Association of Plasma Concentration of Small Heat Shock Protein B7 With Acute Coronary Syndrome

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Background: Heat shock proteins (HSPs) act as chaperones and have a protective function in cardiovascular diseases. The clinical association of a novel small HSPB7 with cardiovascular disease, however, has not been reported. The aim of this study was to investigate the potential biological functions of HSPB7 and its relationship with acute coronary syndrome (ACS).

Methods and Results: A mouse myocardial infarction (MI) model and samples from clinical human subjects were used to determine plasma HSPB7 concentration after acute MI. The associations of plasma HSPB7 concentration with ACS and other risk factors of coronary artery disease were analyzed. Plasma HSPB7 concentration was found to be rapidly elevated in mice after coronary artery ligation. In addition, plasma HSPB7 concentration was significantly higher in patients with ACS than in control patients with non-cardiac chest pain (5.1 ng/ml vs. 2.9 ng/ml, P<0.001). Plasma HSPB7 was detected as early as 1–3 h after the onset of symptoms and remained detectable up to 24 h. Furthermore, in patients presenting to the emergency department with acute chest pain, HSPB7 level was an independent risk factor of ACS (adjusted odds ratio, 7.44; 95% confidence interval: 1.91–28.93, P<0.01).

Conclusions: HSPB7 is a potential early biomarker after MI and serves as an independent risk factor of ACS in patients with acute chest pain. (Circ J 2012; 76: 2226–2233)

Key Words: Acute coronary syndrome; Biomarker; Myocardial infarction; Risk factors; Small heat shock protein

Heat shock proteins (HSPs) are abundant intracellular proteins found in both prokaryotic and eukaryotic organisms. Most HSPs act as chaperones and are involved in protein folding and transport.1–3 HSPs could be grossly classified by molecular mass into the general HSP family (approximately 40–110kDa) or the small HSP family (sHSP, approximately 10–30kDa).4–6 The general HSPs, including HSP60, 70 or 90, are associated with cardiovascular diseases including cardiac hypertrophy,7,8 heart failure,9-12 and ischemia/reperfusion injury.13-16 Therefore, general HSPs may contribute to the protective mechanism during the pathogenesis of cardiac hypertrophy, heart failure, and ischemia/reperfusion injury. The involvement of sHSPs in cardiovascular diseases, however, remains largely unknown.

HSPB7, an sHSP, was first identified by Krief et al and designated as a cardiovascular HSP for its high expression in the heart, low expression in the skeletal muscle but virtual absence in all other tissues examined.17 HSPB7 is characterized by its small molecular mass, approximately 20kDa, and a highly conserved α-crystallin domain.18 Subcellular fractionation and confocal immunofluorescence showed that sHSPs including HSPB7 are localized within the cytosol or are associated with myofilaments in cardiac or skeletal muscle cells.19,20 Despite its abundant cardiac expression, HSPB7 was recently linked to sporadic heart failure on genome-wide association studies.21–24 but whether HSPB7 is involved in the pathogenesis of coronary artery disease (CAD) or acute coronary syndrome (ACS) remains unknown.

We hypothesized that the expression of HSPB7 may be affected and released into the circulation during myocardial ischemia or after myocardial necrosis. The aim of this study was to investigate the potential association of plasma HSPB7 concentration with ACS in both an animal model and human subjects. We found that plasma concentration of HSPB7 is rapidly elevated after myocardial infarction (MI) and is an independent predictor of ACS.
Methods

Animal and Human Protocols
All surgical procedures were performed according to the protocols approved by the Institutional Animal Care and Utilization Committee, Academia Sinica. This study conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Investigations involving human subjects were conducted in accordance with the principles outlined in the Declaration of Helsinki.

Generation of Anti-HSPB7 Monoclonal Antibodies
Splencocytes from BALB/c mice immunized with recombinant GST-HSPB7 (residues 2-170) protein were fused with P3X myeloma cells to produce hybridomas. Hybridomas positive for HSPB7 were identified and cloned. Ascites fluids were prepared, and purified IgG was obtained on protein G chromatography. We obtained 4 independent clones, monoclonal antibody (mAb) 2, 3, 5, and 6, that could specifically recognize the recombinant full-length HSPB7 protein expressed in human embryonic kidney (HEK)-293T cells on western blot (Figure 1). mAbs 2 and 5 were used in the following experiments because of their higher specificity.

