Identification of lncRNA-mRNA regulatory network associated with isolated systolic hypertension and atherosclerotic cerebral infarction

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Background: Increasingly, evidence has shown that long non-coding RNAs (lncRNAs) play an important role in isolated systolic hypertension (ISH). However, a systematic lncRNA-messenger RNA (mRNA) regulatory network is still absent in isolated systolic hypertension and atherosclerotic cerebral infarction patients (ISH & ACI).This research aimed to establish a lncRNA-mRNA co-expression network in patients with ISH & ACI, to probe into the potential functions of lncRNA in such patients.

Methods: Expression profiles of lncRNA and mRNAs were collected and compared, from 8 patients with ISH and 8 patients with ISH & ACI by RNA-seq data. Differentially expressed lncRNAs and mRNAs were screened out via high-throughput sequencing in the plasma of ISH/ACI patients and control ISH patients. Then, a lncRNA-mRNA interaction network was built using the Pearson correlation coefficient by Cytoscape software. The expression levels of the hub genes and lncRNAs were verified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in another 10 ISH/ACI patients and 10 control patients. This study was approved by the responsible institutional review board (IRB) and informed consent was provided by participants.

Results: A total of 2,768 differentially expressed lncRNAs and 747 differentially expressed mRNAs were identified. We identified two hub genes (*CD226* and *PARVB*) and 11 lncRNAs in the lncRNA-mRNA interaction network. The results of qRT-PCR and cell assay verified that lncRNAs ENST00000590604 and CD226 are highly expressed in patients of ISH & ACI. Further, CD226 was associated with vascular endothelial cells growth and stability through the platelet activation and focal adhesion pathway.

Conclusions: We established a novel mRNA-lncRNA interaction network. The lncRNAs ENST00000590604 and CD226 might be the potential biomarkers of ISH & ACI.

Keywords: Isolated systolic hypertension (ISH); atherosclerotic cerebral infarction (ACI); long non-coding RNA (lncRNA); messenger RNA (mRNA); heart and cerebral vessels

Introduction

Isolated systolic hypertension (ISH) is a major risk factor for cardiovascular morbidity and mortality, and the most common form of hypertension in the elderly population (1,2). With an ageing population, the prevalence of hypertension, particularly ISH, is rapidly rising (2). Besides, ISH is associated with cerebrovascular diseases, such as isolated systolic hypertension and atherosclerotic cerebral infarction patients (ISH&ACI) (2).

However, the underlying molecular mechanisms of ISH&ACI have not been elucidated. Therefore, we sought the key molecules involved in ISH&ACI and identified potential diagnostic biomarkers and therapeutic targets for ISH&ACI.

Long non-coding RNAs (lncRNAs) are a class of lncRNA longer than 200 nucleotides. The lncRNAs are involved in many cellular processes, including chromatin remodeling, transcription modulation, and interacting with other RNAs to modulate gene expression (3). With the rapid technological advances in high-throughput sequencing, the study of lncRNAs has made remarkable progress (4). Accumulated experimental evidence shows that lncRNAs play an important role in the development of hypertension and might be the potential key regulators of hypertension (5,6). The lncRNA TUG1/miR-145-5p/FGF10 regulates the proliferation and migration of vascular smooth muscle cell in hypertension by activating the Wnt/β-catenin pathway (6). Down-regulation of MRAK048635_P1 is probably an important factor for vascular remodeling in essential hypertension (7). Nevertheless, the relationship between lncRNAs and ISH has not yet been reported.

In this paper, 946 up-regulated and 1,822 down-regulated differentially expressed lncRNAs, and 288 up-regulated and 519 down-regulated differentially expressed mRNAs were identified between ISH&ACI cases and ISH controls, respectively. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses revealed that differentially expressed lncRNAs and mRNAs were significantly enriched in platelet activation. According to the lncRNA-mRNA co-expression network, two hub genes (*CD226* and *PARVB*) were identified and 9 lncRNAs were predicted to target these two hub genes. Then, the lncRNA-mRNA pairs were verified by real-time polymerase chain reaction (qPCR) in 20 individual samples. Besides, flow cytometer (FCM) was performed to evaluate proliferation and apoptosis rate and qPCR was performed to validate the lncRNA-mRNA pairs in human brain microvascular endothelial cells (HBMECs).

