Genetic Variations in the Growth Arrest–Specific 6 (GAS6) Protein Gene in Patients with Acute Coronary Syndrome

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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. The role of GAS6 in cardiovascular disease, particularly in acute coronary syndrome (ACS) was explored. The study investigated the role of the single nucleotide polymorphism (c.834+7G>A) of GAS6 in ACS. The genotype frequencies for GG, AG, and AA, respectively, in patients with ACS were 57.1% (16/28), 28.6% (8/28), and 14.3% (4/28) and were 20% (4/20), 40% (8/20), 40% (8/20) in the control group. The AA genotype and A allele were less frequent in patients with ACS than in control subjects ($p<0.05$). Our study indicates that the AA genotype and A allele of the GAS6 gene relate to ACS, which may have a protective role against ACS.

Key Words:

- Growth arrest–specific gene 6 GAS6
- Acute coronary syndrome ACS
- Single nucleotide polymorphism SNP
The term acute coronary syndrome (ACS) refers to any group of clinical symptoms compatible with acute myocardial ischemia and covers the spectrum of clinical conditions ranging from unstable angina (UA) to non-ST-segment elevation myocardial infarction (NSTEMI) to ST-segment elevation myocardial infarction (STEMI).

GAS6 protein is a vitamin K–dependent protein encoded by GAS6 gene and secreted by leukocytes and endothelial cells in response to injury (Gibot et al., 2007). It is structurally related to the anticoagulant protein S, the two proteins having 44% amino acid identity.

GAS6 protein is also thought to act as a bridge between apoptotic cells and the phagocytes that ingest them. 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.

GAS6 protein, the product of growth arrest specific (GAS) gene 6 is a ligand for the tyrosine protein kinase receptors Axl, Tyro3 and Mer whose signaling has been implicated in cell growth, survival, adhesion and migration. Although a secreted human vitamin K-dependent protein with close structural similarity with protein S, GAS6 protein does not exhibit anticoagulant properties but rather may be an important regulator of vascular homeostasis and platelet signaling.

GAS6 protein signals via its receptor tyrosine kinases and appears to modulate platelet outside-in signaling via GP alpha(IIb)beta(III), playing a key role in the perpetuation of platelet aggregates and clot retraction.

GAS6 protein is also implicated in foam cell formation and neointimal proliferation in response to vascular injury. Thus GAS6 protein acts at key points in the pathophysiology of atherosclerosis and
thrombosis; two processes implicated in most acute cardiovascular pathology.⁴

Previous studies have reported the genetic structure and allelic variability of the human \textit{GAS6} gene,⁶ indicating that there is an association between stroke and a single nucleotide polymorphism (SNP; c.834+7G>A) in intron 8 of the \textit{GAS6} gene. This suggests a potentially protective role of the AA genotype.⁷

\section*{SUBJECTS AND METHODS}

The present study was conducted on 60 subjects, 42 males and 18 females attending cardiology department of Benha university hospital between the period of February 2013 and February 2014. They were divided into two groups:

The patients' group included 40 patients with CAD (Coronary artery disease) with symptoms of cardiac ischemia that were verified by ECG, diagnostic coronary angiography and elevated levels of biochemical markers (CK-MB and Troponin I).

Their mean age (± SD) was 50.8 years (± 6.3). They were 28 males and 12 females that had been admitted to coronary care unit. Only patients who had experienced their last chest pain within the previous 48 hours were included in the study. Patients were further classified into 3 subgroups: Stable angina pectoris group (SAP), Unstable angina pectoris group (UAP) and acute myocardial ischemia group (AMI).

The control group included twenty apparently healthy individuals of matched age and sex. Only subjects without a clinical history of CVD, with a normal ECG and normal levels of cardiac biochemical markers
were included. Patients with renal or hepatic diseases and patients with chest pain more than 48 hours were excluded.

All procedures were done in accordance with ethical standards, was approved by ethical committee of Benha University and written consent was obtained from each of the study participants.

