Ratio of S-adenosylmethionine to S-adenosylhomocysteine as a sensitive indicator of atherosclerosis

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Abstract. The present study aimed to confirm whether the ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) is a sensitive indicator, and whether it can be used as a biomarker for the clinical diagnosis of atherosclerosis. Apolipoprotein E (ApoE)−/− mice were randomly divided into four groups and fed with a high methionine diet for 15 weeks. Serum levels of homocysteine (Hcy) were determined using an automatic biochemistry analyzer. The concentrations of SAM and SAH were determined using high-performance liquid chromatography. The methylation levels of B1 repetitive elements, adipocyte fatty acid binding protein (FABP4), monocyte chemotactic protein-1 (MCP-1) and extracellular superoxide dismutase (EC-SOD) were analyzed using nested touchdown-methylation-specific-polymerase chain reaction analysis. After 15 weeks, compared with the normal control group, serum concentrations of Hcy were significantly increased by 1.15-, 2.54- and 1.17-fold (P<0.05) in the ApoE−/− control group, Meth group, and Meth-F group, respectively. The sizes of the atherosclerotic lesions were increased in the ApoE−/− control group, Meth group and Meth-F group, by up to 1.44-, 2.40- and 1.45-fold, respectively, compared with the normal control group (P<0.05). The concentrations of SAM and SAH were significantly increased by 3.02-, 3.42- and 2.46-fold in the ApoE−/− control group, Meth group and Meth-F group, respectively (P<0.05). The ratios of SAM/SAH were increased by 1.67- and 2.75-fold in the in ApoE−/− control group and Meth group, respectively, compared with the normal control group. The methylation levels of B1 repetitive elements, FABP4, MCP-1 and EC-SOD were decreased and exhibited hypomethylation. The methylation statuses of these genes were correlated with the ratio of the serum levels of SAM and SAH. These findings suggested that the SAM/SAH ratio is a biomarker and may provide a sensitive indicator for the clinical diagnosis of atherosclerosis.

Introduction

Atherosclerosis (AS) is a leading contributor to mortality rates in developed countries; it is a complex and multifactorial disease and is affected by genetic and environment factors (1). Due to the methods of diagnosing AS being limited and the poor reversibility of the disease, accurate and timely detection of atherosclerotic lesions is particularly important. Several techniques have been used for the analysis of AS clinically, including X-ray angiography, computed tomography, magnetic resonance imaging and intravascular ultrasound. However, the majority of these methods are unable to detect AS until the onset of symptoms of AS (2), and several of the biological changes occur in one event with no prior symptoms. Therefore, the identification of an suitable method of imaging AS prior to an advanced stage is likely to be of significance for the prevention and treatment of AS.

Several retrospective and prospective studies in humans and animals have shown that hyperhomocysteinemia (HHcy) is an independent risk factor for AS, which contributes to atherosclerotic plaque formation and development (3), and the...
toxicity in AS is associated with the metabolism of homocysteine (Hcy). Hcy functions as an intermediate in methionine metabolism and is converted to methionine, the precursor of S-adenosylmethionine (SAM), which is the primary methyl group donor in the majority of biological methylation events, particularly DNA. Following transfer of the methyl group, SAM is converted to S-adenosylhomocysteine (SAH), a potent inhibitor of several SAM-dependent methyltransferases (4). Previous investigations have suggested that DNA methylation may cause disease either by silencing genes through hypermethylation or activating genes through hypomethylation under HHcy (5,6). Aberrant DNA methylation serves as an important mechanism, which controls gene expression in a variety of chronic diseases, particularly AS (7). In addition, associated studies have confirmed the clinical value of certain DNA methylation markers for the early detection of cancer, risk assessment and prediction of therapeutic responses, as altered DNA methylation occurs prior to the onset of symptoms of diseases, including AS (8). Kerins et al (9) found that SAH was a more sensitive indicator of risk in a group of patients with a diagnosis of cardiovascular disease, compared with Hcy, however, other evidence has suggested that the appearance of SAH in the plasma following an oral load of SAM and following an oral load of methionine (10,11). In addition, patients with end-stage renal disease had higher plasma levels of SAM and SAH, as well as Hcy (12). As there are differences among findings, the identification of novel and more sensitive biomarkers for imaging AS prior to the appearance of symptoms is important.