In summary, our results showed that lncRNAs ENST00000590604 regulated the expression of *CD226* through platelet activation and focal adhesion pathway. The lncRNAs ENST00000590604 and *CD226* might be the potential biomarkers of ISH and atherosclerotic cerebral infarction patients.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5176).

Methods

Study samples

A total of 18 ISH&ACI patients were recruited from Affiliated Hospital of Shandong University of Traditional Chinese Medicine from May 2019 to April 2020. We defined ISH and ACI according to the 2018 revised edition of the Chinese guidelines for the prevention and treatment of hypertension (exposure draft) and 2015 China Cerebrovascular Disease Classification. Meanwhile, patients conforming to the following standards were included: (I) aged 18 years or above, no gender limitations; (II) within 2 weeks to 6 months after the occurrence of cerebral infarction; (III) willing to participate in this experiment, cooperate with the doctors, and sign the informed consent in writing. A total of 18 ISH controls were recruited from the same hospital during the same period. We randomly selected 8 ISH&ACI patients and 8 ISH controls from 18 ISH&ACI patients and 18 ISH controls for RNA sequencing, and the remaining samples were used as replication population for the validation of differentially expressed lncRNAs and mRNAs. Among the participants selected this time, 21 were male and 15 were female. All participants were aged about 50–90 years.

The study was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (ethical review approval number: AF/SC-08/03.0). Written informed consent was provided by all participants or their families under the Declaration of Helsinki (as revised in 2013).

RNA extraction, library preparation, and RNA-seq

We extracted RNA from plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The RNA

was then purified and concentrated using the RNeasy MinElute Cleanup Kit (QIAGEN, Germany). The quality of RNA was evaluated using NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA). Sequencing libraries were generated using TruSeq Stranded Total RNA LT - (with Ribo - Zero TM Gold) - Set A (Illumina, San Diego, CA, USA) following the manufacturer's recommendations. The libraries were then sequenced on the Illumina Hiseq 2500 platform and 125 bp/150 bp paired-end reads were generated.

Quantitative real-time PCR validation

Quantitative real-time PCR (qRT-PCR) was used to validate selected lncRNAs and mRNAs in 20 individual samples. Reverse transcription was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (SparkJade Scientific Instruments Co., Ltd., Shandong, China). Then, qRT-PCR was performed using a TB Green® Premix Ex Taq™ II (SparkJade, Shandong, China) and an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher, USA) according to standard methods. Specific primers of lncRNAs and mRNAs are listed in Table S1. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold-change to the house-keeping gene (β-action).

Cell experiments

Cell culture

The HBMECs (Hunan Fenghui Biotechnology Co. Ltd., Hunan, China, Cat.CL0116) were cultured with high glucose (4.5 mg/mL) Dulbecco's modified Eagle medium (DMEM; Gibco, Amarillo, TX, USA, REF.#C11995065BT) and supplemented with 10% fetal bovine serum (FBS; Gibco, REF.10099141) and 1% penicillin/streptomycin solution (P/S; HyClone, Logan, UT, USA, Cat. #SV30010) at 37 ℃ in a humidified 5% CO_2 incubator. Then, HBMECs were intervened by sodium pyruvate solution Ang II (Solarbio Co., Ltd., Beijing, China) and oxygen-glucose deprivation/reoxygenation (OGD/R). We used HBMECs exposed to Ang II and normoxia as controls.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The effect of Ang II and OGD/R on proliferation were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) assay. The HBMECs were seeded at a density of 1,500 cells per well into 96-well

