Plasma Level of Growth Arrest–Specific 6 (GAS6) Protein and Genetic Variations in the GAS6 Gene in Patients With Acute Coronary Syndrome

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Key Words: Growth arrest–specific gene 6; GAS6; Acute coronary syndrome; ACS; Genotype frequencies; Single nucleotide polymorphism; Inflammation

Abstract

Growth arrest–specific gene 6 (GAS6) encodes a vitamin K–dependent protein that regulates inflammation, angiogenesis, and atherosclerotic plaque formation. The level of GAS6 expression is associated with plaque stability and stroke. We explored the role of GAS6 in cardiovascular disease, particularly in acute coronary syndrome (ACS). We determined the plasma levels of GAS6 protein by using an enzyme-linked immunosorbent assay method and investigated the role of the single nucleotide polymorphism (c.834+7G>A) in ACS. The median (interquartile range) plasma GAS6 levels were 16.9 µg/L (13-28 µg/L) in healthy control subjects and 10.65 µg/L (5.7-27.5 µg/L) in patients with ACS. The genotype frequencies for GG, AG, and AA, respectively, in patients with ACS were 66% (37/56), 29% (16/56), and 5% (3/56) and were 35% (14/40), 45% (18/40), 20% (8/40) in the control group. The AA genotype and A allele were less frequent in patients with ACS than in control subjects (P < .001). Our study indicates that GAS6 plasma concentrations at admission reflect the presence of common cardiovascular risk factors and can predict cardiovascular events. In addition, the AA genotype and A allele of the GAS6 gene relate to ACS, which may have a protective role against ACS.

Acute coronary syndrome (ACS) is a common complication of atherosclerosis that is characterized by persistent inflammation resulting from activated immune cells within coronary lesions and the systemic immune response to the lesions.1 The exact underlying mechanisms and risk factors are not fully understood, although extensive efforts have been devoted to this field.

The growth arrest–specific gene 6 (GAS6) has a number of diverse functions and contributes to the regulation of cell survival, proliferation, migration, and adhesion.2 There is increasing interest in studying GAS6 because the vitamin K–dependent protein encoded by this gene is secreted by leukocytes and endothelial cells in response to injury or serum starvation.2 GAS6 is also thought to act as a bridge between apoptotic cells and the phagocytes that ingest them.2 GAS6 apparently exerts many of its effects via receptors in the Axl subfamily of receptor tyrosine kinases composed of Axl, Sky, and Mer.2 In addition, other studies suggest that GAS6 has a signaling function via the PI3K/Akt pathway.3

It has been reported that there is dramatic up-regulation of Axl-GAS6 in the rat carotid artery following balloon injury, and a polymorphism in the GAS6 gene was found to be associated with stroke. GAS6 receptors have also been shown to regulate vascular calcification, and the Axl-Gas6 survival pathway has been implicated in the pathophysiology of atherosclerosis.4 Considering these findings and the roles of GAS6 in inflammation, apoptosis, and migration, we hypothesized that GAS6 has a role in cardiovascular pathophysiology. The purpose of the present study was to compare plasma GAS6 concentrations in patients with ACS with those in healthy subjects to determine the potential role of GAS6 in ACS and to examine its usefulness as a marker for cardiovascular disease.
Materials and Methods

Patients and Healthy Subjects

The study was approved by the institutional review board of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The regulations for human subject protection were strictly followed. The patients were recruited from the Cardiology Department and the Cardiac Care Unit, University Hospital (Union Hospital, Tongji Medical College).

The patient group consisted of 80 unrelated patients ranging in age from 36 to 79 years (mean, 62.9 years); all had symptoms of ischemia that were verified by electrocardiography or by increased levels of biochemical markers (creatinine kinase myocardial binding isoenzyme [CK-MB], >6.6 µg/L [6.6 ng/mL] or troponin I [TnI], >0.3 µg/L [0.3 ng/mL]). Only patients who had experienced their last chest pain within the previous 48 hours were included in the study. The diagnosis of ACS was established on the basis of current definitions: the symptoms of myocardial ischemia associated with ST segment depression (≥0.1 mm), T-wave inversion (≥0.1 mm), or elevation of biochemical marker levels (CK-MB, >6.6 µg/L [6.6 ng/mL] or TnI, >0.3 µg/L [0.3 ng/mL]). Patients were further classified into 3 groups: stable angina pectoris (SAP), unstable angina pectoris (UAP), and acute myocardial ischemia (AMI).

