Original Paper

Identification of Novel LncRNA Biomarkers and Construction of LncRNA-Related Networks in Han Chinese Patients with Ischemic Stroke

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Key Words

Ischemic stroke • LncRNA • Microarray • Biomarker • Network analysis

Abstract

Background/Aims: Long non-coding RNAs (lncRNAs) are potential biomarkers of tumors, cardiac disease, and cerebral disease because of their interaction with coding RNAs. This work focused on ischemic stroke (IS) and aimed to identify novel lncRNA biomarkers and construct lncRNA-related networks in IS. **Methods:** Differentially expressed lncRNAs were identified using Arraystar Human lncRNA Microarray v4.0, and validated with qRT-PCR. A lncRNA–mRNA co-expression network and a lncRNA–miRNA–mRNA regulatory network were constructed. Functional and pathway analyses were then performed. **Results:** In total, 560 up-regulated and 690 down-regulated differentially expressed lncRNAs were found ($P < 0.05$, false discovery rate < 0.05 , absolute fold change ≥ 2). qRT-PCR results confirmed that lncRNA-ENST00000568297, lncRNA-ENST00000568243, and lncRNA-NR_046084 exhibited significant differential expression between IS and controls (all $P < 0.05$). Areas under the curves (AUCs) for these lncRNAs were 0.733, 0.743, and 0.690, respectively, and the combined AUC was 0.843. A coding–noncoding co-expression (CNC) network was constructed based on Pearson's correlation coefficient. A specific lncRNA–miRNA–mRNA regulatory network of ENST00000568297, ENST00000568243, and NR_046084 was also constructed. Functional annotation of the up- and down-regulated mRNAs was performed. Pathway analysis enriched IS-related pathways with mRNAs in the lncRNA–miRNA–mRNA regulatory network. **Conclusion:** lncRNA and mRNA expression profiles in human peripheral blood were altered after IS. ENST00000568297, ENST00000568243, and NR_046084 were identified as novel potential diagnostic biomarkers of IS. Analysis of the CNC network and lncRNA–miRNA–mRNA regulatory network suggested that lncRNAs may participate in IS pathophysiology by regulating pivotal miRNAs, mRNAs, or IS-related pathways.

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Published by S. Karger AG, Basel

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Introduction

Ischemic stroke (IS) is a neurological impairment generated by focal cerebral, spinal, or retinal infarction. IS constitutes about 85% of stroke and is the second leading of cause death and disability worldwide [1]. As reported by the Global Burden of Disease Study 2013, death due to IS in developing countries amounted to 75% of the total morbidity rate worldwide; meanwhile, the disability adjusted of life years (DALY) in developing countries amounted to 81% of the total DALY worldwide [2]. In the years to come, the prevalence of IS and the number of patients are predicted to decrease in high-income countries and double in low- and middle-income countries [3]. China, as a middle-income developing country, bears a heavy burden of stroke. Therefore, research on IS among Chinese is continuing and necessary.

Long non-coding RNAs (lncRNAs) have a long transcription sequence between 200 nucleotides and 100 kb, with minimal coding power and incomplete annotation [4]. Great progress in the field of lncRNAs has been achieved with the development of novel methodologies using a composite algorithm and databases [5]. In the past few years, lncRNAs have emerged as key regulators of various biological or cellular processes [6]. lncRNA profiles have been identified through microarray and high-throughput sequencing analyses to determine the regulatory function of lncRNAs in diseases. Dharap performed microarray analysis of rat cerebral cortex and revealed that lncRNA expression profiles are significantly altered after stroke [7]. Wu [8] and Dharap [9] performed microarray analysis of rat brain tissues to identify and explore the potential function of lncRNA-*N1LR*. Bhattarai performed high-throughput sequencing in rat cerebral cortex and discovered novel stroke-associated lncRNAs [10]. Zhang performed RNA sequencing and confirmed that lncRNA transcriptomic profiles in mouse brain microvascular endothelial cells changed after cerebral ischemia [11]. High-throughput analyses (microarray and sequencing) *in vitro* (oxygen glucose deprivation model in cell) or *in vivo* (middle cerebral artery occlusion model of rat or mouse) indicated that lncRNA probably participates in the pathogenesis of stroke. However, analysis of human samples is essential to explore the association between lncRNAs and stroke in patients with this disease.

Dykstra-Aiello performed microarray analysis and identified 299 and 97 differentially expressed lncRNAs in the peripheral blood of male and female patients with stroke compared with controls ($P < 0.005$, absolute fold change ($|FC|$) > 1.2) [12]. However, qRT-PCR analysis was not performed to verify the expression of the identified differentially expressed lncRNAs. Therefore, distinctly expressed lncRNAs between patients with IS and controls must be identified in human peripheral blood for subsequent determination of lncRNA biomarkers and exploration of functional mechanism.

In this study, microarray analysis was performed to identify the expression profiles of lncRNAs and mRNAs in human peripheral blood of patients with IS and control subjects. Nine differentially up-regulated lncRNAs from the microarray were validated through qRT-PCR analysis. The sample size was expanded for further validation and identification of lncRNA biomarkers for IS diagnosis. A coding–noncoding (CNC) co-expression network and a lncRNA–miRNA–mRNA regulatory network were constructed through bioinformatics analysis.

Materials and Methods

Study subjects and blood collection

Patients with IS were recruited from the Encephalopathy Department of the First Affiliated Hospital of Guangxi University of Chinese Medicine in Nanning from January 2016 to June 2017. The patients were diagnosed by head magnetic resonance imaging (MRI) and computed tomography (CT) scanning with IS diagnostic criteria according to the Fourth Chinese National Conference on cerebrovascular diseases. The patients had no hemorrhagic stroke, transient ischemic attack, cancer, trauma, arterial inflammation, cerebral

vascular malformation, or aneurysm. Healthy controls were also recruited from two health service centers, namely, Health Service Center of Jinzhou Community and Health Service Center of Dongge Community in Nanning. Healthy controls were matched with patients with IS in terms of age and sex composition and had no history of cerebrovascular or autoimmune diseases. All participants were of Chinese Han descent and were not related to each other. This study was conducted according to the Declaration of Helsinki and approved by the Institutional Review Board of Guangxi University of Chinese Medicine. Written informed consent was obtained from all participants prior to the study.

