The potential inhibitory effects of miR-19b on vulnerable plaque formation via the suppression of STAT3 transcriptional activity

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Abstract. Atherosclerotic plaque growth requires angiogenesis, and acute coronary syndrome (ACS) is usually triggered by the rupture of unstable atherosclerotic plaques. Previous studies have identified typically circulating microRNA (miRNA/miR) profiles in patients with ACS. miRNAs serve important roles in the pathophysiology of atherosclerotic plaque progression. The present study aimed to investigate the potential role and mechanism of miR-19b in plaque stability. miRNA array data indicated that 28 miRNAs were differentially expressed in the plasma of patients with unstable angina (UA; n=12) compared with in control individuals (n=12), and miR-19b exhibited the most marked upregulation. Circulating miR-19b levels were further validated in another independent cohort, which consisted of 34 patients with UA and 24 controls, by quantitative polymerase chain reaction. Gene Ontology annotations of the predicted target genes of miR-19b suggested that miR-19b may be involved in endothelial cell (EC) proliferation, migration and angiogenesis, which was confirmed by Cell Counting kit-8, wound healing and tube formation assays in the present study. Finally, the present study indicated that miR-19b may suppress signal transducer and activator of transcription 3 (STAT3) tyrosine phosphorylation and transcriptional activity in ECs, as determined by western blot analysis and luciferase reporter assay. In conclusion, the present study revealed that increased miR-19b expression may delay unstable plaque progression in patients with UA by inhibiting EC proliferation, migration and angiogenesis via the suppression of STAT3 transcriptional activity.

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Introduction

Coronary artery disease (CAD) and its major complication, acute coronary syndrome (ACS), remain the most common causes of morbidity and mortality worldwide (1). ACS comprises two main entities: Unstable angina (UA) and acute myocardial infarction (AMI), which is usually triggered by the rupture of unstable atherosclerotic plaques (2). Vulnerable plaques, also known as unstable plaques, are typically characterized by a thin fibrous cap, large lipid core (>40% of the plaque), inflammatory infiltration, angiogenesis and intraplaque hemorrhage (3). The risk factors of CAD include hypertension, diabetes mellitus, elevated low-density lipoprotein cholesterol levels and cigarette smoke, all of which may lead to endothelial injury. Impaired vascular endothelium has an essential role in the initiation of atherosclerosis and in ultimate formation of unstable plaques (4). Dysfunctional endothelial cells (ECs) exhibit increased proliferation and migration, eventually resulting in re-endothelization and angiogenesis of the atherosclerotic plaque (5); the latter is a major step in promoting plaque destabilization, rupture and thrombus formation (6).

MicroRNAs (miRNAs/miRs) are small non-coding RNA molecules that negatively regulate gene expression, predominantly at the post-transcriptional level. Altered miRNA expression has been identified in the circulation of patients with ACS (7). Furthermore, it is now recognized that miRNAs are involved in almost all steps of atherogenesis, including endothelial damage and dysfunction, monocyte invasion and activation, lipoprotein formation and deposition, and vascular smooth muscle cell and platelet dysfunction, where they exert either beneficial or harmful effects (8). miR-126 is the most abundant miRNA in ECs, which may prompt EC repair by inhibiting inflammation (9). In addition, miR-181b and miR-146a are able to alleviate EC inflammation by regulating the nuclear factor-κB signaling pathway (10-12). Our previous study revealed that miR-19b may function as an antithrombotic protective miRNA in UA by targeting tissue factor in ECs (13). However, it remains unclear as to whether there are other protective roles for miR-19b in UA. It has previously

been suggested that miR-19b may be closely associated with atherosclerosis. miR-19b is downregulated in the aortic walls of apolipoprotein E (apoE) knockout mice, and suppressor of cytokine signaling 3 is a potential target of this miRNA (14). Signal transducer and activator of transcription 3 (STAT3) is an important nuclear transcription factor. The promoters of numerous proliferation, migration and angiogenesis-associated genes, including vascular endothelial growth factor, placental growth factor and SRY-box 18, contain STAT3 consensus sequences (15-17). Therefore, STAT3 activation may induce the expression of these genes and regulate cell proliferation, migration and angiogenesis.

The aim of the study was to evaluate the potential role and mechanism of miR-19b in plaque stability. The present study demonstrated that miR-19b was markedly upregulated in the plasma of patients with UA, and was predicted to be involved in the regulation of cell proliferation, migration and angiogenesis by bioinformatics analysis. Finally, the present study indicated that miR-19b may inhibit EC proliferation, migration and tube formation *in vitro* by suppressing STAT3 transcriptional activity.