Increasing attention has focussed on the mechanisms of AS, and several hypotheses have been accepted, including oxidative stress, inflammation and altered methylation levels of certain typical AS-associated genes (13), including extracellular superoxide dismutase (EC-SOD), adipocyte fatty acid binding protein (FABP4) and monocyte chemoattractant protein-1 (MCP-1) (14-16), which are involved in the mechanisms of HHcy-induced AS. There have been several attempts to elucidate the mechanisms of AS and DNA methylation. Lakshmi et al (17) found that alterations in the methylation level of EC-SOD contributed to increased oxidative stress and increased the susceptibility of cardiovascular disease, particularly AS. Liu et al (18) found that MCP-1 is a potent chemokine and important in AS; in addition to reporting that the hypomethylation of CpG sites in the MCP-1 promoter region may be important in the vascular complications of type 2 diabetes. In our previous studies, it was demonstrated that B1 repetitive element hypomethylation is involved in the pathogenesis of AS (19), in which EC-SOD, FABP4, MCP-1 and B1 repetitive elements were analyzed to determine their DNA methylation levels to confirm the association between the methylation status of these genes and the development of AS.

The present study was designed to examine the correlation between the methylation levels of typical AS-associated genes and the ratio of SAM/SAH in atherosclerotic-based apolipoprotein E (ApoE)−/− mice, and to determine whether the SAM/SAH ratio is a superior biomarker for AS. In addition, the present study aimed to investigate whether the SAM/SAH ratio may be used as a rational factor in diagnosing AS in the future.

Materials and methods

Animals and diets. A total of 48 male ApoE−/− mice (6 weeks old; 18-22 g in weight) were provided by the Animal Center of Peking University (Beijing, China) and were bred in the Animal Center of Beijing University (Beijing China). The mice were housed (6 mice per cage) in a climate-controlled room (22±2°C) with a 12 h light/dark cycle, and provided with free access to water and a pellet diet (Keeoxieli, Beijing, China) for 15 weeks. The animals were divided into the following groups: Normal control group (n=12), wild-type mice with a regular mouse diet; ApoE−/− control group (n=12), ApoE−/− mice with a regular mouse diet; high-methionine group (Meth group; n=12), ApoE−/− mice with a regular mouse diet+1.7% methionine; high-methionine+folic acid and vitamin B12 group (Meth-F group; n=12), ApoE−/− mice with a regular mouse diet+1.7% methionine+0.0066% folic acid+0.0004% vitamin B12. The experimental procedures used on the mice were approved by the Ningxia Medical University Animal Care and Use Committee and were conducted according to the Ningxia Medical University Animal Care Committee guidelines.

Determination of serum levels of Hcy, SAM and SAH. After 15-week experimental diets, mice were euthanized by abdominal injection with 20% ethylcarbamate (5 ml/kg), and blood was immediately collected (~0.8-1.2 ml) to measure serum Hcy, SAM, SAH levels. Blood was collected by cardiac puncture and serum was separated by centrifugation (1,000 x g for 10 min at 4°C). The concentrations of Hcy were measured using an ADVIA 2400 Chemistry System (Siemens AG, Munich, Germany). Serum concentrations of SAM and SAH were measured using high-performance liquid chromatography (HPLC). The supernatant of each sample was filtered through a 0.22 μm filter (EMD Millipore, Billerica, MA, USA) and then loaded into a C18 column (250x4.6 mm ID; 5 μm particles; Shimadzu Corporation, Kyoto, Japan), run by a water HPLC system (D-2000 Elite HPLC system; Hitachi High-Technologies Corporation, Tokyo, Japan) and connected to an ultraviolet detector. The absorption values of the eluted compounds were monitored at (λ) ex=254 nm. Chromatograms were recorded using an HPLC integrator, and quantification was performed by automatic peak area integration. SAM and SAH standards were used to identify the elution peaks. All analyses were performed in triplicate.