The control group consisted of 40 unrelated healthy people. Only subjects without a clinical history of CVD or cardiovascular risk factors and with a normal electrocardiograph and normal blood chemistry values were included.

The patient group included 20% more smokers than the control group. Patients were not enrolled if they were older than 80 years or had liver or kidney disease. All procedures were done in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983), and written consent was obtained from each of the study participants.

Blood Sample Collection and Preparation

Plasma samples were obtained from the 80 patients within 24 hours after admission to the hospital and from the 40 healthy subjects when they visited the outpatient clinic for routine physical examination. In brief, venous blood samples were drawn into citrate tubes, and after centrifugation at 2,300g for 15 minutes at 15°C, plasma was removed and stored at –80°C until analysis. Genomic DNA from patients and control subjects was isolated from peripheral blood lymphocytes (EDTA-anticoagulated blood) by use of the Blood Genomic DNA Extraction Kit (GK1041, Shanghai Generay Biotech, Shanghai, China) and kept at –20°C until analysis.

Enzyme-Linked Immunosorbent Assay for Quantification of Plasma GAS6 Protein Levels

To quantify total plasma GAS6, we used the Human GAS6 ELISA Kit (product No. QRCT-301321113011EIA/UTL, ADL Systems, Sino-US Joint Venture Company, Wuhan, China). The quantification was accomplished according to the manufacturer’s protocol. In brief, the microtiter plates were coated with 50 µL of standards and specimens; 100 µL of Enzyme Conjugated Reagent was added to each well, gently mixed for 15 seconds, and incubated for 1 hour at 36°C. The wells were washed 5 times with washing buffer, and 50 µL of color reagents A and B was added to each well, mixed for 15 seconds, and incubated for 15 minutes at 36°C. The reaction was then stopped by adding 50 µL of Stop Solution, followed by gentle mixing for 30 seconds until all the blue had changed to yellow. The absorbance was measured at 450 nm in a microplate reader within 30 minutes after the addition of Stop Solution. Intra-assay and interassay coefficients of variation of the test were 6.3% and 8.7%, respectively.

SNP Selection and Genotyping

The SNP included in the study was chosen from the SNP database (dbSNP). Because a previous study found that the allele frequencies and genotype frequencies of the 8 GAS6 polymorphisms were similar except for intron 8 c.834+7G>A,6 we chose to focus on this SNP. The expression of intron 8 c.834+7G>A (dbSNP ID rs8191974) was determined by using the following primers: 5’ Near Seq 30 base pairs, TCCCCAGGACA TGACACCTG TGAGGTAGCC, and 3’ Near Seq 30 base pairs, GCCCTGGCC CCGGGGGCCC TTCACCGTGGa (http://www.ncbi.nlm.nih.gov/SNP). Genotyping was performed by Shanghai Generay Biotech (http://www.generay.com.cn).

Data Analysis

Descriptive results of continuous variables are expressed as mean ± SD. Non–normally distributed values, as assessed using the Kolmogorov-Smirnov test, are reported as median (interquartile range). The χ2 test was used to determine the genotype distributions for Hardy-Weinberg equilibrium and to compare the observed allele and genotype frequencies in patients with those in control subjects. Statistical analyses were performed using SPSS for Windows XP13.0 (SPSS, Chicago, IL).
Results

Characteristics of the Study Population

The study included 80 patients with CVD and 40 age- 
and sex-matched healthy control subjects. The main clinical 
and biologic characteristics of the 2 groups are summarized in Table 1. The median (interquartile range) plasma level of 
GAS6 was 16.9 µg/L (13-28 µg/L) in healthy control subjects 
and 10.65 µg/L (5.7-27.5 µg/L) in patients. Other biochemical 
parameters of interest were high-density lipoprotein, low-
density lipoprotein, CK-MB, and C-reactive protein.

