Integration of gene expression and DNA methylation profiles provides a molecular subtype for risk assessment in atherosclerosis

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Abstract. The aim of the present study was to identify an effective method for detecting early-phase atherosclerosis (AS), as well as to provide useful DNA methylation profiles to serve as biomarkers for the detection of AS. A total of 300 individuals (150 AS patients and 150 healthy subjects) were recruited for peripheral blood DNA methylation analyses at 12 gene promoter loci using nested methylation-specific polymerase chain reaction in a test set. Based on the test set, the promoter methylation of TIMP metallopeptidase inhibitor 1 (*TIMP1*), ATP binding cassette subfamily A member 1 (*ABCA1*), and acetyl-CoA acetyltransferase 1 (*ACAT1*) were determined to be candidate biomarkers; demonstrating the highest sensitivity (88%) and specificity (90%). The biomarkers that were candidates for early AS detection were validated in an independent validation set (n=100). In the validation set, the combination of *TIMP1*, *ABCA1* and *ACAT1* methylation achieved sensitivity, specificity and coincidence rate values of 88, 70 and 79%, respectively. In the current pilot study, the patterns of DNA methylation of AS-associated genes were observed to be significantly altered in the peripheral blood of AS patients. Thus, the AS-specific methylation of the three-gene panel (*TIMP1*, *ABCA1*, and *ACAT1*) may serve as a valuable biomarker for the early detection of AS.

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Introduction

Atherosclerosis (AS) of the carotid arteries is a major cause of stroke and transient ischemic attack, which remains the leading cause of mortality in China and is currently the most common cause of mortality worldwide (1,2). The symptoms of AS are not always detected by patients until complications arise and the therapeutic methods to treat AS are poor; therefore, it is particularly important to establish an appropriate, rigorous and efficient detection strategy for early-phase AS, in order to limit disease progression before it results in clinical consequences (3). There are two prevalent methods for diagnosing AS: Doppler ultrasound and computed tomographic angiography (CTA), which have become the standard methods for diagnosing AS (4,5). However, neither of these methods identify the early asymptomatic pathological changes of AS and ultrasound only assesses plaque in the carotid artery. Therefore, it is paramount to develop non-invasive methods for diagnosing high-risk, asymptomatic individuals before the onset of clinical events or symptoms (6,7). Furthermore, data supporting the routine use of Doppler ultrasound to screen for carotid stenosis in an asymptomatic population is considered to be weak, as there is a low overall prevalence of treatable AS in the general asymptomatic population (8). It is widely hypothesized that the next generation of screening tests will be based on molecular biomarkers (9).

DNA methylation, a stable epigenetic mark, is a non-traditional, heritable factor involved in gene transcription regulation (10). DNA methylation occurs at position 5' of CpG dinucleotides; certain regions of DNA are rich in CpG and are termed CpG islands (GCIs), which predominantly locate in the promoter region (11). Changes in patterns of DNA methylation may lead to inappropriate gene expression, and contribute to the development and progression of disease (12). In addition, previous studies have demonstrated aberrant DNA methylation in AS (13). Alterations in DNA

methylation have been identified as an early event in AS; Zhao *et al* (14) found that aberrant methylation may represent an early biomarker for AS, and demonstrated clinical correlations with carotid intima-media thickness (14). Current research primarily focuses on the effect of DNA methylation leading to AS and, to the best of our knowledge, there are few studies that investigate the role of DNA methylation on the early diagnosis of AS. Detection of DNA methylation, at candidate loci in AS, suggests that AS-specific methylation changes could be applied diagnostically. However, DNA methylation affects various factors, including age, living environment, diet and other factors limit the use of methylation changes for diagnosis. Previous studies have shown that simultaneous measure various differentially methylated loci may improve diagnostic use (15). Furthermore, a major limitation towards further developments of DNA methylation in clinical use may be that the majority of studies focus on single genes (16), whereas AS is associated with multiple factors, including genetic and epigenetic alterations (17). Measuring the methylation of individual genes was not as specific or sensitive as using a panel of epigenetic markers for the early detection of breast cancer (18).

In the present study, DNA methylation was profiled in AS and healthy groups of 300 patients using nested methylation-specific polymerase chain reaction (PCR; NT-MSP). Based in previous unpublished data, a biomarker panel capable of differentiating AS patients from healthy individuals, irrespective of AS histology, was identified. The present study provides insight into AS etiology, presents validated tissue-based diagnostic biomarkers, and supplies a framework for the development of DNA methylation-based molecular diagnostics for AS detection in patients.

Materials and methods

Ethics statement. The present study was reviewed and approved by the Ethics Committee of General Hospital of Ningxia Medical University (Yinchuan, China). Written informed consent was obtained from the participants before the collection of any samples and the specimens were irreversibly de-identified.

