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RESEARCH ARTICLE

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Characterization of LncRNA expression profile and identification of functional LncRNAs associated with unstable angina

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Abstract

Background: Increasing evidences suggest that long noncoding RNAs (lncRNAs) play critical roles in the pathogenesis of coronary artery disease (CAD). However, the association between lncRNAs expression profiles and unstable angina (UA) remained poorly known. Thus, the present study aims to investigate expression patterns, biological functions, and diagnostic value of lncRNAs in UA.

Methods: The present study explored the lncRNA and mRNA expression profiles in peripheral blood mononuclear cells (PBMCs) of UA patients and normal coronary artery (NCA) controls using RNA-seq. The biological function of differentially expressed lncRNAs was analyzed using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The expression of the selected lncRNAs was validated in another 44 UA patients and 46 NCA controls. Receiver operating characteristic curve (ROC) was performed to evaluate the diagnostic value of lncRNAs for UA.

Results: A total of 98 lncRNAs and 615 mRNAs were observed differentially expressed in PBMCs of UA patients as compared to NCA controls. The 10 most upregulated lncRNAs were LNC_000226, DANCR, RP1-167A14.2, LNC_002091, LNC_001526, LNC_001165, LNC_002772, LNC_000088, LNC_001226, and FAM157C, and the 10 most downregulated lncRNAs were RP11-734I18.1, RP11-185E8.1, RP11-360I2.1, LNC_001302, LNC_001287, RN7SL471P, LNC_000914, LINC01506, RP11-160E2.6, and LNC_000995. LNC_000226 and MALAT1 have high area under the curve values (AUC) for distinguishing UA from NCA patients (0.810 and 0.799, respectively), and the combination of MALAT1 and LNC_000226 increased the AUC value to 0.878.

Conclusions: The present study added our understanding about the lncRNA expression profile in UA patients and provided potential biomarkers for the diagnosis of UA.

KEYWORDS
biomarker, expression profile, long noncoding RNA, MALAT1, unstable angina

Sudong LiU and Jingyuan Hou, have contributed equally to this work and share first authorship.

1 | INTRODUCTION

Acute coronary syndrome (ACS) is one of the most severe cardiovascular diseases and ranks as the first cause of morbidity and mortality worldwide.[1] ACS refers to a spectrum of ischemic conditions and is comprised of unstable angina (UA) and acute myocardial infarction (AMI).[2] Currently, patients with chest pain, which is the major early symptom of ACS, are examined by risk score algorithms, electrocardiogram (ECG), cardiac enzymes, and occasionally coronary computed tomographic angiography for further diagnosis.[3,4] Due to the absence of myocardial necrosis, patients with UA often undergo prolonged assessment or require hospital admissions.[5] Diagnosis still faces major challenges especially for patients with atypical symptoms and completely normal or dynamic come-and-go ECG changes.[6] Moreover, the current methods for diagnosis of UA are either lacking objectivity or invasive, and discrimination between UA and AMI remains as a knot.[7,8] The recent introduction of high-sensitivity cardiac troponin (hs-cTn) has enhanced the detection rate of non-ST-elevation myocardial infarction (NSTEMI), but it is less effective in distinguishing UA from NSTEMI.[9] To date, there is still a lack of blood-based biomarkers that can be used for quick diagnosis of UA. Thus, it is urgently needed to identify and validate circulating biomarkers to detect reversible ischemic injury as seen in UA patients before progressing to necrosis.

Long noncoding RNAs (lncRNAs) are ncRNAs that longer than 200 nucleotides and incapable of encoding functional proteins.[10] LncRNAs have broad functions in a diverse range of cellular processes, such as epigenetics, genomic imprinting, nuclear organization, alternative splicing, and nuclear import, by regulating gene expression at the transcriptional, post-transcriptional, and epigenetic levels.[11,12] LncRNAs are dysregulated in various diseases, such as cancers,[13] neurological disorders,[14] infectious diseases,[15] and cardiovascular diseases.[16] Myocardial infarction-associated transcript (MIAT) was one of the earliest lncRNAs that was found to be associated with AMI. Researcher found that dysregulation of MIAT increased the susceptibility to AMI.[17] LncRNA ANRIL was widely expressed in endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and macrophages, and increased cell expansion and adhesion, promoting the development of atherosclerosis.[18,19] Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which was first reported to be associated with metastasis of lung tumors, was significantly upregulated under hypoxic condition and played a role in phenotypic switch in endothelial cells.[20] Lu et al. analyzed the expression profiles of lncRNAs from 52 patients with ACS and discovered 7 lncRNAs associated with the progression of ACS. Another study by Li et al.[21] profiled the lncRNA expression patterns in 93 patients with coronary heart disease (CAD) and 48 healthy controls, and identified several lncRNAs with diagnostic value of CAD.[22] However, to the best of our knowledge, the expression profiles of lncRNA in UA patients and diagnostic value of lncRNAs for UA remained poorly known.

The present study performed RNA-seq to profile the lncRNA expression patterns in PBMCs derived from UA patients and NCA controls. The selected differentially expressed lncRNAs were further validated for their expression and diagnostic value in a large scale of UA patients and controls.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was approved by the Ethical Committee of Meizhou People's Hospital (Huangtang Hospital) (NO.: MPH- HEC 2018 -A-36) and was performed in accordance with ethical standards specified by the Declaration of Helsinki and its amendments. Written informed consents were obtained from study participants.

