MEDICAL SCIENCE MONITOR

MiR-206 Suppresses the Progression of Coronary Artery Disease by Modulating Vascular Endothelial Growth Factor (VEGF) Expression

Authors' Contribution:
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Data Collection B
Statistical Analysis C
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Background: We investigated whether microRNA-206 (miR-206) is abnormally expressed in patients with coronary artery disease (CAD). The potential mechanism by which miR-206 may regulate CAD progression was also studied.

Material/Methods: A total of 78 CAD patients in the case group and 65 subjects in the control group were enrolled in this study so that the correlation between miR-206 and CAD could be accurately determined. Serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were detected using a biochemistry analyzer. MiR-206 and vascular endothelial growth factor (VEGF) expression levels were tested using either reverse transcription polymerase chain reaction or western blot. Associations between miR-206 expression and different clinicopathological features of CAD patients were also analyzed. CAD cells were transfected with miR-206 mimic (miR-206), its negative control (miR-NC), miR-206 inhibitor (anti-miR-206), and its negative control (anti-miR-NC), respectively. Flow cytometry was conducted to explore the function of miR-206 in CAD cell apoptosis after transfection. Moreover, transwell assay was carried out to study the migratory ability of endothelial progenitor cells (EPCs) in CAD patients.

Results: MiR-206 expression was enriched in both diseased EPCs and plasma of CAD patients. No significant correlation was found between decrease in miR-206 expression and different clinicopathological features. In addition, miR-206 significantly suppressed the viability and invasion of EPCs in CAD patients, and it promoted the apoptosis of their EPCs. Moreover, we found that miR-206 is able to inhibit VEGF expression.

Conclusions: As suggested by our study, MiR-206 can be a novel benign biomarker for CAD because it may regulate VEGF expression.

MeSH Keywords: Apoptosis • Coronary Disease • Endothelial Cells

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Background

Coronary artery disease (CAD) accounts for the largest proportion of cardiovascular disease (CVD), and its risk factors significantly differ by geographical regions, gender, and ethnicity [1]. A major pathogenic contributor to CAD is atherosclerosis, which accounts for more than 80% cases of sudden cardiac death over the world [2]. Atherosclerosis is primarily characterized by endothelial dysfunction, which triggers the atherosclerotic process by activating homing and adhesion of both circulating blood monocytes and macrophages at the endothelial injury site. Therefore, effective restoration of the endothelial cell monolayer is a key to sustain normal endothelial functions [3]. A number of longitudinal studies have reported that endothelial progenitor cells (EPCs), which are acquired from bone marrow, are related to both angiogenesis and vasculogenesis, and they play an indispensable part in microcirculation repair [4]. Furthermore, some research has identified a negative relationship between the number of EPCs and the development of CAD [3,5,6]. As suggested by clinical evidence, a decrease in EPC counts and defects in EPC activities are associated with increased mortality in CVD patients [7]. Therefore, both neovascularization and endothelial integrity enabled us to suspect that EPCs may possess some protective properties for CAD.

Recent investigations have examined the effect of angiogenic cytokines on EPCs and discovered that reduction in vascular endothelial growth factor (VEGF) expression may explain the decrease in EPC number and systemic circulation activity that is very common in CAD patients [2,8]. VEGF is a known inducer of angiogenesis, which improves the proliferation rate of EPCs and stimulates their activities [9]. As suggested by studies *in vitro*, the proliferation, migration, and neovascularization of EPCs can be significantly improved by transferring the VEGF gene, which undoubtedly confirmed the critical role of VFGF in EPCs [9,10].

Recent studies on microRNAs (miRNAs) have suggested the critical regulatory role of miRNAs in EPC biology [10,11]. MiRNAs are single-stranded RNA molecules of approximately 19–30 nucleotides in length, and they suppress the expression of their target genes through binding with mRNAs at specific sequences [12]. More than 2500 miRNAs have been detected in human genome studies [13], which resulted in numerous contributions to the discovery of novel disease biomarkers and the development of therapeutic drug targets [14]. Indeed, several key miRNAs that are able to repress VEGF expression have also been identified through ongoing research [15]. For instance, MiR-206 has been verified to specifically suppress VEGF expression in various types of cancer cells and smooth muscle cells [16] because it directly interacts with the presumed miR-206 binding site at the 3'-UTR. However, it is still challenging to provide solid evidence for the intrinsic relationship between VEGF and miR-206 in EPCs. Apart from that challenge, whether CAD pathogenesis is triggered by the decrease in VEGF levels that may be caused by miR-206 remains undetermined. As a result, we first hypothesized that decreased VEGF levels contribute to the abundance of anti-VEGF miRNAs in EPCs obtained from CAD patients, and this hypothesis was verified in our study, which explored the relationship between VEGF and miR-206 in EPCs.

