Original Article
Profiling and functional characterization of circulation LncRNAs that are associated with coronary atherosclerotic plaque stability

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Abstract: Background: Accumulating studies have demonstrated that some long non-coding RNAs (lncRNAs) play critical roles in the pathogenesis of atherosclerosis. We aimed to identify circulation lncRNAs that are potential biomarkers to evaluate coronary atherosclerotic plaque stability. Methods and Results: The transcriptomes of blood samples of three patients with stable plaque and three patients with unstable plaque were sequenced by RNA-sequencing. A total of 62 lncRNAs were found to be differentially expressed in patients with unstable plaques. The expressions of four candidate lncRNAs (ANP32A-005, TULP4-005, PDCD4-010, and SNHG7-003) were quantified using blood samples from 15 patients with stable plaques and 15 patients with unstable plaques, subsequently. In addition, the expression levels of these four lncRNAs in LPS (lipopolysaccharide)-activated THP-1 monocytes and THP-1-derived macrophages were measured. LncRNA-SNHG7-003 was validated to be significantly down-regulated in blood samples of patients with unstable plaques and LPS-stimulated monocytes and macrophages. Moreover, plasmid-transfection mediated over-expression of SNHG7-003 markedly inhibited the activation of NF-κB pathway, and reduced the secretion of inflammatory mediators (TNF-α, IL-1β, MCP-1 and MMP-9) in LPS-activated THP-1 monocytes and macrophages. Conclusion: LncRNA-SNHG7-003 inhibits NF-κB activation and regulates inflammatory responses in human monocytes and macrophages. Blood lncRNA-SNHG7-003 is a potential biomarker for evaluating plaque stability in patients with coronary artery diseases.

Keywords: Atherosclerosis, plaque stability, long non-coding RNA, SNHG7-003

Introduction

Coronary artery unstable plaque rupture and thrombosis are the most important pathological basis of acute coronary syndrome (ACS), which is life threatening to patients with coronary artery diseases (CAD) [1]. Even though sometimes unstable plaques might not cause severe arteriostenosis, they could lead to serious cardiac events after rupture. Many patients with coronary artery diseases do not demonstrate evident clinical symptoms, but they have high risk of acute coronary events [2, 3]. Rupture of unstable plaques can lead to myocardial infarction and is the leading cause of morbidity and mortality. Postmortem pathological examinations showed that 60% to 70% of the culprit lesions for ACS demonstrated plaque rupture [4-6], and several studies have revealed the presence of plaque rupture in 14% to 66%

of the culprit lesions from patients with ACS *in vivo* [7-9]. It has been reported that only a few patients, who have suffered from acute myocardial infarction (AMI), are clinically categorized into high risk population before AMI [10]. Hence, early recognition of unstable plaques would be of great value for patients with CAD.

Clinically, Intravascular Ultrasound (IVUS) and its derivatives, as well as Optical coherence tomography (OCT) could provide clinicians with morphological features of coronary artery atherosclerotic plaques, which could help with plaque stability evaluation [11-13]. However, these tools are too invasive and expensive to be widely used for screening of unstable plaques. Strategies targeting circulation biomarkers, such as long non-coding RNAs (lncRNAs), would provide easier ways to evaluate plaque stability in patients with CAD. Identified as non-protein-

coding RNAs with longer than 200 nucleotides in 1992 [14, 15], lncRNAs were found to regulate many biological processes under both physiological and pathological conditions [16, 17]. They are able to regulate gene expression and functions of other molecules through multiple approaches, such as epigenetic regulation, transcriptional regulation, post-transcriptional regulation, molecular sponges, molecular chaperones [18-21]. Some cardiovascular lncRNAs including Novlnc6, Mhrt, MALAT1 and Tie-1-AS, have also been identified to play a role in cardiovascular diseases like AMI and myocardial hypertrophy [22].

Recently, lncRNAs have been demonstrated to be potential key regulators of the inflammatory responses [23]. Since the pivotal roles of inflammation in the pathogenesis of atherosclerosis have been recognized and proved [24-26], this implies a potential link between lncRNA expression and incidence of atherosclerosis. Indeed, some studies have already revealed this correlation. One of the most studied lncRNAs, ANRIL, is an antisense non-coding RNA located in the INK4 locus, which is adjacent to chromosome 9p21 locus (Chr9p21) [22], a strong genetic risk factor for CAD [27, 28]. Further studies demonstrated that lncRNA-ANRIL expression was observed in atherosclerotic tissues and atherogenic cells, such as endothelial cells, smooth muscle cells, and monocyte-derived macrophages [29], and that the expression level of ANRIL was correlated with the severity of atherosclerosis [27, 30]. In addition, MIAT, a lncRNA identified by Ishil *et al.*, was reported to be associated with myocardial infarction and regulate the functions of endothelial cells [31, 32]. Expression levels of MIAT were reported to be reduced in blood mononuclear cells of patients with ST segment-elevation myocardial infarction [33]. Moreover, Lnc-RNA-p21 has been considered to play a role in atherogenesis as it can regulate the proliferation and apoptosis of smooth muscle cells, as well as neointimal hyperplasia [34]. However, whether lncRNAs can be used as reliable circulation biomarkers to distinguish stable and unstable plaque in patients with CAD has not been systematically explored so far.

In this study, we aimed to identify circulation lncRNA molecules related to the coronary atherosclerotic plaque stability by RNA-sequencing (RNA-Seq) of blood samples from patients with CAD, and verified some of the top differentially expressed lncRNAs by RT-PCR. A gain-of-function study was performed to explore the role of validated lncRNA in atherosclerosis-related inflammation in a human monocytic cell line THP-1 and THP-1 derived macrophages through plasmid-mediated over-expression of target gene.

Materials and methods

Study design and ethic statement

Peripheral blood was collected from sex and age matched patients with CAD, in which 3 patients were diagnosed with stable coronary atherosclerotic plaque and 3 patients with unstable plaque. Transcriptomes of these blood samples were sequenced by RNA-Seq to screen out candidate lncRNA molecules that might be related to plaque stability. In order to validate the preliminary results, the expressions of four candidate lncRNAs were quantified in a lager sample size containing blood samples from 15 patients with stable plaques and 15 patients with unstable plaques. In addition, the expression levels of these 4 candidate lncRNAs were quantitated in LPS (lipopolysaccharides)-induced THP-1 monocytes and THP-1-derived macrophages. Over-expression of validated lncRNA in THP-1 monocytes and THP-1-derived macrophage was conducted to explore its role in atherosclerosis-related inflammation. This study was approved by the ethics committee of Shanghai Chest Hospital, China. The study protocol was carefully explained to the participants and participation was fully voluntary. Written informed consent was obtained from all participants.