Whole blood samples were obtained through venipuncture using PAXgene Blood RNA tubes (Pre-AnalytiX, GmbH, Switzerland) to stabilize blood RNA. Samples for lncRNA microarray analysis were obtained from 10 patients with IS and 10 healthy controls. The samples were subjected to the first-stage validation of lncRNA expression level. IS and control subjects ($n > 50$) were used for validation and receiver operating characteristic (ROC) curve analysis. Peripheral blood was collected after symptom onset within 72 h. Fasting blood from healthy controls was also collected strictly. All collected blood samples were kept cold during transport to laboratory within 2 h and stored at -80°C .

RNA extraction, labeling and hybridization

RNA was isolated with PAXgene blood RNA isolation kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol by using TRI Reagent BD (Molecular Research Center, Inc., Cincinnati, OH) for microarray and TRIzol reagents (Invitrogen, Carlsbad, CA) for qRT-PCR analysis. RNA quantity and quality were measured using NanoDrop ND-1000 and TECAN Spark 10M, respectively. Standard denaturing agarose gel electrophoresis was performed to determine RNA integrity. Isolated RNAs were stored at -80°C prior to experiments. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA) with minor modifications. In brief, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre Madison, WI). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias by utilizing a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar Rockville, MD). The labeled cRNAs were purified by RNeasy Mini Kit (Qiagen). Hybridization solution (50 μL) was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated in an Agilent hybridization oven at 65°C for 17 h. The hybridized arrays were washed, fixed, and scanned with the Agilent DNA Microarray Scanner (part number G2505C).

lncRNA and mRNA microarray

Analysis was performed using Arraystar Human lncRNA Microarray v4.0 provided by KangChen Bio-Tech, Inc (Shanghai, China). A total of 40, 173 lncRNAs and 20, 730 coding transcripts were detected. The acquired array images were evaluated using Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). lncRNAs and mRNAs that showed statistically significant differential expression between IS and control groups were identified through P value, false discovery rate FDR, and FC filtering. Hierarchical clustering was performed using GeneSpring GX v12.1.

qRT-PCR

In brief, cDNAs were synthesized from total RNA by using SuperScript III Reverse Transcriptase (Invitrogen) for stage one qRT-PCR or the PrimerScript RT reagent kit with gDNA Eraser (Takara, Beijing, China) for stages two and three qRT-PCR. Primers for lncRNAs were

Table 1. PCR primers of the 9 significantly up-regulated lncRNAs from the microarray

Gene name	Primers
β -actin (H)	F:5' GTGGCCGAGGACTTTGATTG 3' R:5' CCTGTAACAACGCATCTCATATT 3'
T008148	F:5' ATGAGCGAAAGGGGTGGT 3' R:5' TCTGGCTGAAGGGTAGAGTGAC 3'
TCONS_00008492	F:5' GCACCAGAGGGATCAACTCA 3' R:5' TCCTTTGCAACCAAGTCTACTT 3'
ENST00000568297	F:5' GGGAGCTGCTCTATAAATCTTG 3' R:5' AAAGCAAGAAGCAAACTACATC 3'
ENST00000442403	F:5' TATTGAACCTCGCCTCCCA 3' R:5' GAATCTCTGTGAGACCACTCATG 3'
ENST00000565098	F:5' CAGAGGAAACAGATGACTTGAGG 3' R:5' GGGTTGTGGGAAGGAAGTAAA 3'
ENST00000568243	F:5' GCTGCTCCTTATTCTCTCTGTAG 3' R:5' CGCTCTGTTAGGTTGCATGG 3'
ENST00000467369	F:5' ACCCTCTCTGCTGTTCTC 3' R:5' ACAATGGCTCACACCTGTAATG 3'
NR_046084	F:5' GCCAAGGAGACTGTTCTGATG 3' R:5' GGTGTTTCCAAGTGCTGTTT 3'
NR_033861	F:5' GGAGGTGGCATCCAGATT 3' R:5' AAGCAGGGGAAGTTACAAG 3'

designed using Primer Premier v5.0 software and checked with the NCBI Basic Local Alignment Search Tool (Table 1).

Stage one qRT-PCR. SYBR Green 2X Real-Time qPCR Master Mix (Arraystar) was used in the ViiA 7 Real-Time PCR System (Applied Biosystems). Stage one qRT-PCR was performed as follows: 95°C for 10 min; 40 PCR cycles of 95°C for 10 s and 60°C for 60 s for fluorescence collection; amplification at 95°C for 10 s, 60°C for 60 s, and 95°C for 15 s; and slow heating at 99°C (0.05°C/s automatically) to obtain the melting curve.

Stage two and stage three qRT-PCR. Sample collection was expanded to 50 patients with IS and 50 controls for stage two validation. At stage three validation, sample collection was expanded to at least 80 patients with IS and 80 controls. SYBR Premix Ex Taq II (Takara, China) was used in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) or the ABI StepOnePlus Real-Time PCR System (Applied Biosystems). The procedures for stages two and three qRT-PCR were as follows: 95°C for 30 s; 40 PCR cycles of 95°C for 5 s and 60°C for 34 s for fluorescence collection; amplification at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s to obtain the melting curve.

The threshold cycle value (Ct) of each product was determined. Expression levels were calculated using the $2^{-\Delta Ct}$ method and normalized to the internal control of β -actin. lncRNA levels in patients with IS were expressed as FC against the average level of the same lncRNA in controls.

Bioinformatic analysis

GO functional annotation and KEGG pathway analysis. Gene ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using DAVID Bioinformatics Resources v6.8 online tools (<http://david.abcc.ncifcrf.gov/>).

Construction of co-expression network. A CNC network was constructed according to Pearson's correlation coefficient (PCC), which represents the correlation of differentially expressed lncRNAs and mRNAs. PCC was calculated for each pair of genes, and significantly correlated pairs with $PCC \geq 0.90$ and $P < 0.05$ were selected for construction of the CNC network. The open-source bioinformatics software Cytoscape (version 3.4.0) was used to draw the network. Degree centrality (the number of links between nodes) of lncRNA/mRNA within the CNC network determined the relative importance of the nodes.