We began our research by confirming the potential role of miR-206 as a significant biomarker in CAD patients. Then, we explored the mechanism of miR-206 involvement in angiogenesis and vasculogenesis. These findings may provide a profound understanding of CAD and illuminate the feasibility of miR-206 as a benign diagnostic biomarker for diagnosing CAD.

Material and Methods

Patient selection

Peripheral blood samples were collected from both CAD patients (n=78; 19 females and 59 males; age 47–83 years) and healthy volunteers (n=65; 22 females and 43 males; age 48–77 years) at the Affiliated Hospital of Qingdao University between June 2012 and June 2014. Patients and healthy volunteers were matched by age, sex, body mass index (BMI), and history of hypertension, hyperlipidemia, diabetes, or smoking status (Table 1). As suggested by the diagnostic standards of CAD [17], patients were further divided into three groups: stable angina (SA), unstable angina (UA), and acute myocardial infarction (AMI). Patients with other severe diseases or history of thrombolytic therapy were excluded from our study. Hypertension was defined when systolic blood pressure was >140 mm Hg, diastolic blood pressure was >90 mm Hg, or when patients had received a diagnosis together with antihypertensive agent therapy. Diabetes mellitus was defined when fasting blood glucose was >7.0 mmol/L and diet adjustment or antidiabetic drug treatment was presented. Smoking was predefined as consumption of more than 10 cigarettes every day. This study was approved by the Ethics Committee of Affiliated Hospital of Qingdao University, and all participants signed the written informed consent. Peripheral blood samples were collected from all participants. Isolated plasma samples were transferred to RNase-free tubes and stored at –80°C until they were processed with future experiments.

Biochemical analysis

Serum levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TGs) were measured using a Biochemical

Table 1. Clinicopathological features and biochemical indexes of study participants.

Characteristic	Control (n=65)	CHD (n=78)	P
Age (years)	63.4±4.6	65.1±5.8	0.058
Sex			0.212
Male	43	59	
Female	22	19	
BMI	24.71±2.84	24.87±3.25	0.757
Smoking			0.974
Yes	21	25	
No	44	53	
Diabetes			0.855
Yes	15	17	
No	50	61	
Hypertension			0.923
Yes	23	27	
No	42	51	
Hyperlipidemia			0.937
Yes	12	14	
No	53	64	
TC (mM)	4.22±0.09	4.26±0.18	0.105
TG (mM)	1.56±0.12	1.96±0.16	**<0.001**
LDL-c (mM)	2.52±0.21	2.83±0.27	**<0.001**
HDL-c (mM)	1.04±0.21	0.92±0.14	**<0.001**
Subgroups			
SA		21	
UA		34	
AMI		23	

BMI – body mass index; TC – total cholesterol; TG – triglyceride; LDL-c – low density lipoprotein-cholesterol; HDL-c – high density lipoprotein-cholesterol; SA – stable angina; UA – unstable angina; AMI – acute myocardial infarction.

Analyzer (Beckman Coulter, USA). Glucose levels were detected using a glucose oxidase kit (Oxford, UK).

Isolation and cultivation of EPCs

Mononuclear cells were separated from peripheral blood by using Ficoll density gradient centrifugation and then were cultured at 37°C in endothelial basal medium with 20% fetal bovine serum in an incubator with 5% CO_2. We discarded nonadherent cells in four days, and adherent cells were further incubated with fresh medium for subsequent experiments, as in a previous study conducted by Tang et al. [18].

Cell transfection

The oligonucleotides of miR-206 mimic (miR-206), negative control (miR-NC), miR-206 inhibitor (anti-miR-206), and its negative control (anti-miR-NC) were synthesized by Genepharma Co., Ltd. (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer description. All cells were subgrouped into five groups: blank group (cell transfected with empty vector), miR-206 group (cell transfected with miR-206 mimics), miR-NC group (cell transfected with negative control of miR-206 mimics), anti-miR-206 group (cell transfected with miR-206 inhibitor), and anti-miR-NC group (cell transfected with negative control of miR-206 inhibitor). Transfection of EPCs was carried

out under highly stable conditions over a period of 2 weeks, and a follow-up experiment was also conducted.