Patient selection

Patients meeting the following criteria were assigned to the unstable plaque group: (1) Clinically diagnosed as acute coronary syndrome (namely unstable angina, acute ST-segment elevation myocardial infarction, or acute non-ST-segment elevation myocardial infarction); (2) Obvious atherosclerotic lesions with at least 50% diameter stenosis or thrombosis were showed when performing coronary angiography; (3) Intravascular ultrasound indicated that the lesions were unstable coronary atherosclerotic plaques (namely plaque with ulceration, thrombosis, or broken fibrous cap; lipid core > 1 mm^2 or lipid core to plaque ratio >

Table 1. The clinical characteristics of patients whose peripheral blood samples were used for lncRNAs validation

	Stable plaque Group (n = 15)	Unstable plaque Group (n = 15)	*P* value
Age	64.27 ± 5.32	62.00 ± 10.99	0.481
Male	13 (86.7%)	14 (93.3%)	0.543
Hypertension	9 (60%)	9 (60%)	1.000
Diabetes mellitus	2 (13.3%)	4 (26.7%)	0.361
Smoke	3 (20%)	3 (20%)	1.000
Dyslipidemia	11 (73.3%)	6 (40.0%)	0.065
TG	2.35 ± 1.50	1.31 ± 0.93	0.039
TC	4.00 ± 1.17	4.32 ± 1.15	0.481
HDL	1.04 ± 0.26	0.99 ± 0.33	0.661
LDL	2.44 ± 0.78	2.81 ± 0.82	0.235
cTNI	0.01 ± 0.01	27.76 ± 36.30	0.010
LVEF	63.86 ± 2.71	56.50 ± 12.08	0.043
Gensini	7.16 ± 3.81	65.21 ± 37.96	< 0.001

TG, Plasma Total Triglyceride; TC, Plasma Total Cholesterol; HDL, Plasma High Density Lipoprotein; LDL, Plasma Low Density Lipoprotein; cTNI, Plasma Cardiac Troponin I; LVEF, left ventricular ejection fraction.

20%; fibrous cap < 0.7 mm, as previously defined [35]).

Patients meeting the following criteria were assigned to the stable plaque group: (1) Clinically diagnosed as stable angina pectoris; (2) One and only one atherosclerotic lesion with at least 50% diameter stenosis showed when performing coronary angiography; (3) Intravascular ultrasound indicated that the lesion was a stable coronary atherosclerotic plaque (namely plaque without ulceration, thrombosis, and broken fibrous cap; lipid core $\leq$ 1 mm² or lipid core to plaque ratio $\leq$ 20%; fibrous cap $\geq$ 0.7 mm).

Patients with chronic active inflammatory disease, malignant tumor, blood disease, autoimmune disease, and severe hepatic or renal dysfunction were not included. The clinical characteristics of patients whose peripheral blood samples were used for lncRNAs validation are listed in **Table 1**.

Blood collection and total RNA isolation

Blood samples were collected with EDTA-anticoagulant tubes from patients with CAD right after their diagnosis. After collection, 1 ml fresh blood was added immediately to a clean tube containing 2 ml RNAzol® BD (Molecular Research Center, Inc, USA) and 27 µl acetic

acid (Macklin, Inc, China), with vigorously shaking for 30 seconds. A total of 36 samples, comprising 18 samples in the stable plaque group and 18 samples in the unstable plaque group, were collected and stored at -80°C until use. Blood total RNA was extracted using RNAzol® BD (Molecular Research Center, Inc, USA) by following the manufacturer's instructions and was dissolved in RNase-free water. The purity and concentration of the extracted total RNA was tested with NanoDrop ND-1000 (Thermo Scientific, USA). RNA integrity was validated with 1% agarose gel electrophoresis. Three RNA samples in each group from age and sex matched patients were selected for transcriptome sequencing analysis, and the remaining 30 samples were used for subsequent PCR verification.

Preparation of cDNA libraries and sequencing

1~2 µg total RNA of each sample was used to prepare cDNA libraries, as previously described [36]. Briefly, the RiboZero Magnetic Gold Kit (Human/Mouse/Rat) (Illumina, Inc, USA) was used to remove ribosomal RNA from each sample. Then libraries preparation, including steps like RNA fragment, radom primed 1st strand cDNA synthesis, dUTP based 2nd strand cDNA synthesis, adaptor ligation, and PCR amplification, was conducted using KAPA Stranded RNA-Seq Library Prep Kit (Illumina, Inc, USA). The quality of prepared libraries was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Finally, TruSeq SR Cluster Kit v3-cBot-HS (Illumina, Inc, USA) and Illumina HiSeq 4000 instrument were used to conduct sequencing. All procedures were strictly in accordance with manufacturers' instructions.

Analysis of RNA-Seq data

Raw sequencing data were quality controlled and pre-processed, and were aligned with human reference transcriptome (version GRCh37.p13) using Hisat2 (version 2.0.2). Total transcripts were assembled using StringTie (version 1.3.1c). Then the computation of transcripts abundance and screening of differentially expressed transcripts were conducted with the help of Ballgown (version 2.8.4). Transcripts with fold change $\geq$ 1.5 and *P* value <

Table 2. The sequences of DNA primers used for RT-qPCR in this study

Transcripts	Forward	Reverse
GAPDH	5'-TGGACCTGACCTGCCGTCTA-3'	5'-GGAGTGGGTGTCGCTGTTGA-3'
ANP32A-005	5'-ATCGCTCTGGTGTTTGCTCTT-3'	5'-GGCTGCTGTCCTTGGCTTC-3'
TULP4-005	5'-CTCCCAGCCGTGAATAATCT-3'	5'-ACAAGAATGAGCCTGTCTTCC-3'
PDCD4-010	5'-ATCACCTTGTTAAAGAGGCTATTA-3'	5'-CGCTCCAGCACAGAGTATGA-3'
SNHG7-003	5'-CTTCGCCTGTGATGGACTTC-3'	5'-CCTGCCCATCCCTTTATTCC-3'

0.05 between the stable plaque group and the unstable plaque group were considered as differentially expressed transcripts. Hierarchical Clustering was conducted with R (version 3.4.1) to clarify the expression patterns of distinguishable transcripts.

Cell culture and treatment

THP-1 cells (purchased from the Cell Bank of Chinese Academy of Sciences, China) were cultured in RPMI 1640 medium (containing HEPES; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, USA) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), in an atmosphere of 95% air and 5% CO_2 at 37°C. THP-1 monocytes were stimulated with 160 nM PMA (phorbol-12-myristate acetate; Sigma-Aldrich, USA) for 24 h, in order to induce the differentiation of THP-1 monocytes into macrophages.

Plasmid vector (pcDNA3.1+) with enzymatically ligated full length sequence of SNHG7-003 was ordered from Sangon Biotech Co., Ltd., Shanghai, China. Lipofectamine® 2000 Reagent (Invitrogen, USA) was used for transfection of pcDNA3.1+/SNHG7-003 recombinant plasmid or pcDNA3.1+ control plasmid into THP-1 monocytes and macrophages. At 24 hours (THP-1 monocytes) and 30 hours (THP-1 derived macrophages) after plasmid transfection, cells were stimulated with 1 μg/ml LPS (Beyotime, China). After LPS stimulation for 2 hours, cells were harvested for western blot. After LPS stimulation for 24 hours, culture supernatant was harvested for cytokine assay, while cells were harvested for RNA isolation and PCR.

