

## Original Article

# Evaluation of miRNA-133a-3p and miRNA-124-3p expression in atherosclerosis using real-time PCR and immunohistochemical methods

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**Background and objectives:** Atherosclerosis is a chronic disease marked by the build up of lipids and inflammatory cells in arterial walls, leading to vessel narrowing and increasing the risk of serious complications like heart attack and stroke. Recent findings suggest that microRNAs (miRNAs) serve as key regulators in the mechanisms driving atherosclerotic disease. However, the expression levels and functional roles of miRNA-133a-3p and miRNA-124-3p in atherosclerosis remain incompletely understood. The aim of this study was to determine the relationship between the expression levels of miR-124-3p and miR-133a-3p, and the phenotypic changes of S100A4-positive vascular smooth muscle cells in atherosclerosis.

**Methods:** We collected tissue samples from 25 patients with atherosclerosis who underwent coronary artery bypass graft surgery. IMA tissues were used as controls; atherosclerotic aortic tissues as cases. Expression levels of miRNAs were assessed using reverse transcription polymerase chain reaction (RT-PCR). Tissue samples underwent immunohistochemical staining with S100A4 protein to evaluate cellular and structural characteristics.

**Results:** A marked decrease in the expression of miR-133a-3p and miR-124-3p was observed in the atherosclerosis group compared to the control group, and both differences were statistically significant ( $P=0$ ). Additionally, an increase in S100A4 protein immunoreactivity was detected in the atherosclerosis group.

**Interpretations and conclusions:** The downregulation of miRNA-133a-3p and miRNA-124-3p in atherosclerotic tissues, along with the observed increase in S100A4 protein immunoreactivity, suggests that these two miRNAs may play a role in the regulation of inflammatory endothelial phenotypes. Therefore, the interaction between miRNA-133a-3p, miRNA-124-3p, and S100A4 protein may help elucidate a potential mechanism underlying the prevention of atherosclerosis.

**Keywords** Atherosclerosis; miRNA-133a-3p; miR-124-3p; S100A4; Vascular smooth muscle cells

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality.<sup>1</sup> Atherosclerosis is characterized by lipid accumulation, vascular smooth muscle cell (VSMC) proliferation, and chronic inflammation.<sup>1,2</sup> In recent years, various cellular and molecular biomarkers, especially microRNAs (miRNAs), have been investigated in many studies to reduce the complications of atherosclerosis.<sup>3</sup> Among novel biomarkers, miRNAs are increasingly recognized as regulators of endothelial dysfunction, inflammation, and plaque instability.<sup>4</sup> miR-133a-3p has

been shown to regulate myoblast differentiation and act as a tumour suppressor.<sup>5-7</sup> Its role in atherosclerosis remains controversial, with studies reporting both increased and decreased expression levels in vascular tissues and plasma.<sup>8-10</sup> miR-124-3p, primarily expressed in the brain, influences neuronal differentiation and tumour suppression,<sup>11-13</sup> and may also regulate vascular inflammation and plaque stability.<sup>14-16</sup> However, its expression in human atherosclerotic tissue is not well characterised.

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The S100 protein family is a group of 21 proteins found exclusively in vertebrates, playing regulatory roles both inside and outside the cell.<sup>17</sup> S100A4, this member of the S100 protein family, has been identified as a critical regulator of SMC migration, proliferation, and fibrosis, functioning as a marker of the synthetic SMC phenotype.<sup>18</sup> MicroRNAs are critical post-transcriptional regulators of these processes; miR-124-3p directly targets S100A4 to inhibit SMC proliferation, while miR-133a-3p maintains the contractile phenotype and prevents maladaptive remodelling.<sup>19,20</sup> However, the relationship between these miRNAs and S100A4 expression in human atherosclerotic versus non-atherosclerotic tissues remains poorly understood.

This study aimed to evaluate the expression levels of miR-133a-3p and miR-124-3p, along with S100A4 protein immunoreactivity, in paired human tissue samples, in order to better understand their potential interplay in the context of atherosclerosis.