Reverse transcription polymerase chain reaction analysis

Total RNA was extracted from EPCs using TRIzol (Invitrogen) according to the manufacturer instructions, and then cDNA was generated from RNA using SuperScript III (Invitrogen). MiR-206 and VEGF expressions were evaluated using the SYBR green quantitative PCR kit (Takara, China) based on the manufacturer description. Reverse transcription polymerase chain reaction (RT-PCR) was carried out under the conditions that were described by Cai et al. [19]. β-actin and RNU6B were used as an internal control for mRNAs and miRNAs, respectively.

Western blot analysis

Cellular extracts were lysed using the lysis buffer RIPA, which was purchased from KeyGen Biotech Co. Ltd (Nanjing, China), and supernatant was collected after centrifugation. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were blotted onto polyvinylidene difluoride membranes (Bio-Rad, USA). Then, membranes with isolated proteins were blocked for 1 h and detected using primary antibodies including anti-VEGF (1:500) and anti-β-actin (1:100) (Santa Cruz, USA) at 4°C overnight. After that, membranes were washed thrice by Tris-buffered saline with Tween 20 (TBST), and horseradish peroxidase-conjugated secondary antibodies (1:900) were added into the membranes, which were incubated for another 1 h. Finally, membranes were washed thrice again using TBST. Immunobinding signals were tested by the chemiluminescence reagent, which was purchased from KeyGen Biotech Co. Ltd. Relative protein expression was identified through densitometry analysis using the Image-Pro Plus Version 6.0 software and calculated based on the β-actin loading control.

Cell apoptosis analysis

Cell apoptosis pattern was evaluated using the Annexin V-FITC Apoptosis Detection Kit. EPCs were seeded in 24-well plates and were incubated overnight. Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and finally centrifuged. The cell pellet was resuspended in the binding buffer and incubated with Annexin V-FITC. After that, cells were centrifuged again, washed with PBS, and resuspended in the binding buffer containing propidium iodide solution in the dark. Finally, cells were analyzed by using the approach of flow cytometry (Beckman FC400 MPL, USA). Each experiment was independently carried out in triplicate.

Cell migration assay

The migratory ability of EPCs was assessed using the transwell cell migration assay, referred to in a previous study performed by Wang et al. [20]. Briefly, EPCs were plated in a 24-well plate (5×10^6 cells/well) for one-day incubation. Then, EPCs in serum-free medium were placed in the upper chamber, whereas the lower chamber was filled with medium containing serum. Next, EPCs that did not migrate were removed, and migrated EPCs were dried, fixed, and stained. The transmembrane EPCs were calculated using a microscopy, and each experiment was performed in triplicate.

Statistical analysis

SPSS 22.0 was used for statistical analysis. The t-test or one-way analysis of variance was performed to assess differences in continuous variables among groups, whereas the correlation analysis was based on the chi-square test. All continuous data are expressed as mean ± standard deviation, and a *P*-value of less than 0.05 was considered as statistically significant.

Results

Clinical characteristics and biochemical indexes of study participants

For lipid parameters, the case group exhibited higher levels of TGs and LDL-C, and a lower level of HDL-C compared with the control group (all *P*<0.001; Table 1), whereas there was no significant difference in TC between the two groups (*P*=0.105; Table 1). Besides that, no significant difference in age, BMI, sex, diabetes, hypertension, hyperlipidemia, or smoking status was observed between the two groups (all *P*>0.05; Table 1).