Reverse transcription and quantitative PCR (RT-qPCR)

Real-time fluorescence quantitative polymerase chain reaction assays were used to detect relative expression levels of candidate lnc-

RNAs in blood samples and in LPS-stimulated THP-1 monocytes and THP-1-derived macrophages. Blood total RNA was isolated as mentioned above. Total RNA from THP-1 cells was isolated using RNAiso Plus (Takara Biomedical Technology Co., Ltd, Beijing, China). DNase I (Beyotime, China) was used to remove DNA from each RNA sample. After that, cDNA was synthesized from each purified RNA sample using the M-MuLV First Strand cDNA Synthesis Kit (Sangon Biotech, China). Real-time quantitative PCR was performed using 2×SYBR Green qPCR Master Mix (Bimake, USA), with the Applied Biosystems® 7500 Real-Time PCR Systems. All procedures were strictly in accordance with manufacturers' instructions. Expression levels of candidate lncRNAs were calculated by the $\Delta\Delta C_T$ method [37], with normalization to GAPDH. The sequences of primers are shown in **Table 2**.

Western blot

NF-κB activation was evaluated by measuring the phosphorylation of NF-κB p65 (S536) through western blot analysis. Cells were lysed in the RIPA (Radio-Immunoprecipitation Assay) lysis buffer (Beyotime, China), supplemented with the Protease Inhibitor Cocktail (Sigma-Aldrich, USA) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, USA), on ice for 15 minutes. After centrifugation at 12,000 g for 15 minutes at 4°C, whole-cell proteins in the supernatants were collected and quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Each protein sample was mixed with 5×SDS-PAGE Protein Loading Buffer (Beyotime, China), and boiled for 5 minutes. Equal amount (12 μg) of each protein sample was separated using 10% SDS-PAGE and transferred to PVDF membrane (Millipore, USA). The membranes were blocked for 30 minutes in QuickBlock™ Blocking Buffer for Western Blot (Beyotime, China), and then incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: rabbit anti-NF-κB p65 antibody (Abcam, UK), rabbit anti-phosphorylated NF-κB p65 (Ser536) antibody (Abcam, UK), rabbit anti-GAPDH poly-
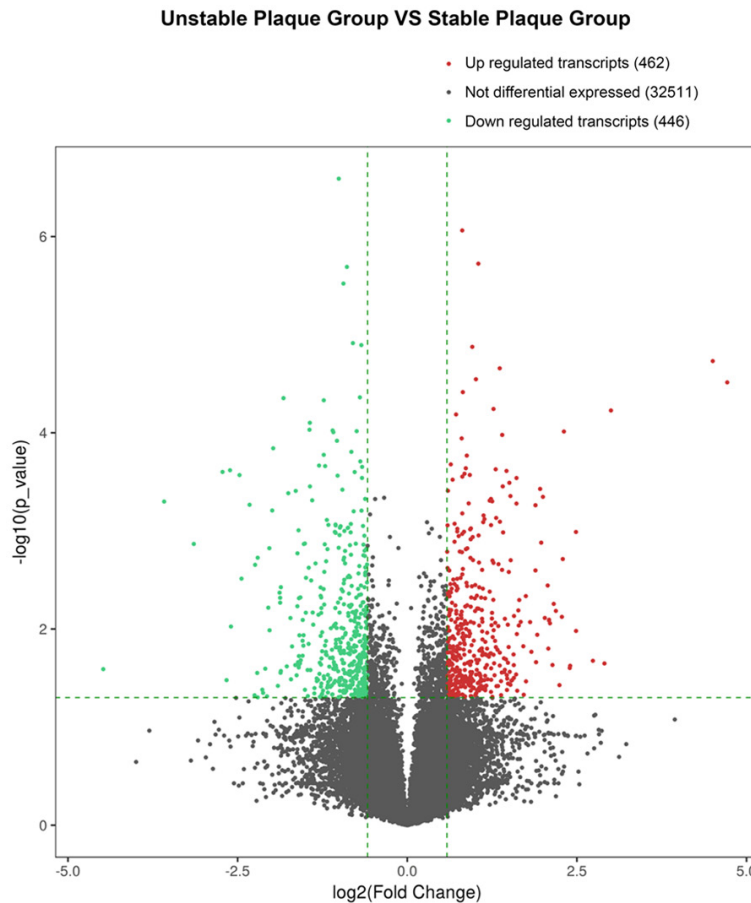
Figure 1. Volcano plot showing the differences of transcripts expression between blood samples of patients with stable plaques and those with unstable plaques. Fold change (unstable/stable) and statistical significance (p value) were two parameters used to screen out differentially expressed transcripts in the volcano plot. X-axis represents $\log_2$ (Fold Change); Y-axis represents $-\log_{10}$ (p value). Two vertical lines represent threshold of up-regulated transcripts (right) and down-regulated transcripts (left), respectively (Fold change ≥ 1.5). The horizontal line represents threshold of statistical significance (p value ≤ 0.05). Red spots represent 462 up-regulated transcripts in the unstable plaque group; green spots represent 446 down-regulated transcripts in the unstable plaque group; grey spots represent 32511 transcripts without differential expression between the two groups.

clonal antibody (Sangon Biotech, China). After incubation overnight, the membranes were washed with TBST Buffer (Sangon Biotech, China) for 3 times, and then incubated with HRP-linked anti-rabbit IgG antibody (Cell Signaling Technology, USA) at room temperature for 2 hours. Afterwards, the membranes were washed again with TBST for 3 times, and then developed using Tanon™ High-sig ECL Western Blotting Substrate (Tanon, China) and Tanon 4600 Automatic Chemiluminescence Imaging System (Tanon, China). The band intensities were quantified by densitometry using ImageJ software (version 1.0, National Institutes of Health, Bethesda, MD, USA), and were normalized to the expression levels of GAPDH.

ELISA (enzyme-linked immunosorbent assay)

The inflammatory responses of THP-1 monocytes and THP-1-derived macrophages were estimated by measuring the levels of inflammatory mediators, including TNF-α (tumor necrosis factor-α), IL-1β (interleukin-1β), MCP-1 (monocyte chemotactic protein 1), and MMP-9 (matrix metallopeptidase 9), secreted in cell culture supernatants through ELISA. ELISA kits used in this study were as follows: Human TNF-α Valukine™ ELISA Kit (Novus Biologicals, USA), Human IL-1β/IL-1F2 Valukine™ ELISA Kit (Novus Biologicals, USA), Human MMP-9 Valukine™ ELISA Kit (Novus Biologicals, USA), Human MCP-1ELISA Kit (Elabscience Biotechnology Co., Ltd, China). All experiments were performed according to the manufacturers' instructions.