## Methods

A total of 25 patients who underwent coronary artery bypass graft surgery using internal mammary artery grafts (IMA) between April 2021 and February 2023 at the department of Cardiovascular Surgery, Faculty of Medicine, Mersin University, were included in the study. The study was approved by the Clinical Research Ethics Committee of Mersin University Faculty of Medicine, and written informed consent was obtained from all patients with atherosclerosis prior to their inclusion in the study.

*Inclusion criteria:* Patients aged 40 to 60 yr; hemodynamically stable; underwent coronary artery bypass surgery using the internal mammary artery as an arterial graft; and had no history of major cardiac surgery, systemic infection, or malignancy were included in the study.

*Exclusion criteria:* Patients with connective tissue diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE); valvular heart disease; heart failure (EF <30%); malignancy; history of acute myocardial infarction (AMI); chronic kidney disease; chronic liver disease; or regular use of immunosuppressive or steroid medications were excluded from the study.

*Sample size:* Using G\*Power version 3.1.9.4 (Heinrich Heine Universität, Düsseldorf, Germany), *a priori* power analysis was conducted with a significance level ( $\alpha$ ) of 0.05, power of 0.8, and an expected effect size of

0.7. Based on these parameters, the estimated sample size was calculated to be 25 participants per group.

*Experimental group structuring based on tissue specimens:* Tissue samples were obtained from patients undergoing coronary artery bypass surgery. From each patient, paired samples were collected to form two groups: the case group included aortic tissue samples from atherosclerotic regions, collected as medical waste during coronary artery bypass graft surgery; the control group consisted of tissue samples from the non-atherosclerotic internal mammary artery (IMA) region of the same patients, also collected as medical waste during the same procedure.

*miRNA isolation and expression analysis:* Total RNAs were isolated from tissue samples stabilised in RNA later solution using the miRNA Isolation Kit (Roche Diagnostics, GmbH, Mannheim, Germany), following the manufacturer's instructions. cDNAs were synthesized from the isolated miRNAs using a cDNA synthesis kit (ABM Inc., Richmond, Canada), and stored at -20°C until further analysis. Expression analyses of miRNA-133a-3p and miRNA-124-3p was performed using a real-time polymerase chain reaction (RT-PCR) system (Roche LightCycler 480). The fold change of miRNA expression for each miRNA was calculated using the  $2^{-\Delta\Delta CT}$  method.<sup>21</sup>

The Ct values of the miRNAs were normalized to SNORD44 (Ella Biotech, Munich, Germany). The primer sequences were as follows: hsa-miR-133a-3p (miRBase accession number: MIMAT0000427) forward primer: 5'-TTTGGTCCCTTCAACCAGC TG-3'; hsa-miR-124-3p (miRbase accession number: MIMAT0000422) forward primer: 5'TAAGGCACG CGGTGAATGCCAA-3'.

*Tissue processing and haematoxylin and eosin staining for tissue examination under light microscopy:* In this study, samples obtained from IMA and atherosclerotic aortic tissues were subjected to tissue processing for histopathological comparison. For fixation, the tissues were kept in formalin solution (neutral buffered, 10%) for 24h, then by routine follow up procedures, embedded in paraffin (Merck, Darmstadt, Germany) and sectioned into 4-5  $\mu$ m slices. Samples slices were then stained with haematoxylin-eosin (H&E) and captured using an Olympus BX53 light microscope equipped with a DP26 digital camera and cellSens Standard software (Olympus Corporation, Tokyo, Japan). The histological procedures were conducted in accordance with established guidelines described by Bancroft and Gamble.<sup>22</sup>

**S100A4-based immunohistochemical analysis:** IMA and atherosclerotic aortic sections were subjected to microwave heat-induced antigen retrieval in citrate buffer (pH 6.0; Thermo Fisher Scientific, USA) for 15 min following deparaffinization and rehydration. After blocking with 3% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) and 5% BSA (Sigma-Aldrich), sections were incubated overnight at 4 °C with rabbit polyclonal anti-S100A4 primary antibody (1:100; Abcam, ab41532). Following incubation with an HRP-conjugated polymer-based secondary antibody system (EnVision+ System; Dako/Agilent) and DAB chromogen (Vector Labs), sections were counterstained with Mayer's hematoxylin (Thermo Scientific).<sup>23</sup> S100A4 staining patterns were evaluated under a light microscope (Olympus, BX53) using a semi-quantitative scoring system based on nuclear/cytoplasmic localization, staining percentage, and intensity.