Tissue preparation and evaluation of atherosclerotic lesions in the aorta. The aortas were excised and placed in plastic processing cassettes to avoid warping of tissues. The aortas were embedded in glycolmethacrylate (Technovit 7100; Kulzer, Wehrheim, Germany). To quantitatively evaluate atherosclerotic plaques by stereological methods, ~10 evenly spaced sections of each aortic segment were sampled. The blocks were exhaustively sectioned at 10 μM and stained with hematoxylin and eosin (H&E; Boster Biological Technology, Ltd., Wuhan, China) (19,20) as follows: The aortas were separated carefully and then put into 4% (w/v) formalin for 24 h. The samples subsequently underwent washing with water, dehydration with a Citadel tissue processor (Thermo Fisher Scientific, Inc.), clearing of surplus liquid from tissues, embedding using a Histostar tissue embed-
Results

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Data were analyzed using one-way analysis of variance, and additional analyses were performed using the Student Newman–Keuls test for multiple comparisons within treatment groups or a t-test for between two groups. The correlation between the SAM/SAH ratio and the methylation levels of the AS-associated genes in the arteries of the ApoE<sup>−/−</sup> mice were analyzed using Pearson correlation and linear regression analyses. P<0.05 was considered to indicate a statistical significant difference.

Serum levels of Hcy and atherosclerotic plaque areas in ApoE<sup>−/−</sup> mice. To confirm whether the atherosclerotic model was successfully established, the serum levels of Hcy and atherosclerotic plaque areas were measured, as shown in Table II. After 15 weeks, the serum concentrations of Hcy were significantly increased by 1.57-, 3.41- and 1.97-fold in the ApoE<sup>−/−</sup> control group, Meth group and Meth-F group, respectively, compared with the normal control group (P<0.05 or P<0.01). These data suggested that a high-methionine diet induced HHcy in the ApoE<sup>−/−</sup> mice. However, the serum concentration of Hcy was decreased by 42.3% in the Meth-F group, compared with the Meth group (P<0.05), which indicated that folate and vitamin B<sub>12</sub> modulated the effect caused by the high methionine diet.

The atherosclerotic lesions were examined and analyzed using a cross-sectional stereological method for the aortic root, as shown in Fig. 1 and Table II. Compared with the normal control group, the sizes of the atherosclerotic lesions were significantly increased in the n ApoE<sup>−/−</sup> control group, Meth group and Meth-F group (P<0.05 or P<0.01). However, the area of the atherosclerotic lesions was decreased by 39.4% in the Meth-F group, compared with the Meth group, (P<0.05). The atherosclerotic lesions in the Meth group were the most severe and widespread, which suggested HHcy may have had an effect in accelerating the development of AS, whereas folate and vitamin B<sub>12</sub> modulated the effect caused by the high methionine diet.

Serum concentrations of SAM and SAH, measured using HPLC, in mice. SAM and SAH are intermediates of methionine metabolism and, for the majority of cellular methyltransferase reactions, SAM is a priority for one-carbon metabolism as the methyl donor. SAM is converted to SAH within the active site of the methyltransferase by transfer of the methyl group (22). As shown in the diagram in Fig. 2, the serum concentrations of SAM and SAH were detected, and the ratio of SAM/SAH was calculated. After 15 weeks on the experimental diets, the concentrations of SAM were increased by 3.02-, 3.42- and 2.46-fold in the ApoE<sup>−/−</sup> control group, Meth group and Meth-F group, respectively, compared with the normal control group (P<0.05 or P<0.01). In addition, the serum concentrations of SAH were 1.08-fold higher in the Meth group, compared with the normal control group (P<0.05). By contrast, the ratio of SAM/SAH, compared with the normal control group, was...
increased by 1.67- and 2.75-fold in the ApoE-/- control group and Meth group, respectively (P<0.05 or P<0.01). These data indicated that the atherogenic diets caused a parallel increase in the levels of SAM and SAH, and the SAM/SAH ratio.