Materials and methods

Study population. The present study was performed in accordance with the Helsinki declaration and was approved by the ethics review board of Peking University People's Hospital (Beijing, China). All individuals recruited to the present study provided written informed consent. Patients who were suspected of CAD with negative angiography were enrolled in the control group (n=36). Patients with typical UA that were angiographically documented as having CAD were enrolled in the UA group (n=46). All individuals, recruited to the present study between April 2012 to November 2013, provided written informed consent. The criteria for the diagnosis of UA were based on the American College of Cardiology Foundation/American Heart Association 2010 guidelines for the management of patients with UA/non-ST-segment-elevation MI (18). Patients presenting elevated troponin I (≥0.04 ng/ml) and/or creatine kinase-MB (≥5 ng/ml) levels, myocarditis, cardiogenic shock, a history of severe hepatic or renal dysfunction, leukemia, leukopenia and ongoing inflammatory malignant disease were excluded from the present study.

Blood collection and RNA extraction. Blood was collected from patients via arterial puncture into tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ, USA) prior to coronary angiography, and was processed for isolation of plasma within 4 h. Blood was centrifuged at 1,300 x g for 10 min at 4°C. miRNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) was used for RNA extraction according to the manufacturer's protocol.

miRNA TaqMan low density array (TLDA). TLDA was used to detect differentially expressed miRNAs in the plasma of patients with UA (n=12) and controls (n=12). Total RNA (~15 ng) was reverse transcribed using the TaqMan miRNA reverse transcription (RT) kit and TaqMan miRNA Multiplex RT assays (human pool) (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RT products were preamplified with TaqMan PreAmp kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), after which preamplification reaction products

were amplified using Human MicroRNA TLDA card A+B version 3.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.), which could detect 754 miRNAs simultaneously.

All steps were performed using a 7900HT Fast real-time polymerase chain reaction (PCR) system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Results were expressed as Cq. Raw data were analyzed using Data Assist software for TaqMan gene expression assays version 3.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.). miRNA expression levels were normalized to RNU6B. Significance analysis of microarrays (SAM) was used to identify differentially expressed miRNAs between the two groups. The miRNAs that exhibited ≥2-fold change, q-value <0.0001% and false discovery rate (FDR) <0.05 were considered differentially expressed.

RT-quantitative (q)PCR. TaqMan miRNA RT kit (cat. no. 4366596), miRNA-specific stem-loop primers (miR-19b, cat. no. 000396; cel-miR-39, cat. no. 000200; RNU6B, cat. no. 001093) and Universal PCR Master Mix (cat. no. 4324018) (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for miRNAs quantification according to the manufacturer's protocol. Real-time PCR reactions were performed on an Applied Biosystem ViiA™ 7 Real-Time PCR system with the following program: 10 min pre-incubation at 95°C, 40 cycles of 15 sec denaturation at 95°C and 60 sec of elongation at 60°C. Values were expressed as $2^{-\Delta\Delta Cq}$ (19). miR-19b levels in the plasma were normalized to a spiked-in control, synthetic *Caenorhabditis elegans* miR-39 (10 fmol/sample; Qiagen, Inc.).

Cell culture and transfection. Cell culture and transfection were performed as previously described (11). Briefly, EA.hy926 cells (fusion cell line derived from human umbilical vein ECs and lung carcinoma cells), obtained from Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai China), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection at a final concentration of 3 μg/ml according to the manufacturer's protocol. miR-19b mimic (cat. no. MC10629) or negative control (NC) mimic (cat. no. 4464058) (Applied Biosystems; Thermo Fisher Scientific, Inc.) were transfected into EA.hy926 cells (70-80% confluence) at a final concentration of 30 pmol/ml for 24 or 48 h.

Western blot analysis. Western blot analysis was performed as previously described (20). The following antibodies were used in the present study: Anti-STAT3 (cat. no. 9132S; 1:1,000 diluted) and anti-phosphorylated-STAT3 (Tyr 705) (cat. no. 9131S; 1:1,000 diluted; both from Cell Signaling Technology, Inc., Beverly, MA, USA). GAPDH (cat. no. SC-32233; 1:3,000 diluted; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. HRP-conjugated goat-anti-rabbit (cat. no. sc-2004)/mouse (cat. no. sc-2005) IgG secondary antibodies (both from Santa Cruz Biotechnology).

Luciferase reporter assay. EA.hy926 cells plated in a 24-well plate at 70-80% confluence were cotransfected with NC or miR-19b mimic (final concentration, 60 pmol/ml) and

Table I. Clinical characteristics of study populations.