microplates in a final volume of 100 μL with 2×10^{-6} mol/L of Ang II, and they were maintained for 12, 24, 48, or 72 h at 37 ℃ in a humidified atmosphere containing 5% CO_2. After that, HBMECs were cultured in Earle's Balanced Salt Solution (EBSS, Solarbio, Cat.H2025) without glucose and FBS and deprived of oxygen by transferring into an anaerobic incubator (0% O_2/5% CO_2/95% N_2) for 4 h. After OGD, cells were returned to a normoxic incubator under 5% CO_2/95% air for 24 h in complete medium. Subsequently, to monitor cell viability, 10 μL of sterile MTT (Solarbio, Lot.#303H0525, Beijing, China) at a final concentration of 5 mg/mL was added to each well for incubation at 37 ℃ for 4 h; this was followed by the administration of 150 μL dimethyl sulfoxide solution (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, Cat. #30072418, Shanghai, China) per well to dissolve the formazan crystals. The optical density (OD) value was measured at 562 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The OD-value indicated the counts of living cells.

Apoptosis rate assay

The effect of Ang II and OGD/R on apoptosis rate was assessed using Annexin V staining by Muse Cell Analyzer (Merck, Darmstadt, Germany Millipore, Burlington, MA, USA), according to the manufacturer's directions.

qRT-PCR

To validate the sequence data, 11 differentially expressed genes(DEGs) related to endothelial dysfunction were chosen for qRT-PCR, including 8 lncRNAs (ENST00000565493, ENST00000606054, ENST00000590604,NONHSAT138949.2, ENST00000566942, ENST00000369385, NONHSAT217189.1, ENST00000540082) and their corresponding target mRNAs (*CD226, PARVB*). After being cultured in 6-well plates at a density of 6,000 cells/cm², total RNAs from model cells and control cells were extracted using SPARKeasy Improved Tissue/Cell RNA Kit (Haosail Science Co., Shandong, China, Lot.YADKZ). PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Cat.#RR047A) and reverse transcription reaction according to the manufacturer's standard operation process was used to synthesize the DNA template. TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Kusatsu, Shiga, Japan, Cat.#RR820A) was used to mplify qRT-PCR following the manufacturer's instructions. The expression of lncRNA was normalized

to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described in the literature. Complementary DNA (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany, Cat. #04379012001), and qRT-PCR was performed by using LightCycler 480 SYBR Green I Master (Roche, Cat. #04707516001), according to the manufacturer's instructions, using a LightCycler480 instrument (Roche). The primer sequences are listed in Table S1 (Sangon Biotech, Shanghai, China). The data were calculated and presented by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analyses were performed using the R 3.6.0 (http://www.R-project.iorg/). The two-tailed Student's t-test was used for normally distributed variables and the Wilcoxon Rank-Sum test was used for abnormally distributed data. Data normality was evaluated using the Shapiro-Wilk test. Differences between the case and control group were considered significant at P value <0.05.

Results

Identification of DEGs and differentially expressed lncRNAs

Read counts obtained from RNA-seq were normalized as fragments per kilobase of transcript per million mapped fragments (FPKM) (8). The FPKM was used to identify the differentially expressed lncRNAs (DELs) and differentially expressed mRNAs in ISH&ACI cases (n=8) and ISH controls (n=8). The DELs and differentially expressed mRNAs met the following cutoff criteria: P value <0.05 and |Fold-Change| >2 (9). A total of 2,768 DELs (946 up-regulated and 1,822 down-regulated) and 747 differentially expressed mRNAs (288 up-regulated and 519 down-regulated) were identified between ISH&ACI cases and ISH controls. Volcano plots were used to visualize the distribution of the DELs (*Figure 1A*) and differentially expressed mRNAs (*Figure 1B*). The hierarchical clustering plots showed clear separation and consistency in the expression profiles of ISH&ACI cases and ISH controls (*Figure 1C,1D*).

GO and KEGG pathway analysis

We performed GO and KEGG enrichment analyses by hypergeometric test to find significantly enriched GO terms and KEGG pathways of DELs and differentially expressed mRNAs. By GO enrichment analysis, DELs and differentially expressed mRNAs were classified into three categories: cellular component (CC), molecular function (MF), and biological process (BP). The potential mRNAs targets of DELs were predicted using cis-specific binding modules and trans-specific binding modules. The intersection of targets of DELs and differentially expressed mRNAs were used to perform enrichment analysis.