2.2 | Blood samples

Patients were diagnosed as UA according to previous guidelines.[23,24] The inclusion criteria were as follows: (1) angina-like chest pain or ischemic equivalent; (2) electrocardiographic abnormalities compatible on at least two contiguous leads; and (3) at least one major pericardial vessel with >70% stenosis. NCA patients who had no obvious stenosis in coronary arteries by quantitative coronary angiography were served as controls. Patients who had impaired left ventricular ejection fraction ≤45%, congestive heart failure, chronic kidney or hepatic disease, and malignant disease were excluded from enrollment. The clinical information and medication records of participants were collected and analyzed.

2.3 | PBMCs isolation and RNA extraction

Peripheral venous blood samples were collected after coronary angiography surgery and placed in EDTA-coated tubes. PBMCs were isolated by density gradient centrifugation using Hypaque-Ficoll (GE Healthcare Biosciences AB) following manufacturer's instruction. The isolated PBMCs were lysed using Trizol (Invitrogen) and stored at −80℃ until used.

Total RNA was extracted from PBMCs using RNeasy kit (TianGen Biotech) following the manufacture's protocol. The concentration and purity of RNA were assessed by Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific).

2.4 | RNA sequencing and bioinformatical analysis

Ribosomal RNA was deprived using the Ribo-zero rRNA removal kit (Epicentre, Madison, WI). The processed RNA was cleaned by ethanol precipitation. Complementary DNA (cDNA) libraries were generated using Ultra II RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's instruction. The cDNA libraries were purified and assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies). Sequencing was performed on the Illumina Hiseq 2,500 platform, and 150 bp paired-end reads were generated.

Raw sequencing data were processed using SOAPnuke to remove adapters, poly-N reads, and low - quality reads. The Q20, Q30, and GC content were calculated and used to check the quality of the clean data. Then, paired-end clean reads were aligned to the human reference genome (version: human.GRCh38/hg38) using TopHat 2. The transcripts were assembled with the mapped reads by reference annotation (version: Gencode.v26) using Cufflink.[25] Hierarchical clustering and volcano plot filtering were used to identify the differentially expressed lncRNAs and mRNAs. Differentially expressed genes with statistical significance were identified with a random variance model, and the *p*-values were determined using paired t tests.

2.5 | GO and KEGG enrichment analysis

Cis and *trans* analysis was performed to discover the target genes of dysregulated lncRNAs. The protein-coding genes neighboring the lncRNAs (<10 kb upstream or downstream) were classified as the *cis* target genes. The *trans* target genes were predicted on the expression levels of coding genes. GO and KEGG analyses were applied to characterize the biological significance of the differentially expressed genes. GO analysis identified potential functions of these genes related to biological processes, molecular functions, and cellular components. KEGG analysis was performed to identify the enriched pathways that were associated with differentially expressed genes. The statistical significance of GO terms and KEGG pathways was evaluated by Fisher's exact tests and Chi-square tests.

2.6 | qRT-PCR assay

We examined the expression of eight most dysregulated lncRNAs in independent set of UA patients and NCA controls by quantitative reverse transcript polymerase chain reaction (qRT-PCR). Total RNA was isolated as described above. The cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (Takara Bio), and quantitative PCR was performed on the Applied Biosystems 7500 (Applied Biosystems) using TB Green Premix Ex Taq II (Takara Bio). Briefly, PCR reactions were performed in triplicate mixture (20 μl) containing 1 μl cDNA, 10 μl 2 × SYBR-Green PCR Mix, 8 μl H_2O, and 0.5 μl each of forward and reverse primers (Table 1). GADPH was used as an internal control. After amplification, a melting curve analysis was performed to confirm reaction specificity. The relative expression of lncRNA was normalized to GAPDH by the $2^{-\triangle Cq}$ method.

2.7 | Statistical analysis

Expression of lncRNAs and mRNAs in peripheral blood samples was compared between UA and NCA controls. A cutoff of 2 was set for up- or down-regulated lncRNAs identification. Student's *t* test was used for gene expression analysis. The area under a receiver operating characteristic (ROC) curve (AUC) was applied to evaluate the predictive value of the lncRNAs in diagnosis of UA. p-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of study subjects

In the present study, RNA sequencing was used to analyze the lncRNA expression profiles of peripheral blood mononuclear cells (PBMCs) from 7 UA patients and 7 NCA controls. There was no difference in the characteristics between the two groups except that UA patients exhibited higher levels of serum triglycerides ($p < 0.05$). For validation of differentially expressed lncRNAs, another 44 UA patients and 46 NCA controls were enrolled. UA patients had higher levels of triglycerides, total cholesterol, and lower levels of HDL ($p < 0.05$). UA patients had higher proportion of medication usage, such as antiplatelet drug, statins, and β-blocker. The basic characteristics of study subjects were presented in Table 2.