Characteristic	Profiles		Validation	
	Controls (n=12)	UA (n=12)	Controls (n=24)	UA (n=34)
General data				
Age (years)	56±9	61±8	61±12	63±9
Gender (males/females)	4/8	6/6	11/13	14/20
BMI (kg/m^2)	26±4	25±3	26±3	25±3
SBP (mmHg)	126±12	131±12	128±13	131±18
DBP (mmHg)	76±6	79±11	79±11	78±10
HR (bpm)	68±6	71±10	70±8	69±9
LVEF (%)	70±6	68±7	68±10	69±6
Medical history				
Hypertension (%)	50	83	58	53
Diabetes (%)	17	33	21	24
Hyperlipidemia (%)	50	58	42	35
Laboratory test				
WBC (10^9/l)	6.00±1.17	6.10±1.65	6.68±1.67	5.93±1.66
LDL-C (mmol/l)	2.11±0.42	2.24±0.51	2.52±0.58	2.19±0.56
HDL-C (mmol/l)	1.02±0.23	0.99±0.30	1.12±0.32	0.96±0.27
TC (mmol/l)	3.71±0.49	3.94±0.63	4.54±0.73	3.73±0.69
Creatinine (μmol/l)	54.63±12.24	70.50±17.13	67.43±13.61	67.91±15.46
Medication (%)				
Aspirin	42	58	63	74
Clopidogrel	25	50	46	50
Statin	58	58	67	68
Calcium antagonist	25	42	33	27
ACEI	17	25	13	15
ARB	17	28	13	27
β-Blocker	47	75	67	47

Data are presented as the mean ± standard deviation. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; SBP, systolic blood pressure; TC, total cholesterol; UA, unstable angina; WBC, white blood cells.

STAT3-driven promoter (2xAPRE) firefly luciferase reporter plasmid (final concentration, 300 ng/ml), as well as the internal control *Renilla* luciferase reporter plasmid (final concentration, 10 ng/ml; phRL-TK; Promega Corporation, Madison, WI, USA) using Lipofectamine 2000 (final concentration, 4 μg/ml). After 24 h, cell extracts were prepared and assessed according to the manufacturer's protocol (Dual Luciferase assay system; Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity in the same well. To construct the STAT3-driven promoter, the 2xAPRE sequence was cloned into the multiple cloning site of pGL3-TATA plasmid, which encodes the firefly luciferase gene containing an upstream TATA element (21).

Cell proliferation assay. Cell proliferation activity was measured using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). EA.hy926 cells in 96-well plates were transfected with miR-19b or NC mimic for 24 or 48 h. Subsequently, 20 μl CCK-8 solution was added to each well with 200 μl culture medium and the plates were incubated for 1 h at 37˚C. The absorbance was measured at 450 nm using a microplate reader.

Wound healing migration assay. EA.hy926 cells in 24-well plates were transfected with miR-19b or NC mimic for 24 h, and were then serum-starved in DMEM containing 1% FBS overnight. Subsequently, the cell monolayer was scratched with a sterile 10-μl pipette tip. The cells were gently washed with DMEM to remove detached cells and were incubated with DMEM containing 1% FBS for 24 h at 37˚C. Images of four injured fields in each well were captured (Olympus IX70; Olympus Corporation, Tokyo, Japan). Cell migration was semi-quantified by measuring the recovered area using ImageJ software (version 1.38; National Institutes of Health, Bethesda, MD, USA) (22).

Tube formation assay. Melted Matrigel (150 μl; BD Biosciences) was applied to 48-well plates and was

Table II. miRNA expression profiles in the plasma of patients with UA compared with controls.

No.	Gene ID	Score (d)	Fold change (UA/controls)	q-value (%)
1	hsa-miR-19b	3.37	17.03	<0.0001
2	hsa-miR-106a	3.09	11.04	<0.0001
3	hsa-miR-20a	3.04	21.22	<0.0001
4	hsa-miR-16	3.00	9.18	<0.0001
5	hsa-miR-17	2.97	9.07	<0.0001
6	hsa-miR-451	2.78	28.65	<0.0001
7	hsa-miR-24	2.72	7.67	<0.0001
8	hsa-miR-223	2.58	10.13	<0.0001
9	hsa-miR-92a	2.53	15.46	<0.0001
10	hsa-miR-146a	2.23	9.01	<0.0001
11	hsa-miR-320	2.05	7.01	<0.0001
12	hsa-miR-19a	1.99	4.84	<0.0001
13	hsa-miR-30a-5p	1.98	37.19	<0.0001
14	hsa-miR-126	1.97	7.16	<0.0001
15	hsa-miR-126*	1.95	18.13	<0.0001
16	hsa-miR-720	1.94	6.82	<0.0001
17	hsa-miR-26a	1.91	22.25	<0.0001
18	hsa-miR-26b	1.88	20.72	<0.0001
19	hsa-miR-191	1.80	4.58	<0.0001
20	hsa-miR-195	1.79	11.81	<0.0001
21	hsa-miR-20b	1.78	23.13	<0.0001
22	hsa-miR-30c	1.77	23.08	<0.0001
23	hsa-miR-1274b	1.76	16.24	<0.0001
24	hsa-miR-30b	1.71	28.93	<0.0001
25	hsa-miR-93	1.54	35.92	<0.0001
26	hsa-miR-484	1.53	4.89	<0.0001
27	hsa-miR-197	1.52	2.52	<0.0001
28	hsa-miR-222	1.39	3.52	<0.0001