As shown in *Figure 2A* and Table S2, GO enrichment analysis identified 42 significant GO terms (P value ≤0.05). In BP, the top 4 GO terms were positive regulation of blood coagulation, positive regulation of coagulation, cellular response to glucagon stimulus, and platelet degranulation; in CC, the top 4 GO terms were platelet alpha granule, platelet alpha granule lumen, secretory granule lumen, and cytoplasmic membrane-bounded vesicle lumen; in MF, the top 2 GO terms were integrin binding and cell adhesion molecule binding. The KEGG enrichment analysis identified 6 significant KEGG pathways (*Figure 2B* and Table S3). The 6 significant KEGG pathways were arrhythmogenic right ventricular cardiomyopathy, platelet activation, extracellular matrix (ECM)-receptor interaction, hypertrophic cardiomyopathy, dilated cardiomyopathy, and focal adhesion. The enrichment analyses showed that the intersection of targets of DELs and differentially expressed mRNAs were mainly associated with platelet activation.

LncRNA-mRNA co-expression network construction and hub genes analysis

We calculated the Pearson correlation coefficient using the expression value of lncRNAs and mRNAs. We identified lncRNA-mRNA co-expression with coefficient great than 0.9 and the P value less than 0.05. According to the results of lncRNA-mRNA co-expression, we then constructed the lncRNA-mRNA network using Cytoscape software (https://cytoscape.org/). We identified 2 hub genes in the network, *CD226* and *PARVB* (*Figure 3*).