**Specimen collection:**

Initial blood samples were collected from patients once admitted to the emergency department and only those with confirmed diagnosis of CVD were included in the study.

i. **First blood sample was seven milliliters of venous blood collected under complete aseptic precautions and divided into three tubes:**

a) 1.8 ml of blood into citrated tubes centrifuged immediately at 3000 rpm for 10 min and assayed for prothrombin Time.

b) 2 ml blood into EDTA-tubes (EDTA anti coagulated blood) to separate genomic DNA from peripheral blood lymphocytes by use of blood genomic DNA extraction kits and then stored at - 20° C untill analysis.

c) The rest of the samples were put into plain test tubes without anticoagulant and left till coagulation. After coagulation, samples were centrifuged (at 3000 rpm for 15 minutes). The separated serum was divided into two aliquots:

   1) One was used for the immediate assay of liver function tests, kidney function tests and cardiac enzymes (CK-MB and troponin I).
2) The second aliquot was stored at -20°C for subsequent assay of HS-CRP.

ii. Second blood sample:

The second venous blood samples (4 ml) were collected from the patients after overnight fasting under complete aseptic precautions into plain tubes without anticoagulant. The plain test tubes were left till coagulation. After coagulation, samples were centrifuged (at 3000 rpm for 15 minutes). The separated serum was used for the assay of lipid profile and FBS. Hemolysed samples were discarded.

Five milimeters of blood were collected from apparently healthy individuals after overnight fasting under complete aseptic precautions, then handled, divided in the same way as the patients' samples.

CK-MB determination

The CK-MB reagent contains an antibody which binds to the M subunit of CK in the serum sample thereby inhibiting the activity of the M subunit. The remaining activity, corresponding to CK-B fraction activity is measured using according to IFCC reference method for measuring CK activity. CK-MB activity is then obtained by multiplying by 2 the remaining activity.

Troponin I determination

Troponin I concentrations were measured on the mini VIDAS instrument using VIDAS Troponin I Ultra (TNIU) kit provided by bioMerieux, Inc. Box 15969, Durham, NC 27704-0969/ USA for determination of human cardiac troponin I in human serum or plasma using the ELFA technique.
**Measurement of high sensitivity C - reactive protein (HS-CRP) ELISA:**

Highly sensitive CRP was measured using STAT-FAX reader using Accu-bind ELISA kits provided by Monbind Inc. lake fores, CA92630 USA. The kit was designed for determination of highly sensitive CRP in human in serum or plasma by microplate immunoenzymatic assay.

**SNP Detection and Genotyping**

**DNA EXTRACTION**

This was done using GeneJET Whole Blood Genomic DNA Purification Mini Kit provided by Thermo Fisher Scientific Inc. Samples were digested with proteinase K in the supplied lysis solution. The lysate was then mixed with ethanol and loaded onto the purification column, where the DNA binds to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the elution buffer.

**Polymerase Chain Reaction Analysis:**

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

The expression of intron8 (c.834+7G>A) of GAS6 gene was determined by using the following primers: GAS6E8F 5’-TTC CCT CAA GAA AGA GCC CG-3’ and GAS6E8R 5’-TCT CAT CCC AAA CCT CCA CA -3’.
Detection of amplified PCR product by Agrose gel electrophoresis:

The amplification products were separated by agarose gel electrophoresis in 2% agarose gel stained with ethidium bromide, and photographed under UV light.

Restriction fragment length polymorphism analysis to detect different genotypes: Determination of GAS6 genotypes was obtained using specific digestion restriction enzyme (ALWN1) enzyme digest PCR product covering exon 8 and part of intron 8 (481bp) and it is used to differentiate between A and G alleles of GAS6 gene as following A allele digested into 2 fragments (345bp and 136bp), but G allele remains uncutted.

Data ANALYSIS

Statistical analysis was performed using the computer program SPSS (Statistical package for social science) version 16. Descriptive statistics were calculated for the data in the form of Mean ± SD. Student’s t-test Used to compare between mean of two groups of numerical (parametric) data.