**Statistical analysis:** All data were analysed using SPSS version 21.0 software (IBM Corporation, Armonk, USA) and are presented as median with interquartile range (25th–75th percentile), mean ± standard deviation (SD), and percentages (%), as appropriate. The normality of continuous variables, including miRNA values and histopathological semiquantitative data, was assessed using the Shapiro-Wilk test. Median miRNA levels were compared using the Wilcoxon signed-rank test. An independent samples t-test was used for normally distributed data, and the Chi-square test was applied for the comparison of categorical variables. Spearman correlation analysis was used to assess associations between miRNA expression changes and continuous clinical variables. The Mann–Whitney U test was used to compare miRNA expression changes according to categorical clinical variables when the assumption of normality was not fulfilled. A *P* value of <0.05 was considered statistically significant.

## Results

The study included 25 patients with atherosclerosis who underwent coronary artery bypass graft surgery using IMA. Most of the patients were male, comprising 80% of the study population. Approximately half of the patients were current or former smokers (44%), and 56% had type 2 diabetes mellitus (**Table I**).

The mean expression level of miRNA-133a-3p was significantly decreased by 0.35-fold, while miRNA-124-3p decreased by 0.25-fold in the patient group compared to control group (**Figure**).

**Table I. Baseline assessment of demographic and clinical parameters in patients with atherosclerosis**

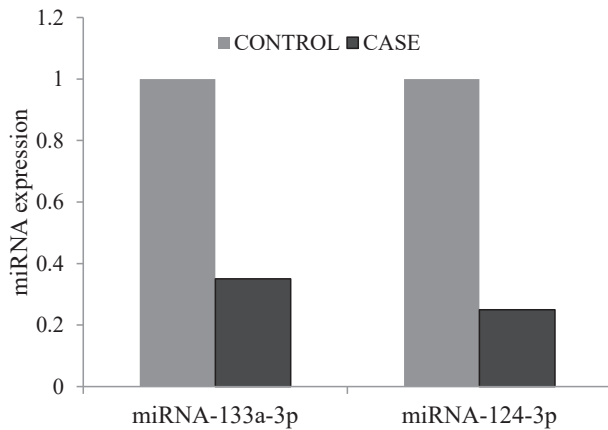
	Category	Frequency (n)	Percentage (%)
Age (yr)	40-50	2	8
	51-60	23	92
Gender	Male	20	80
	Female	5	20
BMI, (kg/m <sup>2</sup> )	<25	6	24
	25-30	11	44
	>30	8	32
Smoking	Yes	11	44
	No	14	56
LDL, (mg/dL)	< 100	15	60
	100-160	8	32
	>160	1	4
HDL, (mg/dL)	< 40	18	72
	40-60	7	28
	>60	-	-
TG, (mg/dL)	< 150	15	60
	150-175	1	4
	> 175	9	36
AIP	<0.1	9	36
	0.1-0.21	2	8
	>0.21	14	56
Fasting blood glucose, (mg/dL)	70-100	4	16
	101-125	2	8
	>125	19	76

AIP, atherogenic index of plasma; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, Triglycerides

The medians of miRNA expression values were compared between the atherosclerosis group and the IMA control group. As shown in **Table II**, the atherosclerosis group showed a significant downregulation of two miRNAs compared to the control group, with a *P* value of 0 for each.

When the relationship between miRNAs and demographic parameters in atherosclerosis was examined, no significant correlation was found between miRNA-133a-3p, miRNA-124-3p, and demographic risk factors (*P* > 0.05, data not shown).