Construction of the predicted lncRNA-miRNA-mRNA regulatory network. A comprehensive search in five databases (DIANA-LncBase v2.0, lncRNASNP v2, lncREG, RegRNA v2.0, and LNCipedia v4.1) was performed to predict target miRNAs of lncRNAs. We searched the DIANA-Tarbase v7.0 database (<http://www.microrna.gr/tarbase>) to obtain the targeted mRNAs of the predicted miRNAs.

Statistical analysis

Data analysis and plotting were performed with IBM SPSS Statistics 18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA), respectively. Comparison among groups of quantitative variables was performed using the independent-samples *t* test (normal distribution) or the Mann-Whitney *U* test (abnormal distribution). Comparison among groups of enumeration data was performed using the χ^2 test. Correlation analysis between lncRNA expression and clinical parameters was performed using Pearson's correlation. The area under the receiver operating characteristic (ROC) curves and the calculated area under the ROC curves (AUC) were estimated using SPSS Statistics 18.0 with sensitivity (Se) and specificity (Sp) at the cut-off point to determine the feasibility of diagnosing IS in peripheral whole blood. Significance was assigned at the $P < 0.05$ level.

Results

Clinical characteristics

Demographic data are shown in Table 2. Healthy controls were comparable with patients with IS. However, the patients and the controls significantly differed in terms of systolic blood pressure (SBP) ($P = 0.002$).

lncRNA and mRNA Expression profiles of IS

lncRNA expression profile of IS. A total of 1, 250 differentially expressed lncRNAs obtained from peripheral blood were compared between patients with IS ($n = 10$) and healthy controls ($n = 10$) by using the criteria of absolute fold change ($|FC| \geq 2$, P value < 0.05 , and false discovery rate (FDR) < 0.05 . Among the differentially expressed lncRNAs between the IS patients and healthy participants, 560 were up-regulated and 690 were down-regulated. The top 25 (sorted by $|FC|$, P , and FDR) differentially expressed lncRNAs in overall samples are shown in Table 3. Another top 25 (sorted by $|FC|$, P , and FDR) differentially expressed lncRNAs in male/female samples are shown in Table S1-S2 (For all supplemental material see www.karger.com/10.1159/000495058/). Hierarchical clustering analysis was performed in 20 samples (10 patients with IS and 10 healthy controls). The results showed distinguishable lncRNA expression profiles (Fig. 1A). Fig. 1B and C present the scatter and volcano plots which were visualized to assess lncRNA expression variation between the two sample groups.

A forward analysis of distinguished lncRNAs based on their categorization was performed. All lncRNAs were classified into seven groups. Except for the undefined lncRNAs (66.21%), the highest proportion of lncRNAs belong to intergenic groups (21.81%), followed by natural antisense (4.95%), intronic antisense (4.07%), intron sense-overlapping (1.44%), bidirectional (1.12%), and exon sense-overlapping (0.40%) groups (Fig. 2A). The pie

Table 2. Clinical characteristics of participants. Data are mean value $\pm$ standard deviation. IS, ischemic stroke; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein. * To test the statistical significance of the comparisons between groups (healthy controls vs. IS patients), the independent-samples t test (normal distribution) or the Mann-Whitney U test (abnormal distribution) were used for quantitative variables and the χ^2 test was used for enumeration data

Terms	Samples for microarray and stage one validation		
	Healthy control (n=10)	IS patients (n=10)	P*
Age $\pm$ years	69.3 $\pm$ 5.9	63.2 $\pm$ 10.7	0.132
Sex (male/female)	7/3	7/3	1.000
Systolic blood pressure (mmHg)	128.0 $\pm$ 12.1	156.2 $\pm$ 21.9	0.002
Diastolic blood pressure (mmHg)	78.4 $\pm$ 8.8	88.2 $\pm$ 12.6	0.059
TC (mmol/L)	5.0 $\pm$ 0.9	5.2 $\pm$ 1.3	0.766
TG (mmol/L)	1.6 $\pm$ 0.8	1.9 $\pm$ 1.1	0.510
HDL (mmol/L)	1.2 $\pm$ 0.2	1.1 $\pm$ 0.3	0.766
LDL (mmol/L)	3.1 $\pm$ 0.8	3.4 $\pm$ 0.8	0.420
Glucose (mmol/L)	4.6 $\pm$ 0.6	6.2 $\pm$ 3.0	0.073

Table 3. Top 25 of differently expressed lncRNAs in IS patients compared to healthy controls (sorted by $|FC|$). $|FC|$, absolute fold change; FDR, false discovery rate