DNA methylation levels of B1 repetitive elements, FABP4, EC-SOD, and MCP-1, and their correlation with SAM, SAH and SAM/SAH. To confirm whether the methylation levels of the AS-associated genes were correlated with the ratio of SAM/SAH, the present study measured the methylation levels of B1 repetitive elements, FABP4, EC-SOD and MCP-1. As shown in Fig. 3A, the methylation levels of the B1 repetitive elements were decreased by 40% in the Meth group, compared with the normal control group (P<0.05), however, the methylation levels of the B1 repetitive elements were increased by 1.54-fold in the Meth-F group. These data indicated that the B1 repetitive elements were hypomethylated, which accounted for the majority of gene transcription activity in the pathogenesis of AS, induced by HHcy. The levels of B1 methylation were negatively correlated with levels of SAH (P=0.0212; \( \gamma = -0.3634 \)); however, as shown in Fig. 3D, there was a positive correlation between the methylation levels of the B1 repetitive elements and the ratio of SAM/SAH (P=0.0210; \( \gamma = -0.3638 \)). The B1 methylation levels were correlated with the concentration of serum SAM; however, the results showed that the correlation between the B1 methylation levels and the SAM/SAH ratio was more marked.

FABP4 is important in metabolic deterioration and the development of AS (23). As shown in Fig. 4A, the methylation levels of FABP4 detected by nt-MSP decreased by 5.02% in the Meth group, compared with the normal control group (P<0.05). In addition, FABP4 methylation levels were negatively correlated with the ratio of SAM/SAH (P=0.00158; \( \gamma = -0.3792 \)). These findings indicated that FABP4, a crucial gene in lipid metabolism and inflammatory reactions exhibited with hypomethylation in the AS model induced by HHcy.

EC-SOD regulates baseline cardiac morphology and protects the heart from fibrosis, apoptosis and loss of function following oxidative injury caused by HHcy (24). As shown in Fig. 5A, the methylation status of EC-SOD showed marked hypermethylation. Compared with the normal control group, the methylation of EC-SOD in the Meth group was increased by 1.08-fold (P<0.05). Additionally, as shown in Fig. 5D, the methylation levels of EC-SOD were significantly negatively correlated with the ratio of SAM/SAH (P=0.0117; \( \gamma = -0.3949 \)). These findings indicated that EC-SOD, a crucial gene in lipid metabolism and inflammatory reactions, exhibited with hypomethylation in the AS model induced by HHcy.

MCP-1 is important in inflammation and is a basic mediator in a biological system, which is valuable in the formation of AS (25). Following feeding with the respective experimental diets for 15 weeks, the status of MCP-1 exhibited hypomethylation in the PBMCs. In the Meth group, the methylation levels were increased by 1.67- and 2.75-fold in the ApoE-/- control group and Meth group, respectively (P<0.05 or P<0.01). These data indicated that the atherogenic diets caused a parallel increase in the levels of SAM and SAH, and the SAM/SAH ratio.