Inclusion and exclusion criteria. Patients with AS were diagnosed by Doppler ultrasound examination of the carotid artery. The healthy control group comprised of age- and gender-matched healthy individuals that were not exhibiting AS risk factors. The exclusion criteria were as follows: Individuals with moderate or severe valvular heart disease, chronic renal failure or diabetes; smokers; obese individuals; and those exhibiting disordered lipid metabolism.

NT-MSP analysis of DNA methylation. The following candidate genes formed the panel for early detection of AS: ATP binding cassette subfamily A member 1 (*ABCA1*), acetyl-CoA acetyltransferase 1 (*ACAT1*), peroxisome proliferator activated receptor α (*PPARA*), platelet derived growth factor subunit B (*PDGFB*), arachidonate 5-lipoxygenase (*ALOX5*), LDL receptor related protein 1 (*LRP1*), lecithin-cholesterol acyltransferase (*LCAT*), TIMP metallopeptidase inhibitor 1 (*TIMP1*), matrix metallopeptidase 9 (*MMP9*), intercellular adhesion molecule 1 (*ICAM1*), vascular endothelial growth factor A (*VEGFA*) and nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (*NFKB1*). These genes contained CGIs, were expressed in peripheral blood cells, and were correlated with the occurrence and development of AS (19-24). DNA methylation of these candidate genes was examined in two independent test sets (total of 300 peripheral blood samples) and genomic DNA was isolated from the peripheral blood mononuclear cells using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). An EZ DNA Methylation-Gold™ kit (Zymo Research Corporation, Irvine, CA, USA) was used to detect the DNA methylation patterns, and integrates the DNA denaturation and bisulfite conversion processes into one step. The DNA was modified by sodium bisulfite (Zymo Research Corporation, Irvine, CA, USA) in which unmethylated cytosine residues were converted to thymine, whereas methylated cytosine residues were retained as cytosine; this difference was then utilized to specifically amplify either methylated or unmethylated DNA. NT-MSP, which consists of a two-step PCR amplification, was then used to detect methylation (25). The first step of NT-MSP uses an outer primer pair, which does not contain any CpGs. The second step of PCR was conducted with conventional PCR primers serving as inner primers. The primers for NT-MSP amplification were designed according to the bioinformatics program, MethPrimer (www.urogene.org/methprimer/index.html) and are presented in Table I. PCR products were gel purified with an agarose gel DNA fragment recovery kit, according to the manufacturer's instructions, and were sequenced by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). To reduce mispriming and to increase efficiency, touchdown (TD) PCR was used for amplification. Samples were subjected to 30 cycles in a TD program (94˚C for 5 min, 94˚C for 30 sec; 56˚C for 30 sec and 72˚C for 1 min, followed by a decrease of 0.5˚C in the annealing temperature every second cycle). After completion of the TD program, 20 cycles were subsequently run (94˚C for 30 sec, 56˚C for 30 sec and 72˚C for 60 sec), terminating with a 7-min extension at 72˚C. The PCR products were separated by electrophoresis through a 2% agarose gel (Borunlaite Science & Technology, Beijing, China) containing ethidium bromide (Tokyo Chemical Industry Co., Ltd., Shanghai, China) for 30 min at 100 V. The DNA bands were visualized using ultraviolet light. The following formula was used: Methylation %= methylation / (methylation + unmethylation) x100%.

Reverse transcription-quantitative PCR (RT-qPCR) for mRNA. The total RNA was extracted from the peripheral blood mononuclear cells using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Inc.). Primer Premier 5 software was used to design the primers (Table II). The expression levels of candidate genes were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward, 5'-AGAAGGCTGGGGCTCATTTG-3', and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. RNA was reverse transcribed using the PrimeScript Real-Time PCR reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA then underwent