Up-regulated lncRNAs					Down-regulated lncRNAs				
Seqname	Gene symbol	$ FC $	P	FDR	Seqname	Gene symbol	$ FC $	P	FDR
T008148	G001747	8.187	0.001	0.010	NR_027076	LINC00928	17.393	<0.001	0.001
TCONS_00008492	XLOC_003959	8.040	0.003	0.022	NR_033851	SERPINB9P1	7.227	<0.001	0.005
ENST00000568297	RP11-386M24.6	7.868	0.011	0.047	TCONS_00015989	XLOC_007368	7.079	0.002	0.018
ENST00000442403	AC007064.24	7.726	0.001	0.012	TCONS_00029193	XLOC_013852	6.350	<0.001	0.002
T285334	G066582	6.738	0.011	0.047	ENST00000596781	CTB-50E14.4	6.327	<0.001	0.001
ENST00000565098	RP11-52401.4	6.432	<0.001	0.004	T250117	G057505	6.221	<0.001	0.001
T361606	G085412	6.221	0.005	0.028	NR_105049	C5orf66-AS1	5.974	<0.001	0.002
ENST00000424837	AP000282.3	6.092	0.002	0.015	T377137	G089185	5.714	<0.001	0.001
T234995	G054048	5.824	0.005	0.028	NR_110052	LOC101929106	5.707	0.001	0.012
ENST00000568243	RP11-408H20.1	5.641	0.007	0.034	T246988	G056794	5.556	<0.001	0.004
T024358	G005305	5.460	<0.001	0.004	NR_121674	ERICH6-AS1	5.509	0.003	0.021
T348942	G082295	5.447	0.008	0.037	T081704	G018886	5.489	<0.001	0.002
T162386	G037535	5.397	<0.001	0.003	T221263	G051177	5.462	<0.001	0.012
TCONS_00000918	XLOC_000160	5.350	0.007	0.036	NR_034023	LOC339807	5.454	<0.001	0.037
ENST00000404689	RP11-127B16.1	5.322	<0.001	0.006	TCONS_00015176	XLOC_007223	5.355	<0.001	0.014
T076452	G017605	5.053	<0.001	0.004	NR_117097	LINC01353	5.311	0.002	0.016
T268717	G062219	5.002	<0.001	0.007	ENST00000598616	AC007193.8	5.305	<0.001	0.002
T172822	G040151	4.996	0.011	0.046	T290706	G067949	5.055	<0.001	0.006
uc.402+	uc.402	4.807	0.002	0.016	uc.363-	uc.363	4.951	0.012	0.049
T202060	G046681	4.802	0.005	0.027	NR_033999	LOC100129148	4.930	<0.001	0.002
T184924	G042451	4.614	<0.001	0.003	ENST00000456932	DNM1P51	4.882	<0.001	0.002
T273869	G063597	4.521	<0.001	0.005	T276638	G064277	4.875	<0.001	<0.001
T055645	G012808	4.489	<0.001	0.004	T001293	G000157	4.773	<0.001	0.001
NR_120309	LOC102724467	4.478	<0.001	0.003	T299627	G070053	4.752	<0.001	<0.001
T074200	G017046	4.378	0.003	0.021	NR_121634	FOXO3-AS1	4.586	<0.001	0.004

Fig. 1. Differential expression of lncRNAs in IS patients ($n = 10$) and healthy controls ($n = 10$). (A) Heatmap of 1,250 differentially expressed lncRNAs between the two groups. (B) Scatter plot of differential lncRNA expression. X-axis: Test, Y-axis: Control. (C) Volcano plot of differential lncRNA expression. X-axis: $\log_2$ fold change (FC) (Test vs Control), Y-axis: $-\log_{10}$ (P value) for each probe. Each lncRNA is represented by a dot, up-regulated as red dots ($FC \geq 2$, $P < 0.05$, $FDR < 0.05$) and down-regulated as green dots ($FC \leq -2$, $P < 0.05$, $FDR < 0.05$). The black dots in the center area represent the remaining lncRNAs ($-2 < FC < 2$, $P < 0.05$, $FDR < 0.05$).

charts show the components of the up-/down-regulated lncRNAs in each category (Fig. 2B, C). The chromosome and length distribution of dysregulated lncRNAs are shown in Fig. 3A and B. lncRNAs concentrated in few chromosomes and possessed length of less than 4,000 nt. The distribution of the up-regulated and down-regulated lncRNAs was parallel.

Fig. 2. Classification of lncRNAs into 7 categories (bidirectional, exon sense-overlapping, intergenic, intron sense-overlapping, intronic antisense, natural antisense, and unknown) according to their relationships with protein-coding genes. (A) Numbers of identified lncRNAs in different categories. (B) Pie charts showing the components of up-regulated lncRNAs in each category. (C) Pie charts showing the components of down-regulated lncRNAs in each category.

Expression profile of mRNA in IS. In the same cohort of patients with IS ($n = 10$) and healthy controls ($n = 10$), we screened 733 significantly differentially expressed mRNAs through microarray analysis based on the criteria of $|FC| \geq 2.0$, $P < 0.05$, and $FDR < 0.05$ (Fig. 4A). Of these 733 mRNAs, 249 were up-regulated and 484 were down-regulated. The top 25 differentially expressed mRNAs in all samples are presented in Table 4. In addition, the top 25 (sorted by $|FC|$, P , and FDR) differentially expressed mRNAs in male and female samples are shown in Table S3 and S4. Fig. 4B and C show scatter and volcano plots, respectively, for assessment of variation in mRNA expression between the patients with IS and the healthy controls. The potential functions of differentially expressed lncRNAs were predicted by functional annotation for dysregulated mRNAs. Significantly up-regulated and down-regulated mRNAs in the microarray (setting $|FC| \geq 2$, $P < 0.05$, $FDR < 0.05$) were

Fig. 3. Chromosome and length distribution of dysregulation lncRNAs. (A) Chromosome distribution of up- and down-regulated lncRNAs. (B) Length distribution of up- and down-regulated lncRNAs.

Fig. 4. Differential expression of mRNAs in IS patients ($n = 10$) and healthy controls ($n = 10$). (A) Heatmap of 733 differentially expressed mRNAs between the two groups. (B) Scatter plot of differential mRNA expression. X-axis: Test, Y-axis: Control. (C) Volcano plot of differential mRNA expression. X-axis: $\log_2$ FC (Test vs Control), Y-axis: $-\log_{10}$ (P value) for each probe. Each mRNA is represented by a dot, up-regulated as red dots ($FC \geq 2$, $P < 0.05$, $FDR < 0.05$) and down-regulated as green dots ($FC \leq -2$, $P < 0.05$, $FDR < 0.05$). The black dots in the center area represent the remaining lncRNAs ($-2 < FC < 2$, $P < 0.05$, $FDR < 0.05$).

included to enrich GO functions (Fig. 5A-F) and KEGG pathways (Fig. 5G, H) by using DAVID bioinformatics online tools. Genes related to the up-/down-regulated mRNAs probably affected the IS process through significant GO categories or KEGG pathway ($P < 0.05$, that is $-\log_{10}P\text{-value} > 1.3$) (Table S5).

