Altered Plasma MicroRNAs as Novel Biomarkers for Arteriosclerosis Obliterans

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Aim: Arteriosclerosis obliterans (ASO) of the lower extremities is a major cause of adult limb loss worldwide. A timely diagnosis in the early stages of the disease determines the clinical outcomes, however lacking of palpable symptoms remains the biggest obstacle. This study aimed to screen a cluster of microRNAs (miRNAs) that can be used as biomarker for the ASO in the earlier stages.

Methods: Plasma from 3 patients with ASO and 3 healthy controls were profiled to screen altered miRNAs by microarray, then Real time PCR was further used to confirm the changes in 55 ASO patients and 54 controls. We also analyzed the correlation of miRNAs level with Fontaine stages and the influence of T2DM which is a common complication with ASO on the level of miRNAs.

Result: Twenty-four aberrantly expressed miRNAs were screened in the plasma of ASO patients. Real time PCR verified that the level of miR-4284 was significantly increased, while levels of miR-4463, miR-4306 and miR-221-3p were significantly decreased both in the plasma and in the sclerotic samples compared with the controls. Interestingly, we revealed a time and stage specific expression manner, as shown that expression of miR-4284 increased at the stage I of ASO and maintained the tendency to stage IV, while miR-4463 expression decreased at every stage of ASO; however, the expression of miR-4463 showed opposite changes in ASO patients with or without T2DM.

Conclusion: Altered expressions of miR-4284 and miR-4463 are novel characteristics and may serve as potential biomarkers for the early diagnosis of ASO.

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Key words: miRNA, Arteriosclerosis obliterans (ASO), Biomarker

Introduction

Arteriosclerosis obliterans (ASO) of the lower extremities is a vascular disease that results in adult limb loss around the world. It derives and develops from atherosclerosis1). Surgery and endovascular treatment are the major approach for the treatment of ASO but restenosis usually relapses in 1-2 years after therapy2,3). Most patients with ASO have no apparent clinical symptoms in the early stages, which leads to delay in treatment. Thus, a diagnosis at the early stage is essential to prevent progression.

MicroRNAs (miRNAs) are a novel class of endogenous, small noncoding RNAs approximately 22 nucleotides in length that regulate approximately 30% of the encoding genes of the human genome. miRNAs regulate gene expression by accelerating mRNA degradation or inhibiting translation at the posttranscriptional level by binding the 3'-untranslated region (UTR) of their target mRNAs4-6). Numerous studies have revealed that some miRNAs play a significant role in atherosclerosis. The microRNA profile in human ASO arteries has been described by Mian et al. who found that miR-21 was the most...
upregulated miRNA localizing mainly in the arterial smooth muscle cell (ASMC) of ASO and inhibiting the proliferation and migration of human ASMC. MiR-145 and miR-143 regulate the fate and plasticity of VSMC. MiR-210 overexpression stimulates tubulogenesis and migration. The inhibition of miR-126 increases leukocyte adherence to TNF-α stimulated endothelial cells.

Studies have revealed that miRNAs are remarkably stable and can be readily quantified in serum and plasma. More importantly, circulating miRNAs demonstrate significant dynamic change in some pathological conditions and can partly reflect the disease state, such as cancers, heart diseases, diabetes, and cardiovascular diseases. Patients have specific patterns of circulating microRNA levels, often in the early disease progress. These discoveries have laid the ground work for identifying circulating miRNAs as non-invasive biomarkers of many diseases. However, they just selectively detected miRNAs that could be used as new biomarkers for the diagnosis of ASO.

In this study, microRNAs profile in the plasma samples of patients with ASO and controls was determined to screen altered miRNAs. Twenty-four miRNAs were significantly changed more than two fold. Moreover, several dysregulated miRNAs were further verified in the plasma and intima samples by qPCR. The purpose of this study is to find a cluster of miRNAs that could be used as new biomarkers for ASO.