MiR-206 levels in study participants and EPCs

Our data revealed that two of the three CAD subgroups (UA, AMI) had substantially up-expressed miR-206 compared with the control group (all *P*<0.05; Figure 1A), whereas subgroup SA exhibited an insignificant elevation. Among the three CAD subgroups, AMI patients displayed the uppermost level of miR-206; however, no statistical differences in miR-206 levels were found among the three subgroups (Figure 1A). In addition, the level of miR-206 in EPCs was measured using RT-PCR analysis. Our results showed that miR-206 was highly expressed in EPCs obtained from CAD patients compared with those in the control group (*P*<0.05; Figure 1B). Moreover, CAD patient EPCs transfected with miR-206 were associated with a remarkable increase in miR-206 expression, whereas CAD patient EPCs transfected with anti-miR-206 exhibited a significant decline in miR-206 expression (both *P*<0.05; Figure 1B). The above
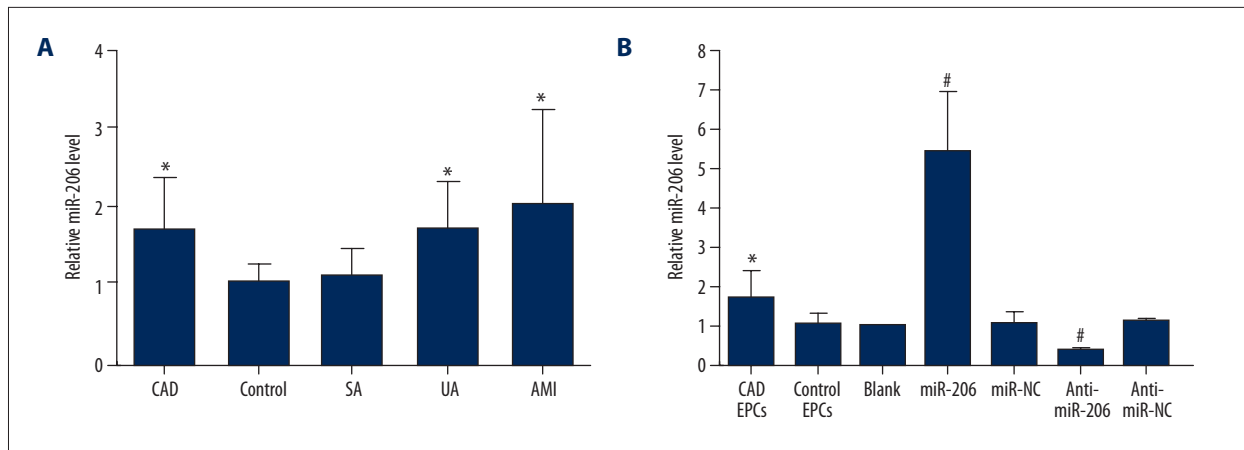
Figure 1. Relative miR-206 level in plasma specimens. EPCs of CAD patients and healthy subjects were analyzed by reverse transcription polymerase chain reaction. (**A**) Plasma specimens; * $P<0.05$ vs. control group. (**B**) EPCs of CAD patients. AMI – acute myocardial infarction; CAD – coronary artery disease; EPCs – endothelial progenitor cells; SA – stable angina; UA – unstable angina. * $P<0.05$ vs. control EPCs. # $P<0.05$ vs. blank group.

evidence suggested that miR-206 may contain some protective roles in CAD.

Association between miR-206 expression and clinicopathological features of CAD patients

The median of miR-206 expression (1.77) was selected as the threshold that was used to divide CAD patients into two groups: a high-expression group in which miR-206 expressions were over 1.77 and a low-expression group in which miR-206 expressions were less or equal to 1.77. As suggested by Table 2, no significant correlation was identified between miR-206 expression and clinicopathological features of CAD patients.

VEGF levels in study participants and EPCs

As shown in Figure 2A, the relative levels of plasma VEGF in the three CAD subgroups were significantly different from those in the control group (all $P<0.05$; Figure 2A). In addition, VEGF was markedly down-expressed in CAD patient EPCs compared with those in normal EPCs ($P<0.05$; Figure 2B). Furthermore, CAD patient EPCs transfected with miR-206 exhibited a prominent decrease in VEGF, while CAD patient EPCs transfected with anti-miR-206 showed a remarkable decrease in VEGF level compared with the blank group (both $P<0.05$; Figure 2B).

MiR-206 inhibited cell viability of CAD patient EPCs

The viability of EPCs in CAD patients was assessed using a MTT assay. Our findings suggested that the optical density at 570 nm (OD_{570}) value of EPCs in CAD patients was markedly decreased once miR-206 transfection had been carried out for 72 and 96 hours ($P<0.05$; Figure 3), while the anti-miR-206 transfected group exhibited an increase in the OD_{570} value after 72-hour

and 96-hour transfection ($P<0.05$; Figure 3). By contrast, there were no significant changes in the survival rate during the culture process when diseased EPCs were transfected with miR-NC or anti-miR-NC ($P>0.05$; Figure 3).

MiR-206 reduced cell migration of CAD patient EPCs

As suggested by the transwell assay, the number of invaded cells per field was significantly decreased when diseased EPCs were transfected with miR-206 compared with the miR-NC group (85 ± 6 vs. 180 ± 13; $P<0.05$). On the other hand, the number of migrated cells in the anti-miR-206 group was substantially increased compared with that in the anti-miR-NC group (402 ± 26 vs. 179 ± 13; $P<0.05$) (Figure 4A, 4B). Moreover, diseased EPCs in the miR-NC group or anti-miR-NC group exhibited similar migration patterns without significant difference (Figure 4A, 4B). Hence, our results illustrated that miR-206 suppressed the invasion of CAD EPCs.