lncRNA qRT-PCR validation and ROC curves

At the first stage, nine differentially expressed lncRNAs were selected based on the criteria of absolute $FC \geq 2$, $P < 0.05$, and $FDR < 0.05$ (sorting by FC , P , and FDR with consideration of RNA source and length). qRT-PCR analysis was performed to determine the expression level of these lncRNAs obtained from peripheral blood samples of patients with IS ($n = 10$) and healthy controls ($n = 10$) (Fig. 6A). The significantly and differentially up-regulated lncRNAs were *ENST00000467369* (alias name: *HCP5*; $P = 0.005$, $FC = 2.667$), *ENST00000568297* (alias name: *RP11-386M24.6*; $P = 0.036$, $FC = 7.868$), *ENST00000568243*

Table 4. Top 25 of differently expressed mRNAs in IS patients compared to healthy controls (sorted by |FC|). |FC|, absolute fold change; FDR, false discovery rate

Up-regulated mRNAs					Down-regulated mRNAs				
Seqname	Gene Symbol	FC	P	FDR	Seqname	Gene Symbol	FC	P	FDR
NM_006865	LILRA3	20.570	0.005	0.031	uc009vmq.3	mir-34	9.228	<0.001	0.006
NM_002125	HLA-DRB5	20.143	0.007	0.039	NM_001753	CAV1	8.685	<0.001	0.004
NM_001099293	KIF4B	10.693	<0.001	0.001	NM_017805	RASIP1	7.273	0.004	0.027
ENST00000589616	LPHN1	8.814	<0.001	0.006	NM_001178139	TFDP2	6.669	<0.001	0.005
NM_152402	TRAM1L1	6.509	<0.001	0.006	NM_019008	MIEF1	6.647	<0.001	0.001
NM_001925	DEFA4	5.763	0.001	0.013	NM_001304388	GOLGA6L2	6.570	0.001	0.011
NM_031475	ESPN	5.611	<0.001	0.002	NM_145804	ABTB2	6.495	<0.001	<0.001
NM_001911	CTSG	5.125	0.003	0.024	NM_001282477	TMEM210	6.403	<0.001	0.004
NM_145286	STOML3	4.649	<0.001	0.005	NM_006404	PROCR	6.231	<0.001	0.006
NM_153269	C20orf96	4.480	<0.001	0.004	NM_001105247	ARMCS	5.765	0.004	0.027
NM_001008784	CD200R1L	4.358	<0.001	0.005	NM_012232	PTRF	5.571	0.003	0.023
NM_021069	SORBS2	4.222	0.009	0.045	NM_003881	WISP2	5.531	<0.001	0.003
NM_020747	ZNF608	4.016	<0.001	0.007	NM_002632	PGF	5.381	0.001	0.008
NM_001136493	MFS2A	4.011	<0.001	0.003	NM_022475	HHIP	5.300	0.006	0.036
NM_138787	C11orf74	3.977	0.010	0.04	NM_000424	KRT5	5.175	0.001	0.013
NM_144973	DENND5B	3.772	<0.001	0.002	NM_017950	CCDC40	5.108	<0.001	0.004
NM_002237	KCNG1	3.735	0.007	0.039	NM_017659	QPCTL	4.902	<0.001	<0.001
NM_006235	POU2AF1	3.631	0.001	0.013	NM_004995	MMP14	4.769	<0.001	0.001
NM_030764	FCRL2	3.514	<0.001	0.006	NM_178510	ANKK1	4.657	<0.001	0.004
NM_013363	PCOLCE2	3.507	0.001	0.012	NM_001077693	ECSCR	4.600	0.002	0.017
NM_004084	DEFA1	3.474	0.001	0.014	NM_001101401	SBK2	4.569	0.003	0.023
NM_002343	LTF	3.456	0.003	0.023	uc022bgl1	AX748309	4.563	<0.001	0.006
NM_001816	CEACAM8	3.394	0.001	0.009	uc003wfu.3	AF035281	4.555	0.003	0.024
NM_001126128	PROK2	3.386	<0.001	0.004	ENST00000529166	NPIPA2	4.549	0.001	0.009
NM_001098833	ATXN7L3	3.368	<0.001	<0.001	NM_001039840	CHIC1	4.502	<0.001	0.003

(alias name: *RP11-408H20.1*; $P < 0.001$, $FC = 2.395$), and *NR_046084* (alias name: *SH3BP5-AS1*; $P < 0.0001$, $FC = 2.175$). At the second stage, we expanded samples to validate the four significant lncRNAs by using 50 patients with IS and 50 controls. *ENST00000568297* ($P = 0.025$, $FC = 2.965$), *ENST00000568243* ($P = 0.017$, $FC = 1.302$), and *NR_046084* ($P = 0.004$, $FC = 1.902$) were significantly up-regulated in patients with IS compared with controls (Fig. 6B, C, D). The qRT-PCR results of these three lncRNAs, but not *ENST00000467369* ($P = 0.414$, $FC = -1.268$), are consistent with the microarray findings. We expanded the sample size to further validate the up-regulation of *ENST00000568297* ($P < 0.0001$, $FC = 7.300$), *ENST00000568243* ($P < 0.0001$, $FC = 1.841$), and *NR_046084* ($P < 0.0001$, $FC = 2.265$) in patients with IS compared with controls (Fig. 6E, F, G). These results were also consistent with the microarray analysis. Further, relative expression levels of the three significantly expressed lncRNAs were analyzed in sex subgroups (Fig. S1). These lncRNAs were also significantly up-regulated in male/female IS cases compared with male/female controls (*ENST00000568297*: $P = 0.0006/P = 0.0005$, $FC = 6.98/FC = 5.32$; *ENST00000568243*: $P < 0.0001/P < 0.0001$, $FC = 3.37/FC = 3.36$; *NR_046084*: $P < 0.0001/P = 0.0132$, $FC = 2.46/FC = 1.87$). Descriptions of the three significantly expressed lncRNAs are shown in Table 5.

As shown in Fig. 7A, B, and C, the ROC analysis indicated that the AUC values of *ENST00000568297* ($Sp = 63.6\%$, $Se = 64.8\%$), *ENST00000568243* ($Sp = 69.5\%$, $Se = 70.5\%$), and *NR_046084* ($Sp = 69.2\%$, $Se = 61.5\%$) were 0.733, 0.743, and 0.690, respectively. The AUCs of the combined ROC curves of *ENST00000568297* and *ENST00000568243* (Fig. 7D); *ENST00000568297* and *NR_046084* (Fig. 7E); *ENST00000568243* and *NR_046084* (Fig. 7F); and *ENST00000568297*, *ENST00000568243*, and *NR_046084* (Fig. 7G) were 0.776, 0.719, 0.787, and 0.843, respectively. Finally, the specificity (80.0%) and sensitivity (82.8%) were significantly improved; hence, the combination of the three whole lncRNAs was employed for IS diagnosis.

