial tissue was not studied. Further mechanistic studies are needed to investigate how HSP regulates inflammation and atrial structure. An association between low HSP27S level and AF-related structural and electric remodeling was shown, but a causative role could not be determined by the present study. Because sinus rhythm was resumed only after pulmonary vein isolation and ablation of complex fractionated atrial electrograms in the patients with nonparoxysmal AF, the atrial voltage might not reflect the baseline voltage before catheter ablation. Therefore, voltage mapping was not regularly detected in those with nonparoxysmal AF.

Conclusions

The HSP27S levels were correlated with LAD, left atrial voltage, and fractionated intervals, and predicted AF recurrence after catheter ablation, which might be considered as a prognostic factor of AF progression or outcome after catheter ablation. The mechanisms could be related to inflammation.

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Disclosures

None.

References

This case-control study (atrial fibrillation [AF] group, n=114; control group, n=100) was conducted to determine the clinical relationship between serum heat shock protein 27 (HSP27S) and AF. A low baseline HSP27S was independently associated with AF. In patients with AF, those with paroxysmal AF (PAF) had higher baseline HSP27S levels compared with those without PAF. In patients with PAF, a lower baseline HSP27S was associated with larger left atrial diameter, lower atrial voltage, and more nonpulmonary vein ectopic beats. In non-PAF, the mean fractionated interval had a good correlation with baseline HSP27S. After catheter ablation, a high baseline HSP27S level predicted sinus rhythm maintenance in the patients with PAF. Baseline HSP27S was also correlated with IL-10 and TNF-α levels. Analysis of Buffy coat mRNA levels showed the same correlations. In conclusion, HSP27S levels were correlated with left atrial diameter, left atrial voltage, fractionated intervals, and predicted AF recurrence after catheter ablation. The mechanisms could be related to inflammation.
Electrophysiological Correlation and Prognostic Impact of Heat Shock Protein 27 in Atrial Fibrillation

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SUPPLEMENTAL MATERIAL

Electrophysiological Correlation and Prognostic Impact of Heat Shock Protein 27 in Atrial Fibrillation

Yu-Feng Hu, MD,‡ Hung-I Yeh, MD, PhD,§‡ Hsuan-Ming Tsao, MD,† Ching-Tai Tai, MD, ‡ Yenn-Jiang Lin, MD, ‡ Shih-Lin Chang, MD, ‡ Li-Wei Lo, MD, ‡ Ta-Chuan Tuan, MD, ‡ Kazuyoshi Suenari, M.D, † Cheng-Hung Li, MD,*, Tze-Fan Chao, MD,* Shih-Ann Chen, MD‡

Methods

Contact electro-anatomical mapping and signal analysis

These techniques have been described in our previous work (14-15). In brief, each patient underwent an electrophysiological study and catheter ablation in the fasting, non-sedated state after written informed consent was obtained. A sequential contact voltage map was constructed in all patients during sinus rhythm before radiofrequency ablation. The bipolar electrograms were filtered between 32 to 300 Hz and recorded digitally. The absolute peak was selected as the detection setting to determine the point of activation in the waveform. The electrodes of a coronary sinus catheter were used to provide the timing reference signal during the mapping procedure. A 4-mm tipped ablation catheter (EP Technologies Boston Scientific Inc., MA) was selected as the roving catheter. The signal from the roving catheter was used to build a sequential map. The average bipolar mapping sites either in the right and
left atria were more than two hundred points for each patient. After completion of the sequential map, the bipolar mapping points were collected and analyzed by the off-line software. The mean peak-to-peak voltage throughout both atria was calculated. The voltage mapping was performed only during sinus rhythm.