Histopathological examination revealed distinct morphological differences between IMA tissues (control group) and atherosclerotic aortic tissues (case group). In IMA tissues, the Tunica (T.) intima



**Figure.** Fold change in tissue miRNA expression between in two groups.

**Table II.** 2<sup>-ΔΔCT</sup>-based analysis of miRNA-133a-3p and miRNA-124-3p expression levels

	Case	Control
miRNA	[25th-75th]	[25th-75th]
miRNA-133a-3p	[28.635-31.330]	[3.223-17.783]
miRNA-124-3p	[28.295-30.775]	[3.11-10.57]

exhibited a thin structure and was lined with a single layer of uniformly arranged endothelial cells. The T. media consisted of regularly organized smooth muscle cells and intact elastic laminae, indicating preserved structural integrity. The T. adventitia presented a histologically normal appearance, characterised by a loose connective tissue structure, vasa vasorum, and nerve fibres. No significant inflammatory cell infiltration was detected. In atherosclerotic aortic tissue samples, the boundaries of the vessel wall layers were disrupted and prominent pathological changes were observed. The T. intima was markedly thickened, with lipid accumulation, infiltration of foam cells, proliferation of fibrous tissue, and occasional necrotic lipid cores observed in this area. In the T. media, a pronounced loss of structural integrity, irregularities, and segmental fragmentation of the elastic laminae were observed. These alterations are consistent with elastic fibre degeneration and matrix structural changes associated with the atherosclerotic process. In addition, a disorganised architecture characterized by an irregular arrangement of smooth muscle cells was observed. This indicates a transition of smooth muscle cells from a contractile phenotype to a synthetic phenotype and a remodelling process in the vascular structure. The T. adventitia was notable for chronic inflammatory cell infiltration, predominantly lymphocytes and

macrophages, concentrated especially around the vasa vasorum. These findings suggest that the inflammatory process is not limited to the intima but also extends into the deeper layers of the vessel wall (**Supplementary Fig. 1**).

In the immunohistochemical analysis using the S100A4 protein, IMA tissues exhibited limited and physiological levels of immunoreactivity. The distribution of the staining pattern across the different layers of the vessel wall was evaluated as follows: In the intima layer, endothelial integrity was preserved, and the cellular composition remained within normal limits. No significant immunoreactivity for S100A4 protein was detected in this region. S100A4 positivity was observed at a level suggesting low infiltration of dendritic or other immune cells. The media layer was typically composed of smooth muscle cells, which exhibited a mild level of S100A4 immunoreactivity. In the T. media, no inflammatory cells were detected, and the structural integrity was observed to be preserved. S100A4 positivity was observed at a mild to moderate level. In contrast, positive immunoreactivity for the S100A4 protein was detected in the adventitia layer. These findings, however, support the existence of neuronal elements that are physiologically located in the T. adventitia. Moreover, S100A4 positivity appeared in the connective tissue components as well as in the perivascular area.

In the immunohistochemical examination of aortic tissues from regions associated with atherosclerotic lesions (case group), variable levels of immunoreactivity were observed in different vascular layers following staining with the S100A4 protein. S100A4-positive cells were identified within the cellular components accompanying the atheromatous plaque in the intima layer. Marked S100A4 immunoreactivity was observed especially in the group of cells located around the necrotic core and morphologically resembling dendritic cells. This finding suggests that antigen-presenting cells, probably dendritic cells, are present in this region as part of the intimal inflammatory response. Smooth muscle cells in the media layer showed positive immunoreactivity for S100A4. In some cases, due to pathological conditions, there is a transition among VSMCs from a physiological contractile state to a synthetic and proinflammatory phenotype. A large number of S100A4 positive cells were found in the medial layer, and it was thought that these cells may be associated with an inflammatory response. In the adventitia layer, strong S100A4 protein staining was observed, consistent with the presence of nerve