Table I. Primer sequences for DNA methylation analysis.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
ABCA1		
Outer	GAAAGTAGGATTTAGAGGAAGTAAAT	AATTCAATCACTCAACAAAAAACAC
Methylation	AATTTTATTGGTGTTTTTGGTTGTC	ATATCTTAAAATCCGCGATCTACG
Unmethylation	AATTTTATTGGTGTTTTTGGTTGTT	CAAATATCTTAAAATCCACAATCTA
ACAT1		
Outer	AGGAAGATTTAGAGATTTAGAAGTAA	TCAATAATCACTCAAACACACAA
Methylation	TTAATTAGTTTGGTTTTTGGTCGTT	CTTATATACCAAATGCGAACT
Unmethylation	TTAATTATGTGTGTTTGGTTTTTTG	TATCCAAATTAACCAAACTCCATA
PDGFB		
Outer	TTTTTTTGTTTTGAAATTTTGGTTAA	CAAAATCCCAACAAAAATCTCC
Methylation	TTTGGAAATTAATGATAAGTTAGGC	AAGCATCATAAAAAAAAACGCATC
Unmethylation	TTGGAAATTAATGATAAGTTAGGTGA	AAACATCATAAAAAAAAACACATCA
ALOX5		
Outer	TTATATTTTCGTCGTTTTACGTACG	AATATAAAAAAAATTTCGCGCG
Methylation	TATATTTTTGTTGTTTTATGTATGG	AAATATAAAAAAAATTACACACT
Unmethylation	AGATGTTATAGGGATTTTGTTGTTT	TCTAAAACACAAAAACTCTCAAC
LRP1		
Outer	TTTGTTGTTTAGGTTGGAGTGTAGT	TAAAAAAATCACTTTTAAAAATTC
Methylation	TGTTTAGGTTGGAGTGTAGTGGTAC	AAAAAATCACTTTTCAAAATTCGA
Unmethylation	TGTTTAGGTTGGAGTGTAGTGGTAT	AAAAATCACTTTTCAAAATTCAAA
LCAT		
Outer	GAGTTTTTTGTTTTGTTTTGGTTTC	AATCTTAATCAATCTATCTACCGC
Methylation	GAGTTTTTTGTTTTGTTTTGGTTTT	ATCTTAATCAATCCATCTACCACA
Unmethylation	TTTGGTTGTTTTTTTGTATTATTTG	TCAATCTTAATCAACTCATCTACC
TIMP1		
Outer	TTTTTTTTAATGTTTATTTATTTATT	CCAAACACTCACAATTTATCTCAC
Methylation	TTAGGTAGTTTTTGTTTGAATTTCG	ATAAATACCGTTCTTATTCCCGTT
Unmethylation	TAGGTAGTTTTTGTTTGAATTTTGG	ATAAATACCAATTCTTATTCCCATT
MMP9		
Outer	GGTTATATAGTTGGAAATGGTAGAGT	ATTCAAACAATTCTCTACCTCAAC
Methylation	TTTTAAATATAGTTTATTGGGTCGG	TTTAATAAAAATAAAATTTCACTA
Unmethylation	TTTTTTAAATATAGTTTATTGGGTTGG	AATAAAAATAAATTTCACTACATT
ICAM1		
Outer	GTAAGAGTTTAGTGGAATTCGTTCG	GAATCACTAACCATCCAAAAACG
Methylation	TAAGAGTTTAGTGGAATTTGTTTGA	AAATCACTAACCATCCAAAAACAC
Unmethylation	TAAGAGTTTAGTGGAATTTGTTTGA	CCTAAAACTTTCCTATTATAAAAC
VEGFA		
Outer	TAGTTAGAGTCGGGGTGTGTAGAC	GAAAAACCGAACAAAAACGAA
Methylation	TAGTTAGAGTTGGGGTGTGTAGATG	AACAAAAAACCAACAAAAACAAA
Unmethylation	GGAGTAAATTTTTTTTATTTTTTTT	ATTGTGGTTTTTGGTTTAGTTTTG
PPARA		
Outer	AGTATAGTGGTATAGGGTATTGGTAG	TAAAACTCTACAAAACAAAAAAAA
Methylation	TAGTGGTAGGTATAGTTGGTAGCGG	ACCAATAACGAAAATAAAAAAAC
Unmethylation	TAGTGGTAGGTATAGTTGGTAGTGG	CAATAACAAAATAAAAAAACACC
NFKB1		
Outer	TATAGATGAGTTTTATTTATTTGGTA	AAACTCTAACTCCTAACAAAAC
Methylation	TTGATTGGGTTCGGTAGGC	GCACTTCTAAAAAGCTATACGCC
Unmethylation	TTATTGATTGGGTTTGGTAGGT	CCCACACTTCTAACACTATACACC

Table II. Primers used in quantitative polymerase chain reaction for mRNA analysis.

Gene	Primer	Sequence (5'-3')	Product size (bp)
ACAT1	Left	ACGCTGCTGTAGAACCTATTGA	116
	Right	GGCTTCATTTACTTCCCACATT	
ABCA1	Left	ATCAAGGGCATCGTGTATGAG	227
	Right	AGGATTGTCACCACAGCAAAC	
PPARA	Left	ATCACGGAACACGCTTTCAC	100
	Right	CGATGTTCAATGCTCCACTG	
TIMP1	Left	ATACTTCCACAGGTCCCACAAC	194
	Right	GGATGGATAAACAGGGAAACAC	
ALOX5	Left	AGTTCCAGCAAGGGAACATT	204
	Right	CATCCGAAGGGAGGAAAATAG	