Statistics

Statistical analyses were performed using the SPSS software (version 23.0; IBM, USA). Results were presented as mean ± SD (standard deviation), and comparisons between groups were performed using paired t-test or unpaired t-test where appropriate. P value < 0.05 was considered statistically significant.

Results

Screening of differentially expressed lncRNAs between the stable plaque group and the unstable plaque group by RNA-Seq

In order to screen for potential lncRNAs that are associated with the plaque stability of coronary atherosclerosis, we performed RNA-Seq analy-

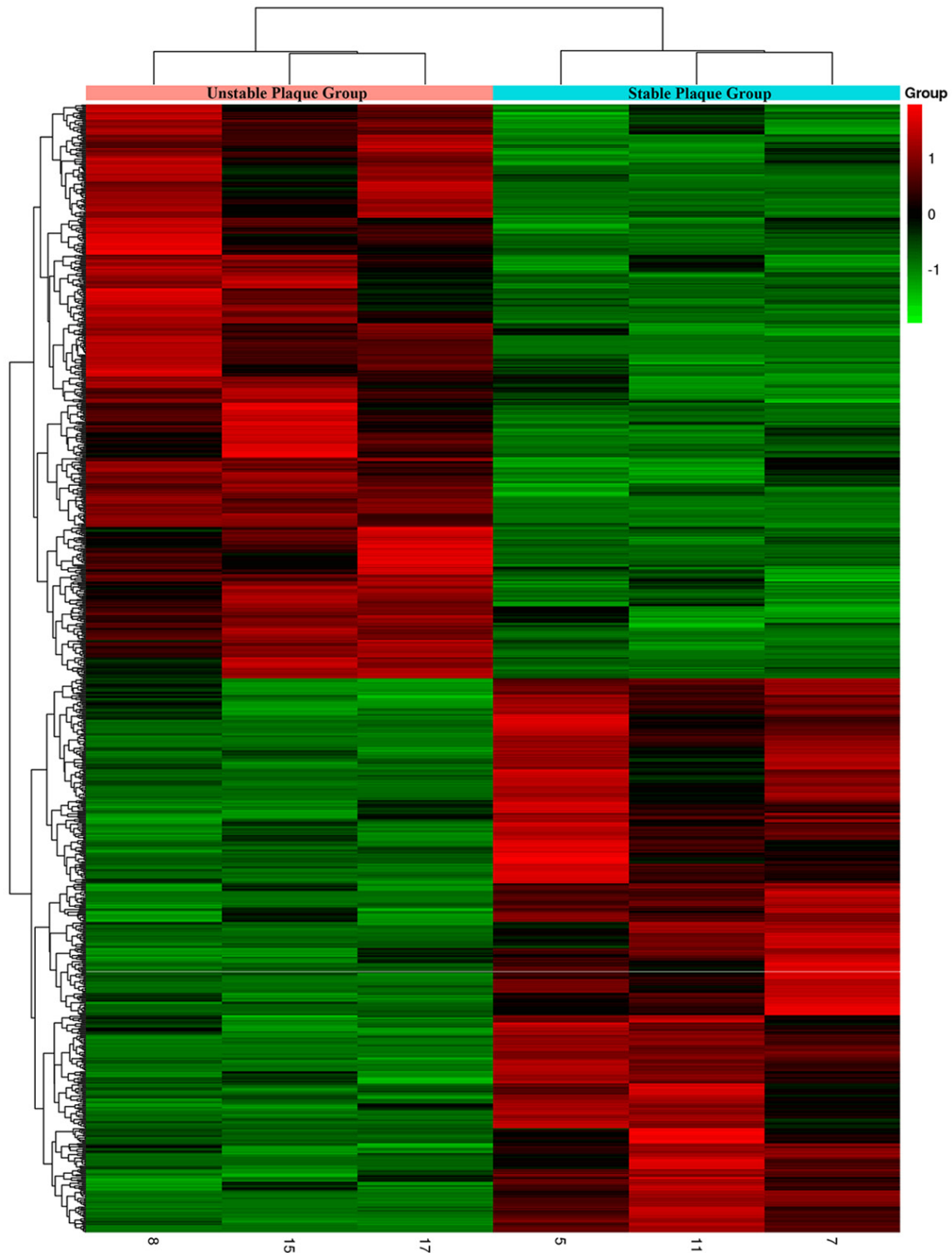## LncRNAs associated with coronary atherosclerotic plaque stability

Figure 2. Heat map showing hierarchical clustering of differently expressed transcripts between blood samples of patients with stable plaques and those with unstable plaques. Each column represents a sample; each row represents a transcript. The left three samples are in the unstable plaque group; the right three samples are in the stable plaque group. Transcripts expressions are depicted using a color scale. Red indicates up-regulated, while green indicates down-regulated.

ses of blood samples from patients. Totally, 28325 transcripts were identified in blood sam-

ples of patients in the stable plaque group, while 28489 transcripts were identified in the

Table 3. List of differentially expressed lncRNAs in blood samples of patients from the unstable plaque group and the stable plaque group

Up regulated			Down regulated		
lncRNA	Fold change	P value	lncRNA	Fold change	P value
ANP32A-005	4.08	0.008	ATP5C1-006	0.16	0.033
TBC1D5-021	3.71	0.001	TMCC2-004	0.17	0.009
TULP4-005	3.36	0.005	PDCD4-010	0.23	0.045
MSN-006	3.29	0.047	SNHG7-003	0.27	0.028
GDI2-006	3.04	0.029	RAD51C-009	0.28	0.000
HIST4H4-004	2.89	0.034	ANP32A-007	0.32	0.030
POLR2J3-007	2.87	0.037	C17orf62-032	0.33	0.006
PMS2P3-004	2.77	0.042	SGK1-022	0.34	0.021
C9orf78-006	2.57	0.000	CTD-2561J22.5-004	0.34	0.013
SCAF11-011	2.51	0.016	RP11-820I16.4-001	0.36	0.003
SERINC3-003	2.46	0.009	DDX11L1-002	0.40	0.032
VCL-007	2.42	0.018	PPIF-005	0.40	0.034
AC093323.3-002	2.38	0.007	CROCCP3-006	0.40	0.007
VPS39-004	2.37	0.009	RP11-185E8.1-001	0.41	0.004
AC005154.6-015	2.36	0.033	CHIT1-004	0.44	0.017
MORC3-010	2.35	0.001	DCAF17-002	0.44	0.018
LINC01128-004	2.33	0.018	CTD-3203P2.3-001	0.45	0.035
CANX-016	2.33	0.018	GS1-124K5.3-001	0.46	0.047
GS1-124K5.3-002	2.32	0.004	GS1-279B7.2-001	0.47	0.020
ZFAND5-007	2.26	0.011	FAM228B-002	0.47	0.000
STAG3L5P-002	2.26	0.023	RBM39-027	0.47	0.023
KLHL20-003	2.22	0.005	GDAP2-003	0.48	0.009
PAPSS1-008	2.20	0.039	DUSP22-010	0.48	0.034
LEPROT-005	2.16	0.041	PPP1R37-006	0.48	0.001
FHL3-005	2.15	0.008	CNDP2-030	0.48	0.001
RBM17-011	2.15	0.018	LINC01151-001	0.48	0.014
PRR5L-013	2.15	0.011			
EIF4H-005	2.14	0.016			
ERICH1-006	2.13	0.005			
ARF1-011	2.09	0.034			
ADARB1-001	2.09	0.004			
FXR2-006	2.08	0.009			
BRD7-003	2.08	0.021			
MYLK-016	2.07	0.001			
VRK3-012	2.02	0.029			
RP11-20D14.6-001	2.01	0.047			