MiR-206 promoted the apoptosis of CAD patient EPCs

As suggested by the flow cytometric analysis, the percentage of apoptotic diseased EPCs in the miR-206 group was prominently higher than that in the blank group, whereas the anti-miR-206 group exhibited a remarkable decrease in this figure (both $P<0.05$; Figure 5A, 5B), indicating that miR-206 stimulated the apoptosis of CAD EPCs.

MiR-206 modulated VEGF expression in CAD patient EPCs

Western blot testing was conducted to unveil the relationship between miR-206 and VEGF in EPCs obtained from CAD patients. Our results revealed that reduced VEGF expression was identified in diseased EPCs that were transfected with miR-206

Table 2. Association between microRNA-206 expressions and different clinicopathological features of CAD patients.

| Characteristic | MicroRNA-206 expression | | P |
	High	Low	
Age (years)			0.648
≤65	23	21	
>65	16	18	
Sex			0.792
Male	30	29	
Female	9	10	
BMI			0.255
≤25	24	19	
>25	15	20	
Smoking			0.808
Yes	13	12	
No	26	27	
Diabetes			0.784
Yes	8	9	
No	31	30	
Hypertension			0.475
Yes	12	15	
No	27	24	
Hyperlipidemia			0.555
Yes	8	6	
No	31	33	
Subgroups			0.22
SA	9	12	
UA	15	19	
AMI	15	8	

BMI – body mass index; SA – stable angina; UA – unstable angina; AMI – acute myocardial infarction; CAD – coronary atherosclerotic heart disease.

(P<0.05; Figure 6); hence, a negative correlation between miR-206 level and VEGF in EPCs of CAD patients was suggested.

Discussion

CAD contributes to a large number of cardiovascular deaths, which are usually caused by the formation and accumulation of atheromatous plaque resulting from lipoprotein accumulation, endothelial damage, and inflammation [21]. Endothelium damage starts the mobilization of circulating EPCs derived from the bone marrow; these EPCs target the damage site, differentiate into mature endothelial cells, integrate into the endothelium, and replace apoptotic or injured cells [22,23]. Therefore, circulating EPCs that contribute to endothelial functions play an indispensable role in endogenous refurbishment and CVD pathogenesis. Our study reported that miR-206 promoted CAD progression by regulating VEGF expression. Apart from that, we discovered a high expression level of miR-206 and a low expression level of VEGF in both plasma and EPCs of CAD patients. More importantly, we concluded that miR-206 could inhibit cell viability, reduce cell invasion, promote the apoptosis of EPCs, and modulate VEGF expression in EPCs obtained from CAD patients.
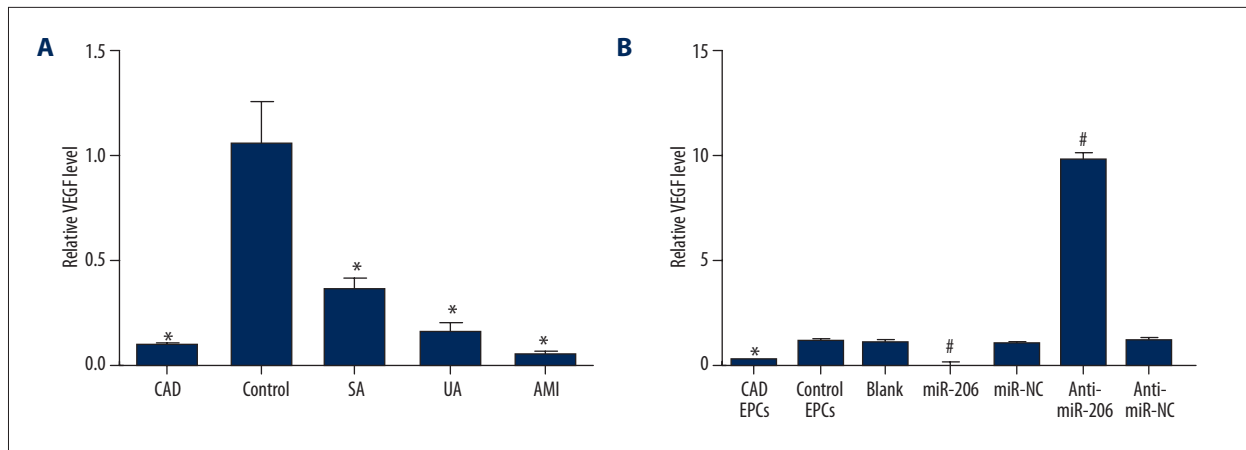
Figure 2. Relative VEGF level in plasma specimens. EPCs of CAD patients and healthy subjects were analyzed by reverse transcription polymerase chain reaction. (**A**) Plasma specimens; * *P*<0.05 *vs.* control group. (**B**) EPCs of CAD patients. AMI – acute myocardial infarction; CAD – coronary artery disease; EPCs – endothelial progenitor cells; SA – stable angina; UA – unstable angina. * *P*<0.05 *vs.* control EPCs. # *P*<0.05 *vs.* blank group.
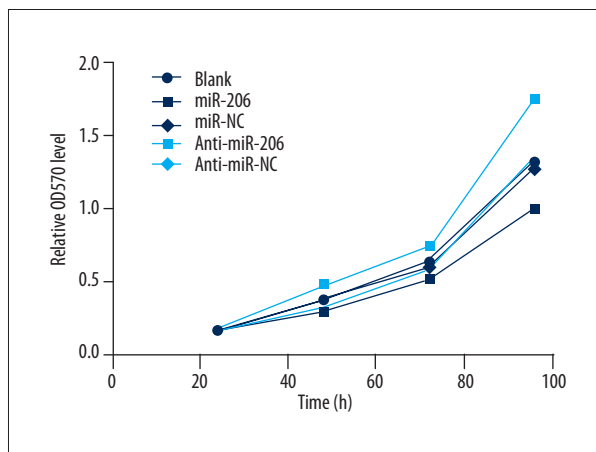