Circulating miRNAs exhibited significant alterations in patients with UA (n=12) compared with controls (n=12). Only miRNAs with >2-fold change and false discovery rate <0.0001% are presented. miR/miRNA, microRNA; UA, unstable angina.
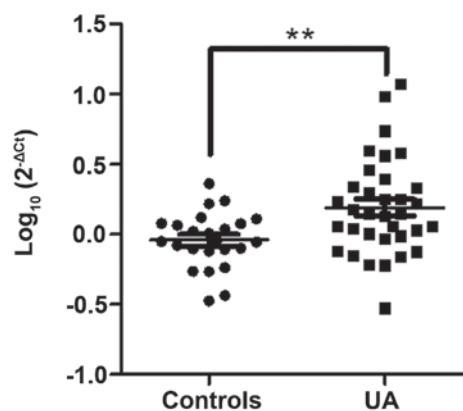

Figure 1. Validation of circulating miR-19b levels in patients with UA. Reverse transcription-quantitative polymerase chain reaction analysis of miR-19b levels in plasma samples from patients with UA (n=34) and controls (n=24). miR-19b levels were normalized to the spiked-in *Caenorhabditis elegans* miRNA, cel-miR-39. Data are presented as the mean ± standard error of the mean. **P<0.01 compared with the control group. miR, microRNA; UA, unstable angina.

endothelial target genes from CGAP SAGE via Database for Annotation, Visualization and Integrated Discovery (DAVID) platform (23). The target genes of miR-19b in the endothelium were classified into functional groups using DAVID according to the biological process (BP) classification of Gene Ontology (GO) (24). As input, the GenBank ID of each gene target was used. As output, significant processes (GOTERM) with P<0.05 were generated. All the details can be freely found at http://david.abcc.ncifcrf.gov/.

Statistical analysis. Quantitative data are presented as the mean ± standard deviation or standard error of the mean. For continuous variables, statistical significance was calculated using one-way analysis of variance followed by the Tukey multiple comparisons test. For comparisons between two groups, Student's t-test was conducted. For categorical variables, statistical significance was calculated using the χ^2 test for the comparison of two groups. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-19b levels were increased in plasma samples from patients with UA. The present study initially detected the miRNA expression profiles in plasma samples from patients with UA (n=12) and controls (n=12) by TLDA. The clinical characteristics of the 24 subjects are summarized in Table I. Analysis of array data using SAM algorithm identified 28 miRNAs that were significantly upregulated in patients with UA compared with in the controls, among which miR-19b exhibited the most marked upregulation (Table II).

The present study further validated circulating miR-19b levels in another independent cohort (Table I), which comprised 34 patients with UA and 24 controls, by RT-qPCR. The results indicated that the expression levels of miR-19b were increased in the UA group compared with in the control group (P<0.01; Fig. 1); this finding was consistent with the miRNA array data.

incubated at 37˚C for 30 min. EA.hy926 cells were transfected with miR-19b mimic or NC mimic for 24 h. Transfected cells (4x10⁴/well) were seeded in the gel and incubated at 37˚C for 16 h to allow tube formation. The effects of miR-19b on EA.hy926 cell differentiation stimulated by EC growth supplement (contained within Matrigel) at 37˚C were observed under a microscope (Olympus IX70) and images of four representative fields were captured. Average total tube numbers in four representative fields were determined.

Functional enrichment analysis. The target genes of miR-19b were predicted using DIANA-microT (http://www.microrna.gr/microT-CDS), TargetScan (http://www.targetscan.org/) and miRanda (http://34.236.212.39/microrna/getDownloads.do) databases. Target genes simultaneously predicted by all three databases were selected to obtain the vascular

Table III. Top ten enriched GO terms of microRNA-19b potential target genes in vascular endothelial cells.

| ID	Definition	Gene count	P-value
GO:0042127	Regulation of cell proliferation	33	1.60×10^{-6}
GO:0001944	Vasculature development	16	4.36×10^{-5}
GO:0048514	Blood vessel morphogenesis	14	4.36×10^{-5}
GO:0019220	Regulation of phosphate metabolic process	22	4.65×10^{-5}
GO:0042981	Regulation of apoptosis	30	4.90×10^{-5}
GO:0051270	Regulation of cell motion	13	7.87×10^{-5}
GO:0042325	Regulation of phosphorylation	21	7.94×10^{-5}
GO:0030334	Regulation of cell migration	12	1.04×10^{-4}
GO:0001525	Angiogenesis	11	1.58×10^{-4}
GO:0048545	Response to steroid hormone stimulus	12	3.18×10^{-4}