**Table III. Mean±SD and P values of immunoreactivity results of IMA (CONTROL) and Atherosclerotic Aorta (CASE) groups**

Comparison of vascular total immunoreactivity scores between groups					
IMA (control)		AORTA (case)		P value	
Mean±SD	N	Mean±SD	N		
3.072±0.500	24	4.927±0.286	24	< 0.0001*	
Comparison of immunoreactivity of vascular layers between groups					
IMA (control)		AORTA (case)		P value	
Mean±SD	N	Mean±SD	N		
T. Intima	0.436±0.151	24	1.466±0.098	24	< 0.0001*
T. Media	1.018±0.340	24	1.866±0.196	24	< 0.0001*
T. Adventitia	1.581±0.239	24	1.766±0.222	24	=0.0079*

sheath structures. Additionally, S100A4 positivity was detected in cells located in the connective tissue of the adventitia and around small-caliber vessels, which, based on their morphological features, suggest dendritic cells. This finding indicates that the adventitia may also play an active role in inflammatory and immunological processes (**Supplementary Fig. 2 and Table III**).

The correlation analysis results obtained in this study revealed strong associations between S100A4 immunopositivity scores and the expression levels of miRNA-133a-3p and miRNA-124-3p. Specifically, the analyses demonstrated a very strong negative correlation between S100A4 and miR-124-3p ( $r = -0.84$ ,  $P < 0.001$ ), and a strong negative correlation between S100A4 and miR-133a-3p ( $r = -0.77$ ,  $P < 0.001$ ). These findings suggest that the downregulation of miRNA-133a-3p and miRNA-124-3p, along with the upregulation of S100A4, may contribute to the phenotypic switch of VSMCs from a contractile to a synthetic/proinflammatory state (**Supplementary Fig. 3**).

### Discussion

MicroRNA expression profiles involved in atherosclerotic processes such as endothelial dysfunction, plaque progression, instability, and rupture provide valuable insights into the underlying biological mechanisms and hold potential as biomarkers for prognosis, diagnosis, and treatment.<sup>24,25</sup> In this study, we found no association between miRNA-133a-3p, miRNA-124-3p, and demographic risk factors. However, we determined that miR-133a-3p and miR-124-3p were downregulated, while S100A4 protein expression was upregulated in human atherosclerotic

tissues. Therefore, our findings support the hypothesis that miRNAs dysregulation actively contributes to inflammatory remodelling in atherosclerosis.

A study by Carmona-Maurici *et al*<sup>26</sup> reported an association between miR-133a-3p and BMI, while Escate *et al*<sup>27</sup> found a correlation between this miRNA and cholesterol levels. Both findings are in contrast with our results. On the other hand, Andiappan *et al*<sup>9</sup> did not observe any association between miR-133a-3p and triglyceride, HDL, LDL, or glucose levels, which is consistent with our findings. In contrast to our results, de Ronde *et al*<sup>5</sup> reported that high miR-124-3p expression was associated with an increased risk of subclinical atherosclerosis in smokers.

The relationship between miRNA-133a-3p and Coronary Artery Disease (CAD), examined in this study, has also been investigated in previous studies to identify biomarkers for early diagnosis and treatment.<sup>8,9,26,27</sup> While a plasma-based study reported a 28-fold increase in miR-133a levels in CAD patients<sup>9</sup>, Shi *et al*<sup>8</sup> observed decreased miR-133a-3p expression in blood and vascular plaques; consistent with this, our tissue-based study also found reduced miR-133a-3p levels in atherosclerotic samples. We interpreted the downregulation of miR-133a-3p in vascular tissue as a potential indicator of increased atherosclerotic risk, in contrast to plasma studies<sup>26,27</sup> showing the opposite. The roles of miR-124-3p, another miRNA examined in our study, in atherosclerosis have been investigated in previous studies.<sup>14,16,28</sup> Decreased miR-124 levels, consistent with our findings, have been reported in CAD patient serum, atherosclerotic tissues of high-fat diet-fed ApoE<sup>-/-</sup> mice, and RAW264.7 macrophages.<sup>16</sup> miR-124-3p also downregulates *CETP*, *PCK9*, *MTTP*, and *APOB* genes, reducing cell viability and suggesting therapeutic potential.<sup>28</sup> Conversely, it inhibits P4HA1 in vascular smooth muscle cells (VSMCs), decreasing collagen synthesis and plaque stability.<sup>14</sup> Our findings demonstrate that S100A4 expression is markedly increased in atherosclerotic aortic tissue compared to IMA, while both miR-124-3p and miR-133a-3p levels are significantly reduced. This inverse relationship supports the concept of VSMC phenotypic switching, in which the loss of contractile markers (*e.g.*,  $\alpha$ -SMA, MYH11) is paralleled by the upregulation of S100A4, promoting migration, proliferation, and fibrotic remodelling.<sup>29</sup> Importantly, miR-124-3p has been shown to directly target S100A4, thereby limiting VSMC proliferation and neointimal formation<sup>19</sup>, while miR-133a-3p plays a central role in preserving the