Materials and Methods

Controls and Patient Samples

We enrolled 55 patients diagnosed with ASO of the lower extremities and 54 age and sex-matched controls from the Affiliated Hospital of Luzhou Medical College. This study was approved by the Ethic Committee Board of Luzhou Medical College, and informed consent was provided by all patients. The investigations were performed in accordance with the Declaration of Helsinki principles. The intermittent claudication and ischemic rest pain were used as the main criteria, and the estimations of the Ankle/Brachial Index (ABI) and pulse wave velocity (PWV) were used as complementary parameters. Fontaine classification was conducted by at least three vascular surgeons. All ASO patients had the characteristic complaints of chronic limb ischemia, intermittent claudication, and rest pain as confirmed by angiography.

Twenty newly diagnosed type 2 diabetes mellitus (T2DM) patients who had not been diagnosed with diabetes 3 years ago were enrolled. T2DM was diagnosed according to the fasting glucose level ≥ 7.0 mmol/L or 2-h postprandial plasma glucose level ≥ 11.1 mmol/L. All patients who had received surgical interventions or who were critically ill within the last 6 months were excluded, such as cancer, serious infection, renal failure, connective tissue diseases, and hormone replacement therapy. Individuals without any of the above symptoms and other diseases were enrolled as controls. The patient characteristics are summarized in Table 1. Blood samples were collected from all the patients. Plasma was isolated within 2 h by centrifugation at 3000 × g at 4°C for 20 min. The plasma samples were aliquoted into 1.5 mL RNase-free Eppendorf tubes and stored at −80°C. Tissue samples were obtained from amputation patients. Sclerotic arteries were collected, and the normal arteries near the edge of the sclerotic arteries were used as the control. The sclerotic and normal intimas were separated and preserved in RNAlater storage solution until RNA extraction.

Extraction of MicroRNAs from Plasma and Tissues

Total RNA, including miRNAs, was isolated from the plasma using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the manufacturer’s instructions with the following modifications: 400 μL plasma was lysed with 2 mL QIAzol Lysis Reagent; 400 μL synthetic Spike-In (Caenorhabditis elegans RNA39, cel-miR-39) of 1.6 × 10^8 copies/μL was added as internal calibrator to monitor extraction efficiency. The remainder of the extraction was performed according to the manufacturer’s instructions. The eluted RNA was used for further analysis immediately. Furthermore, because the yield of RNA could not be accurately measured by NanoDrop spectrophotometer (Thermo Scientific), we used a fixed volume for every step during the RNA isolation and reverse transcription. In addition, miRNAs from the tissues were extracted by the miRNeasy Mini Kit (QIAGEN) according to the instructions.

Microarray Analysis of Plasma

The RNA from plasma samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array kit and hybridized on the miRCURY™ LNA Array (v.18.0) according to array manual. Then the slides were scanned by the Axon GenePix 4000B microarray scanner (Axon). GenePix Pro 6.0 software (Axon) was used to analyze the data. miRNAs whose intensities < 30 were abandoned. miRNA data were normalized.
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using the Median normalization and selected for differentially expressed miRNAs screening.

Analysis of miRNA Expression

Input RNA (5 μL) was polyadenylated by poly (A) polymerase and reverse transcribed to cDNA using miScript II RT Kit (QIAGEN) according to the manufacturer’s instructions. The expression levels of mature miRNAs were measured using the miScript SYBR Green PCR kit in ABI StepOne Plus PCR system (Applied Biosystems). The miRNA-specific primers were bought from QIAGEN. Real-time PCR reactions were performed in triplicates. In our experiment, the threshold cycle (Ct) value over 35 was excluded. The relative expression levels were calculated by the 2^(-ΔΔCt) method with cel-miR-39 as the reference gene in the plasma samples and U6 in the artery samples.

Bioinformatic Analyses

The miRNA target genes were predicted by TargetScan. Functional classification of the target genes was conducted by Gene Ontology (GO) analysis. We selected the mRNA target following such criteria as far as possible: 1) there are at least 7 binding sites in the 3’-UTR of predicted target mRNA with miRNA. 2) previous researches reported that these genes may participate in angiogenesis, intimal hyperplasia, or vascular restenosis.