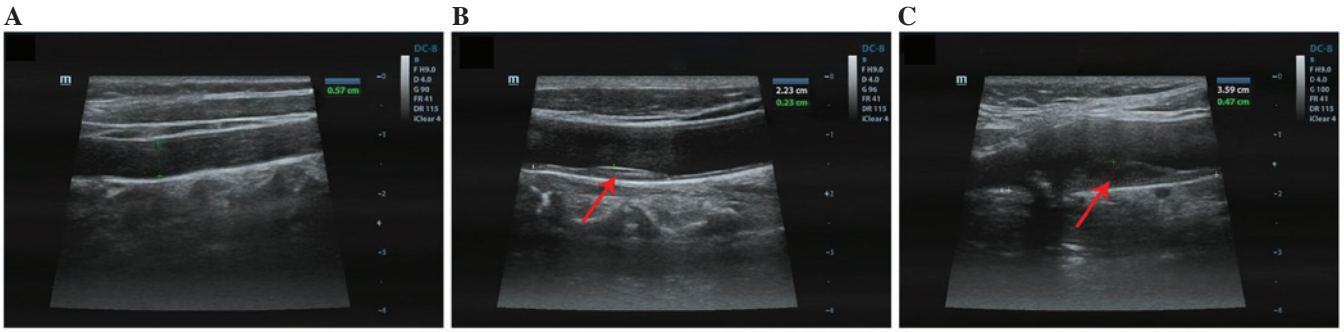

Figure 1. Carotid artery plaques detected by Doppler ultrasound. (A) Carotid artery in the healthy control group. (B) Carotid atherosclerosis plaque sonograms from patients with (B) minor (plague area, 0.23x2.23 cm) and (C) severe (plague area, 0.47x3.59 cm) lesions. Sonograms were performed on the right side of the carotid artery to atherosclerosis plaques. Plaques are demonstrated by the red arrows.

cDNA synthesis and PCR amplification using the PrimeScript Real-Time PCR reagent kit according to the manufacturer's instructions. The qPCR reaction was performed following the manufacturer's instructions, as follows: Denaturation at 94˚C for 10 min, 50 cycles at 94˚C for 15 sec, annealing at 53˚C for 30 sec and extension at 72˚C for 30 sec. The RNA level of every gene was acquired from the value of the quantitation cycle (Cq) of the qPCR normalized to that of GAPDH using the following formula: $\Delta_{Cq} = Cq_{GAPDH} - Cq_{gene}$. The final results were expressed as the N-fold differences in the target gene expression and was relative to the calibrator, termed N_{target}, which was determined as follows: $N_{target} = 2^{\Delta Cq(sample)-\Delta Cq(calibrator)}$, where Δ_{Cq} values of the calibrator and sample were determined by subtracting the Cq value of the target gene from the Cq value of GAPDH.

Statistical analysis. The patient characteristics were compared with those of the control subjects using the χ^2 test and the independent-samples *t*-test was used to detect significantly different methylation levels between the AS patients and matched controls. Receiver-operating-characteristics (ROC) curves were calculated to evaluate the diagnostic performance of different marker combinations. Furthermore, sensitivity and specificity analyses were performed to assess different marker combinations for AS detection. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Doppler ultrasound of carotid artery samples. All participants in the test and validation sets were verified by Doppler ultrasound. Images of carotid arteries of the healthy control subjects are presented in Fig. 1A, the samples demonstrate intima-media with no plaques and normal blood flow. Conversely, Doppler flow imaging of the patients with AS demonstrated anterior wall filling defects in the carotid artery, as shown in Fig. 1B and C. Furthermore, the plaque area was 0.23x2.23 cm in Fig. 1B and 0.47x3.59 cm in Fig. 1C.

Subject recruitment and collection of blood samples. A total of 300 peripheral blood samples were assessed in this case-control study, which included samples from AS patients and healthy control subjects. Between June 2011 and March 2012 150 patients and 150 healthy subjects (who had undergone Doppler ultrasound) were enrolled from the General Hospital of Ningxia Medical University for the test study. All participants were human immunodeficiency virus-, hepatitis B virus-, and hepatitis B virus-negative and did not exhibit inflammation or liver and kidney diseases. Furthermore, they did not have any previous history of cancer and were not pregnant. AS is often accompanied by lipid abnormalities; in the present study, AS patients demonstrated higher levels of

Table III. Demographic characteristics of study subjects.