In addition, for the three validated lncRNAs that were up-regulated in IS patients compared to controls, correlation analysis with common clinical parameters (e.g. blood pressure, blood lipids, blood glucose) was performed in overall, male, and female samples. Most clinical parameters, including age, SBP, diastolic blood pressure, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, and glucose, were not significantly correlated with expression of the three lncRNAs ($P > 0.05$). Nevertheless, some significant correlations were found. Details are described in Table S6.

Fig. 5. Functional annotation of 249 up-regulated and 484 down-regulated genes from microarray. (A) Enriched GO terms ($-\log_{10} P \text{ value} > 1.3$) related to BP of up-regulated mRNAs. (B) Enriched GO terms ($-\log_{10} P \text{ value} > 1.3$) related to CC of up-regulated mRNAs. (C) Enriched GO terms ($-\log_{10} P \text{ value} > 1.3$) related to MF of up-regulated mRNAs. (D) Enriched GO terms ($-\log_{10} P \text{ value} > 1.3$) related to BP of down-regulated mRNAs. (E) Enriched GO ($-\log_{10} P \text{ value} > 1.3$) terms related to CC of down-regulated mRNAs. (F) Enriched GO terms ($-\log_{10} P \text{ value} > 1.3$) related to MF of down-regulated mRNAs. (G) Enriched KEGG pathways ($P < 0.05$) of up-regulated mRNAs. (H) Enriched KEGG pathways ($P < 0.05$) of down-regulated mRNAs.

Construction of CNC co-expression network

We constructed a co-expression network consisting of differentially expressed lncRNAs and their corresponding mRNAs (Section 3.2) to determine their potential functions in IS progression. The CNC network contains 679 lncRNAs (165 up-regulated and 514 down-regulated) and 561 mRNAs (136 up-regulated and 425 down-regulated). Fig. S2 and S3 show up-regulated CNC network and down-regulated CNC network, respectively. Fig. 8 shows the targeted CNC network of the three significantly expressed lncRNAs (*ENST00000568297*, *ENST00000568243*, and *NR_046084*). We performed GO functional annotation with mRNAs related to up-regulated lncRNAs within the CNC network. GO terms such as “regulation of branching morphogenesis of a nerve” and “positive regulation of dopamine receptor signaling pathway” were enriched. The mRNAs that interacted with *ENST00000568297*, *ENST00000568243*, or *NR_046084* significantly enriched several GO terms (Table S7).

Fig. 6. Relative expression level of (A) the 9 differentially up-regulated lncRNAs; (B, E) ENST00000568297; (C, F) ENST00000568243; (D, G) NR_046084. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$, **** represents $P < 0.0001$. (Notice: at stage one, only ENST00000568243 was performed with the ABI StepOnePlus Real-Time PCR System using stage two/three methods).

Table 5. Information of the 3 differentially expressed lncRNAs in qRT-PCR. FDR, false discovery rate; |FC|, absolute fold change; Reg, regulation; Chr, chromosome

Name	Gene Symbol	Source	P	FDR	FC	Reg	Length	Chr	Strand	Start	End
ENST00000568297	RP11-386M24.6	GENCODE	0.011	0.047	7.868	up	374	chr15	-	93138789	93139692
ENST00000568243	RP11-408H20.1	GENCODE	0.007	0.034	5.641	up	554	chr18	-	28638805	28639359
NR_046084	SH3BP5-AS1	RefSeq	<0.001	0.003	2.175	up	5592	chr3	+	15295690	15306005

Construction of lncRNA-miRNA-mRNA regulatory network

Using the three validated distinctly up-regulated lncRNAs (ENST00000568297, ENST00000568243, and NR_046084), we constructed a comprehensive lncRNA-miRNA-mRNA regulatory network to interact with 81 predicted miRNAs and 7,312 targeted mRNAs. Fig. S4 shows the three lncRNAs, 81 miRNAs, and 824 mRNAs (each of which interacted with at least five miRNAs). Specifically, lncRNA-ENST00000568297 targeted to 33 miRNAs, lncRNA-ENST00000568243 targeted to 21 miRNAs, and lncRNA-NR_046084 targeted to 28 miRNAs. Furthermore, THBS1 was regulated by 10 miRNAs, AKT2 was regulated by 9 miRNAs, PTEN was regulated by 7 miRNAs, AKT3 and MAPK1 were regulated by 5 miRNAs, and SMAD2 and SMAD3 were regulated by 6 miRNAs. The KEGG pathways (Fig. 9) were enriched with 824 mRNAs (each of which interacted with at least five miRNAs) in the lncRNA-miRNA-mRNA regulatory network. A total of 43 significant pathways ($P < 0.01$) were enriched using DAVID Bioinformatics Resources v6.8 online tools and included six IS-related pathways (Table 6): “PI3K-Akt signaling pathway”, “p53 signaling pathway”, “AMPK signaling pathway”, “MAPK signaling pathway”, “Wnt signaling pathway”, and “TGF-beta signaling pathway”. As shown in Fig. S5 and Table S9, the lncRNA-miRNA-mRNA-pathway regulatory network revealed indirect relations between the three distinguished lncRNAs and the six important KEGG pathways.