Statistical Analysis

Data are expressed as the mean ± SD. All data were processed using the SPSS software 19.0. The Student’s t-test was used to statistically evaluate significant differences when the 2 groups were compared and one way ANOVA for multiple comparisons. P < 0.05 was considered statistically significant.

Results

miRNA Levels in Plasma

To investigate the levels of circulating miRNAs in ASO, plasma from 3 patients with ASO and 3 healthy controls were profiled. Detailed information about the patients and the healthy controls is listed in Table 2. After evaluation of the relative expression of these miRNAs compared with the control group, 24 miRNAs were gathered with a change of more than 2 fold (P < 0.05), in which 4 miRNAs were upregulated.

Table 1. The baseline clinical characteristics of the ASO patients and controls

<table>
<thead>
<tr>
<th></th>
<th>ASO (n = 55)</th>
<th>Control (n = 54)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>76.52 ± 10.41</td>
<td>74.36 ± 7.22</td>
<td>0.316</td>
</tr>
<tr>
<td>Male/Female (n/n)</td>
<td>33/22</td>
<td>34/20</td>
<td>0.845</td>
</tr>
<tr>
<td>Smoker n(%)</td>
<td>29*</td>
<td>11</td>
<td>0.030</td>
</tr>
<tr>
<td>Wine n(%)</td>
<td>18</td>
<td>11</td>
<td>0.418</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>130.8 ± 20.1</td>
<td>120.8 ± 14.1</td>
<td>0.089</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>78.6 ± 10.8</td>
<td>80.6 ± 11.8</td>
<td>0.312</td>
</tr>
<tr>
<td>WBC (x10^3/L)</td>
<td>9.40 ± 4.67*</td>
<td>6.80 ± 1.80</td>
<td>0.031</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>5.42 ± 1.23</td>
<td>5.11 ± 1.04</td>
<td>0.490</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.45 ± 1.14</td>
<td>4.31 ± 0.69</td>
<td>0.145</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.56 ± 0.93</td>
<td>1.01 ± 0.42</td>
<td>0.542</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.34 ± 0.43</td>
<td>1.35 ± 0.27</td>
<td>0.193</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.47 ± 0.87</td>
<td>2.35 ± 0.55</td>
<td>0.312</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>33.87 ± 15.43</td>
<td>26.82 ± 13.43</td>
<td>0.236</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>24.06 ± 16.37</td>
<td>19.39 ± 9.74</td>
<td>0.453</td>
</tr>
<tr>
<td>HCY (μmol/L)</td>
<td>17.01 ± 7.91*</td>
<td>11.69 ± 2.25</td>
<td>0.005</td>
</tr>
<tr>
<td>Cys C (mg/L)</td>
<td>1.73 ± 0.85*</td>
<td>0.87 ± 0.21</td>
<td>0.011</td>
</tr>
<tr>
<td>Crea (μmol/L)</td>
<td>95.92 ± 55.00</td>
<td>78.53 ± 26.89</td>
<td>0.126</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>2.26 ± 0.13</td>
<td>2.22 ± 0.11</td>
<td>0.314</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>104.11 ± 5.07</td>
<td>107.13 ± 7.53</td>
<td>0.102</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>4.27 ± 0.52</td>
<td>4.11 ± 0.43</td>
<td>0.210</td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>141.43 ± 5.23</td>
<td>140.99 ± 2.56</td>
<td>0.678</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; HCY, homocysteine; Cys C, Cystatin C. *p < 0.05, **p < 0.01 versus control. Data are expressed as mean ± SD.
samples of ASO patients could be a sign of vascular abnormality. The seven miRNAs with significant changes in plasma including miR-221-5p, miR-432, miR-4463, miR-4284, miR-124, miR-4306, and miR-221-3p were selected to be examined in the intima samples in 24 patients with ASO (Fig. 3). Our results showed that the level of miR-4284 was significantly increased \((p < 0.05)\), while the levels of miR-4463, miR-4306, and miR-221-3p were significantly decreased in the sclerotic samples compared with the normal samples. There was no significant difference in the expression changes of the other miRNAs when compared with the normal samples.