Results

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

To determined whether c.834+7G>A SNP is associated with an increased risk of CVD; all subjects were genotyped as shown in Table (1) and Figure (1). It was found that the c.834+7G>A GG, AG, and AA genotype frequencies, respectively, in 40 patients with ACS were 55% (n =22), 30% (n =12), and 15% (n =6) and in 20 control subjects were 20% (n = 4), 40% (n = 8), and 40% (n = 8). The GG genotype was the most prevalent, and the AA genotype was less frequent in ACS (6[15%]) than
in control subjects (8 [40%]). The A allele frequency was particularly low in patients with ACS.

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

A significantly lower percentage of the AA genotype was observed in the UAP subgroup compared with the control group and patients with SAP or AMI Table 2 and Figure (2). In fact, the percentage of patients with UAP with the AA genotype (5.6% [1/18]) was lower that of the control group (40% [8/20]). These findings suggest that the AA allele may protect patients from UAP. Also there was no significant difference in GAS6 genotypes distribution in patients with SAP and AMI versus control group (p value >0.05). The percentage of patients with SAP with the AA genotype (16.7% [2/12]) and the percentage of patients with AMI with the AA genotype (30% [3/10]) than was slightly lower that of the control group (40% [8/20])

In UAP group, patients with GG genotype were 24 times more risky than those with AA genotype and the frequency of G allele was 3.8 more than A allele.

In SAP group, patients with GG genotype were 6 times more risky than those with AA genotype and the frequency of G allele was 2.2 more than A allele. In AMI group, patients with GG genotype were 2.7 times more risky than those with AA genotype and the frequency of G allele was 1.6 more than A allele. In CAD group, patients with GG genotype were 7.3 times more risky than those with AA genotype and the frequency of G allele was 2.5 more than A allele Table (3).

Table (1): Comparison between patients and controls as regard genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n=40)</th>
<th>Control (n=20)</th>
<th>Total</th>
<th>X²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>AA</td>
<td>6</td>
<td>15.0%</td>
<td>8</td>
<td>40.0%</td>
<td>14</td>
</tr>
<tr>
<td>AG</td>
<td>12</td>
<td>30.0%</td>
<td>8</td>
<td>40.0%</td>
<td>20</td>
</tr>
<tr>
<td>GG</td>
<td>22</td>
<td>55.0%</td>
<td>4</td>
<td>20.0%</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure (1): Comparison between patients and control as regard genotypes.

Table (2): Comparison between patients and controls as regard genotype.

<table>
<thead>
<tr>
<th>CASES</th>
<th>AA (NO)</th>
<th>AA (%)</th>
<th>AG (NO)</th>
<th>AG (%)</th>
<th>GG (NO)</th>
<th>GG (%)</th>
<th>X²*</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP</td>
<td>2.0</td>
<td>16.7</td>
<td>4.0</td>
<td>33.3</td>
<td>6.0</td>
<td>50.0</td>
<td>3.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>UAP</td>
<td>1.0</td>
<td>5.6</td>
<td>5.0</td>
<td>27.8</td>
<td>12.0</td>
<td>66.7</td>
<td>10.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AMI</td>
<td>3.0</td>
<td>30.0</td>
<td>3.0</td>
<td>30.0</td>
<td>4.0</td>
<td>40.0</td>
<td>1.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ACS (UAP+AMI)</td>
<td>4.0</td>
<td>14.3</td>
<td>8.0</td>
<td>28.6</td>
<td>16.0</td>
<td>57.1</td>
<td>7.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CONTROL</td>
<td>8.0</td>
<td>40.0</td>
<td>8.0</td>
<td>40.0</td>
<td>4.0</td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure (2): Comparison between patients and controls as regard genotypes.