Transcripts of lncRNAs with fold change (unstable/stable) ≥ 2 and P value < 0.05 were considered as differentially expressed transcripts. n = 3 for each group.

protein-coding RNAs, were down-regulated in the unstable plaque group. Hierarchical clustering of these differentially expressed transcripts showed that patients with unstable plaques could be clearly distinguished from patients with stable plaques based on centered Pearson correlation (**Figure 2**). A total of 62 differentially expressed lncRNAs (fold change ≥ 2) between the stable plaque group and the unstable plaque group are listed in **Table 3**. We therefore designed a series of primers for the top 5 most up-regulated and top 5 most down-regulated lncRNAs and attempted to verify their expression by qPCR. However, only the primers for ANP32A-005, TULP4-005, PDCD4-010, and SNHG7-003 were proven to be specific and effective. Hence, these 4 lncRNAs with valid qPCR primers were selected as candidate lncRNAs for subsequent investigations.

Verification of selected candidate lncRNAs by comparing their expression levels in blood samples by qPCR

The expression levels of candidate lncRNAs (ANP-32A-005, TULP4-005, PD-CD4-010, and SNHG7-003) in 30 blood samples (15 patients in the stable plaque group and 15 patients in the unstable plaque group) were determined using RT-qPCR. These patients whose peripheral blood samples were used for lncRNAs validation were age and sex matched, and have largely comparable incidence of hypertension and diabetes mellitus, as well as smoking ratio (**Table 1**), which potentially excluded the interference of other
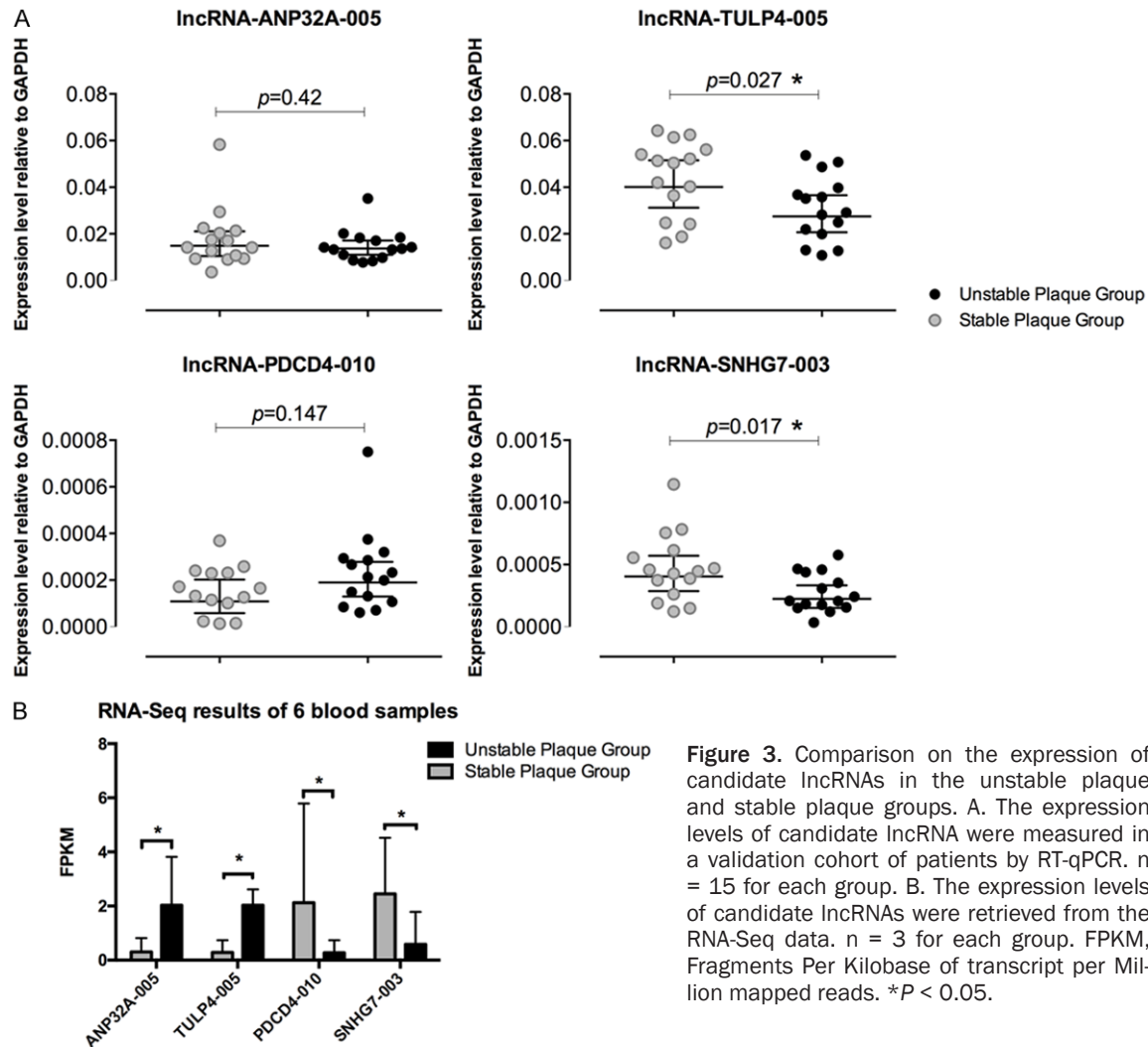
unstable plaque group. As shown in **Figure 1**, 908 transcripts were found differentially expressed between the two groups. Among them, 462 transcripts, including 119 lncRNAs and 343 protein-coding RNAs, were up-regulated in the unstable plaque group, while 446 transcripts, including 112 lncRNAs and 334

Figure 3. Comparison on the expression of candidate lncRNAs in the unstable plaque and stable plaque groups. A. The expression levels of candidate lncRNA were measured in a validation cohort of patients by RT-qPCR. n = 15 for each group. B. The expression levels of candidate lncRNAs were retrieved from the RNA-Seq data. n = 3 for each group. FPKM, Fragments Per Kilobase of transcript per Million mapped reads. *$P < 0.05$.