miRNAs Expression in ASO Intima Samples

Because the circulating miRNAs were released from tissues, the changes of miRNAs in the plasma samples of ASO patients could be a sign of vascular abnormality. The seven miRNAs with significant changes in plasma including miR-221-5p, miR-432, miR-4463, miR-4284, miR-124, miR-4306, and miR-221-3p were selected to be examined in the intima samples in 24 patients with ASO (Fig. 3). Our results showed that the level of miR-4284 was significantly increased \((p < 0.05)\), while the levels of miR-4463, miR-4306, and miR-221-3p were significantly decreased in the sclerotic samples compared with the normal samples \((p < 0.05)\). There was no significant difference in the expression changes of the other miRNAs when compared with the normal samples. Among these seven miRNAs, the expression of miR-4284 was the highest in both the plasma and intima samples.

Table 2. The information of 3 ASO patients and 3 healthy controls

<table>
<thead>
<tr>
<th>Group</th>
<th>NO.</th>
<th>Gender</th>
<th>Age</th>
<th>T2DM</th>
<th>Hypertension</th>
<th>Hyperlipidemia</th>
<th>Smoke (years)</th>
<th>Alcohol (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASO</td>
<td>1</td>
<td>M</td>
<td>79</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>40Y</td>
<td>40Y</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>82</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>F</td>
<td>77</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Healthy control</td>
<td>1</td>
<td>M</td>
<td>78</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td></td>
<td>2</td>
<td>M</td>
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<tr>
<td></td>
<td>3</td>
<td>F</td>
<td>76</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

ASO, arteriosclerosis obliterans; T2DM, type 2 diabetes mellitus; F, female; M, male; Y, Yes; N, No

Fig. 1. miRNA profile of plasma in patients with ASO vs healthy controls

A heat map diagram is shown clustering the differentially expressed miRNAs with fold change \(>2\) and \(P < 0.05\). C1, C2, and C3 represent controls. P1, P2, and P3 represent patients with ASO. ASO: arteriosclerosis obliterans

while 20 miRNAs were downregulated (Fig. 1). The expression levels of 9 microRNAs (miR-124, miR-5004, miR-4284, miR-432, miR-221-5p, miR-221-3p, miR-4463, miR-4306, and miR-4301) were selected to be confirmed by qRT-PCR in 55 ASO patients and 54 controls. The levels of miR-124, miR-4284, and miR-221-5p were significantly increased, while miR-432, miR-4463, miR-4306, and miR-221-3p were significantly decreased in the ASO plasma samples compared with the control (Fig. 2). No statistical difference was observed for miR-5004 and miR-4301.
The levels of miR-4284, miR-4463, miR-4306, and miR-221-3p were significantly altered both in the plasma and intima samples from ASO patients. To test whether they can serve as biomarkers for ASO at an early stage, we analyzed the four miRNAs expression levels in the plasma of ASO patients at different Fontaine stages (Fig. 4). We found that the miR-4284 level was elevated at stage I of ASO and maintained a high level at stage IV, while miR-4463 level was suppressed from stage I to stage IV. The expression of miR-4306 and miR-221-3p was gradually decreased with the development of ASO but showed no specific prediction at the early stage of ASO. However, the low level of miR-4306 and miR-221-3p may implicate a severe process.
The expression levels of miR-4284, miR-4463, miR-221-3p, and miR-4306 were examined in the plasma samples from 55 ASO patients (Fontaine I, n = 8; Fontaine II, n = 16; Fontaine III, n = 19; and Fontaine IV, n = 12) and 54 controls. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, compared with control. #p < 0.05, compared with each other.