3.2 | LncRNA expression profiles in PBMCs of UA patients and NCA controls

A total of 4,837 lncRNAs were identified in our study, including 3,585 annotated lncRNAs and 1,252 novel lncRNAs. Meanwhile, a number

TABLE 1 Primers for validation of lncRNAs expression by RT-qPCR

Sequence name	Forward primer	Reverse primer
RP11-734I18.1	GTGTCAGGAAATGGTCGCCT	GGGTTAGGTGGGTAGTCTGC
LNC_000226	CTTTACGACGATAGGCCTC	CACACTTTCTGTAGAATCTG
DANCR	AATGCAGCTGACCCTTACCC	GGCTTCGGTGTAGCAAGTCT
RP11-185E8.1	GCTGTCACTCAGGTTTTCTGC	TCCAGGTTTTGGTGAAGGGT
RP1-167A14.2	ACAAGATCGCCGGAGTCATA	GTGAGTTACCACCACCACCG
LNC_002091	GCACTGAGTGACGACAGGAA	CTGCGGACCAAATGGAAAGC
LNC_001526	AAGGGAGTGAGCTCAGACGA	GCACAGGCTGTTGACTACCA
MALAT1	ATGAGCCACTGGGTGTACCA	CAGACCAACCCCCAGTTCAA

TABLE 2 Clinical characteristics of UA patients and NCA controls for RNA sequencing and Variables

	RNA-Seq		Validation	
Variables	**UA ($n = 7$)**	**NCA ($n = 7$)**	**UA ($n = 44$)**	**NCA ($n = 46$)**
Age, y	56.0 ± 8.5	57.0 ± 9.2	60.4 ± 12.3	60.1 ± 9.4
Male, *n* (%)	5 (71.4)	4 (57.1)	30 (68.2)	25 (56.8)
Smokers, *n* (%)	3 (42.9)	2 (28.6)	17 (38.6)	12 (27.3)
Drinker, *n* (%)	3 (42.9)	2(28.6)	2 (4.5)	1 (2.3)
Hypertension, *n* (%)	4 (57.1)	1(14.3)	19 (43.2)	16 (36.4)
Diabetes, *n* (%)	4 (57.1)	1(14.3)	15 (34.1)	3 (6.8)*
Triglycerides, mmol/L	2.08 ± 0.64	1.19 ± 0.56*	2.22 ± 1.67	1.20 ± 0.46**
Total Cholesterol, mmol/L	4.26 ± 0.64	4.32 ± 0.77	5.29 ± 1.30	4.75 ± 1.14*
HDL, mmol/L	1.08 ± 0.20	1.27 ± 0.38	1.22 ± 0.31	1.32 ± 0.29*
LDL, mmol/L	2.40 ± 0.53	2.29 ± 0.42	2.91 ± 1.01	2.61 ± 0.78
Antiplatelet drug, *n* (%)	6 (85.7)	2 (28.6)	36 (81.8)	9 (20.5)**
Nitrate esters drug, *n* (%)	1 (14.3)	0 (0.0)	8 (18.2)	1 (2.3)*
Statins, *n* (%)	6 (85.7)	3 (42.9)	39 (88.6)	15 (34.1)**
ACEI/ARB, *n* (%)	5 (71.4)	2 (28.6)	30 (68.2)	20 (45.5)*
β-blocker, *n* (%)	4 (57.1)	2 (28.6)	37 (84.1)	19 (43.2)**
$Ca2^+$ antagonist, *n* (%)	3 (42.9)	1 (14.3)	7 (15.9)	8 (18.2)

Abbreviations: ACEI/ARB, angiotensin-converting enzyme inhibitors/angiotensin antibody; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*$p < 0.05$.; **$p < 0.001$.