Animal Model of MI
Coronary artery ligation was used as an animal model of MI as described. The incision landmark is the left armpit. An oblique 8-mm incision is made 2 mm away from the left ster nal border toward the left armpit. The muscles are separated without damaging blood vessels. After the opening of the chest cavity, the chest retractor is applied for better visualization of the pericardium. The left anterior descending (LAD) coronary artery can be visualized after opening the pericardium. The LAD artery is ligated 1–2 mm with a 7-0 silk ligature below the tip of the left auricle in its normal position. Occlusion is confirmed by the change of color of the myocardium. The chest wall was closed layer by layer. Mice in the sham control group underwent the entire procedure except for ligation of coronary artery. Blood samples were collected by laparotomy via the inferior vena cava for HSPB7 assay at indicated time points after MI.

Patient Enrollment
The collection of patient samples was approved by Institute Review Board of Chang-Gung Memorial Hospital, Taoyuan, Taiwan (97-0606C) and the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan (AS-IRB01-10050 (07010)). Adult patients (aged ≥18 years old) presenting to the emergency department with chest pain as the chief complaint were evaluated for eligibility. Exclusion criteria included idiopathic cardiomyopathic conditions, significant valvular heart disease, any malignancy, hematologic or rheumatic disease, and chronic kidney disease (serum creatinine kinase ≥3 mg/dl). Patients gave informed consent to be in the study. Blood samples were collected for laboratory testing for blood count, lipid profile and creatinine kinase-MB isoform (CK-MB), cardiac troponin I (cTnI), and HSPB7. Patients were assigned to the non-car-
Diabetic chest pain group, stable angina (SA) group, or ACS group according to American College of Cardiology/American Heart Association guidelines for the management of ST-elevated MI (STEMI) and unstable angina/non-ST-elevated MI (UA/NSTEMI).

**Plasma HSPB7 Assay**

Heparinized blood samples were centrifuged at 2,500 \( \times \) g, and plasma was stored at \(-80^\circ\mathrm{C}\) for further analysis. An in-house capture enzyme-linked immunosorbent assay (ELISA) using anti-HSPB7 mAb 2 and 5 was developed as described. The minimum detection limit of this method was 2 ng/ml. All assays were performed in batch by another investigator blinded to the clinical diagnoses. The intra-assay and inter-assay covariance of this assay was <10%.

**Statistical Analysis**

Demographic data are presented as mean±SD for continuous variables and n (%) for binominal variables. Analysis of the baseline subject characteristics followed a case-control design, namely, ACS versus control subjects and SA versus control subjects. The chi-squared and 2-sample t-test/Mann-Whitney rank sum tests were used for analysis. Circulating HSPB7 levels had a lognormal distribution, and therefore data were transformed (log10) before logistic regression analysis. Multiple logistic regression analysis was used to evaluate the association(s) between HSPB7 concentration and ACS or risk factors, with appropriate adjustments for covariates. A 2-tailed \( P<0.05 \) was considered statistically significant. SAS v9.1 (SAS Institute, Cary, NC, USA) was used for statistical analysis.

**Results**

**Production of Anti-HSPB7-Specific mAbs**

To investigate the clinical association of cardiac HSPB7 at the protein level, we first generated mAbs against human HSPB7 protein. Spleen cells from BALB/c mice immunized with the GST-HSPB7 fusion protein containing residues 2-170 of human HSPB7 were used to prepare the mAb with use of a standard hybridoma technique. After screening on ELISA and subcloning, 4 specific mAb clones (2, 3, 5, and 6) against human HSPB7 were obtained. The specificity of these mAbs was tested on western blot with recombinant human HSPB7 or HSPB9 protein (the most closely related sHSP family member) expressed.
in HEK-293T cells. As shown in Figure 1A, these mAbs detected only human HSPB7 and did not cross-react with human HSPB9. In addition, these mAbs also recognized mouse HSPB7 protein on western blot or validated its expression in the cardiomyocytes on immunohistochemistry (Figures 1B, C).