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Table I. Primer sequences of FABP4, EC-SOD, MCP-1 and B1 repetitive elements.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primer sequence (5'→3')</th>
<th>Size (bp)</th>
<th>Amplification temperature (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FABP4-O</td>
<td>F: ATTTAATTTAGGGGAGAAGAAA</td>
<td>134</td>
<td>60.7</td>
</tr>
<tr>
<td></td>
<td>R: TCACATCTCAAAAAATCCTAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FABP4-M</td>
<td>F: AAGTTGGAAGTTTTTTTGTAAACG</td>
<td>150</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>R: CCTTTACATATATTGGTCGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FABP4-U</td>
<td>F: AGTTGGAAGTTTTTTTGTAAAGG</td>
<td>140</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>R: TTTACCTATATTTACTTCTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC-SOD-O</td>
<td>F: TTGTGAGAATGTTTTAAGGTTT</td>
<td>543</td>
<td>55.4</td>
</tr>
<tr>
<td></td>
<td>R: AACCAAATCAAAAATTTCAATCAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC-SOD-M</td>
<td>F: AGTTAAGTGGAGAGGTTAGGGTTT</td>
<td>110</td>
<td>64.0</td>
</tr>
<tr>
<td></td>
<td>R: AAATAAAACAAAAACACTCTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC-SOD-U</td>
<td>F: GTAATGGAAGAGGTTAGGGTTT</td>
<td>110</td>
<td>64.0</td>
</tr>
<tr>
<td></td>
<td>R: AAAATAAAACAAAAACACTCTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1-O</td>
<td>F: TTGTGGAATGTTTTAGTTTTTTCGTT</td>
<td>137</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>R: AACTCTCTACCTATTTCTTCTCGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1-M</td>
<td>F: TTGTGGAATGTTTTAGTTTTTTCGTT</td>
<td>137</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>R: AAATAAAACAAAAACACTCTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1-U</td>
<td>F: TTGTGGAATGTTTTAGTTTTTTCGTT</td>
<td>137</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>R: AAATAAAACAAAAACACTCTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-O</td>
<td>F: ATAGAAGTGGAGTTATAGTTAGTTATTG</td>
<td>269</td>
<td>64.7</td>
</tr>
<tr>
<td></td>
<td>R: CACTCCAACCTTTTTAACCCTAAAC</td>
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<tr>
<td>B1-M</td>
<td>F: GTTAGTGATGGATGGTTATACGG</td>
<td>139</td>
<td>62.1</td>
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<td></td>
<td>R: TACACTAATAAAAACAAACTCCGAA</td>
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<tr>
<td>B1-U</td>
<td>F: GTTAGTGATGGATGGTTATACGG</td>
<td>139</td>
<td>62.1</td>
</tr>
<tr>
<td></td>
<td>R: TACACTAATAAAAACAAACTCCGAA</td>
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<td></td>
</tr>
</tbody>
</table>

FABP, fatty acid binding protein; EC-SOD, extracellular superoxide dismutase; MCP-1, monocyte chemoattractant protein-1; O, outer primer; M, methylated primer; U, unmethylated primer; F, forward; R, reverse.
Table II. Serum levels of Hcy and atherosclerotic lesion area.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hcy (µmol/l)</th>
<th>Atherosclerotic lesion area (10^2/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6.51±0.21</td>
<td>0</td>
</tr>
<tr>
<td>ApoE–/– control</td>
<td>10.25±0.86*</td>
<td>3.99±0.45b</td>
</tr>
<tr>
<td>Meth</td>
<td>22.22±1.54b</td>
<td>6.22±0.49b</td>
</tr>
<tr>
<td>Meth-F</td>
<td>12.82±1.80c</td>
<td>4.41±0.57c</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± standard error of the mean (n=12). *P<0.05 and **P<0.01, compared with the normal control group; ‘P<0.01, compared with the Meth group. Normal control group, wild-type mice fed a regular diet; ApoE–/– control group, ApoE–/– mice fed a regular diet; Meth group, ApoE–/– mice fed a high-methionine diet; Meth-F group, ApoE–/– mice fed a high-methionine diet containing folic acid and vitamin B₁₂. ApoE, apolipoprotein E; Hcy, homocysteine.

decreased by 6.18%, compared with the normal control group (P<0.05), as shown in Fig. 6A. In investigating the association between MCP-1 methylation and SAM/SAH ratio, a negative correlation was found (P=0.0496; γ=-0.3125).

Taken together, the data of the present study indicated that there was a marked correlation between the SAM/SAH ratio and the methylation levels of atherosclerotic-associated genes, which were considered a biomarker of AS. These findings provided evidence that the ratio of SAM/SAH may be a more sensitive indicator for AS.

Discussion

Numerous epidemiological investigations and case-control studies have demonstrated that elevated plasma Hcy is associated with an increased risk of vascular disease (26-28). However, other case-control studies have not demonstrated such a causal association, and the mechanism of Hcy-induced AS remains to be fully elucidated (29,30). In addition, whether Hcy itself is a major factor or an indirect metabolic marker of certain biochemical processes, which occur in AS is unclear. The present study aimed to identify an effective and reliable biomarker of Hcy-induced AS.