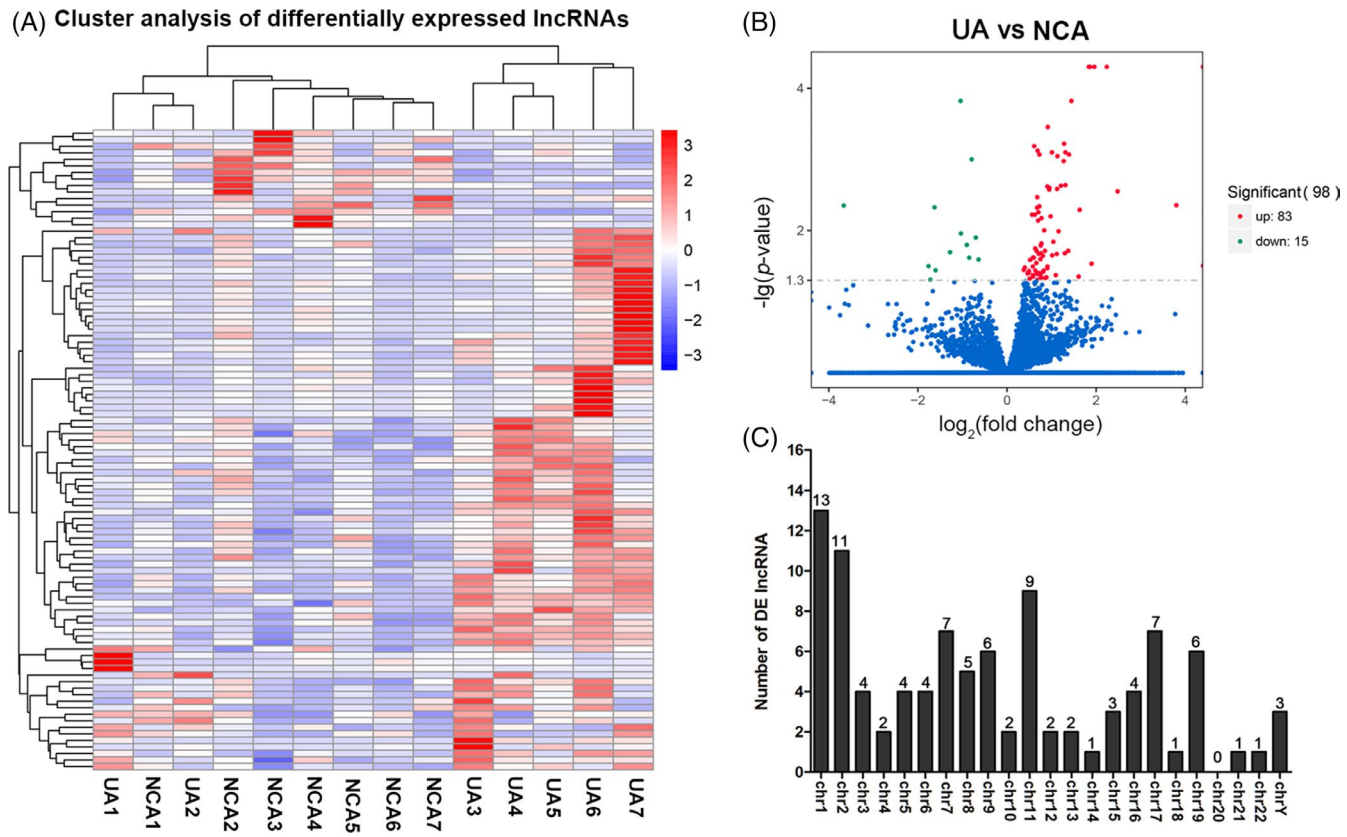


FIGURE 1 The expression profile of lncRNA in UA patients and NCA controls. (A) Hierarchical clustering of differentially expressed lncRNAs. (B) Volcano plot of differentially expressed lncRNAs in UA patients compared with NCA controls. (C) Distribution of differentially expressed lncRNAs in chromosome

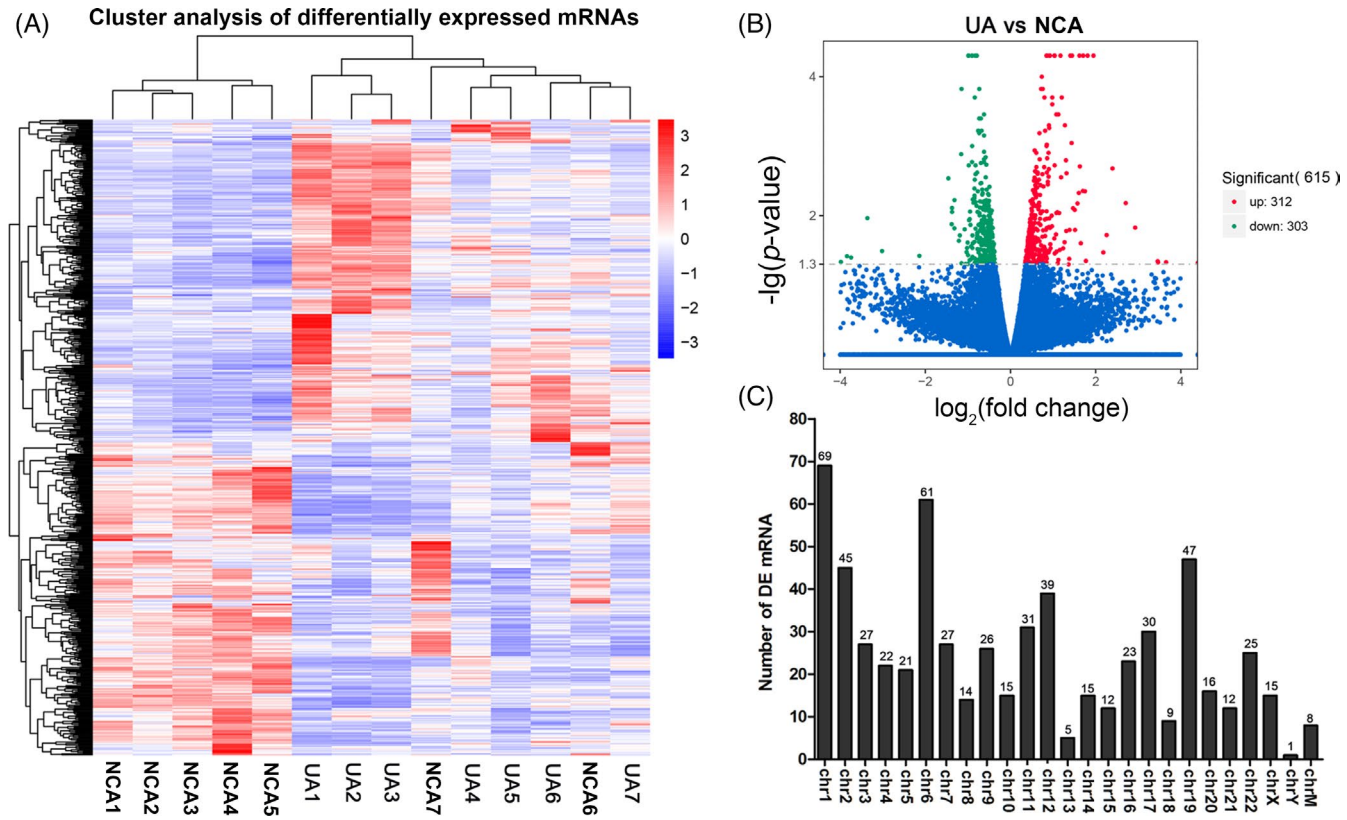


FIGURE 2 The expression profile of mRNAs in UA patients and NCA controls. (A) Hierarchical clustering of differentially expressed mRNAs. (B) Volcano plot of differentially expressed mRNAs in UA patients compared with NCA controls. (C) Distribution of differentially expressed mRNAs in chromosome