The Fast-Fourier transform method has been described previously. A 6.82-second data segment was exported to external software. Frequency analysis (sampling rate=1200 Hz, resolution=0.14 Hz, with a Hanning window function) was performed from all recording sites. For fractionation mapping, NavX mapping parameters were set to the CFE-mean (fractionation interval, FI), an interval-analysis algorithm that measures the average index of the fractionation at each site and produces a color CFE distribution map. Sites of continuous CFEs indicated the most fractionated sites with a local mean FI <50 msec and recording duration >5 seconds. Sites with shorter mean FI indicated high temporal stability of the fractionated electrograms. Non-CFEs were defined as FI >120 msec. The consistency of fractionated electrograms over time has been validated previously (16).

**Catheter ablation of AF**

In paroxysmal AF, we first tried to find the spontaneous onset of the ectopy triggering the AF. The short-duration (eight beats) burst pacing from the right atrium, coronary
sinus, and PVs was used to facilitate spontaneous AF with or without an isoproterenol infusion (up to 4 µg/min). The pulmonary vein (PV) ostia were identified by fluoroscopy and marked on the 3-dimensional map of the LA. Continuous circumferential lesions were created encircling the right and left PV ostia guided by the NavX system using 4 mm irrigated tip ablation catheters (Therapy Cool Path, St Jude Medical). Radiofrequency ablation in the left atrium was performed at 30W on the anterior wall, and 25W on the posterior wall. The tip of the catheter was irrigated with heparinized saline at the rate of 17mL/min. After completion of the circumferential lesion set, the ipsilateral superior and inferior PVs were mapped carefully by a circular recording catheter (Spiral, AF Division, St. Jude Medical, Inc., Minnetonka, MN) during sinus rhythm or CS pacing. After successful isolation of all four PVs, which was confirmed by PV circumferential mapping, high current (3-5 times the pacing threshold) and wide (8 msec) pulse duration stimulation from the proximal and distal CS was performed (in 10-ms decrements from 250 to 150 ms, with a duration of each pacing cycle length [CL] of 5-10 s), and repeated 3-5 times. If sustained AF/flutter was still induced, cardioversion was performed to restore sinus rhythm.

In non-paroxysmal AF, PV isolation was performed as the first step. If AF did not stop, an additional ablation of the complex fractionated atrial electrogram (CFAE)
sites was performed sequentially based on the results of the CFAE maps after the PVI.

The end point of the ablation of the CFAE sites was to obtain a prolongation of the

cycle length, eliminate the CFAE, or abolish the local fractionated potentials (bipolar

voltage < 0.05 mV). If the AF still did not stop after additional ablation of the CFAEs,

sinus rhythm was restored by electric cardioversion.

Methods of the identification of the atrial ectopic beats were described in our previous

publications (17). Identification of pulmonary vein and non-pulmonary vein ectopies

was performed in paroxysmal AF patients. In patients with paroxysmal AF, we

attempted to find the spontaneous onset of atrial ectopic beats or repetitive episodes of

short runs or sustained AF and to predict the location of the initiating foci at baseline.

Mapping of the pulmonary veins was guided by the venous phase of selective

pulmonary artery angiography, with the first pair of electrodes straddling the ostium;

the catheters were first put into superior pulmonary veins and then the inferior

pulmonary veins if the ectopic focus was suspected to be from the inferior pulmonary

veins. If the initiating focus of AF was considered to be from the right atrium, we put

one duodecapolar catheter (1-mm electrode length and 2-mm interelectrode spacing)

along the crista terminalis to reach the atriocaval junction area and the superior vena
cava for simultaneous mapping of the pulmonary veins and superior vena cava. The

superior vena cava mapping catheter was advanced to the site with the most distally
recorded. In patients with ectopic beats from the ligament of Marshall (LOM), double potentials (DPs) are present at the orifice of or inside the left PVs, and distal coronary sinus (CS) pacing can help differentiate the LOM potential from the PV musculature potential. If the second deflection (D2) of DPs is attributable to activation of LOM, the CS ostium to D2 interval will be shorter during distal CS pacing compared with sinus rhythm.

**Supplemental figure 1.** ROC curve of HSP27S levels for classifying the recurrence after catheter ablation for AF.