Characteristic	Groups		P-value
	Matched control (%)	AS (%)	
Gender ratio (male/female)	70/80	60/90	0.1359
Age (years)			
30-40	63	18	
41-49	55	50	0.3791
51-60	32	87	
Alcohol	25	45	0.2852
Smoking	48	55	0.5721
Total triglyceride	6 (4)	66 (44)	0.0091
Total cholesterol	12 (8)	39 (26)	0.0082
High-density lipoprotein	6 (4)	88 (72)	0.0063
Low-density lipoprotein	45 (30)	25 (41)	0.0489
Apolipoprotein A	81 (54)	99 (66)	0.0894
Apolipoprotein B	24 (36)	75 (51)	0.0739

P<0.05 was considered to indicate a statistically significant difference. AS vs. controls (n=150). AS, atherosclerosis.

Table IV. Methylation levels of AS-associated genes in the two groups.

Gene	Healthy group	AS group	P-value
ACAT1	0.6103±0.01082	0.4357±0.02562	0.0024[a]
ICAM1	0.2812±0.02119	0.2530±0.02335	0.3722
ALOX5	0.3091±0.01589	0.2770±0.00573	0.0325[b]
LRP1	0.6977±0.01104	0.6893±0.01261	0.6213
MMP9	0.6246±0.02744	0.5585±0.02754	0.0944
TIMP1	0.5103±0.00276	0.5924±0.00782	0.0031[a]
VEGFA	0.5607±0.00841	0.5565±0.01386	0.8073
PDGDB	0.5493±0.01008	0.5217±0.01175	0.0812
ABCA1	0.4855±0.006517	0.6708±0.01609	0.0011[a]
LCAT	0.7087±0.01502	0.6827±0.01682	0.2544
PPARA	0.9944±0.00215	0.9786±0.00147	0.0266[b]
NFKB1	0.5709±0.01952	0.5631±0.007049	0.7090

[a]P<0.01, [b]P<0.05 vs. healthy group. Values are presented as the mean + standard deviation. AS, atherosclerosis.

the following factors: Total cholesterol (TC; P=0.0082), total triglycerides (TG; P=0.0091), high-density lipoprotein (HDL; P=0.0063), apolipoprotein A (P=0.0895) and apolipoprotein B (P=0.0739) compared with the matched control subjects, however, the low-density lipoprotein (LDL) is decreased. The two groups were matched according to gender, age, smoking habit and alcohol consumption; Table III presents the clinical profiles of the patients.

Blood lipid levels. The 300 samples were evaluated using an automatic biochemical analyzer and the blood lipids levels are presented in Fig. 2. Compared with the healthy control group, the concentrations of TG and TC increased by 2.4- and 1.2-fold, respectively in the AS patients (P<0.01 and P<0.05), while, the HDL level was reduced by 43.8% in the AS group (P<0.05).

Methylation frequencies of target genes in the AS and matched control subjects in the test set. DNA methylation of *ABCA1*, *ACAT1*, *PPARA*, *PDGFB*, *ALOX5*, *LRP1*, *LCAT*, *TIMP1*, *MMP9*, *ICAM1*, *VEGFA* and *NFKB1* were initially assessed by NT-MSP (Fig. 3; Table IV). The figure demonstrates the patterns of hyper- and hypomethylated genes in the AS patients and matched control subjects. Methylation frequencies of *ACAT1* (P<0.01), *ALOX5* (P<0.05) and *PPARA* (P<0.05) were decreased whereas *TIMP1* (P<0.01) and *ABCA1* (P<0.01)were significantly increased in AS compared the matched controls. However, no statistically significant differences were identified

Table V. Diagnostic performance of five genes in AS group versus the matched controls in the test set.

	AS group		Matched control group					
Gene	Sensitivity (%)	Pos./total	Specificity (%)	Pos./total	AUC	PPV	NPV	P-value
TIMP1	78	117/150	84	24/150	0.891	0.83	0.79	<0.0001
ACAT1	68	102/150	84	24/150	0.830	0.81	0.72	<0.0001
ABCA1	62	90/150	78	33/150	0.711	0.74	0.67	<0.0001
ALOX5	48	72/150	60	60/150	0.547	0.55	0.54	0.0325
PPARA	44	66/150	72	42/150	0.535	0.61	0.56	0.0380

AS, atherosclerosis; AUC, area under the curve; Pos., presented positive; PPV, positive predictive value; NPV, negative predictive value. n=150.

Table VI. Diagnostic performance of different panels in the AS and matched groups.

	AS group		Matched control group			
Genes	Sensitivity (%)	Pos./total	Specificity (%)	Pos./total	PPV	NPV
ACAT1, *TIMP1*	84	126/150	78	33/150	0.79	0.81
ABCA1, *TIMP1*	76	114/150	94	9/150	0.93	0.80
ACAT1, *ABCA1*	76	114/150	74	39/150	0.75	0.76
ACAT1, *ABCA1*, *TIMP1*	88	132/150	90	15/150	0.90	0.88

AS, atherosclerosis; Pos., presented positive; PPV, positive predictive value; NPV, negative predictive value. n=150.

between the remaining genes (*PDGFB*, *LRP1*, *LCAT*, *MMP9*, *ICAM1*, *VEGFA* and *NFKB1*) in the AS patients and matched control subjects, thus, these genes were excluded. The methylation of *TIMP1*, *ACAT1* (P<0.01), *ABCA1*, *ALOX5* and PPARA were significantly different in AS; thus, these genes may be used as a clinical tool for the early diagnosis of AS.