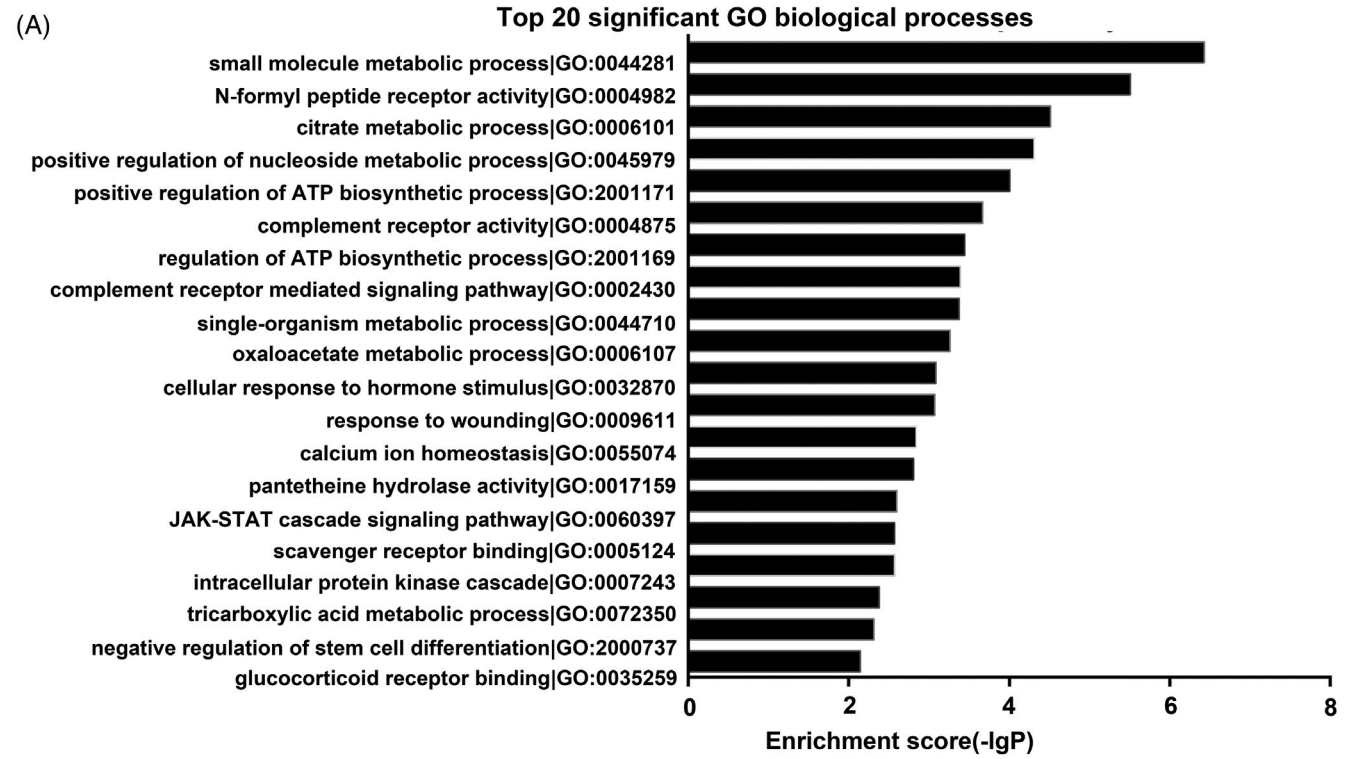
of 31,183 mRNAs were detected in our analysis. Hierarchical clustering was applied to group lncRNAs and mRNAs and display the expression patterns of each subject on heatmap (Figure 1A and Figure 2A). It was observed the lncRNA or mRNA expression profiles were distinctly different between UA patients and NCA controls. The differentially expressed lncRNAs or mRNAs were visualized on volcano plot. A total of 98 lncRNAs were specifically dysregulated in UA patients, including 83 upregulated and 15 downregulated (FC > 1.3, $p < 0.05$) (Figure 1B). A total of 615 mRNAs were specifically dysregulated in UA patients, including 312 upregulated and 303 downregulated (FC > 1.3, $p < 0.05$) (Figure 2B). The distribution of differentially expressed lncRNAs and mRNAs in chromosomes was shown in Figure 1C and Figure 2C.