possible factors on lncRNA expression. As shown in **Figure 3A**, the expression level of lncRNA-SNHG7-003 was significantly lower in blood samples of patients in the unstable plaque group, which was consistent with the RNA-Seq results (**Figure 3B**). The expression levels of ANP32A-005 and PDCD4-010 were not significantly different between the unstable plaque group and the stable plaque group. The expression level of lncRNA-TULP4-005 in the unstable plaque group was also lower. However, this was opposite to the RNA-Seq results, which showed an up-regulated expression of lncRNA-TULP4-005 in patients with unstable plaques (**Figure 3B**). The contradictions between our qPCR and RNA-Seq assays were probably due to the big variation in the RNA-Seq assay with a much smaller sample size. Therefore, the candidate lncRNA-SNHG7-003 was validated to be the lncRNA with differentially down-regulated

expression in the blood of CAD patients with unstable plaque.

lncRNA-SNHG7-003 was down-regulated in LPS induced inflammatory cell models

Considering the critical roles of inflammation in the pathogenesis of atherosclerosis [24-26], we examined the expression levels of candidate lncRNAs (ANP32A-005, TULP4-005, PD-CD4-010, and SNHG7-003) in the human monocytic cell line THP-1 cells. LPS stimulation was employed to mimic the inflammatory signal in THP-1 monocytes and THP-1-derived macrophages. Compared with the THP-1 cells without LPS treatment, lncRNA-SNHG7-003 was significantly down-regulated in LPS stimulated THP-1 monocytes (**Figure 4A**) and macrophages (**Figure 4B**), while the expression of ANP32A-005, TULP4-005, and PDCD4-010
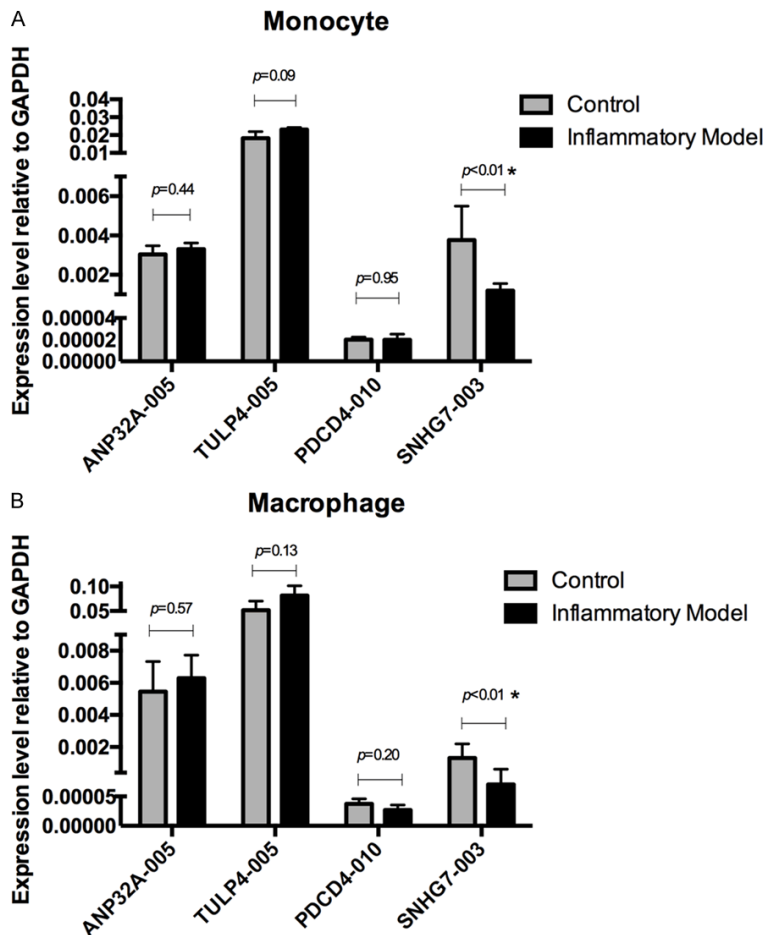
Figure 4. Comparison on the expression levels of candidate lncRNAs in LPS induced inflammatory cell models. (A, B) The expression levels of indicated lncRNAs were quantitated by RT-qPCR in THP-1 monocytes (A) and THP-1-derived macrophages (B) without LPS treatment (Control) or with stimulation by 1 μg/ml LPS for 24 hours (Inflammatory Model). n = 3 for each group; *P < 0.01. Data represent one of three experiments with similar results.
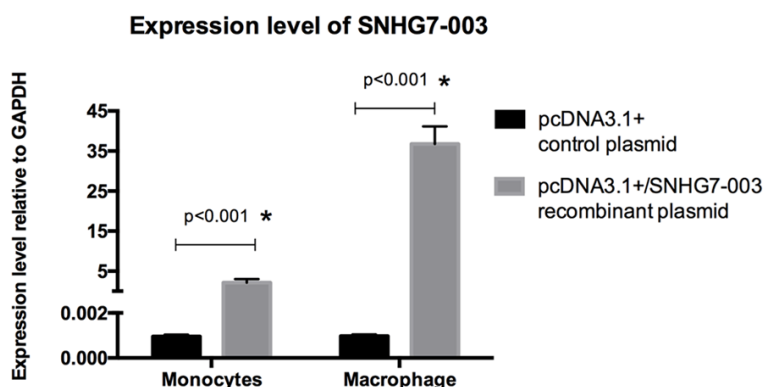

Figure 5. Verification of SNHG7-003 over-expression in THP-1 cells. The expression levels of SNHG7-003 in THP-1 monocytes or THP-1-derived macrophages transfected with pcDNA3.1+/SNHG7-003 recombinant plasmid or pcDNA3.1+ control plasmid were quantitated by RT-qPCR. n = 3 for each group; *P < 0.001. Data represent one of three experiments with similar results.

showed no difference between the inflammatory cells and control cells.

Activation of NF-κB in LPS stimulated monocytes and macrophages was inhibited by over-expression of lncRNA-SNHG7-003

To further elucidate the molecular mechanisms of lncRNA-SNHG7-003 in regulating inflammation, we explored the impacts of over-expression of lncRNA-SNHG7-003 on inflammatory responses of THP-1 cells. First, we determined the overexpression efficiency of lncRNA-SN-HG7-003 by RT-qPCR analysis. As shown in **Figure 5**, the expression levels of SNHG7-003 in THP-1 monocytes or macrophages transfected with pcDNA3.1+/SNHG7-003 recombinant plasmid were much higher than those transfected with pc-DNA3.1+ control plasmid, which indicated that SNHG7-003 was successfully overexpressed in THP-1 monocytes and macrophages.