The hub gene *CD226* (also known as DNAM-1, PTA-1, or TLiSA-1) is an immunoglobulin-like glycoprotein. It was shown to be expressed on the platelet membrane and involved in platelet activation (10,11). Platelets play a crucial role in the control of acute hemorrhage and thrombosis, and thus are involved in atherosclerosis and myocardial infarction (12). It may be that *CD226* participates in atherosclerotic cerebral infarction. The other hub gene, *PARVB* (also known as beta-parvin and affixin) is a member of the parvin family of
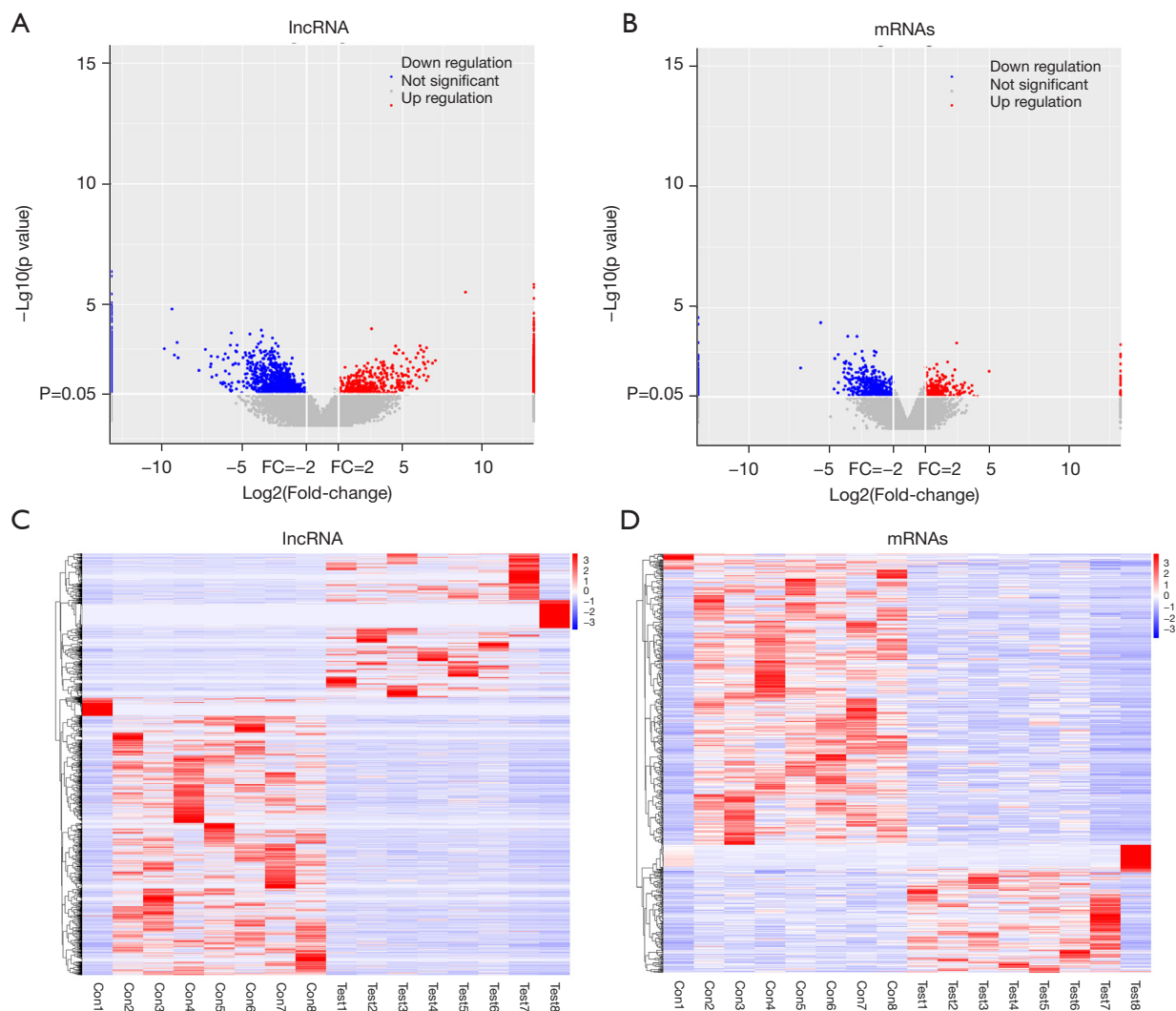
Figure 1 Differentially expressed lncRNAs and mRNAs in ISH&ACI cases (n=8) and ISH controls (n=8). (A) Volcano plots of differentially expressed lncRNAs. The X-axis is log2 Fold-Change and the Y-axis is log10p-value. Red (up-regulated) and blue (down-regulated) points indicated the differential expression lncRNA; (B) Volcano plots of differentially expressed mRNAs; (C) differentially expressed lncRNAs hierarchical clustering, each column represents a sample and every row represents a lncRNA. Red indicates high relative expression and blue indicates low relative expression; (D) differentially expressed mRNAs hierarchical clustering. ISH&ACI, isolated systolic hypertension and atherosclerotic cerebral infarction; DEGs, differentially expressed genes; DELs, differentially expressed lncRNAs; lncRNAs, long non-coding RNAs; mRNAs, messenger RNAs.

actin-binding proteins, which plays a role in cytoskeleton organization and cell adhesion (13). It was shown to be associated with coronary restenosis, hematoma volume, and spontaneous intracerebral hemorrhage (14,15).