3.3 | Function prediction of differentially expressed lncRNAs in UA patients

The lncRNAs are known to exert their function through regulating the expression of associated protein-coding genes. Thus, we performed GO terms and KEGG pathway analysis to explore the functions of differentially expressed lncRNAs between UA patients and NCA controls. The top 20 significant enriched GO terms were exhibited in Figure 3A. The most enriched GO terms were small molecule metabolic process (GO: 0044218) and N-formyl peptide receptor activity (GO: 0004982), and both were significant after statistical adjustment (adj_$p < 0.05$). After KEGG analysis, the top 20 enriched pathways associated with differentially expressed lncRNAs were shown in Figure 3B. Fourteen out of these 20 pathways were observed significant, but the significance disappeared after adjustment. To be noted, some pathways that have been reported to be associated with pathogenesis of ACS were observed in our results, such as HIF-1 signaling pathway and JAK-STAT signaling pathway.

3.4 | Validation of differentially expressed lncRNAs by qRT-PCR

Among the differentially expressed lncRNAs from RNA-seq, 8 lncRNAs (RP11-734I18.1, LNC_000226, DANCR, RP11-185E8.1, RP1-167A14.2, LNC_002091, LNC_001526, and MALAT1) were selected for qRT-PCR validation in another independent set of subjects including 44 UA patients and 46 NCA controls. These lncRNAs were selected based on FKPM, FC, *p*-value, or predicted target genes. The relative expression of lncRNAs was normalized to GAPDH. As shown in Figure 4, all of these 8 lncRNAs were differentially expressed between UA and NCA patients. Two lncRNAs, namely RP11-734I18.1 and RP11-185E8.1, were decreased in UA patients, while the other six lncRNAs were increased in UA patients. These data were inconsistent with that observed in RNA-seq. Considering



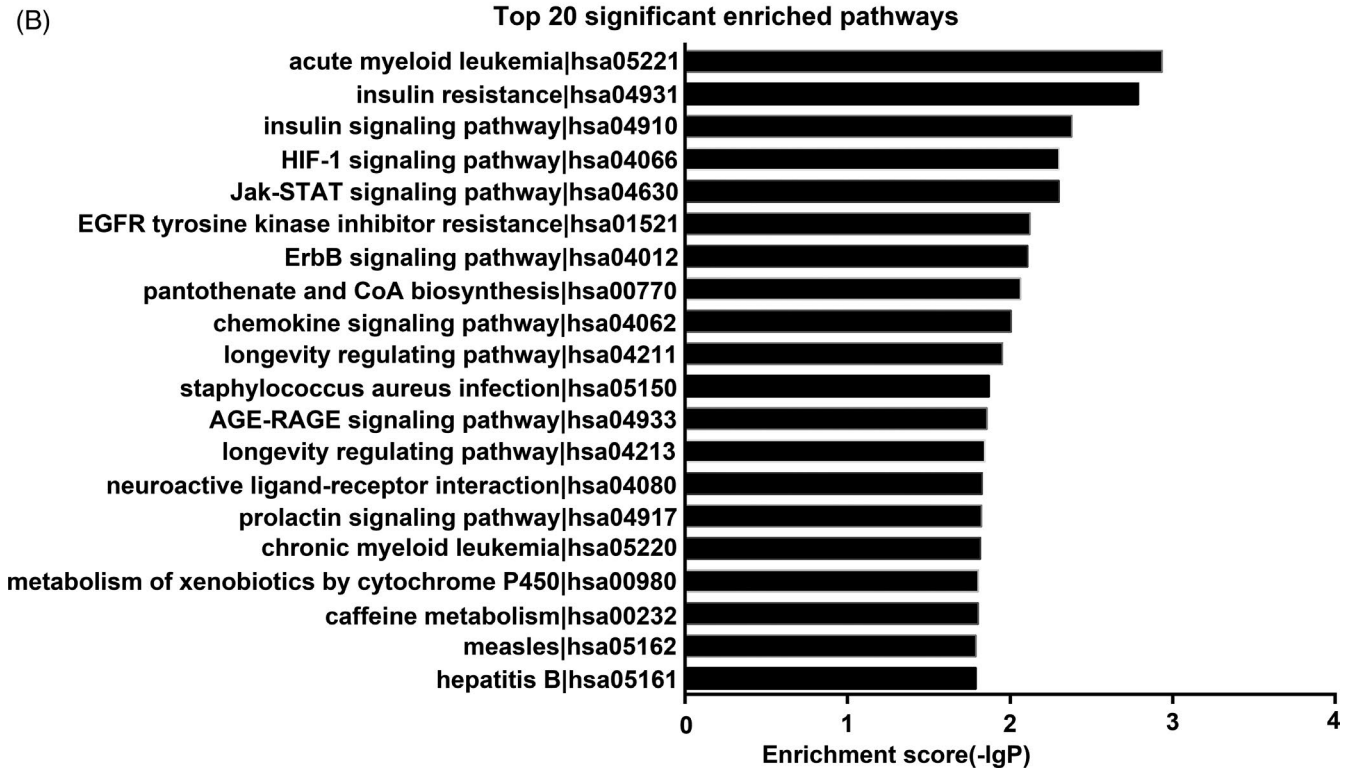


FIGURE 3 GO classification and KEGG pathway analysis of differentially expressed lncRNAs. (A) Top 20 biological processes enriched for differentially expressed lncRNAs. (B) The top 20 pathways enriched for differentially expressed lncRNAs

the practical utility, the lncRNAs with mean expression less than 0.1 of GAPDH were removed from the analysis. Thus, only MALAT1, LNC_00226, and LNC_001526 were selected for subsequent evaluation as biomarkers.