GO, Gene Ontology.
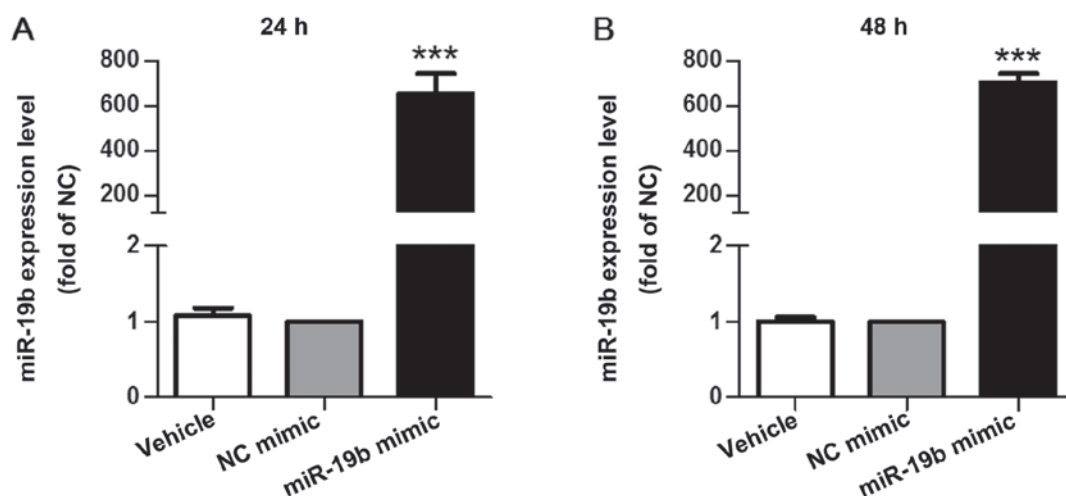
Figure 2. miR-19b overexpression in EA.hy926 cells. EA.hy926 cells were transfected with miR-19b mimic or NC mimic and were collected after (A) 24 or (B) 48 h. miR-19b levels were measured by reverse transcription-quantitative polymerase chain reaction and were normalized to RNU6B. Data are presented as the mean ± standard error of the mean, n=4. ***P<0.001 compared with the NC mimic group. miR-19b, microRNA-19b; NC, negative control; Vehicle, Lipofectamine 2000.

Functional enrichment analysis of miR-19b. Dysfunctional endothelium is an important pathological basis of unstable plaque formation, which triggers the clinical symptoms of UA. Therefore, the present study performed a functional enrichment analysis for miR-19b in ECs, in order to reveal the potential role of miR-19b in patients with UA. GO annotations of 276 predicted target genes in ECs demonstrated that miR-19b may be involved in regulating cell proliferation, migration and angiogenesis, which are closely associated with the formation of unstable plaques (Table III).

Effects of miR-19b on EC proliferation, migration and angiogenesis. To determine the effects of miR-19b on EC proliferation, miR-19b mimic was transfected into EA.hy926 cells for 24 or 48 h. The degree of miR-19b overexpression was detected 24 h (fold change, 653±90; P<0.001; Fig. 2A) and 48 h (fold change, 704±40; P<0.001; Fig. 2B) post-transfection compared with the NC mimic. Furthermore, the results of a CCK-8 assay demonstrated that miR-19b significantly inhibited cell proliferation at the two time points (P<0.001; Fig. 3).

Wound healing assay was conducted to investigate the effects of miR-19b on EA.hy926 cell migration. As presented in Fig. 4, EA.hy926 cell migration was markedly inhibited by 50% post-transfection with miR-19b mimic for 24 h compared with in the NC group (P<0.001).

Tube formation assay was performed to determine the role of miR-19b in angiogenesis. The results demonstrated that compared with in the NC group, EA.hy926 cells tube formation was decreased by ~70% in the miR-19b mimic group (P<0.001; Fig. 5).

All together, these findings indicated that miR-19b may act as an inhibitor of unstable plaque formation by negatively regulating EC proliferation, migration and angiogenesis.