Patients with ACS were further divided into groups with 
UAP and AMI. The patient risk factors and their relevance to 
the risk of cardiovascular events are summarized in Table 2. 
GAS6 concentrations were significantly lower in the SAP, 
UAP, and AMI groups than in the control group (P < .001) 
(Table 2). There was a trend toward differential 
expression of GAS6 in the different subtypes of CVD, but no 
statistical difference between the UAP and SAP groups (10.99 
vs 10.56 µg/L; P = .082) or between the UAP and AMI groups 
(10.99 vs 10.87 µg/L; P = .317) was found.

Patients with CVD differed significantly from control 
subjects in several risk factors for CVD such as older age, 
sex, current smoking, hypertension, and diabetes mellitus. 
Among patients with SAP, there was a higher proportion of 
ST depression at admission (P = .006). Patients with AMI had

![Figure 1](https://academic.oup.com/ajcp/article-abstract/131/5/738/1766589/1766589)
a higher proportion of ST depression at admission (P < .001) and of dyslipidemia (P = .01). Logistic regression analyses were adjusted for these factors.

**GAS6 Intron 8 c.834+7G>A Polymorphism in Patients With ACS and SAP**

To determine whether the c.834+7G>A SNP is associated with an increased risk of CVD, we first genotyped all patients. As shown in Table 3, we found that the c.834+7G>A GG, AG, and AA genotype frequencies, respectively, in 56 patients with ACS were 66% (n = 37), 29% (n = 16), and 5% (n = 3) and in 40 control subjects were 35% (n = 14), 45% (n = 18), and 20% (n = 8). The GG genotype was the most prevalent, and the AA genotype was less frequent in the SAP (2 [8%]) and ACS (3 [5%]) groups than in control subjects (8 [20%]). The A allele frequency was particularly low in patients with ACS, particularly in patients with UAP (0.18).

The **GAS6 Intron 8 c.834+7G>A SNP Is Associated With a Decreased Risk of CVD**

A significantly lower frequency of the AA genotype was observed in the UAP subgroup compared with the control group and patients with SAP or AMI (Table 4 and Table 5). In fact, the percentage of patients with UAP with the AA genotype (4% [2/48]) was almost a quarter of that of the control group (20% [8/40]), and the A allele frequency in the UAP group (0.18) was significantly lower than that in the control group (0.42). These findings suggest that the AA

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**Table 3**

<table>
<thead>
<tr>
<th>Genotype Frequencies in Control Subjects and Patients With SAP and ACS*</th>
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<tbody>
<tr>
<td>A</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Control subjects (n = 40)</td>
</tr>
<tr>
<td>Patients with CVD (n = 80)</td>
</tr>
<tr>
<td>ACS (n = 56)</td>
</tr>
<tr>
<td>SAP (n = 24)</td>
</tr>
</tbody>
</table>

A, allele A frequency; ACS, acute coronary syndrome; CVD, cardiovascular disease; SAP, stable angina pectoris.

* Data are given as number (percentage) unless otherwise indicated. The P value (χ² test) compares the CVD, SAP, and ACS groups with the control group.

**Table 4**

<table>
<thead>
<tr>
<th>Genotypes and Frequencies in Control Subjects, Patients With SAP, and ACS Subgroups*</th>
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<tr>
<td>Allele A Frequency</td>
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<td>-------------------</td>
</tr>
<tr>
<td>Control subjects (n = 40)</td>
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<tr>
<td>UAP (n = 48)</td>
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<tr>
<td>AMI (n = 8)</td>
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<tr>
<td>SAP (n = 24)</td>
</tr>
</tbody>
</table>

AMI, acute myocardial ischemia; CVD, cardiovascular disease; SAP, stable angina pectoris; UAP, unstable angina pectoris.

* Data are given as number (percentage) unless otherwise indicated. See Table 6 for additional data analysis.