Fig. 8. CNC network with PCC ≥ 0.90 and $P < 0.05$. Triangles represent up-regulated lncRNAs; circles represent up-regulated mRNAs; lncRNAs and mRNAs in the co-expressed network are expressed in the same direction. Details are presented in Table S8.

apoptotic process”, which are related to biological process (BP); and “protein kinase activity”, which is related to molecular function (MF). In the lncRNA-miRNA-mRNA regulatory network, KEGG pathway analysis indicated that lncRNAs may regulate relative mRNAs via “PI3K-Akt signaling pathway”, “p53 signaling pathway”, “AMPK signaling pathway”, “MAPK signaling pathway”, “Wnt signaling pathway”, and “TGF-beta signaling pathway” to perform functions.

lncRNAs are dysregulated in several diseases, such as obesity, ischemic heart failure, moyamoya disease, and stroke [12-15]. Dykstra-Aiello explored the lncRNA expression profile of IS and stroke risk loci by using human peripheral blood obtained from American and Canadian subjects; the results identified 299 and 97 differentially expressed lncRNAs in males and females, respectively ($P < 0.005$, $|FC| > 1.2$) [12]. Affymetrix GeneChip Human Transcriptome 2.0 arrays ($> 40,000$ lncRNAs and $> 245,000$ coding transcripts) were used to detect significantly altered lncRNAs in the Dykstra-Aiello study; meanwhile, we used Arraystar Human lncRNA Microarray v4.0 (40,173 lncRNAs and 20,730 coding transcripts) in the present study. However, Dykstra-Aiello performed a microarray analysis without qRT-PCR validation, and therefore the microarray analysis may yield false-positive results. Experimental techniques such as qRT-PCR are essential to validate the microarray result. We identified differentially expressed lncRNAs according to strict and common criteria of $|FC| \geq 2$, $P < 0.05$, and $FDR < 0.05$. Recently, Deng identified novel lncRNA diagnostic biomarkers for acute ischemic stroke (AIS) in peripheral blood mononuclear cells (PBMCs) using Human lncRNA Array v4 (CapitalBio Technology, China) [16]. A total of 70 up-regulated and 128 down-regulated lncRNAs were identified by microarray to be differentially expressed in five AIS patients compared to five controls with a criterion of $|FC| > 2$ and $P < 0.05$. Deng included a total of 206 IS and 179 healthy control samples for the study and finally determined three lncRNAs (linc-DHFRL1-4, SNHG15, and linc-FAM98A-3) as IS diagnostic biomarkers. By comparison, although relatively fewer samples were included in the current study, potential lncRNA diagnostic markers were also established and further preliminary bioinformatics analysis was performed to explore potential functions of the three lncRNAs in IS pathology.

Circulating lncRNAs are a novel category of promising biomarkers and play key roles in screening, diagnosis, process, treatment, and prognosis of many diseases [17-19]. Serum lncRNA-*RP11-445H22.4* has been confirmed as a biomarker for detecting breast cancer and exhibited AUC of 0.904 (Sp = 74%, Se = 92%) [20]. Ying Zhang revealed lncRNA-*ZFAS1* and lncRNA-*CDRIAS* as novel predictors for acute myocardial infarction (AMI) in human whole blood; the AUC was 0.664 or 0.671 when AMI was diagnosed with lncRNA-*ZFAS1* or lncRNA-*CDRIAS* alone and increased to 0.691 when the two lncRNAs were combined [21]. We first evaluated the diagnostic value of lncRNAs in human peripheral blood samples for IS based on lncRNA microarray screening and qRT-PCR validations. The AUCs were as follows: *ENST00000568297* alone, 0.733; *ENST00000568243* alone, 0.743; *NR_046084* alone, 0.690; *ENST00000568297* and *ENST00000568243*, 0.776; *ENST00000568297* and *NR_046084*, 0.719; *ENST00000568243* and *NR_046084*, 0.787; combination of all, 0.843. The AUC of the combination of the three lncRNAs is significantly increased compared with that of each single lncRNA. At the optimal cut-off points, the sensitivity values of *ENST00000568297*, *ENST00000568243*, *NR_046084*, *ENST00000568297* and *ENST00000568243*, *ENST00000568297* and *NR_046084*, *ENST00000568243* and *NR_046084*, and the combination of all are 64.8%, 70.5%, 61.5%, 75.9%, 75.9%, 70.7%, and 82.8%, respectively; the specificity values are 63.6%, 69.5%, 69.2%, 75.0%, 67.5%, 75.0%, and 80.0%, respectively. The sensitivity (82.8%) and specificity (80.0%) of the combination of the three lncRNAs is significantly improved compared with single *ENST00000568297* or single *NR_046084*. This finding suggests that the combination of the three lncRNAs will provide higher diagnostic performance for IS diagnosis. Based on the correlation analysis these three potential lncRNA diagnostic biomarkers for IS were independent of some common clinical risk factors for IS, which may suggest the lncRNA biomarkers could be used for IS diagnosis independently.

Protein coding and non-coding RNAs regulate each other to perform their functions. For example, non-coding and coding RNAs connect to each other in the CNC network; as such, non-coding RNAs affect protein expression by regulating the effects of coding RNAs on diseases. Thus, gene functional annotation and pathway analyses are essential. Based on GO analysis, several gene functions related to BP, cellular composition (CC), and MF were enriched. With up-regulated mRNAs in the microarray, “positive regulation of c-Jun N-terminal kinase (JNK) cascade” was enriched and regarded as a significant GO term related to BP. JNK has been reported to play a critical role in ischemic brain injury [22]. The up-regulated mRNAs in the microarray and mRNAs related to up-regulated lncRNAs within the CNC network both enriched the GO term “positive regulation of dopamine receptor signaling pathway”, related to BP. An increased level of dopamine D2 receptor, which as a part of the dopamine receptor signaling pathway acts as a key target in the regulation of neuroinflammatory response, had a neuroprotective effect in OGD-treated astrocytes and MCAO-treated mice [23]. Focused on the three validated lncRNAs (*ENST00000568297*, *ENST00000568243*, and *NR_046084*), several GO categories that have been reported to be associated with the pathological mechanism of IS were enriched significantly. Interestingly, the category named “cellular response to oxidative stress” supported that lncRNAs participate in the process of oxidative stress in cells [24], probably through regulating mRNAs such as *SLC25A24*, *LRRK2*, and *SETX*. Kim reviewed and indicated that lncRNAs play important roles in cellular responses to oxidative stress; for example, cytoplasmic lncRNA *LINK-A* interacts with *LRRK2* and was able to enhance phosphorylation of HIF-1 α at Ser797 in signal transduction under normoxic conditions [25]. In addition, the three lncRNAs-interacted mRNAs within the up-regulated CNC network could also enrich some other categories of importance, such as “MAPK cascade” and “protein phosphorylation”. Apoptotic stress induced *ASK1* (alias: *MAP3K5*) activation is promoted by Trx1siRNA, performing as *ASK1*/Trx1 binding complex, and ischemic reperfusion destroyed the complex and led to attenuation of the *ASK1*-JNK/p38 signaling pathway (part of the MAPK cascade) [26]. As well, protein phosphorylation plays key roles in MAPK signaling activation.