The validation of expression profiles of lncRNAs

We performed qRT-PCR for further validation of 9 selected differentially expressed lncRNA-mRNA pairs in 20 individual samples (*Table 1*). We found that the expression of *CD226* and lncRNAs ENST00000590604 were significantly up-regulated in the cases compared to controls (*Figure 4*). The results of lncRNAs ENST00000590604 and *CD226* were consistent with the results of RNA-seq data. The hub gene *CD226* was significantly upregulated in ISH&ACI patients.
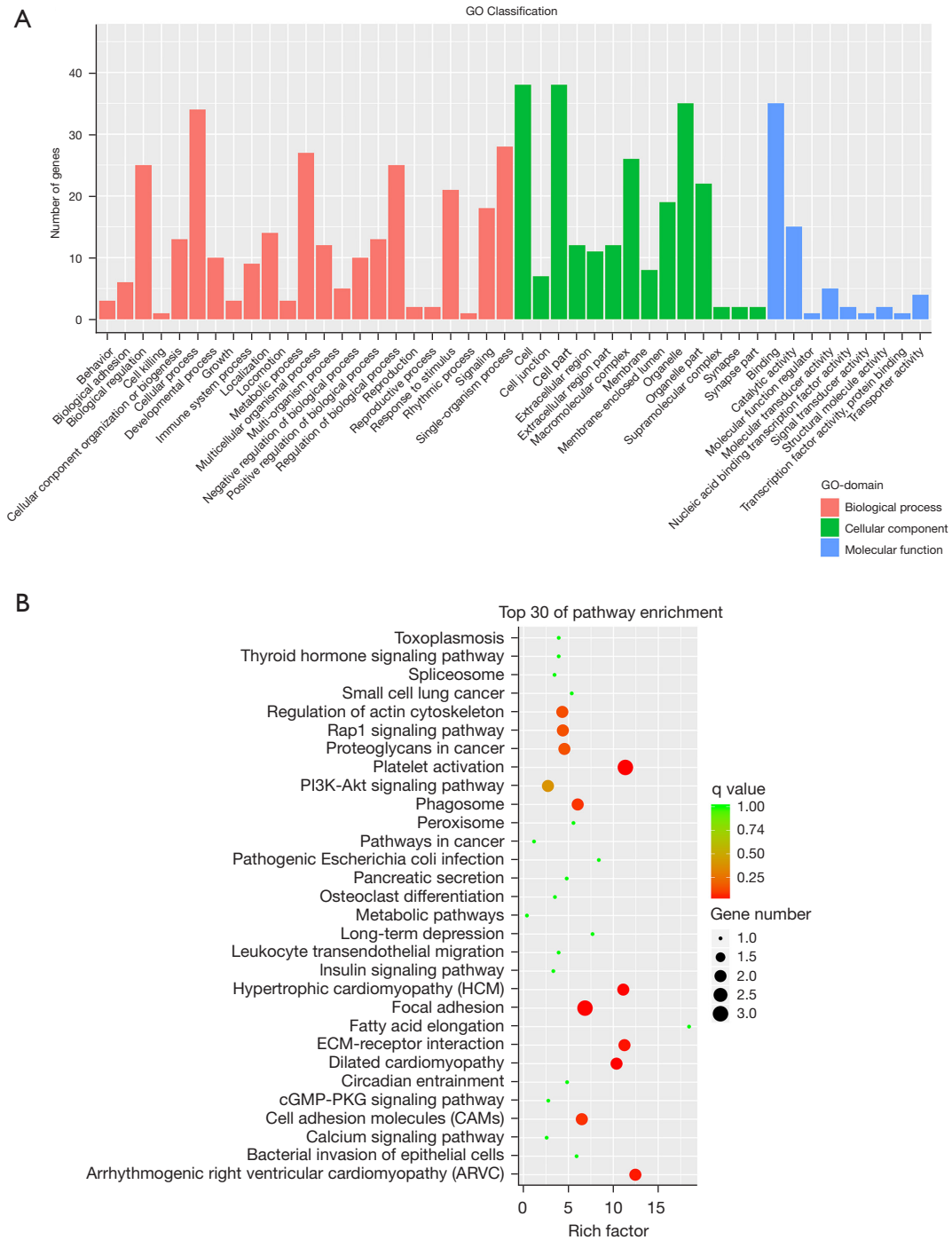
Figure 2 GO and KEGG pathway enrichment analysis. (A) GO enrichment contains 3 terms: cellular component, molecular function, and biological process; (B) the top 30 enriched KEGG pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
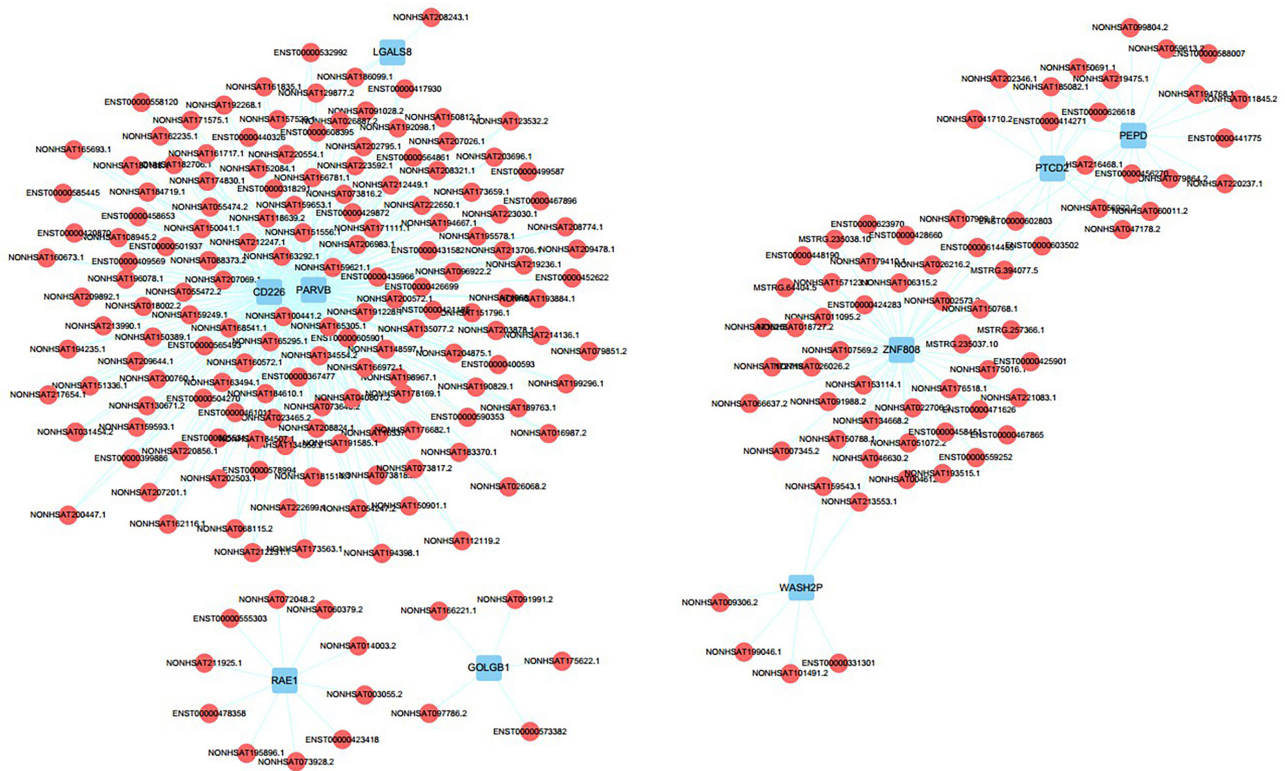
Figure 3 LncRNA-mRNA co-expression network. Red indicates lncRNAs and blue indicates mRNAs. The top 2 hub genes are CD226 and PARVB. lncRNAs, long non-coding RNAs; mRNAs, messenger RNAs.