Next, we evaluated the effects of SNHG7-003 over-expression on NF-κB activation in LPS induced THP-1 monocytes and macrophages by western blot analysis. As shown in **Figure 6**, the levels of phosphorylated NF-κB p65 (S5-36), a hallmark of NF-κB activation, were significantly reduced in LPS stimulated THP-1 monocytes and macrophages when lncRNA-SNH-G7-003 was over-expressed. On the contrary, over-expression of SNHG7-003 didn't show effects on NF-κB activation in THP-1 monocytes and macrophages without LPS stimulation. Moreover, the expression levels of basal NF-κB p65 in THP-1 monocytes
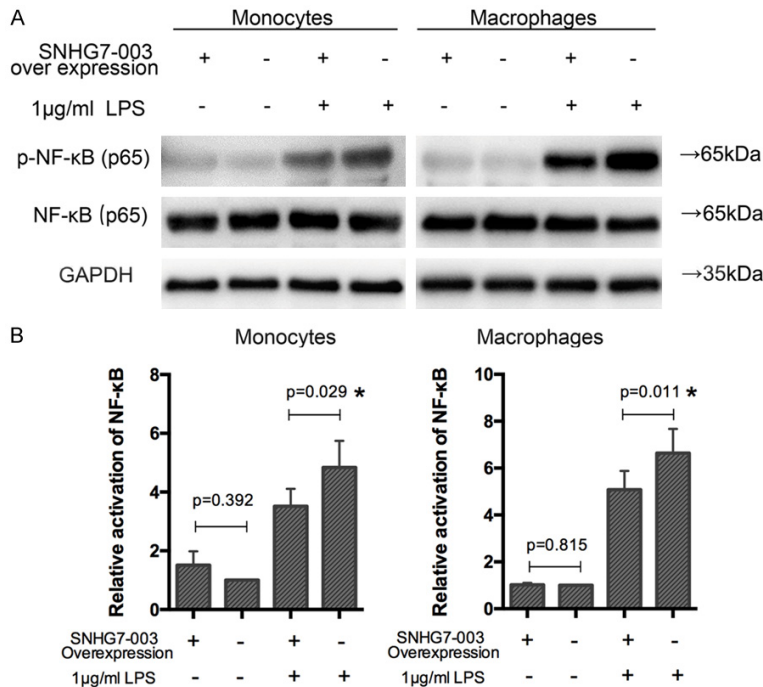
Figure 6. Activation of NF-κB in LPS stimulated monocytes and macrophages was inhibited by over-expression of lncRNA-SNHG7-003. A. Representative western blot results showing effects of SNHG7-003 over-expression on NF-κB activation in LPS stimulated THP-1 monocytes and THP-1-derived macrophages. B. Summarized results on the relative expression levels of phosphorylated NF-κB (p65). n = 3 for each group; *$P < 0.05$. Data represent one of three experiments with similar results.

and macrophages were not influenced by either SNHG7-003 over-expression or LPS stimulation (**Figure 6**).

Inflammatory mediators were reduced by over-expression of lncRNA-SNHG7-003 in LPS stimulated monocytes and macrophages

We also evaluated the effects of SNHG7-003 over-expression on the production of inflammatory mediators in LPS stimulated THP-1 monocytes and macrophages by ELISA. These soluble inflammatory factors included TNF-α, IL-1β, MCP-1 and MMP-9, which are considered as major driving forces of atherosclerotic lesion development [38, 39]. As shown in **Figure 7**, LncRNA-SNHG7-003 over-expression in both monocytes and macrophages significantly reduced the secretion of LPS induced inflammatory mediators (TNF-α, IL-1β, MCP-1 and MMP-9) to cell culture supernatants. However, over-expression of SNHG7-003 didn't show significant effects on the production of these inflammatory factors in THP-1 monocytes and macro-

phages without LPS stimulation (**Figure 7**).

Discussion

Cardiovascular disease (CVD) is the leading cause of global death [40], and ACS is one of the most life-threatening CVDs, with an estimated mortality rate of one third worldwide [41]. Early and timely recognition of unstable plaques would help with effective intervention in patients with CAD, and thus significantly improve their prognosis. In this study, we profiled lncRNAs in blood samples from CAD patients with unstable and stable plaques by RNA-Seq, and identified a series of lncRNAs that were differentially expressed in peripheral blood of patients with unstable plaques. LncRNA-SNHG7-003 was validated to be significantly down-regulated in blood samples of patients with unstable plaques and LPS-stimulated THP-1 monocytes and THP-1-derived macrophages. Over-expression of SN-HG7-003 markedly inhibited the activation of NF-κB pathway, and reduced the secretion of inflammatory mediators (TNF-α, IL-1β, MCP-1 and MMP-9) in LPS-activated THP-1 monocytes and macrophages. Our work suggests that LncRNA-SNHG7-003 is a potential blood biomarker for evaluating plaque stability.

Increasing studies have demonstrated that many circulation biomarkers could reflect inflammatory reaction and oxidative stress during atherogenesis, such as C-reactive protein, fibrinogen, interleukin-6, and interleukin-18 [42]. Additionally, molecules promoting the development and rupture of unstable plaques, such as matrix metalloproteinases (MMPs, especially MMP-9), myeloperoxidase (MPO) and MCP-1, also showed uncertain sensitivity and specificity in predicting cardiac events [42]. In recent years, some microRNAs were found to be related to plaque stability, such as miR-21, miR-100, miR-127, miR-133, miR-143/145,
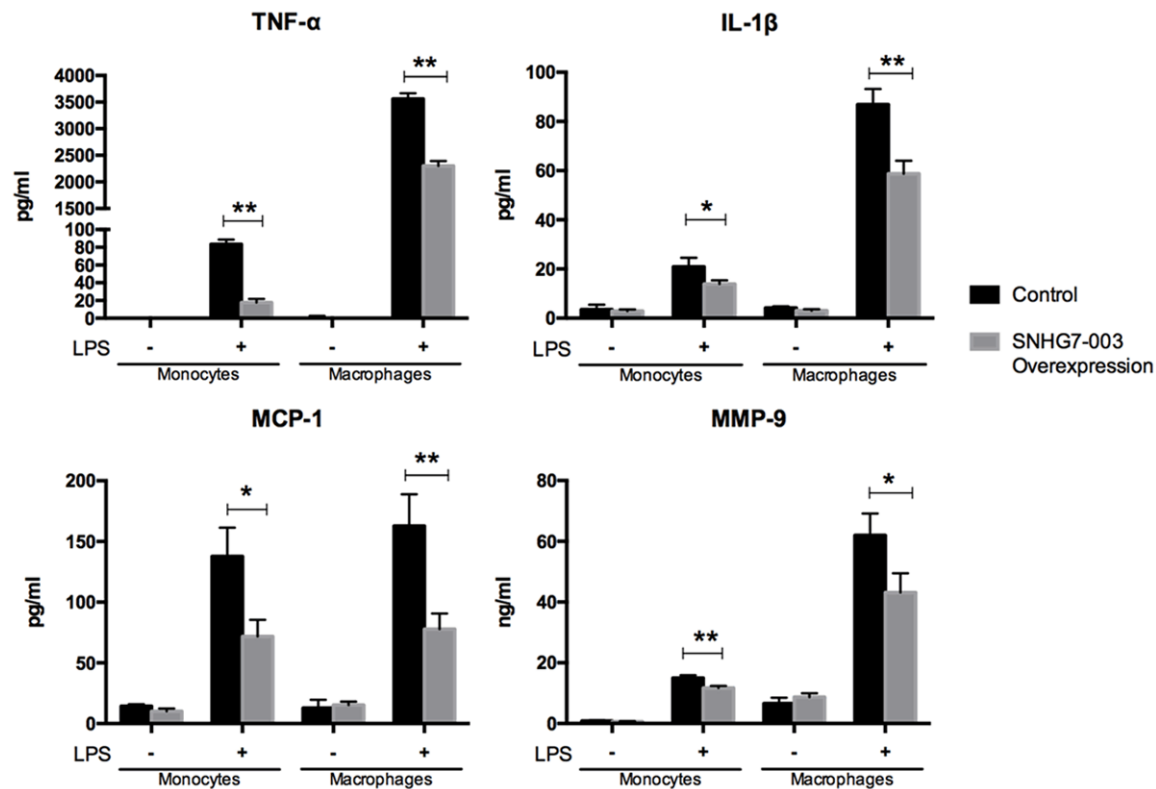
Figure 7. ELISA results showing the effects of SNHG7-003 over-expression on the production of inflammatory mediators in THP-1 cells. The levels of soluble inflammatory factors, including TNF-α, IL-1β, MCP-1 and MMP-9, secreted from THP-1 monocytes and THP-1-derived macrophages with or without LPS stimulation were quantiated by ELISA. n = 3 for each group; *$P < 0.05$, **$P < 0.01$. Data represent one of at least three experiments with similar results.