Analysis of the KEGG pathway related to 824 mRNAs (each of which interacted with ≥ 5 miRNAs, targeted to lncRNA-*ENST00000568297*, lncRNA-*ENST00000568243*, and lncRNA-*NR_046084*) in the lncRNA-miRNA-mRNA regulatory network enriched several interesting items. In particular, the following six stroke-related signaling pathways were outstanding: “PI3K-Akt signaling pathway”, “p53 signaling pathway”, “AMPK signaling pathway”, “MAPK signaling pathway”, “Wnt signaling pathway”, and “TGF-beta signaling pathway”. These pathways were enriched by some prominent mRNAs, such as *AKT2*, *AKT3*, *MAPK1*, *PTEN*, *SMAD2*, *SMAD3*, and *THBS1*, which are known to be associated with IS.

The MAPK signaling pathway has been implicated in inflammatory responses and cell apoptosis [27, 28]. Anti-*CKLF1* antibody inhibited neutrophil influx into the ischemic region via the MAPK signaling pathway, and the inhibition of *CKLF1* activity significantly protected against ischemia/reperfusion injury [29]. In rat brains, CBSA-PEG-TIIA-NPs (cationic bovine serum albumin-conjugated tanshinone IIA PEGylated nanoparticles) displayed distinguished neuroprotective effects on IS by modulating MAPK signaling pathways involved in the neuroinflammatory cascade [30]. Yaning Li reported the *SDF-1 α* -*CXCR4* interaction to activate *AKT*, *ERK*, and p38 MAPK signaling pathways in post-acute ischemic mice; *SDF-1 α* could promote neurogenesis and angiogenesis without eliciting inflammatory responses [31]. Lin revealed that the p38 MAPK/calpain 1 signaling pathway participated in endogenous neurorepair and spontaneous recovery after IS, mediated by *IL-17A* from astrocytes [32]. Both the MAPK and PI3K/Akt signaling pathways are involved in cerebral ischemic injury associated with *TRPV4*-induced apoptosis [33]. The PI3K/Akt/mTOR signaling pathway mediates *ZSTK474* (a *PI3K* inhibitor) to inhibit inflammatory responses, reduce infarct volume, and exert neuroprotection in IS mice [34]. Akt phosphorylation was found to mediate the neuroprotective activity of melatonin, and melatonin inhibited p53 phosphorylation through the PI3K/Akt signaling pathway to increase neuronal survival [35]. The up- and down-stream components of PI3K/Akt signaling pathways, such as *PTEN* and *mTOR*, were

also activated. TGF- β acted as an anti-inflammatory factor. Myeloid TGF β signaling has been suggested to be utilized for interference with stroke etiology-associated inflammation or vascular diseases [36]. The central intracellular mediator of TGF- β signaling, namely, *SMAD4*, has been implicated in maintaining cerebrovascular integrity [37].

In the pathophysiology of stroke, p53 acts as master regulator in cell death [38]. Balaganapathy demonstrated that the Notch intracellular domain (NICD)-p53 interaction contributed to IS pathogenesis and enhanced the expression of pro-apoptotic genes following IS [39]. In rat cerebral cortical neurons, the ANXA1-p53 interaction in the nucleus was enhanced following oxygen-glucose deprivation-reoxygenation (OGD/R) and induced cell death [40]. The Wnt signaling pathway is involved in the development of the blood-brain barrier, contributed to stimulating neurogenesis, consolidating the blood-brain structure, and recovering cognitive brain functions after central nervous system injury [41-43]. Chang indicated that the Gpr124-Wnt signaling axis is essential for protecting blood-brain barrier function and vascular integrity [44]. In general, the AMPK signaling pathway is considered important in treatment of IS; metformin activated the AMPK signaling pathway to down-regulate *ICAM-1* expression, thereby effectively alleviating neutrophil infiltration to prevent brain injury induced by ischemia [45].

The regulatory mechanism between the upper coding and non-coding RNAs remains undetermined. We inferred that the three potential diagnostic biomarkers (*ENST00000568297*, *ENST00000568243*, *NR_046084*) participated in the pathophysiological processes of IS, possibly by regulating IS-related signaling pathways or pivotal miRNAs and mRNAs.

Limitations

MiRNAs included in the regulatory network were predicted by algorithmic model in comprehensive databases, and experimental validations in Han Chinese were performed well. We performed some of the basic bioinformatics analyses. The CNC co-expression network and the lncRNA-miRNA-mRNA regulatory network were constructed; however, further experiments (*in vivo* or *in vitro*) must be performed to validate the functions of the three lncRNAs. The three lncRNAs are potential biomarkers for diagnosis of general IS patients. Further analysis such as exploring the association of IS subtypes and the lncRNAs is required. Thus, we will explore the ceRNA mechanism of the lncRNA-miRNA-mRNA regulatory network by using experimental technologies, such as luciferase report gene, RNA RIP, and RNA pull down.

Conclusion

This study found that the lncRNA and mRNA expression profiles in human peripheral blood were altered after IS. Three IS-associated lncRNAs, namely, *ENST00000568297*, *ENST00000568243*, and *NR_046084*, were validated and identified as novel potential biomarkers for diagnosis of IS. These lncRNAs probably play important roles in IS pathophysiology by regulating IS-related pathways, mRNAs in the CNC co-expression network, or pivotal miRNAs and mRNAs within lncRNA-miRNA-mRNA regulatory networks.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (No. 81860822, No. 81473670 and No. 81573756).

Disclosure Statement

All authors declare no conflicts of interest.

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