Figure 3. MTT assay: MiR-206 inhibited the proliferation of CAD patient EPCs. Cell growth curve of EPCs at 24 h, 48 h, 72 h, or 96 h of transfection was plotted.
CAD – coronary artery disease; EPCs – endothelial progenitor cells.

MiRNAs are highly conserved, endogenous, small noncoding RNAs of 19–30 nucleotides in length, and they regulate gene expressions via targeting specific miRNAs [12,24]. So far, increasing evidence has suggested that miRNAs play an important role in modulating various biological and pathological processes [25,26]. Several miR subtypes are involved in vascular integrity, angiogenesis, endothelial repair capacity, increased chronic inflammation, and atherosclerosis-associated vascular remodeling [27]. Zhang et al. provided solid evidence for the dysfunction of several miRNAs, such as miR-126, miR-130a, miR-221, miR-222, and miR-92a, in EPCs that are derived from CAD patients [28].

MiR-206, which is located on chromosome 6 in a bicistronic cluster that belongs to a skeletal muscle-specific myomiR,

is specifically expressed in muscles and it is believed to be an important regulator for myogenic differentiation [29,30]. As suggested by growing evidence, miR-206 has been linked with many human cancers including breast cancer, colon cancer, endometrial and endometrioid carcinoma, gastric cancer, lung cancer, glioma, laryngeal cancer, and rhabdomyosarcoma [31–34]. Besides that, miR-206 may have an indispensable role in cardiovascular diseases. Shan et al. conducted a pioneer study linking miR-206 with heart diseases, and they constructed a mouse model with myocardial infarction simulation that demonstrated that miR-206 levels were up-regulated in introcurved tissues [35]. Limana et al. also observed increasing miR-206 expression in a rat model in which heart failure was simulated, and a significant rise in miR-206 expression was seen in rats that were treated with HMGB1 [36]. On top of that, our study provided parallel evidence for comparing miRNAs contained in both plasma and EPCs isolated from CAD patients with those from healthy volunteers. We discovered that miR-206 expressions were significantly increased in both plasma and EPCs of CAD patients. This trend was confirmed by a study carried out by Tang et al., who indicated a role of miR-206 along with its target PIK3C2a in EPC function and potential pathogenesis of CAD [18].