Aberrant methylation profiles in AS. Among the 12 genes, the methylation frequencies of five genes, *TIMP1*, *ACAT1*, *ABCA1*, *ALOX5* and PPARA, were significantly different between the AS patients and the matched control subjects. ROC curves were constructed for each of the five genes to classify AS patients and the matched control subjects. The area under the curve (AUC) of the ROC curve for *TIMP1* was 0.891 (P<0.0001), which was the largest among the five genes. The sensitivity and specificity of *TIMP1* were 78 and 84%, respectively, in the diagnosis of AS (Table V). When used separately to diagnose AS, the sensitivity of each gene ranged from 44 to 78% and the specificity ranged from 60 to 84%, the AUC of the ROC curves for the other four genes (*ACAT1*, *ABCA1*, *ALOX5* and *PPARA*) ranged from 0.547 to 0.830.

Combining various markers is a common strategy to improve diagnostic sensitivity when investigating clinical biomarkers. Based on the ROC curve for each gene, the most sensitive for the diagnosis of AS was *TIMP1*, which was found to be methylated in 117 of the 150 cases of AS patients, displaying a sensitivity of 78%; *TIMP1* was also identified in 126 of the 150 cases of matched controls, displaying a specificity of 84% (Table V). The more frequently methylated gene

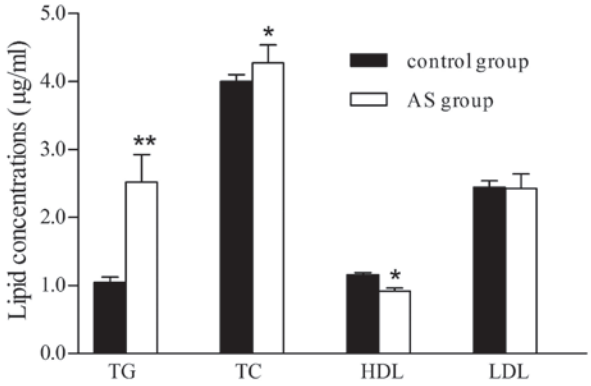

Figure 2. Blood lipid concentrations. *P<0.05, **P<0.01 vs. control group. TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

was *ACAT1* (68%; 102/150), followed by *ABCA1* (62%; 93/150), *ALOX5* (48%; 72/150) and *PPARA* (44%; 66/150). The AUC of *ALOX5* (0.547) and *PPARA* (0.535) was the smallest among the five genes, and the methylation frequencies of *ALOX5* and *PPARA* demonstrated no significant difference between the AS and matched control groups, ranging from 44 to 48% and from 60 to 72% in the in AS and matched control groups, respectively. Three genes, *TIMP1*, *ACAT1* and *ABCA1*, were highly specifically methylated in AS; therefore, these constituted the first combination (P<0.01), which was the highest among the five genes for the diagnosis of AS. According to this analysis, combinations of different markers were examined to

Table VII. AS-associated gene mRNA expression in the two groups.

Gene	Healthy group	AS group	P-value
ACAT1	0.1027±0.02326	0.1585±0.03378	0.0348[a]
ABCA1	0.4492±0.12360	0.1046±0.02010	0.0156[a]
ALOX5	0.07529±0.01171	0.2545±0.05715	0.0040[b]
TIMP1	0.1477±0.01251	0.05062±0.00502	0.0001[b]
PPARA	0.0861±0.02322	0.33960±0.06839	0.0025[b]

[a]P<0.05, [b]P<0.01 vs. healthy group. Values are presented as the mean + standard deviation. AS, atherosclerosis.