3.5 | Diagnostic value of lncRNAs for UA

To identify the potential novel biomarkers for UA, ROC curve analysis was performed to evaluate the diagnostic value of lncRNA MALAT1,

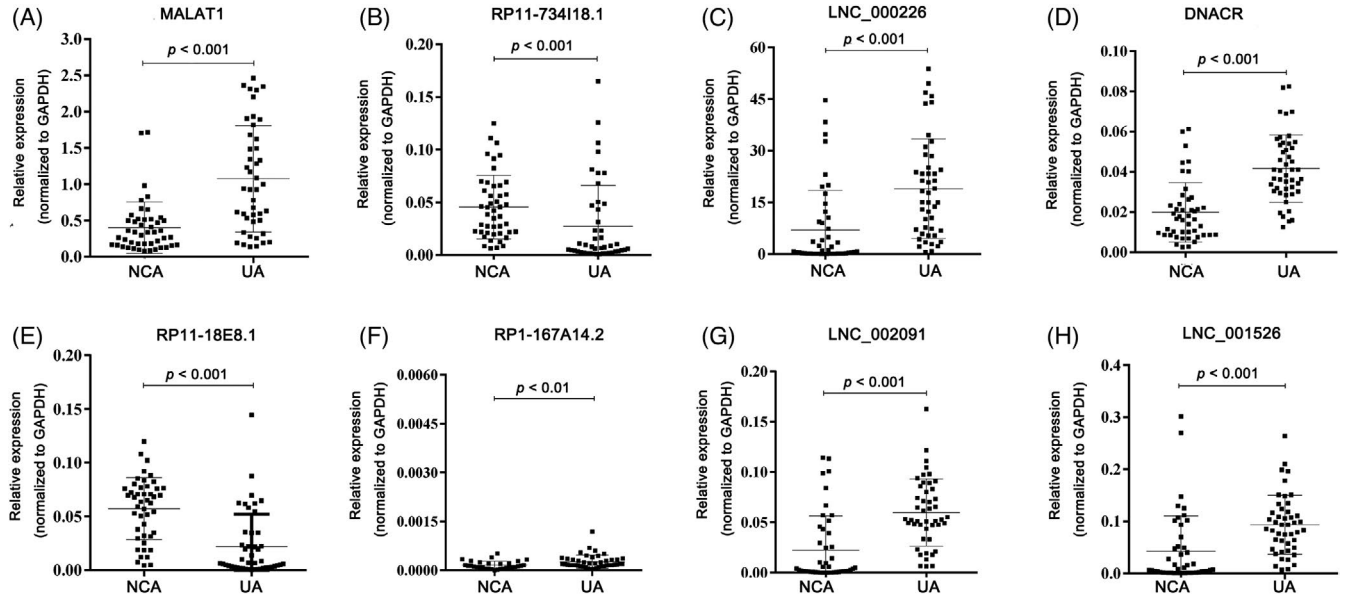


FIGURE 4 Validation of the differentially expressed lncRNAs using RT-qPCR. The expression of the selected lncRNAs was validated by RT-qPCR in UA patients ($n = 44$) and NCA controls ($n = 46$)

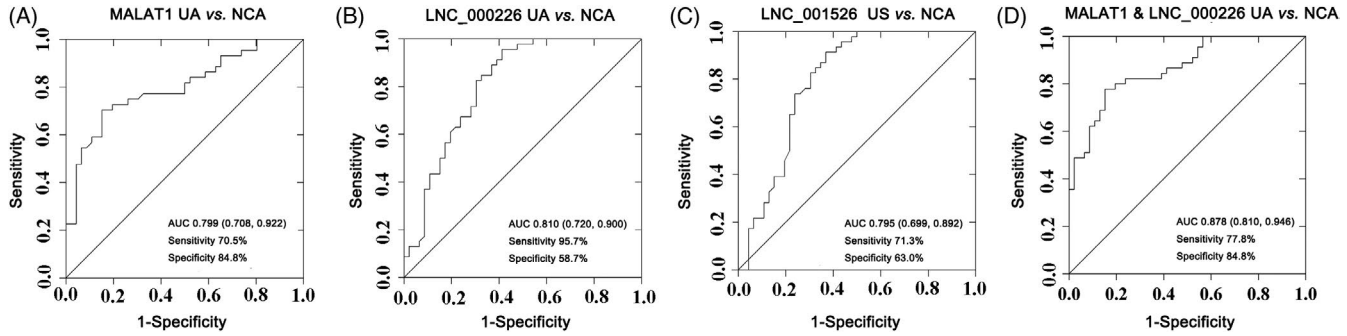


FIGURE 5 ROC curve analysis of lncRNA MALAT1, LNC_000226, and LNC_001526. ROC curves for discrimination of UA patients from NCA controls using (A) MALAT1, (B) LNC_000226, (C) LNC_001526, and (D) combination of MALAT1 and LNC_000226

LNC_00226, and LNC_001526. The results were shown in Figure 5. The AUC values, sensitivity, and specificity were 0.799, 70.5%, and 84.8% for MALAT1 (Figure 5A); 0.810, 95.7%, and 58.7% for LNC_000226 (Figure 5B); and 0.795, 91.3%, and 63.0% for LNC_001526 (Figure 5C). We also evaluated the combined effect of lncRNAs. It was observed that the AUC, sensitivity, and specificity for combination of MALAT1 and LNC_000226 were 0.878, 77.8%, and 84.8%.