Effects of miR-19b on STAT3 transcriptional activity. STAT3 is an important nuclear transcription factor and a major regulator of cell proliferation, migration and angiogenesis. In order to investigate whether the inhibitory role of miR-19b in ECs was mediated by STAT3, EA.hy926 cells were transfected with miR-19b mimic for 24 h and STAT3 tyrosine phosphorylation was detected by western blot analysis. As shown in Fig. 6A,
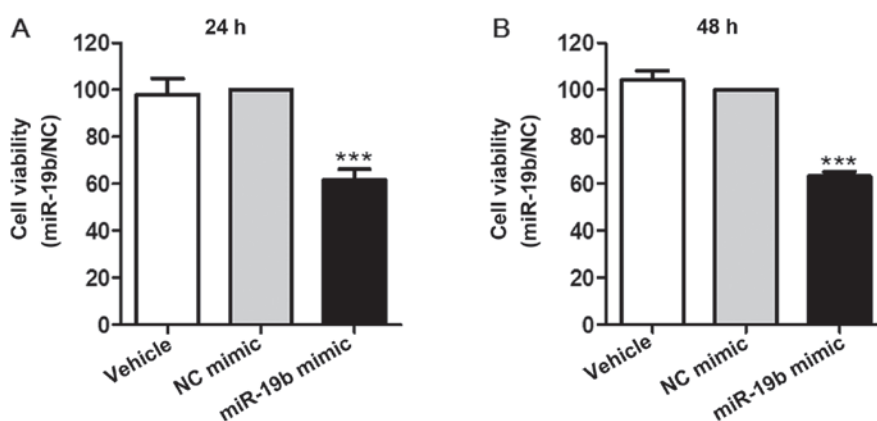
Figure 3. miR-19b decreases proliferation of EA.hy926 cells. EA.hy926 cells were transfected with miR-19b mimic or NC mimic (final concentration, 30 pmol/ml) for (A) 24 or (B) 48 h, and cell viability was measured using the Cell Counting kit-8 (n=4). Data are presented as the mean ± standard error of the mean. ***P<0.001 compared with the NC mimic group. miR-19b, microRNA-19b; NC, negative control; Vehicle, Lipofectamine 2000.
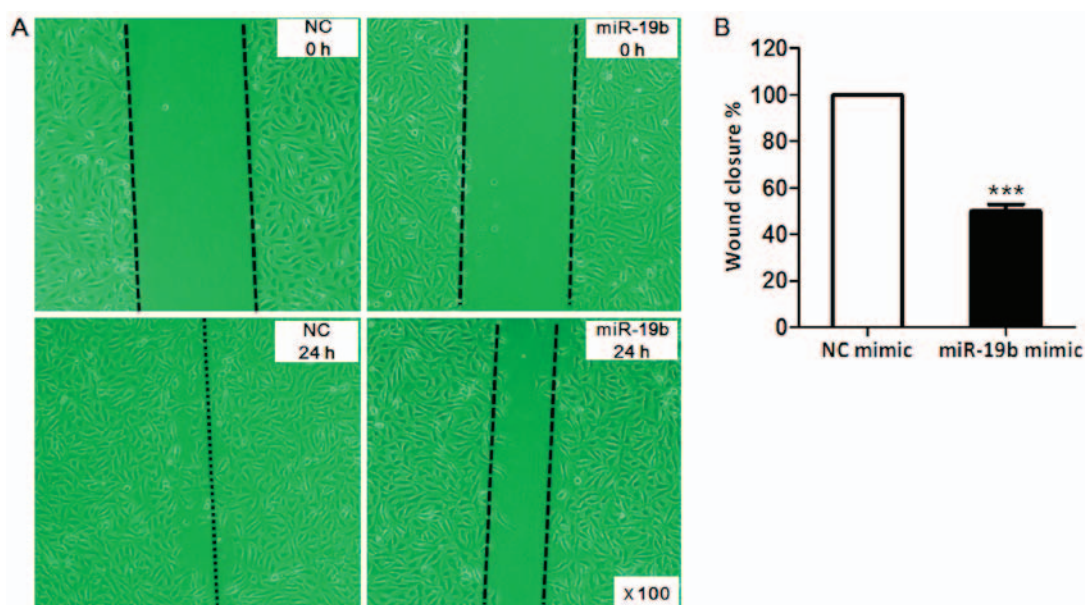

Figure 4. miR-19b inhibits migration of EA.hy926 cells. EA.hy926 cells were transfected with miR-19b mimic or NC mimic (final concentration, 30 pmol/ml) for 24 h and then underwent a scratch assay. (A) Images were acquired at 0 and 24 h. Dotted lines define the area lacking cells. (B) Semi-quantification of cell migration was conducted by measuring the recovered area (n=3). Data are presented as the mean ± standard error of the mean. ***P<0.001 compared with the NC mimic group. miR-19b, microRNA-19b; NC, negative control.

STAT3 phosphorylation was markedly decreased by ~40% in the miR-19b mimic group compared with in the NC group (P<0.01).

STAT3 transcriptional activity was also examined by transfecting STAT3-driven promoter luciferase plasmid into EA.hy926 cells. Consistent with the results of STAT3 tyrosine phosphorylation analysis, miR-19b significantly inhibited the transcriptional activity of STAT3 by ~48% compared with in the NC group (P<0.05; Fig. 6B).