Profile of Released HSPB7 in Mouse MI Model
We then examined whether myocardial HSPB7 can be released into the circulation in a mouse model of MI induced by ligation of the LAD coronary artery. In MI mice, the plasma HSPB7 level was significantly elevated as early as 1 h after LAD ligation, peaked at 6 h and remained detectable up to 12 h after MI (Figure 2). Plasma HSPB7 concentration returned to the minimal detection threshold level at 24 or 48 h after MI. In the sham control animals, however, HSPB7 concentration was below or around the minimal detection level at all time points (Figure 2A). In addition, the expression of myocardial HSPB7 protein remained basically unaltered before or after MI as determined on western blot (Figure 2B) or immunohistochemical staining for HSPB7 (Figure 2C), respectively. Together, these data suggest that increased plasma HSPB7 level after MI is likely caused by passive release instead of active synthesis from the myocardium after injury.

Plasma HSPB7 Concentration in ACS
We further investigated the association of plasma HSPB7 levels and ACS in 186 patients presenting to the emergency department with chest pain: 77 patients with non-cardiac chest pain (control) group, 36 with SA, and 73 with ACS. Demographic characteristics are listed in Table 1. As compared with the non-cardiac chest pain group, patients in the ACS and SA groups were of similar age and sex and had similar risk factors, including diabetes mellitus, hypertension, current smoking, and CAD family history. In the ACS group, however, the detection rate for HSPB7 was much higher (62% vs. 14% or 22%, P<0.001; Table 1) or its plasma concentration was significantly elevated than in the SA or non-cardiac chest pain groups (5.1 ng/ml vs. 2.9 ng/ml, P<0.001; Figure 3). The ACS and non-cardiac chest pain groups differed in levels of cTnI, CK-MB, total cholesterol, low-density lipoprotein (LDL) and triglycerols, as well as leukocyte count, whereas the SA and non-cardiac chest pain groups differed in only leukocyte count and LDL level. Furthermore, plasma HSPB7 levels were positively correlated with the concentrations of markers for myocardial necrosis (Figure 4): cTnI (r=0.55, P<0.0001) or CK-MB (r=0.66, P<0.0001). The number of patients with STEMI, NSTEMI, and UA was 25, 25, and 23, respectively (Table S1). Plasma HSPB7 was detect-
able in >70% and >50% of patients with NSTEMI and STEMI, respectively, and was detectable in 5 of the 23 patients with UA (Table S1). Plasma HSPB7 level correlated with cTnI, which was highest in the NSTEMI group (Table S1).

### Plasma HSPB7 Concentration and Time After Symptom Onset

Plasma HSPB7 level was detectable as early as 1–3 h after the onset of symptoms in patients with ACS (Figure 5). Most patients with a detectable HSPB7 concentration experienced a short interval between symptom onset and blood sampling, usually <24 h, but plasma HSPB7 level was not detectable in patients presenting to the emergency department 24 h after the onset of symptoms.

### Plasma HSPB7 Concentration as a Risk Factor for ACS

Compared with traditional risk factors of CAD, plasma HSPB7 concentration was found to be a significantly predictor of ACS in the present patients (adjusted odds ratio [OR], 7.44; 95% confidence interval [CI]: 1.91–28.93, P<0.01; Table 2). Current smoking was the only traditional risk factor predicting ACS (adjusted OR, 3.54; 95%CI: 1.49–8.42, P<0.01). Male sex, age, body mass index, hypertension, and diabetes mellitus were not predictors of ACS in the present patient cohort.

HSPB7 level was detectable in some patients in the non-cardiac chest pain group. We examined whether a high HSPB7 concentration was associated with increased risk of ACS by dividing patients into 2 groups according to the 75th quartile of HSPB7 concentration (3.94 ng/mL; Table S2). Patients with ACS were more likely than others to have high HSPB7 concentration after adjusting for other possible risk factors (adjusted OR, 4.31; 95% CI: 1.80–10.33, P=0.0033).

Because current smoking was the only traditional risk factor associated with ACS in the present patients, we then analyzed...
the cumulative predictive effect of current smoking and HSPB7 concentration for ACS. Patients with ACS were more likely than others to have both risk factors (adjusted OR, 20.08; 95% CI: 2.21–182.32, P=0.0077; Table S3). With either risk factor alone, the risk was lower but still significant (adjusted OR, 3.87; 95% CI: 1.84–8.1), Furthermore, patients with ACS were more likely than others to be current smokers and have high HSPB7 (adjusted OR, 18.17; 95% CI: 1.96–168.77, P=0.0097; Table S4).