**Table 5**

<table>
<thead>
<tr>
<th>ORs and CIs for Genotypes and Frequencies in Control Subjects, Patients With SAP, and ACS Subgroups*</th>
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<tbody>
<tr>
<td>Unadjusted OR (95% CI)</td>
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</tr>
<tr>
<td>Control subjects (n = 40)</td>
</tr>
<tr>
<td>All patients with CVD (n = 80)</td>
</tr>
<tr>
<td>UAP (n = 48)</td>
</tr>
<tr>
<td>AMI (n = 8)</td>
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<tr>
<td>All patients with CVD (n = 80)</td>
</tr>
<tr>
<td>AMI (n = 8)</td>
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<tr>
<td>SAP (n = 24)</td>
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<tr>
<td>AA vs GG/AG</td>
</tr>
</tbody>
</table>

AMI, acute myocardial ischemia; CVD, cardiovascular disease; CI, confidence interval; ND, not done; OR, odds ratio; SAP, stable angina pectoris; UAP, unstable angina pectoris.

* Logistic regression model adjusted for risk factors.
allele may protect patients from UAP, although it apparently has no protective effect against SAP. Of interest, patients with AMI expressed the A allele at a frequency similar to or slightly greater than patients with SAP. We also noted after univariate analysis that the GAS6 c.834+7A genotype was associated with a decreased risk for ACS (odds ratio [OR], 0.623; 95% confidence interval, 0.097-3.988) [Table 6].

Even after adjusting the OR for CVD risk factors, a statistically significant association was obtained between GAS6 c.834+7G>A and ACS (OR, 0.706; 95% confidence interval, 0.129-3.868) and with ACS subtypes (Tables 4 and 5). Thus, the GAS6 c.834+7G>A SNP was revealed as a protective factor for ACS.

**Discussion**

In the present study, we found that the GAS6 plasma concentration seems to correlate with CVD, especially in patients with ACS, a significant correlation between the degree of the disease, reflecting the destabilization of atherosclerotic plaque and GAS6 plasma levels, although the difference between these patients and control subjects did not reach the level of statistical difference. We also evaluated the distribution of the c.834+7G>A genotype between patients with ACS and patients with SAP. Although the different GAS6 genotypes (GG, AG, and AA) were all expressed in patients with CVD, the expression of the A allele was lower in patients with ACS than in patients with SAP. Our results indicate that the GAS6 c.834+7G>A polymorphism is associated with a lower risk for CVD, particularly for the subtypes affecting atherosclerotic plaque destabilization.

Several studies have examined GAS6 plasma concentrations under different conditions, such as anticoagulant therapy and septic shock. The regulation of GAS6 expression by vascular cells seems to be important for their function, and high concentrations of GAS6 protein have been reported to inhibit granulocyte adhesion to endothelial cells, suggesting an anti-inflammatory effect of the Axl-GAS6 interaction in endothelial cells. This finding is in agreement with GAS6/Axl interactions having been shown to have a role in the development, organization, and maintenance of atherosclerotic plaques. GAS6 has also been shown to promote platelet aggregation and the formation of thromboses in certain models of injury. All of these vascular effects are postulated to be results of GAS6 expression in the vessel wall following cellular damage or stress, where the local concentration of GAS6 and its receptors is increased.

There are also other possible mechanisms of action. For example, treatment of vascular smooth muscle cells with 100 µg/L of GAS6 decreased their level of apoptosis. GAS6 also protects endothelial cells from apoptosis and from tumor necrosis factor α–mediated cytotoxicity, 2 processes that may be involved in inflammation and atherogenesis, and that, when superimposed on the fibrous plaques of atherosclerotic arteries, might contribute to the cycle of broken plaques, inflammatory responses, new plaque tears, and the ultimate perpetuation of atherosclerosis. GAS6 has also been implicated in foam cell formation and neointimal proliferation in response to vascular injury. GAS6 may, thus, have a novel role in the pathogenesis of ACS via an upstream inflammation-related signaling pathway.