miRNAs Expression in Patients with ASO, T2DM, and ASO Combined with T2DM

T2DM is related to a significant increase in the risk of atherosclerosis. There is a general agreement that diabetics develop ASO in the legs more frequently and at an earlier age than nondiabetics, which results in a higher risk of critical limb ischemia and limb loss. T2DM is a common complication with ASO; therefore, we next investigated whether the miRNA changes were specific in patients with ASO compared with those with ASO and T2DM. We additionally determined four miRNA expressions in the plasma of 12 patients with ASO only, patients with ASO and T2DM, and 20 patients with T2DM. It turned out that the miR-4284 level was increased in the three groups but with no significant difference in the patients with ASO combined with T2DM versus control group. MiR-4463 was significantly increased in the patients with T2DM and with ASO combined T2DM while it was significantly decreased in the ASO only patients (p < 0.01). MiR-4306 was suppressed in both the ASO patients and the patients with ASO and T2DM but increased sharply in T2DM patients. An obvious suppression in the miR-221-3p expression was observed in the three group patients. These results indicated that the high expression of miR-4284 and the low expression of miR-4463 were specific indicators for the discrimination of ASO and ASO combined with T2DM.

Effect of Clinical Indexes on miRNAs Expression

Patients with ASO were more likely to have a higher homocysteine (HCY), cystatin C (Cys C), and white blood cell (WBC), and no difference in other biochemical indexes (Table 1). Previous researches have reported that smoking is a recognized risk factor for ASO. To explore whether HCY, Cys C, and smoking affect the expression of miR-4284, miR-4463, miR-4306, and miR-221-3p, ASO patients were grouped according to HCY, Cys C levels, and smoke habit to compare the 4 miRNAs levels. The level of miR-221-3p was lower in the high HCY group, while the level of miR-4463 was lower in the high Cys C group (Fig. 6). In addition, the levels of miR-4284 and miR-221-3p were higher in the smoking group than the nonsmoking group. However, no obvious change was observed in other miRNAs.

Bioinformatic Analyses

The predicted target genes of miR-4463, miR-4284, miR-4306, and miR-221-3p were analyzed using the databases from TargetScan (www.targetscan.org). The predicted target genes of miR-4463, miR-4284, miR-4306, and miR-221-3p that may be related to the formation and development of ASO are shown in Table 3. According to the functional analysis result, miR-4463 is involved in regulating the cell polarity and cell migration via targeting the AMOT gene; MiR-4284 and miR-4306 may regulate smooth muscle cell proliferation and miR-221-3p participates...
in several signaling pathways.

**Discussion**

At present, the diagnosis of ASO depends on several clinical tests, such as angiography, estimations of ankle/brachial Index (ABI), and pulse-wave velocity (PWV), as well as the measurement of circulating hs-CRP levels. However, these examinations can only be detectable when ASO already developed, and...
changes of miR-130a and miR-21 were opposite, which could be interpreted by different sample types. In general, we identify 4 miRNAs, miR-4284, miR-4463, miR-4306, and miR-221-3p, which showed a positive correlation in the intima samples with plasma. More importantly, miR-4284 and miR-4463 was promptly altered at the stage I of ASO. Because miR-NAs are remarkably stable and can be readily quantified in the plasma, these data suggest that miR-4284 and miR-4463 become potential predictors of ASO.

MiR-221-3p has been reported to play a crucial role in arteriosclerosis. In an early study, Liu et al. found that miR-221-3p localized in VSMCs in the injured vascular walls and regulated VSMC proliferation and neointimal hyperplasia by targeting p27 and p57 in vitro. Li et al. found that miR-221 significantly decreased in the sclerotic samples compared with the normal samples. Our results coincide with Li’s report and show that miR-221-3p expression is significantly decreased both in the plasma and in the intima from ASO patients. These observations indicate that the abnormal expression of miR-221-3p in the plasma and intima is a characteristic of ASO and may be an indicator for ASO.

With regard to miR-4463, miR-4284, and miR-4306, few studies have been reported till now. AMOT is a predicted target of miR-4463 by Target Scan, which is an angiostatin binding protein regulating cell migration, polarity, and tube formation. Therefore miR-4463 may participate in regulating the progression of ASO via AMOT. Wang et al. found that miR-4284 was depleted in the cyst fluids derived from invasive carcinomas in pancreatic cancer. MiR-4306 was significantly upregulated in 83% of pancreatic cancer serum-exosomes. In our study, we found that miR-4284 was the most abundant miRNA expressed in the plasma and intima. However, the there was no predictable markers for ASO in its earlier stages.