These results suggested that the negative regulation of miR-19b in vulnerable plaque formation may be mediated by inhibiting STAT3 signaling.

Discussion

Angiogenesis serves a critical role in the development and ultimate rupture of unstable plaques, which is considered to be responsible for the majority of acute coronary artery events (25). Previous studies have suggested that miRNAs have an important role in regulating atherosclerotic diseases (26), and some miRNAs have been reported to regulate the angiogenic response to various pathological stimuli (27). The present study demonstrated that miR-19b may serve as a regulator of unstable plaque formation in patients with UA by inhibiting EC proliferation, migration and angiogenesis.

There are specific circulating miRNA profiles associated with various diseases. The present study revealed that circulating miR-19b was significantly upregulated in patients with UA compared with in controls (Fig. 1); this finding was consistent with our previous study (13). Zeller *et al* also reported that miR-19b levels were increased in plasma samples from patients with UA (28). Furthermore, a recent study observed that the levels of plasma miR-19b were significantly increased in early stage AMI (29). Our previous study indicated that miR-19b acted
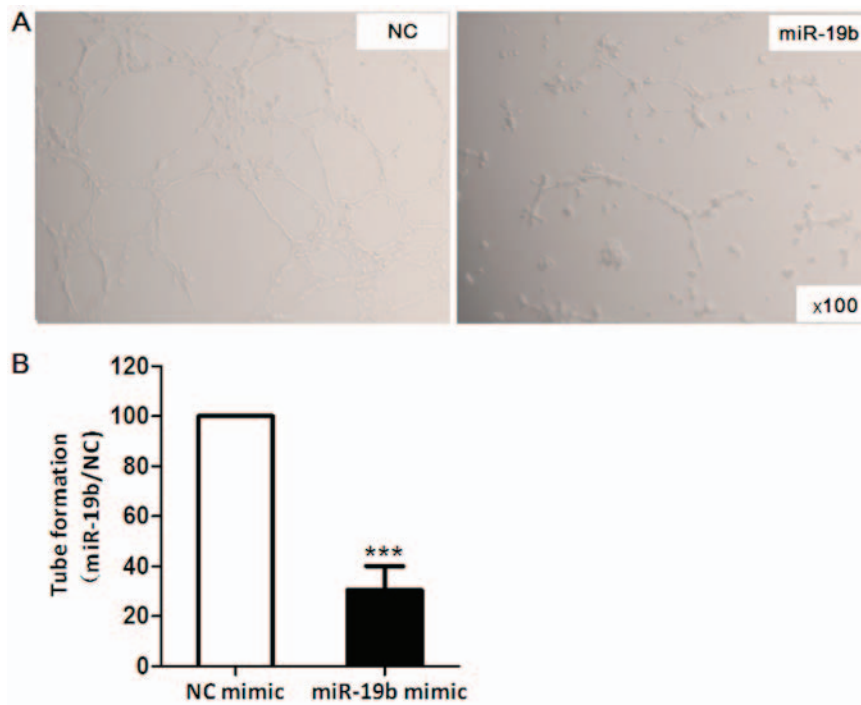
Figure 5. miR-19b inhibits proangiogenic functions of EA.hy926 cells. (A) EA.hy926 cells transfected with miR-19b mimic or NC mimic (final concentration, 30 pmol/ml) for 24 h were subjected to tube formation assay in Matrigel. (B) Number of tube-like networks were calculated (n=5). Data are presented as the mean ± standard error of the mean. ***P<0.001 compared with the NC mimic group. miR-19b, microRNA-19b; NC, negative control.
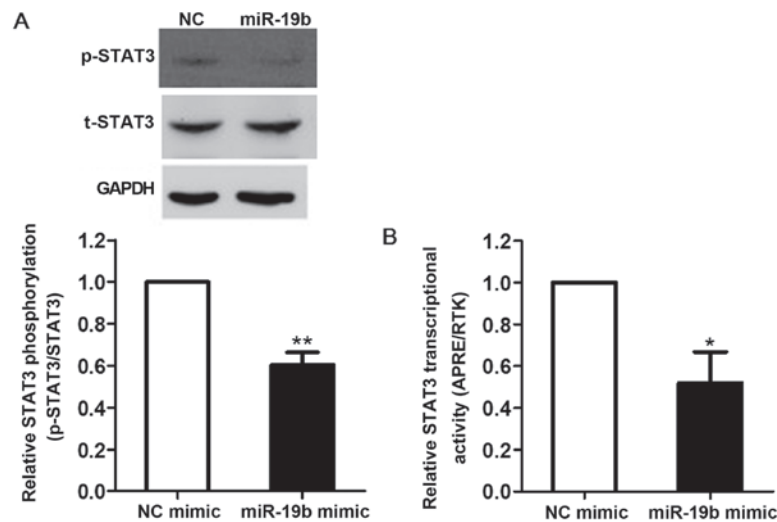