In the processes of methionine metabolism, there are several byproducts, including Hcy, SAM and SAH, and previous studies have demonstrated that alterations in the SAM/SAH ratio may be more directly associated with vascular damage, compared with Hcy or the other intermediates (31-33). A number of studies have described that increased levels of Hcy are always followed by higher concentrations of SAM, and it may be an important mediator of toxicity in the body. In addition, the SAM/SAH ratio is considered to be an indicator of the transfer of methyl groups from SAM to the numerous methyl acceptors in cells, and thereby reflects its methylation potential (34). The significance of this observation is associated with the hypothesis that SAM and SAH may be key components in the pathophysiology of the Hcy-vascular disease axis. Although the hypothesis that Hcy is directly linked to damage in the vascular endothelium has not been validated, studies have suggested that there is an association between the SAM/SAH ratio and vascular injury, and a decrease in the SAM/SAH ratio is often regarded as an indicator of reduced cellular methylation capacity (35). In the present study, ApoE–/– mice were fed a high methionine diet, and it was found that the serum concentrations of Hcy were increased, particularly in the Meth group. Compared with the normal group, the serum concentrations of Hcy were increased by 1.15-, 2.54- and 1.17-fold in the ApoE–/– control group, Meth group and Meth-F group, respectively. In addition, as the concentration of Hcy increased, the atherosclerotic lesion was more severely widespread. The present study also found that folate and vitamin B₁₂ suppressed the effect of Hcy-induced AS, and the sizes of the atherosclerotic lesions were significantly increased in the ApoE–/– control group, Meth group and Meth-F group, by up to 1.44-, 2.40- and 1.45-fold, respectively, compared with those of the normal control group. Taken together, these data suggested that establishment of the AS model induced by HHcy was successful. As the intermediates of methionine metabolism, the concentrations of SAM and SAH also increased, as did the ratio of SAM/SAH.

Several studies have confirmed that alterations in the methylation levels of specific genes are usually associated with the occurrence and development of cancer (36). AS is similar to cancer in certain aspects and has been referred to as a cancer-like disease (37,38); AS and cancer are chronic and progressive, and the inducing factors are very similar. In addition, the pathological mechanisms are similar, involving genetic and environmental elements. In the present study, the methylation statuses of specific genes associated with AS were examined. Cash et al (39) first suggested that DNA hypomethylation is involved in the development of cardiovascular disease, and evidence from animal studies has suggested that global DNA hypomethylation is associated with atherosclerotic lesions. A number of previous reports have focused on alterations in the DNA methylation of specific genes associated with AS (40,41). Global hypomethylation affects CpG dinucleotides in repetitive elements and in certain gene-specific promoter CpG islands (42). In the present study, it was found that the methylation levels of pivotal AS-associated genes were altered. B1 repetitive elements are the sequences of short interspersed nucleotide elements in mice, corresponding to the Alu sequence in humans (19). Increasing evidence suggests that B1 repetitive element hypomethylation is associated with gene transcriptional activity in the pathogenesis of cardiovascular diseases, particularly AS (43). In the present study, it was also found that B1 repetitive elements exhibited hypomethylation in the blood cells of the AS model. FABP4 has been identified as a potential circulation marker for metabolic syndrome,
and is increased in individuals with diabetes and metabolic syndrome due to the development of subclinical inflammation, which leads to AS (44). The present study is the first, to the best of our knowledge to report that the methylation levels of FABP4 were closely associated with AS. EC-SOD protects arteries against the deleterious effects of superoxide anions.
and the development of AS. Laukkanen et al (45) reported that the altered methylation of EC-SOD is associated with the development of AS, and suggested that it may affect the structures and functions of EC-SOD and other genes, which may be involved in the development of atherosclerotic lesions in mice. The present study found that EC-SOD was hypermethylated, particularly in the Meth group; the methylation of EC-SOD was increased by 1.08-fold, compared with that in the normal control group. It has been reported that CpG site hypomethylation in the MCP-1 promoter region may be important in the AS of type 2 diabetes (18). The present study also found that, in the Meth group, MCP-1 was hypomethylated, and the methylation levels were decreased by 6.18%, compared with the normal control group. Therefore it was hypothesized that the DNA methylation levels of FABP4, EC-SOD, MCP-1 and B1 repetitive elements may be an indicator of AS.