The aberrant versions of miRNA have been widely reported in different vascular diseases. Circulating miRNAs play important roles in extracellular communication between different cells and tissues through microparticles or HDL. Alterations of miRNAs in the serum or plasma in cardiovascular disease have been reported in ample studies. These findings support the diagnostic and therapeutic values of these small molecules for cardiovascular diseases. However, as to ASO, most references are mainly focused on miRNAs that are abnormally expressed in the artery tissues or detecting known miRNAs expression in the serum by qPCR directly. Li et al. reported that the levels of miR-130a, miR-27b, and miR-210 significantly increased in the sclerotic samples and serum samples. In addition, Wang et al. demonstrated that miR-21 markedly increased to 7.98 fold and miR-125b significantly decreased in ASO arteries. In the current study, we focused on screening the aberrantly expressed plasma miRNAs as significant new biomarkers for ASO and successfully identified 7 miRNAs significantly altered in the plasma of ASO patients. Among them, only 4 miRNAs, miR-4284, miR-4463, miR-4306, and miR-221-3p changed in the intima samples with significant differences, indicating the discrepant miRNAs expression profile between plasma and intima samples. This could be easily understood because the secretion of miRNA from cytoplasm to plasma was influenced by many factors and only part of miRNAs in the tissues can be released to the plasma. In accordance with Li’s result, the microarray result showed a similar tendency of miR-210, miR-27b, and miR-125b in the plasma sample but with no statistical difference. However, the Table 3. Target genes that may be related to the formation and development of ASO

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Symbol</th>
<th>GO description</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-4463</td>
<td>AMOT</td>
<td>establishment of cell polarity involved in ameboidal cell migration</td>
</tr>
<tr>
<td>hsa-miR-4284</td>
<td>AMOTL2</td>
<td>tight junction</td>
</tr>
<tr>
<td></td>
<td>CALCRL</td>
<td>positive regulation of smooth muscle cell proliferation</td>
</tr>
<tr>
<td>hsa-miR-4306</td>
<td>IGFBP5</td>
<td>negative regulation of smooth muscle cell proliferation/negative regulation of insulin-like growth factor receptor signaling pathway</td>
</tr>
<tr>
<td>hsa-miR-221-3p</td>
<td>KSR1</td>
<td>positive regulation of MAPK cascade</td>
</tr>
<tr>
<td></td>
<td>P27</td>
<td>negative regulation of epithelial cell proliferation involved in prostate gland development/phosphorylation</td>
</tr>
</tbody>
</table>

, name abbreviation of the regulated gene; , brief introduction of the protein translated from the gene.
higher in the smoking group than the nonsmoking group. Those data demonstrated that HCY, Cys C, and smoking may be associated with the level of miRNAs in ASO.

In conclusion, the present study screened the aberrantly expressed miRNAs in the plasma of three patients with ASO and three healthy controls by microarray. We screened out 24 miRNAs with expression fold change more than 2 folds. We confirmed that the level of miR-4284 was significantly increased while the levels of miR-4463, miR-4306, and miR-221-3p were significantly decreased in the plasma and intima in ASO patients using qPCR. Furthermore, we found that miR-4284 promptly increased at the stage of ASO and maintained the tendency to stage IV, while miR-4463 dropped at every stage of ASO. More interestingly, we found that the expression of miR-4463 was significantly decreased in the ASO patients, while it showed opposite changes in ASO patients combined with T2DM. However, it is regretful that the relevant studies have not yet been published, and the reason for these changes needs to be further investigated. In conclusion, miR-4284 and miR-4463 may be potential and promising molecular markers for ASO because of their specificity. Future studies will be aimed at the function of miR-4284 and miR-4463 to have a better understanding of their unique nature, and an in-depth study regarding the difference of miR-4463 in the ASO patients and ASO patients combined with T2DM needs to be conducted.

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Conflict of Interest

All authors disclose that there is no financial relationship with a biotechnology manufacturer, a pharmaceutical company, or any other commercial entity that has an interest in the subject matter or materials discussed in the manuscript. All authors declare no conflict of interest.
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