**Discussion**

HSPB7 is an abundant protein selectively expressed in myocardial muscle cells, but the biological function and clinical association of HSPB7 is not clear. In this study, we demonstrated that plasma HSPB7 level is associated with ACS in both an animal model and human subjects. Because plasma HSPB7 concentration was detectable as early as 1–3 h after LAD ligation in the animal model or after MI in human patients (Figures 1,4) and because we found a positive correlation between plasma HSPB7 and 2 cardiac biomarkers of necrosis, cTnI and CK-MB (Figure 3), HSPB7 might be rapidly released from cardiomyocytes into the circulation as a result of myocardial necrosis according to western blotting and immunohistochemical staining (Figure 2). We found that HSPB7 level, especially high HSPB7, is an independent risk factor for ACS in patients presenting to the emergency department with acute chest pain. Thus, HSPB7 not only shows potential as an early biomarker of MI but also as an independent risk factor for ACS in patients with acute chest pain. This study establishes the basis for further investigations into the role of HSPB7 in ACS.

Previous studies have demonstrated that overexpression of sHSPs such as HSP20 or HSP27 prevents apoptosis by ischemia-reoxygenation in myocardial cells and that overexpression of sHSP in transgenic mice protected the heart against the damaging effects of ischemia-reperfusion.20–31 Furthermore, a recent report showed that HSPB7 is the most active member of the sHSP family in preventing aggregation of disease-associated proteins with an expanded polyQ stretch.32 Because myocardial HSPB7 translocates from the cytosol to the Z- or I-band of myofibrils upon ischemia injury,20 HSPB7 may have a protective function by preventing misfolding or refolding of the denatured cytoskeletal proteins under acute stress as in MI, but further studies are needed to clarify its specific role in ACS.

The current biomarkers used to detect MI are cardiac-specific troponins (cTnI or cTnT). Measurement of cTnI or cTnT provides accurate, sensitive, and specific determination of myocardial injury but also has prognostic value.33–35 and they have replaced CK-MB as the preferred marker for detection of myocardial necrosis.36,37 Troponins have some disadvantages, however. For example, plasma troponin concentration cannot be detected until at least 4–6 h after the onset of symptoms. Thus, patients presenting to the emergency department with typical chest pain must undergo a second troponin measurement (usually 8–12 h after the onset of symptoms) if the initial measurement showed no elevation. Chest pain units/centers have been developed as a model of patient care in the emergency department in order to decrease the cost of patient care and the adverse events.38,39 Recent multi-center studies showed that high-sensitivity cTn assays can improve the early diagnosis of acute MI and risk stratification,40,41 regardless of the time of chest pain onset. Because plasma HSPB7 is detectable as early as 1–3 h after the onset of symptoms (Figures 1,4), it is tempting to speculate that HSPB7 level could serve as another early myocardial necrosis biomarker that may further increase diagnostic specificity when combined with sensitive cTn assays. cTn is not elevated solely in the presence of myocardial injury. Plasma cTn may be elevated in sepsis, hypovolemia, atrial fibrillation, renal failure, and so on, without thrombotic event.42 Given that HSPB7 is detectable only in cardiac muscles, it might be utilized for the diagnosis of ACS with better specificity in some of the aforementioned conditions, but additional studies are needed to verify this issue. HSPB7 is detected in some patients with SA and non-cardiac chest pain. Further study is needed to verify if these patients should receive the same management protocol as those with ACS. This result demonstrates the potential clinical importance of plasma HSPB7 level to identify more patients with CAD. Elevated HSPB7 levels were reported to be associated with increased risk of CAD.43 Other scores are used for risk stratification in patients with ACS.44 Risk factors of CAD are diabetes mellitus, older age, male sex, dyslipidemia, hypertension, obesity, and CAD family history. These factors help physicians stratify risk for patients presenting with acute chest pain. We were unable to determine whether patients with non-cardiac chest pain had CAD because no stress test was performed. We then examined plasma HSPB7 concentration as a risk factor for ACS instead of CAD and found that plasma HSPB7 concentration and current smoking were 2 independent risk factors of ACS in patients with chest pain. The cumulative effects were also significant. In the emergency department, presenting with a risk factor for ACS is more important than presenting with a risk factor for CAD. ACS requires emergency treatment including percutaneous coronary intervention, thrombolytic therapy, and intensive care unit admission. Of note, we found high HSPB7 levels in some patients with SA, and some ACS patients had elevated HSPB7 but not cTn. Therefore, HSPB7 may represent a more sensitive biomarker, at least in a subgroup of patients with low or undetectable cTn, in detecting myocardial necrosis, but this remains to be further investigated.