A previous study reported that GAS6 can inhibit angiogenesis in endothelial cells via its primary receptor, Axl, at concentrations as low as 1 µg/L. Therefore, the level of GAS6 expression observed in our study (>10 µg/L) suggests that the protein would be capable of signaling through its receptor. GAS6 may exert its function in the pathogenesis of ACS by activating STAT1 via the Axl receptor, leading to the induction of SOCS1 and SOCS3. GAS6 also inhibits toll-like receptors (TLRs), which mediate the inflammatory response implicated in autoimmune and inflammatory diseases. It is possible that GAS6 may exert its anti-inflammatory and antiangiogenic activity by signaling through the STAT1-SOCS-TLR pathway. In support of this hypothesis, elevated levels of GAS6 have been detected in patients with severe sepsis. Our study also supports this hypothesis. Because inflammation has a major role in the various phases of atherosclerotic plaque formation, including the rupture of fissures of the plaque associated with ACS, the increased plasma levels of GAS6 in patients with ACS supports a protective role for GAS6 in inflammation and CVD.

SNPs are the most common genetic variations in the genome and may influence factors that are involved in atherosclerosis. We found that the AA genotype was expressed at a lower frequency in patients with ACS compared with patients with SAP, which may suggest a protective role for this GAS6.

**Table 6**

GAS6 Intron 8 c.834+7G>A Genotype Distribution in Patients With Cardiovascular Disease

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
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<tbody>
<tr>
<td>All (n = 80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG/AG</td>
<td>0.650 (0.113-3.730)</td>
<td>0.744 (0.074-7.520)</td>
</tr>
<tr>
<td>ACS (n = 56)</td>
<td></td>
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<tr>
<td>AA vs GG</td>
<td>0.527 (0.079-3.515)</td>
<td>0.700 (0.116-3.232)</td>
</tr>
<tr>
<td>AA vs GG/AG</td>
<td>0.623 (0.097-3.988)</td>
<td>0.706 (0.129-3.868)</td>
</tr>
<tr>
<td>SAP (n = 24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG</td>
<td>0.625 (0.222-1.754)</td>
<td>0.657 (0.159-6.195)</td>
</tr>
<tr>
<td>AA vs GG/AG</td>
<td>0.667 (0.243-1.946)</td>
<td>0.688 (0.088-4.250)</td>
</tr>
</tbody>
</table>

ACS, acute coronary syndrome; CI, confidence interval; GAS6, growth arrest–specific 6 protein; OR, odds ratio; SAP, stable angina pectoris.
variant in ACS and indicates that GAS6 may be a candidate susceptibility gene for this disease. Therefore, GAS6 polymorphisms that would result in differences in GAS6 levels could influence the regulation of atherogenesis or the activation of endothelial cells and vascular smooth muscle cells by this protein.\textsuperscript{6} To improve the validity of SNP results, future study should use a larger sample.

Two limitations of this study should be pointed out. First, we found a trend toward differential expression of GAS6 in the subtypes of CVD but no statistical difference between the UAP and SAP groups (10.99 vs 10.56 µg/L; \(P = .082\)) or between the UAP and AMI groups (10.99 vs 10.87 µg/L; \(P = .317\)). We considered that the reason was an insufficient sample size. Future study with a larger sample with power analysis is needed to confirm our results. Second, in the original experimental design, we gave attention to the plasma levels and genotype frequency independently. The relationship between the plasma levels and genotype frequency has no statistical significance in our study, and we propose to make the subgroups dependent on concentration levels and genotype frequency in a future study.

Our study indicates that GAS6 is associated with CVD and provides further evidence that the AA genotype of the c.834+7G>A polymorphism may have a protective role against ACS. These results support the hypothesis that modulation of GAS6 activity may provide an important point for intervention. GAS6 and its receptor(s) represent a new class of therapeutic targets. Understanding the nature of the Axl-Gas6 interaction would ultimately help in the development of novel small molecules or neutralizing monoclonal antibodies for therapeutic applications for diseases in which the interaction between GAS6 and its receptors contributes to their progression or pathology.\textsuperscript{16}

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Supported by a grant from the Cardiology Department and CCU of Union Hospital, Wuhan.

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Acknowledgment: We thank Dong Wei for helpful comments and advice on statistical analysis.

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