Table (3): OR and CI for genotypes and frequencies in control subjects, patient with SAP and ACS groups.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In all patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG</td>
<td>7.3</td>
<td>1.6-32.9</td>
</tr>
<tr>
<td>AA vs AG</td>
<td>2</td>
<td>0.5-8.4</td>
</tr>
<tr>
<td>GG vs AG</td>
<td>3.6</td>
<td>0.9-14.7</td>
</tr>
<tr>
<td>A allele vs G allele</td>
<td>2.5</td>
<td>0.9-6.45</td>
</tr>
<tr>
<td><strong>UAP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG</td>
<td>24</td>
<td>2.3-255.9</td>
</tr>
<tr>
<td>AA vs AG</td>
<td>5</td>
<td>0.5-52.9</td>
</tr>
<tr>
<td>GG vs AG</td>
<td>4.8</td>
<td>0.97-23.5</td>
</tr>
<tr>
<td>A allele vs G allele</td>
<td>3.8</td>
<td>1.1-12.5</td>
</tr>
<tr>
<td><strong>SAP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG</td>
<td>6</td>
<td>0.8-44.4</td>
</tr>
<tr>
<td>AA vs AG</td>
<td>2</td>
<td>0.3-14.2</td>
</tr>
<tr>
<td>GG vs AG</td>
<td>3</td>
<td>0.5-17.2</td>
</tr>
<tr>
<td>A allele vs G allele</td>
<td>2.2</td>
<td>0.6-7.8</td>
</tr>
<tr>
<td><strong>AMI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA vs GG</td>
<td>2.7</td>
<td>0.4-18.2</td>
</tr>
<tr>
<td>AA vs AG</td>
<td>1</td>
<td>0.2-6.5</td>
</tr>
<tr>
<td>GG vs AG</td>
<td>2.7</td>
<td>0.4-18.2</td>
</tr>
<tr>
<td>A allele vs G allele</td>
<td>1.6</td>
<td>0.4-5.8</td>
</tr>
</tbody>
</table>
Discussion

The present study was designed to evaluate the role of the single nucleotide polymorphism (c.834+7G>A) of GAS6 in cardiovascular disease, particularly in acute coronary syndrome (ACS).

In the present study hypertension is the most common risk factor of ACS (61.7%) followed by smoking (53.4%). Diabetes mellitus came at the last (43.3%). In a similar study hypertension was the commonest risk factor of acute coronary syndrome followed by cigarette smoking, diabetes mellitus came at the last. Other several studies reported that smoking as a risk factor for ACS, came after hypertension and diabetes mellitus, which differs from the results of the present study probably, because all female patients were nonsmokers.

Dyslipidemia was one of the nine major risk factors (smoking, diabetes, hypertension, visceral obesity, psychosocial stress, sedentary life, low fruit and vegetable consumption and alcohol consumption), and alone accounted for more than 50% of population attributable risk. Assessment of lipid profile parameters revealed that there was a statistically significant elevation in serum levels of TC, LDL-C, TG, in ACS patients compared with the control subjects while regarding HDL-C it was significantly low in ACS patients compared to the control subjects (p < 0.001).

A similar study reported that dyslipidemia, manifested by elevated levels of total and low density lipoprotein cholesterol (TC, LDL-C), low levels of high density lipoprotein cholesterol (HDL-C) and high levels of triglycerides (TG), is an important risk factor for CAD. Also several studies reported increased TC, TG, LDL-C and decreased HDL-C levels in patients with ACS than controls.

Data from the present study revealed that there was no significant difference between patients with MI, UAP and SAP regarding all lipid
profile parameters and this is against the idea which reported that low levels of HDL-C were significantly low in subjects with MI compared to those with UAP.\textsuperscript{15,16}

CK-MB normally exists in the cellular compartment and leak out into the plasma during myocardial injury due to disintegration of contractile elements and sarcoplasmic reticulum. Troponin I is protein of the troponin regulatory complex involved in cardiac contractility. It has very high myocardial tissue specificity, are not detectable in the blood of healthy persons and offers an improved sensitivity and specificity for AMI versus a combination of ECG and traditional biochemical markers.\textsuperscript{17}