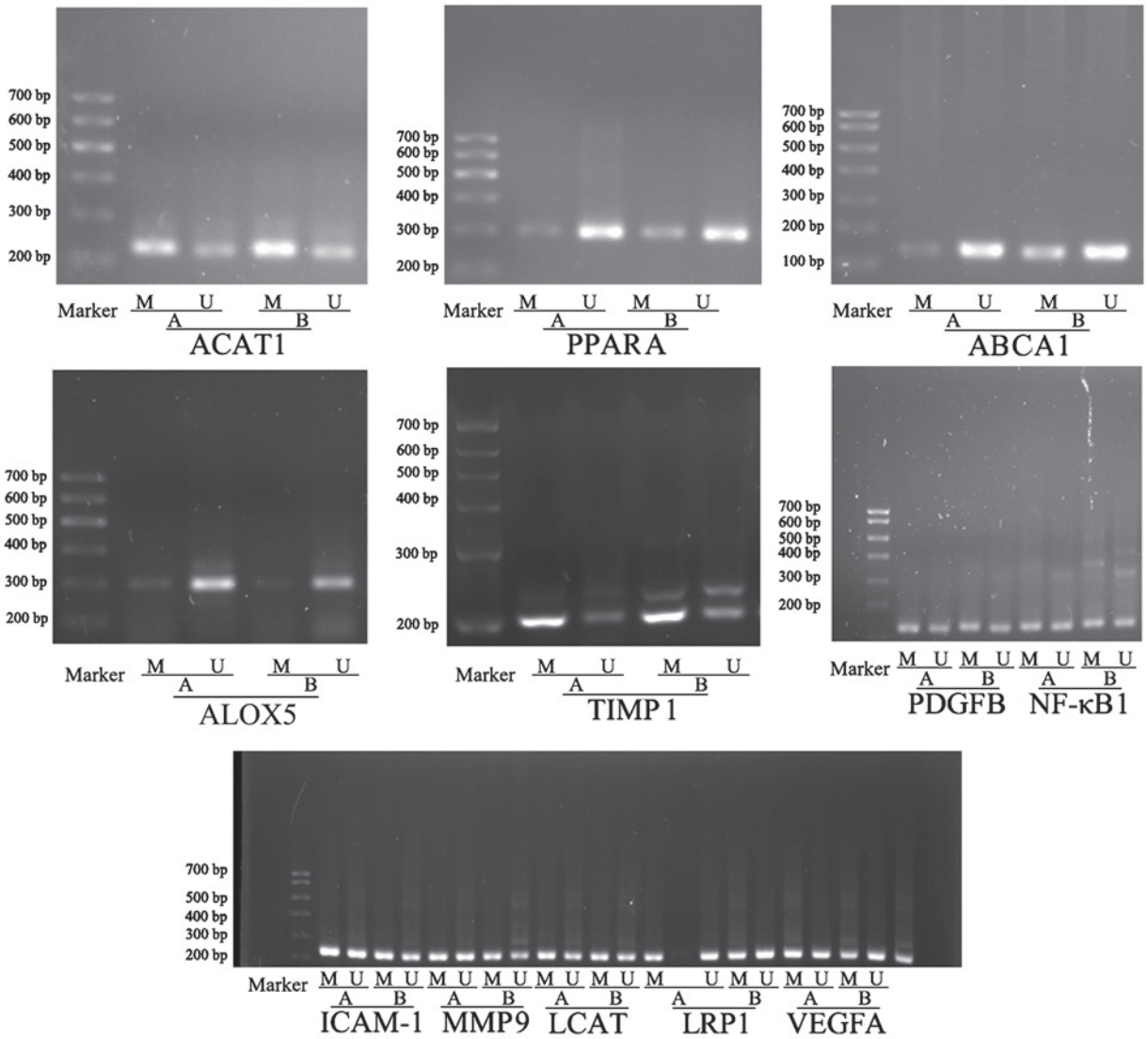

Figure 3. Methylation of AS-associated genes. Nested touchdown methylation-specific polymerase chain reaction was performed following bisulfite modification of DNA from samples of peripheral blood from the healthy and AS groups. Lane M, methylation; lane U, unmethylation; lane A, healthy group; lane B, AS group. AS, atherosclerosis.

maximize specificity and sensitivity (Table VI). The combination of *TIMP1*, *ACAT1* improved the sensitivity to 84%, which is higher than *TIMP1* or *ACAT1* alone, while the specificity dropped to 78%. Using this method, a total of three panels of genes were analyzed. The sensitivity and specificity of the three-gene panel (*TIMP1*, *ACAT1* and *ABCA1*) was 88 and 90%, respectively and thus was identified to be the optimal combination of markers in the present study.

Expression levels of TIMP1, ACAT1, ABCA1, ALOX5 and PPARA in the AS and matched control groups. DNA methylation is often correlated with changes in the accessibility of DNA

to transcriptional activators, which is crucial in the regulation of gene expression (26). RT-qPCR analysis of these five genes (*TIMP1*, *ACAT1*, *ABCA1*, *ALOX5* and *PPARA*) was performed in the AS and matched control groups on samples of peripheral blood for which total RNA was available. Compared with the healthy group, the expression levels of *PPARA*, *ALOX5* and *ACAT1* were increased by 3.94-, 3.38- and 1.54-fold in the AS group, respectively (Table VII). These data suggest that these AS-associated genes were markedly altered in AS patents.