**Study Limitations**

The present study contained some limitations. First, we could not exclude the possibility of protein degradation because plasma samples were stored at −80°C until analysis. The samples were thawed only once, however, and HSPB7 data were similar in fresh and frozen samples. Second, patients presented to the emergency department with the chief complaint of chest pain, typical or atypical. In addition, patients with non-cardiac chest pain did not undergo standard examinations (ie, stress test or coronary angiography) to exclude the presence of CAD.

**Table 2. Risk Factors for ACS**

<table>
<thead>
<tr>
<th>log HSPB7 (ng/ml)</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.44</td>
<td>1.91–28.93</td>
<td>0.0038</td>
<td></td>
</tr>
<tr>
<td>Current smoking</td>
<td>3.54</td>
<td>1.49–8.42</td>
<td>0.0042</td>
</tr>
<tr>
<td>Male</td>
<td>1.02</td>
<td>0.45–2.28</td>
<td>0.9667</td>
</tr>
<tr>
<td>Age ≥65 years</td>
<td>1.37</td>
<td>0.61–3.04</td>
<td>0.4450</td>
</tr>
<tr>
<td>BMI ≥25 kg/m²</td>
<td>1.04</td>
<td>0.50–2.17</td>
<td>0.9198</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.82</td>
<td>0.38–1.77</td>
<td>0.6044</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.19</td>
<td>0.54–2.62</td>
<td>0.6598</td>
</tr>
<tr>
<td>CAD family history</td>
<td>1.30</td>
<td>0.61–2.78</td>
<td>0.4980</td>
</tr>
</tbody>
</table>

HSPB7 levels were log-transformed before analysis. CI, confidence interval; BMI, body mass index; OR, odds ratio. Other abbreviations as in Table 1.
The diagnosis was made by clinical judgment, which might suggest sampling bias. A gold standard to exclude CAD in patients with chest pain should be used in future studies. The sensitivity, specificity, and receiver operating characteristic analysis of HSPB7 in predicting ACS is compared with cTnI in Figure S1, suggesting that HSPB7 has less but still acceptable predictive value than cTnI. It was not reported in the results because the reference diagnosis (NSTE/UA) is determined by the presence of positive cTnI or not. This verification bias made it difficult to generate another biomarker with better or equivalent diagnostic accuracy on the basis of the current study design. Furthermore, the current ELISA for plasma HSPB7 concentration has detection sensitivity limited at 2 ng/ml. Given improvements in assay sensitivity, the clinical significance and implications of plasma HSPB7 concentration <2 ng/ml can be further elucidated. Finally, because the relatively small number of deaths or non-fatal cardiac events prevented prognostic evaluation during 6-month follow-up, further studies on whether HSPB7 level can predict clinical outcome in a larger number of ACS patients is warranted.

Conclusions

We found that plasma HSPB7 levels rise and fall rapidly after the onset of ACS in an animal model and clinical human subjects, and such levels may be an early marker of myocardial injury after MI. High HSPB7 level was significantly associated with increased risk of ACS after adjustment for traditional risk factors. Thus, plasma HSPB7 level may be an independent risk factor of ACS in patients presenting to hospital emergency departments with acute chest pain.

Acknowledgments

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References


**Supplementary Files**

**Supplementary File 1**

- **Table S1.** Plasma HSPB7 Level in ACS Patients
- **Table S2.** ORs for ACS vs. HSPB7 Level
- **Table S3.** Cumulative Effect of HSPB7 Level and Current Smoking on Risk of ACS
- **Table S4.** Combined Effects of Current Smoking and HSPB7 Level on Risk of ACS
- **Figure S1.** Receiver operating characteristic (ROC) analysis of HSPB7 in NSTEMI patients.

Please find supplementary file(s): http://dx.doi.org/10.1253/circj.CJ-12-0238