Several studies investigating biomarkers of AS have been performed, and have reported that Hcy may be a sensitive

Figure 3. Methylation of B1 repetitive elements, concentrations of serum SAM and SAH, and their correlation. (A) Methylation levels of B1 repetitive elements were detected using nested touchdown-methylation specific polymerase chain reaction analysis. Concentrations of serum (B) SAM and (C) SAH were measured using high-performance liquid chromatography. (D) Correlation between B1 methylation and SAM/SAH ratio. Results are presented as the mean ± standard error of the mean. *P<0.05, vs. normal control group. (A) Normal control group; (B) ApoE−/− control group of ApoE−/− mice fed a regular diet; (C) Meth group of ApoE−/− mice fed a high-methionine diet (D) Meth-F group of ApoE−/− mice fed a high-methionine diet containing folic acid and vitamin B12. ApoE, apolipoprotein E; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; M, methylated primer; U, unmethylated primer.
biomarker for AS (46-48). It has also been reported that plasma SAH is a more sensitive marker of clinical cardiovascular disease than plasma Hcy (49). However, the data of the present study suggested that the SAM/SAH ratio may be a more sensitive biomarker of AS. By causing feedback inhibition of SAM-dependent methyltransferases, the accumulation of SAH can affect the DNA methylation pattern and lead to the promotion of chronic diseases, and the SAM/SAH ratio is the prime regulator of the activities of the majority of methyltransferases in the cell (50). SAH is the metabolic precursor of Hcy in a reaction which catalyzes SAM by SAH hydrolase. Due to this reaction, changes in the concentrations of SAM and SAH are
always equal, but the ratio usually does not change significantly. A study by Loehrer et al (10) described the levels of SAH in the plasma following an oral load of SAM and following an oral load of methionine. In the present study, SAM and SAH concentrations were determined by HPLC. Serum Hcy concentrations were measured by an automatic biochemical analyzer. HPLC has high sensitivity and accuracy, and so the ratio of S-adenosylmethionine to S-adenosylhomocysteine can be used as early diagnostic indicators. Therefore, the present study performed correlation analyses of FABP4, EC-SOD, MCP-1 and B1 repetitive elements, respectively, with serum levels of SAM and SAH, and the SAM/SAH ratio, to confirm

Figure 5. Methylation of EC-SOD, concentrations of serum SAM and SAH, and correlation analysis. (A) Methylation levels of EC-SOD were analyzed using nested touchdown-methylation specific polymerase chain reaction analysis. Concentrations of serum (B) SAM and (C) SAH were measured using high-performance liquid chromatography. (D) Correlation of EC-SOD methylation levels and SAM/SAH ratio. Results are presented as the mean ± standard deviation. **P<0.01, vs. normal control group. (A) Normal control group; (B) ApoE-/- control group of ApoE-/- mice fed a regular diet; (C) Meth group of ApoE-/- mice fed a high-methionine diet (D) Meth-F group of ApoE-/- mice fed a high-methionine diet containing folic acid and vitamin B12. ApoE, apolipoprotein E; SAM, S-adenosylmethionine SAH, S-adenosylhomocysteine; EC-SOD, extracellular superoxide dismutase. M, methylated primer; U, unmethylated primer.
which is the optimal biomarker of AS. The results of the present study showed that the methylation levels of these AS-associated genes were significantly correlated with the ratio of SAM/SAH in the ApoE<sup>-/-</sup> mice.

In conclusion, FABP4, EC-SOD and MCP-1 are important in the formation and/or development of AS, and previous studies have confirmed that DNA methylation is a diagnostic biomarker of AS (51-53). On this basis, the present study analyzed the correlation between DNA methylation of FABP4, EC-SOD, MCP-1, and B1 repetitive elements and the serum SAM/SAH ratio to confirm the hypothesis that the serum SAM/SAH ratio may be a more sensitive biomarker of AS, and
may be used for the clinical diagnosis of AS. Further investigations are required to support and confirm this conclusion, to determine its potential for clinical usage.

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