Analysis of sensitivity and specificity of methylation profiles. The novel panel, consisting of the genes *TIMP1*, *ACAT1* and *ABCA1*, initially exhibited a combined sensitivity of 88% for the AS group and specificity of 90% for the matched control group, the performance of the novel panel was higher when compared with that of the individual genes for the detection of AS. The novel epigenetic (epi)-panel was compared with the previously used Doppler ultrasound method to identify AS in the validation data analysis. The validation set consisted of 100 samples that were also obtained from the General Hospital of Ningxia Medical University, including 50 patients and 50 healthy individuals, who had been excluded from the AS group via Doppler ultrasound diagnosis.

To study the correlation between methylation levels obtained by NT-MSP and Doppler ultrasound (which is a diagnostic criteria for AS) samples of the validation set were reexamined by qualitative NT-MSP. In order to increase the analytical sensitivity and to yield a higher reproducibility, primer binding sites in the test set and the validation set were located in the same genomic promoter region. By contrasting Doppler ultrasound with *TIMP1*, *ACAT1* and *ABCA1* promoter methylation, a significant, positive correlation was identified. The combination of *TIMP1*, *ACAT1* and *ABCA1* methylation revealed a sensitivity of 88% for the detection of AS and a specificity of 70% in healthy subjects, respectively. The novel panel also revealed a coincidence rate of 79%, which refers to consistency between the results of the novel panel and the Doppler ultrasound.

Discussion

The incidence of AS is increasing and represents a significant health issue, thus, there is a clear requirement for the development of prognostic markers in AS to provide risk-adjusted treatment and surveillance management. DNA methylation biomarkers have obvious applications in diagnostics (27). In the present study, the performance of a blood-based PCR assay for methylated DNA of 12 potential biomarkers was determined in two independent test sets with a total of 300 samples. The data confirmed the high performance of certain previously identified DNA methylation markers. Furthermore, the methylation frequencies of *ACAT1*, *ABCA1*, PPARA, *TIMP1* and *ALOX5* of AS peripheral blood were identified to be significantly changed when compared with that of the matched controls, the novel epi-panel consisting of *TIMP1*, *ACAT1* and *ABCA1* exhibited a higher sensitivity and specificity than any of the individual biomarkers.

These genes have previously been reported to be significantly involved in preventing or promoting AS. Aberrant methylation of *ABCA1* and *ACAT1* has been identified in the progression of AS (28). In addition, *TIMP1* expression has been associated with a negative prognosis in AS (29). To the best of our knowledge the present study is the first to demonstrate *TIMP1* methylation in AS, and that methylation of *ACAT1* and *ABCA1* has never previously been reported in AS.

Methylation changes in AS are often heterogeneous and, as yet, to the best of our knowledge, no single gene has been identified to be methylated in every AS patient specimen. Furthermore, in the majority of studies investigating methylation levels of only single genes the sensitivity was low. Therefore, it is considered to be advantageous to use a panel of genes for disease screening procedures. As a result of the comparative analysis, a biomarker panel with the best values for AS specificity was defined first in a test set. *TIMP1*, *ACAT1* and *ABCA1* proved to be candidate genes, showing a significant specificity of the methylation pattern in the matched control and AS groups. Furthermore, *TIMP1*, *ACAT1* and *ABCA1* methylation achieved 88% sensitivity, with a high specificity of 90% in the test set, this indicated that the panel had the potential to perform as effectively, potentially more effectively, than the single markers, indicating that the methylation of three genes may be useful when screening high risk individuals.

In an independent validation set, promoter methylation of the *TIMP1*, *ACAT1* and *ABCA1* combination enabled significant discrimination of AS from various control conditions. ROC analysis of three-gene panel methylation revealed a sensitivity of 88% with a specificity of 70%. Combining these with other detection methods may provide a robust epi-panel with a high sensitivity for AS detection. Compared with ultrasound, a reliable biomarker panel for the early detection of AS, revealed that the coincidence rate was 79%; thus, *TIMP1*, *ACAT1* and *ABCA1* methylation analyses may be used as a supplement to mammography, which has a low sensitivity in the early detection of AS.

In conclusion, methods for AS screening should be easy to perform, non-invasive and provide a benefit to patients. Blood-based biomarkers fulfill these three requirements. Quantifying promoter methylation of AS-associated genes in peripheral blood is a rapidly growing research topic for early AS detection. In the current study, 12 potential biomarkers were evaluated in two independent test sets, and promoter methylation of the *TIMP1*, *ACAT1* and *ABCA1* gene combination exhibited a high sensitivity and specificity in peripheral blood DNA from patients with AS. It is proposed that such a blood-based screening method would be convenient for the patient and reduce costs for health care providers.

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