Figure 6. miR-19b inhibits STAT3 transcriptional activity in EA.hy 926 cells. (A) p-STAT3 levels were detected in EA.hy926 cells transfected with miR-19b mimic or NC mimic (final concentration, 30 pmol/ml) for 24 h by western blot analysis. Densitometry was performed and levels were normalized to t-STAT3 expression (n=3). (B) EA.hy926 cells were transfected with STAT3-driven promoter (2xAPRE) firefly luciferase reporter plasmid, alongside miR-19b mimic or NC mimic. Luciferase activities were normalized to *Renilla* activities (n=4). Data are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01 compared with the NC mimic group. miR-19b, microRNA-19b; NC, negative control; p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; t-, total.

as a potential antithrombotic miRNA in patients with UA by targeting tissue factor in ECs (13). A single miRNA may serve various biological roles by targeting numerous genes; therefore, the present study aimed to explore the possible effects of miR-19b upregulation on patients with UA. By bioinformatics analysis, the present study demonstrated that miR-19b may be involved in EC proliferation, migration and angiogenesis (Table III), which is closely associated with unstable atherosclerotic plaque formation. Subsequently, numerous assays were conducted to determine the effects of miR-19b on ECs. Consistent with the

bioinformatics prediction, the experimental results indicated that miR-19b could inhibit EC proliferation, migration and tube formation *in vitro* (Figs. 3-5). In addition, Han *et al* reported that miR-19b was downregulated in aortic tissues of apoE gene knockout mice compared with in healthy C57BL/6 (B6) mice (14). Tang *et al* suggested that miR-19b alleviated EC apoptosis, which was also predicted in the functional enrichment analysis of the present study (Table III) (30). These results suggested that atherosclerosis may be delayed by supplementing miR-19b mimic. However, miR-19b has also been reported to

prompt macrophage cholesterol accumulation and aortic athero-sclerosis by targeting ATP-binding cassette transporter A1, whereas diosgenin could inhibit atherosclerosis via suppressing the effects of miR-19b (31,33). Therefore, it is unfeasible to prevent the development of atherosclerosis by delivering a miR-19b mimic in a systemic manner. Previous studies have demonstrated success at penetrating the vascular endothelium of the vessel wall and peripheral blood mononuclear cells (10,11). Delivery of a miRNA mimic (or inhibitor) in a targeted cell- or tissue-specific manner may represent a novel strategy to suppress the progression of atherosclerosis.

STAT3 inhibitor could suppress STAT3-mediated human EC proliferation, migration and tube formation (17). However, it remains unclear as to whether the inhibitory effect of miR-19b on EC proliferation, migration and tube formation is mediated by STAT3. In the present study, overexpression of miR-19b in ECs markedly decreased STAT3 tyrosine phosphorylation (Fig. 6A). The inhibitory effects of miR-19b on STAT3 transcriptional activity were further confirmed by STAT3-driven promoter luciferase assay (Fig. 6B). These results suggested that the role of miR-19b in regulating EC proliferation, migration and tube formation may be mediated by the STAT3 signaling pathway. Furthermore, miR-19b has also been reported to inhibit the migration and angiogenesis of human umbilical vein ECs by targeting the proangiogenic protein, fibroblast growth factor receptor 2, and by suppressing the expression of cyclin D1. However, this previous study failed to observe the inhibitory role of miR-19b on cell proliferation (33), which may be due to differences in cell culture.

In patients with UA, differentially expressed circulating miRNAs may serve opposite effects in regulating angiogen-esis. miR-19b belongs to the miRNA-17-92 cluster, which comprises miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. This miRNA cluster is highly expressed in human ECs and is upregulated by ischemia (34). In the present study, miR-17-5p, miR-19a, miR-20a, miR-19b and miR-92a were all upregulated in patients with UA compared with in controls. It has also been reported that members of the miRNA-17-92 cluster exhibit an antiangiogenic effect in ECs (34). Upregulated miR-106a, miR-222, miR-320 and miR-451 were also observed to inhibit angiogenic activity in ECs (35-42). Conversely, miR-30a/b/c, miR-93, miR-126, miR-146a and miR-24 may promote proangiogenic activity (43-47). These circulating miRNAs may influence vulnerable plaque formation by forming complex regula-tory networks in patients with UA. Our future studies aim to determine the key miRNAs in the network using systems biology, in order to identify effective therapeutic targets.

In conclusion, the present findings suggested that miR-19b may exert protective effects on plaque stability by modulating the STAT3-mediated signaling pathway. These findings may provide information regarding vulnerable plaque formation intervention in patients with UA.

Acknowledgements

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