### शोध-संदेश

यह अध्ययन एथिरोस्क्लेरोसिस के विकास में माइक्रो-आरनए की भूमिका को समझने पर केंद्रित है। एथिरोस्क्लेरोसिस (atherosclerosis) धमनियों की दीवारों में वसा और सूजनकारी कोशिकाओं के जमाव से उत्पन्न एक दीर्घकालिक रोग है जो हृदयाघात व स्ट्रोक का जोखिम बढ़ाता है। शोध का उद्देश्य एथिरोस्केरोटिक ऊतकों में miRNA-133a-3p और miRNA-124-3p की अभिव्यक्ति तथा S100A4- पॉजिटिव धमनियों की स्मूथ मसल कोशिकाओं के फेनोटाइपिक परिवर्तनों के बीच संबंध का मूल्यांकन करना था। अध्ययन में पाया गया कि एथिरोस्क्लेरोसिस में miRNA-133a-3p और miRNA-124-3p के स्तर में कमी आती है, जबकि S100A4 प्रोटीन की अभिव्यक्ति में वृद्धि होती है, जो सूजनकारी एंडोथीलियल फेनोटाइप के नियमन में इन माइक्रो-आरनए की संभावित भूमिका का संकेत देती हैं। अतः miRNA-133a-3p, miRNA-124-3p और S100A4 प्रोटीन के बीच पारस्परिक सम्बन्ध एथिरोस्क्लेरोसिस की रोकथाम से जुड़े संभावित सूक्ष्म कारणों को समझने में सहायक हो सकते हैं।

contractile phenotype and its downregulation facilitates pathological remodelling.<sup>20</sup> Taken together, these data suggest that the miR-124/133–S100A4 axis represents a critical regulatory pathway in atherosclerosis.

This study offers novel insights into the regulatory axis involving miR-133a-3p and miR-124-3p, and S100A4 protein in the context of atherosclerosis. On the other hand, there were several limitations. First, our study was not designed to directly assess clinical outcomes (such as the degree of atherosclerosis, major cardiovascular events, or survival). Therefore, larger-scale, prospective clinical studies are needed to demonstrate the clinical prognostic value of our findings. Second, the sample size is limited, which particularly limits the power of subgroup analyses. Furthermore, our study focused solely on miR-133a-3p and miR-124-3p and did not include an analysis of gene regulatory pathways involved in the pathogenesis of atherosclerosis. In conclusion, this study demonstrates the downregulation of miR-133a-3p and miR-124-3p expression levels alongside increased S100 protein expression in human atherosclerotic tissues. Our findings support the hypothesis of miRNA-S100 interplay in vascular inflammation. This relationship may offer a novel perspective for the treatment of atherosclerotic cardiovascular diseases and stimulate continued scientific exploration in this field. However, further large-scale studies are required to confirm the potential diagnostic implications.

**Author contributions:** NE: Conceptualization, methodology, validation, formal analysis, investigation, manuscript writing, visualization, project administration; LB: Conceptualization, methodology, validation, formal analysis, investigation, manuscript writing, visualization; AB: Conceptualization, investigation, manuscript writing; NS: supervision, conceptualization, methodology, investigation, manuscript writing, project administration, funding acquisition. All authors read and approved the final printed version of the manuscript.

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