4 | DISCUSSION

In the present study, we investigated the expression profiles of lncRNA and mRNA in UA patients and NCA controls by RNA-seq. To characterize the functional significance of differentially expressed lncRNAs for UA, we performed GO and KEGG analyses and found some associated biological processes and signal pathways, that is, HIF-1 signaling pathway and JAK-STAT signaling pathway. We validated the expression of 8 lncRNAs and examined the diagnostic value for UA in clinical samples.

In the past decades, though the diagnosis and management for cardiovascular disease have greatly improved, it still remains the most deadly disease worldwide.[26,27] Especially with the advent of elder surge, the burden and damage of cardiovascular disease would enlarge in the future. Evidences showed that the mortality rate of patients with UA was lower than patients with AMI,[28] thus highlighted the practical significance of early diagnosis of UA. It was urgently needed to identify novel biomarkers to improve the prediction of UA. Noncoding RNAs such as miRNAs have been proved to be promising circulating biomarkers for AMI, heart failure, and other cardiovascular diseases. Another class of noncoding RNAs and lncRNAs participate in various biological processes and are promising novel molecular biomarkers of many diseases.[29,30] Vausort et al.[31] assessed the expression levels of five lncRNAs in peripheral blood cells of 414 AMI patients and 86 healthy volunteers. The study showed that the expression of lncRNA HIFα, KCNQ1OT1, and MALAT1 was higher in AMI patients than in healthy volunteers, while expression of ANRIL was lower in AMI patients. Li et al. reported the profiles of lncRNAs in PBMCs from

93 CAD patients and 48 healthy volunteers, and identified two novel lncRNAs ENST00000444488.1 and uc010yfd.1 which would serve as biomarkers for diagnosing CAD.[22] Yan et al.[32] studied the lncRNAs expression in plasma from CAD and controls and identified that lncRNA CoroMarker was a stable, sensitive, and specific biomarker for CAD. In the current study, we found that lncRNA expression profiles were distinct in UA patients in comparison with NCA controls. We observed 98 lncRNAs dysregulated in UA patients, and some of these lncRNAs were first identified in our study. Functional enrichment analysis suggested that differentially expressed lncRNAs were involved pathways that associated with inflammation, cytokine, and metabolism, like small molecule metabolic process, HIF-1 signaling pathway, JAK-STAT signaling pathway, and AGE-RAGE signaling pathway. Inflammation and dyslipidemia are common symptoms and well-known risk factors of ACS. In accord with functional pathways, we observed that the UA patients in the present study presented higher levels of triglycerides, total cholesterol, and lower levels of HDL. Hypoxia plays an important role in the development of cardiovascular disease, as manifested by the activation of HIF-1 signaling pathway.

We further validated the expression of selected lncRNAs in another set of UA patients and NCA controls. The results were inconsistent with RNA-seq. However, five lncRNAs were a very low expression levels as normalized to housekeep gene GAPDH, which might compromise the value as biomarkers. Finally, we evaluated the predictive ability of MALA1, LNC_000226, and LNC_001526 with ROC analysis. The AUC values for these lncRNAs were between 0.795 and 0.810, while it elevated to 0.878 with the combination of MALAT1 and LNC_000226. A previous similar study by Lu et al.[21] examined the lncRNA expression profiles of ACS patients and identified 7 candidate lncRNA biomarkers for predicting the risk of myocardial infarction. The lncRNA-based classifier achieved a discriminatory power of 0.976 as measured by AUC. In a word, lncRNAs proved to be a promising biomarker of cardiovascular disease.

Previous studies showed that MALAT1 was profoundly dysregulated in endothelial cells (ECs) under condition like hypoxia, high glucose, and oxidative stress.[33,34] Furthermore, MALAT1 was also found to regulate multiple pathophysiological processes such as proliferation, apoptosis, autophagy, and pyroptosis in ECs.[35] In the present study, we observed that the expression of MALAT1 was significantly increased in UA patients. LNC_000226 and LNC_001526 were novel lncRNAs first identified in the present study. These lncRNAs were selected as potential candidates because they were of rich abundance and their expression significantly increased in UA patients.

There are several limitations to our study. First, sample size for RNA-seq was relatively small. A large-scale and multicenter study was needed for a reliable and reproducible validation of lncRNA biomarkers. Second, various confounding factors could affect the lncRNA levels, such as sex, age, and cardiovascular risk factors. Third, the molecular mechanism underlying the dysregulation of MALAT1, LNC_000226, and LNC_001526 in UA patients remained unclear.

5 | CONCLUSIONS

The present study investigated the lncRNA expression profiles in UA patients and NCA controls by RNA-seq. The expression of 8 selected lncRNAs was validated by qRT-PCR. Our data suggest that MALAT1, LNC_000226, and LNC_001526 are potential diagnostic biomarkers for UA. However, further large-scale validation is needed to confirm the diagnostic value of these lncRNAs.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Zhixiong Zhong conceived and designed the experiments. Sudong Liu wrote the manuscript and Jingyuan Hou helped edit manuscript; Xiaodong Gu enrolled patients and analyzed data; Ruiqiang Weng collected the samples and conducted the experiments. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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