VEGF is a critical factor for regulating the formation of atherosclerosis that is able to enhance atherosclerosis in rat models of cardiac allografts [37]. As suggested by Moulton et al., VEGF is a momentous regulator that may account for inflammation and revascularization in atheromatous plaques [38]. Hypoxia-inducible factor 1-alpha (HIF-1α) inhibits infarct size via stimulating both vascular remodeling and angiogenesis in human heart ischemia, which is related to the pathway of increasing steady-state levels of VEGF mRNA [39]. Moreover, Lee et al. suggested that increasing angiopoietin-2 (Ang-2), Tie-2,
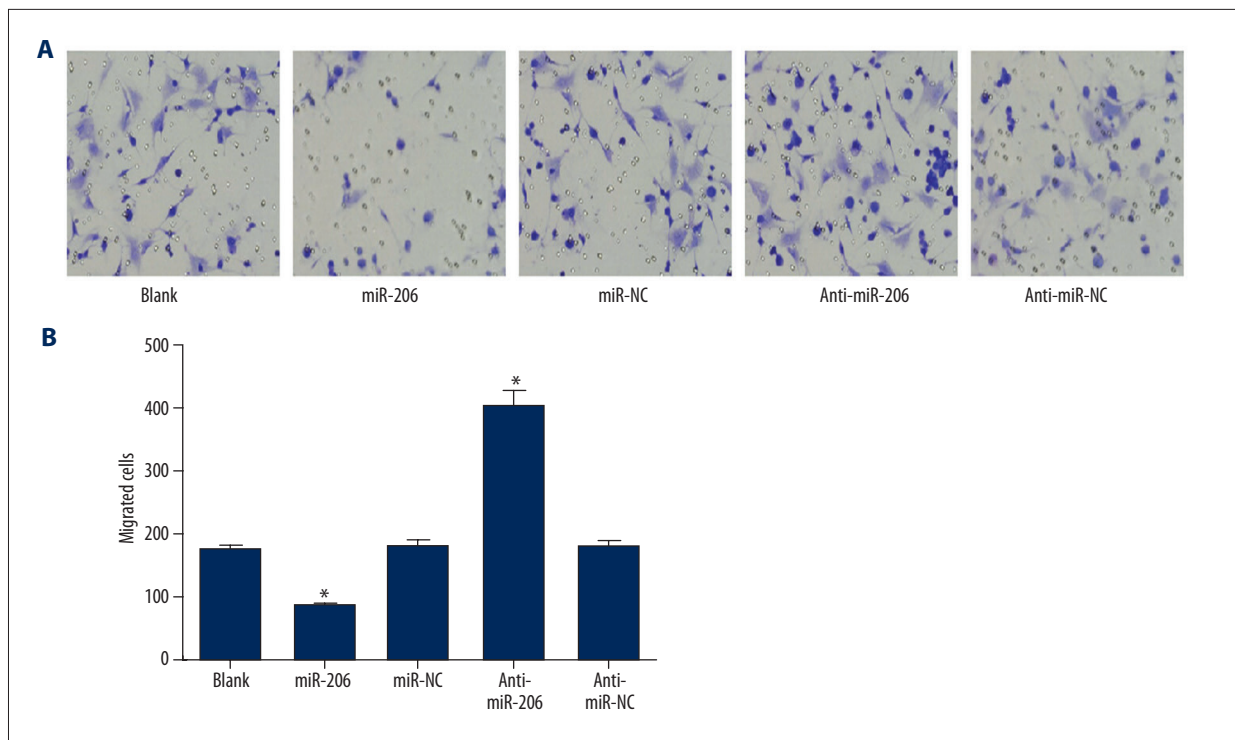
Figure 4. Transwell assay: MiR-206 inhibited the invasion of CAD patient EPCs. (**A**) Representative photographs of invaded cells after transfection using the transwell experiment among the five different groups. (**B**) The invaded cells of each field among the five different groups after transfection were assessed by transwell chamber experiment. CAD – coronary artery disease; EPCs – endothelial progenitor cells.* *P*<0.05 *vs.* blank group.
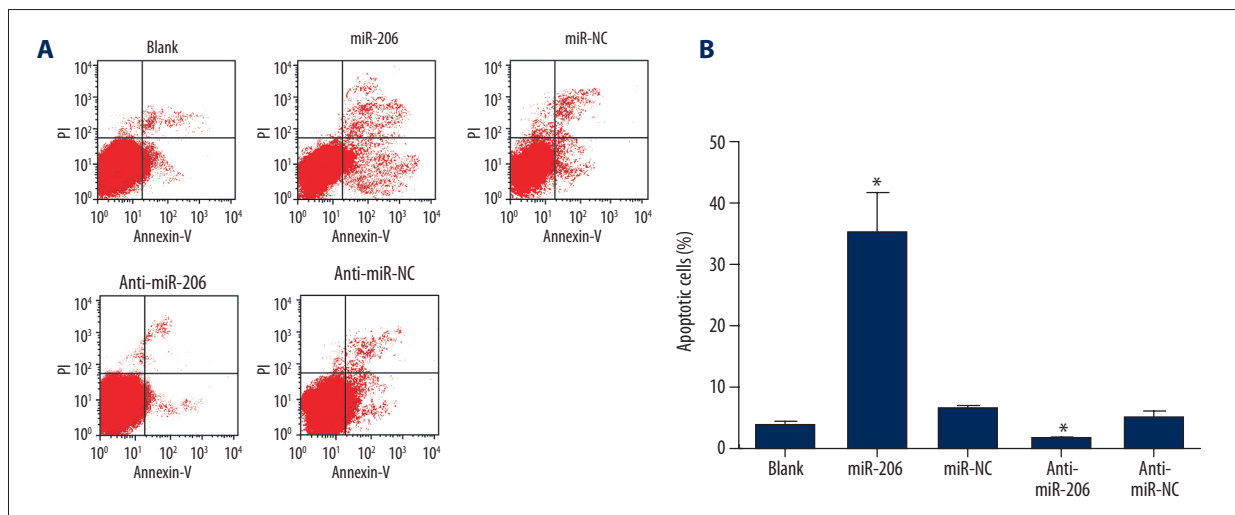