The results of the present study showed a statistically highly significant increase in troponin I and CK-MB mean levels in patients with acute coronary syndrome versus control group. These results were in agreement with CK, CK-MB, troponin T and troponin I levels were significantly increased (p<0.001) in subjects suffering from MI and IHD compared with control subjects.\textsuperscript{18}

A subgroup analysis revealed that there was a statistically significant increase in the mean troponin I & CKMB concentration in patients with AMI vs UAP groups (P1<0.05), and AMI vs SAP groups (P2<0.05), but there was no statistically significant difference in the mean troponin I & CKMB concentration between UAP vs SAP groups (P3>0.05) and this is accepted with that patients with unstable angina and AMI had significantly higher concentrations of troponin I and CK-MB than patient with stable angina or controls.\textsuperscript{19}

A similar study found that Serum TnT and CK-MB levels were also significantly high in AMI patients as compared to controls.\textsuperscript{17} Possible explanation for elevated levels of CK-MB and troponin I is myocardial necrosis.\textsuperscript{20}

The degree of coronary obstruction may affect degree of myocyte injury and hence troponin I and CK-MB levels. An occlusive thrombus in the absence of significant collateral vessels most often results in STEMI. Angiographic studies had shown that evidence of thrombus; complex lesions and reduced TIMI (Thrombolysis in Myocardial Infarction) flow
grade were more common in patients with elevated troponin levels than in those with normal levels.  

High sensitive C-reactive protein is a positive acute phase protein synthesized by the liver cells, its level rises in response to inflammation. Hs-CRP release is triggered by various pro inflammatory stimulants as cytokines, oxidized LDL, and infectious agents. In patients with coronary artery disease (CAD), increased level of Hs-CRP is regarded as an important prognostic indicator for risk stratification in acute and recurrent attack as it is directly and actively participates in both atherogenesis and atheromatous plaque disruption. There are many researches describing that elevation of serum C-reactive protein (CRP) levels is related to increased risk of myocardial infarction.

CRP possesses activation of peripheral leukocytes with producing plaque-destabilizing mediators and results in infectious diseases trigger manifestations of atherosclerosis, in which CRP elevation might lead to the onset of cardiovascular events.

The earliest research from Italy found that elevated CRP predicts a poor outcome in patients with unstable angina. There was a highly significant difference between patients and control group as regard high sensitive – CRP level, and this is supported by another study which demonstrated higher level of Hs-CRP in patients with ACS versus control group. 

Also in a subgroup analysis there was significant increase in the mean HS–CRP concentration in patients with AMI vs UAP groups (P1<0.05) and AMI vs SAP groups (P2<0.05) and UAP vs SAP groups (P3<0.05) and this is supported by another study which found significant elevation in all patients.

This conflicted with another study which demonstrates that Hs-CRP level was not significantly increased in patients with UA.

There was a significant difference between patients and control group as regard Gas 6 genotypes; AA genotype was less frequent in cases versus control group (15%) vs (40%) respectavily (p value <0.05)
and in contrast, the GG genotype was more frequent in cases versus control group (55%) vs (20%) respectively (p value <0.05).

A similar study 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 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 protein levels could influence the regulation of atherogenesis or the activation of endothelial cells and vascular smooth muscle cells by this protein.

Other study stated that GAS6 is associated with CVD and provides further evidence that the AA genotype of the c.834+7G>A GAS6 polymorphism may have a protective role against ACS.

A subgroup analysis of the present study as regard AA,AG and GG genotypes revealed that there was no statistically significant difference between either patients with SAP and with AMI versus control groups (p value >0.05) and this is in supported by another study which found no difference in allelic frequency and genotype distribution of Gas 6 gene between the AMI and control groups, with a similar distribution of GG, GA and AA genotypes.

Hence, the current study revealed that the GAS6 c.834+7G>A polymorphism is associated with a lower risk of CVD. The G allele of GAS6 gene is the risky allele, which is significantly more frequent in patients with ACS than in controls. The results also suggest a protective role of the AA genotype.

**In Conclusion**

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 and controls. The results also 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.
References


population with acute coronary syndrome. Cardiovascular Diabetology; 5:15.