miR-221/222, and miR-494 [43]. Considering the pivotal role of inflammatory reaction on unstable plaques formation and rupture, many studies evaluated their predictive value for cardiac events among CAD patients. However, the results were controversial and their predictive values were far from comprehensive [42]. Therefore, effective biomarkers for unstable plaque, like troponin for myocardial infarction and BNP for heart failure, are still absent.

With the springing up of research in lncRNAs, the roles of lncRNAs in the process of atherosclerosis are gradually uncovered. Some studies have reported that several lnc-RNAs, such as OTTHUMT00000387022, ENST0000051-2246.1, AC100865.1, H19 and LIPCAR, might be potential diagnostic markers of coronary artery diseases [44-47]. However, lncRNAs related to coronary artery atherosclerotic plaque stability have never been identified so far. Our results showed that SNHG7-003 was significantly down-regulated in blood samples of patients with unstable plaque comparing with those with stable plaque, which was in accor-

dance with the RNA-Seq results. Inconsistent results were observed between RTqPCR verification and initial RNA-Seq screening for other 3 candidate lncRNAs, including ANP32A-005, PDCD4-010 and TULP4-005, and this was probably due to the small sample size and large variations. Due to the technical limitation on design for specific and effective primers, we only selected 4 candidates among the very top differentially expressed lncRNAs for validation. With more improvements on primer design and larger sample sizes, more potential candidates might be validated to be clarified for their correlation with coronary artery atherosclerotic plaque stability. Nevertheless, we could, at least so far, conclude that lncRNA-SNHG7-003 is a potential circulating biomarker for evaluating plaque stability.

Atherosclerosis is a chronic inflammatory disease occurs at the arterial wall [48]. Inflammatory reaction in the plaques is the key factor leading to formation and rupture of unstable plaques [1, 49]. Monocytes and monocytes-derived macrophages are two major cells involved

in plaque inflammation, and play important roles in occurrence and development of unstable plaques [1, 50]. In this study, the samples we used for RNA-Seq screening and RT-qPCR validations were from peripheral blood, in which monocytes are included. Acquisition of RNA samples from total peripheral blood is more convenient, which could facilitate the fast diagnosis of unstable plaques without cell purification. However, purified monocytes could be a more ideal population for studying the association between lncRNAs and inflammation. Therefore, we compared the expression levels of candidate lncRNAs in LPS stimulated THP-1 monocytes and THP-1-derived macrophages to figure out their relationship with atherosclerosis-related inflammation. Interestingly, lncRNA-SNHG7003 was also significantly down-regulated in these LPS activated monocytes and macrophages, which indicated that SNHG7-003 was potentially involved in the inflammatory reactions in monocytes and macrophages.

LPS could activate THP-1 monocytes and macrophages through Toll-like receptor 4 (TLR4) and its downstream pathways (such as NF-κB pathway), which leads to the expression and release of inflammatory factors (such as TNF-α, IL-1β and MCP-1) and matrix-degrading enzymes (such as MMP-9), and thus facilitates inflammation [51-53]. These inflammatory factors and matrix-degrading enzymes have been shown to contribute to unstable plaques formation and rupture during atherosclerosis [48, 49, 54, 55]. Therefore, in order to validate our speculation on the role of SNHG7-003 in atherosclerosis-related inflammation, we determined NF-κB activation and expression levels of inflammatory mediators in monocytes and macrophages with SNHG7-003 over-expression. Our results showed that activation of NF-κB and expression of inflammatory mediators (TNF-α, IL-1β, MCP-1 and MMP-9) were inhibited by over-expression of SNHG7-003 in LPS stimulated monocytes and macrophages. Recently, lncRNA-SNHG7 has been reported to promote the proliferation of lung cancer cells, glioblastoma cells and colorectal cancer through multiple mechanisms [56-58]. Therefore, the reduced production of inflammatory mediators was not likely due to the decreased proliferation of LPS-stumulated monocytes and macrophages. Taking all these into account, we could at least conclude that lncRNA-SNHG7-003 can substantially weaken the inflammatory responses of LPS activated monocyte and macrophage, although the underlying molecular mechanisms still remain to be fully elucidated.

The current investigation has several strengths and limitations. First, to the best of our knowledge, this is the first study on identifying circulation lncRNAs related to coronary atherosclerotic plaque stability. Moreover, we were the first to link lncRNA-SNHG7-003 and plaque stability. Furthermore, we clarified a novel role of SNHG7-003 in LPS-activated monocytes and macrophages. However, there are some limitations in our study. First, blood samples used for RNA-Seq screening and candidate lncRNAs verification were limited and the variations were large. Further verifications are required to support the clinical use of SNHG7-003 for plaque stability evaluation. In addition, the specificity and sensitivity of SNHG7-003 in diagnosing unstable plaques were not investigated, which should be addressed in future studies.

In conclusion, we provided transcriptome-wide overview of aberrantly expressed lncRNAs in CAD patients with unstable and stable plaques, and identified SNHG7-003 as a novel lncRNA biomarker for diagnosing unstable plaques. SNHG7-003 could inhibit LPS-induced activation of NF-κB pathway, and regulate inflammatory response in human monocytes and macrophages. Blood lncRNA-SNHG7-003 has the potential to be a circulation lncRNA biomarker for evaluating plaque stability in patients with CAD.

Acknowledgements

Disclosure of conflict of interest

None.

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