Figure 5. Flow cytometry: MiR-206 promoted the apoptosis of CAD patient EPCs. (**A**) Representative photographs of apoptotic cells after transfection among the five different groups. (**B**) The apoptotic cells among the five different groups after transfection were assessed using flow cytometry. CAD – coronary artery disease; EPCs – endothelial progenitor cells. * *P*<0.05 *vs.* blank group.

and VEGF levels were discovered in patients with acute coronary syndrome [40]. Importantly, the increased VEGF in plasma of CAD patients may be considered as a significant indicator for revascularization [41]. In addition, VEGF was able to

ameliorate myocardial perfusion, angiographic collaterals, myocardial diastole, and contractile function in early nonrandomized and uncontrolled trials that used replacement therapy together with recombinant human VEGF (rhVEGF) or VEGF gene
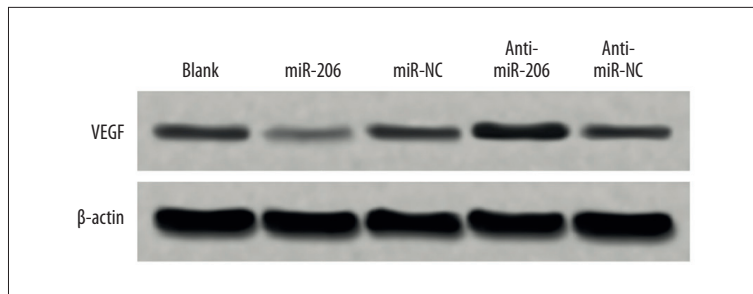
Figure 6. Western blot analyses: MiR-206 suppressed vascular endothelial growth factor expression in EPCs isolated from CAD patients. CAD, coronary artery disease; EPCs, endothelial progenitor cells.

therapy [42,43]. Administration of rhVEGF could impede the development of atherosclerotic plaque in mice with deficient apolipoprotein E as well as in rabbits that were fed with cholesterol [44]. As suggested by double-blind and placebo-controlled trials, replacement therapy with rhVEGF is a potentially effective strategy for refractory CAD in human beings [45]. However, the effectiveness of VEGF gene therapy was not confirmed by some randomized and place-controlled trials, and this discrepancy may be explained by the fact that different levels of VEGF in angiogenesis are likely to be influenced by VEGF polymorphisms.

Nevertheless, our study contained some limitations that should be addressed in future studies. First, it is still unclear what factors contribute to the down-expression of VEGF, and the correlation between VEGF polymorphisms and CAD pathogenesis should be profoundly studied. Second, we had a relatively small sample size, which restricted the statistical power of our study. We were unable to determine the optimal sample size for our study because estimating the true effect size of our parameters is extremely challenging. As a result of this, a

study with the optimal sample size should be carried out to confirm the results from our study.

Conclusions

In summary, miR-206 not only inhibited cell proliferation and invasion of EPCs obtained from CAD patients but also stimulated the apoptosis of their EPCs. Apart from that, miR-206 could improve the pathological state of CAD patients, which is possibly linked with VEGF pathway regulation. Our study provided solid evidence that it is feasible to consider miR-206 as a biomarker for CAD, and this may further assist researchers in developing alternative therapeutic and preventive interventions for tackling CAD.

Acknowledgement

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