Table 1 Differentially expressed lncRNA-mRNA pairs

Target	lncRNAs
CD226	ENST00000565493, ENST00000606054, ENST00000590604, NONHSAT217189.1, ENST00000566942
PARVB	ENST00000565493, NONHSAT138949.2, ENST00000369385, ENST00000540082

The effects of Ang II and OGD/R on HBMECs

We performed MTT assay and FCM to evaluate proliferation and apoptosis rate. The results indicated that, compared with the control group, HBMECs' growth was inhibited and apoptosis rate was increased, which were treated with OGD/R (*Figure 5*).

qRT-PCR validation of significant DELs and differentially expressed mRNAs of HBMECs

The qRT-PCR results using HBMECs were consistent with the RNA-Seq results using plasma of patients, including lncRNA ENST00000590604 and *CD226* (*Figure 6*).

Discussion

Summary

Hypertension has become one of the most important risk factors for cardiovascular and cerebrovascular accidents in the world today and contributes to an elevated risk of stroke, myocardial infarction, heart failure, and renal failure (16,17). Globally, hypertension affects 31.1% of adults (18). The cost of hypertension has an enormous public health burden. In elderly men and young adults, ISH is the most common type of hypertension (19).

The emerging evidence indicates that lncRNAs play a critical role in various biological functions and disease processes (20). For example, HOTAIR (HOX transcript
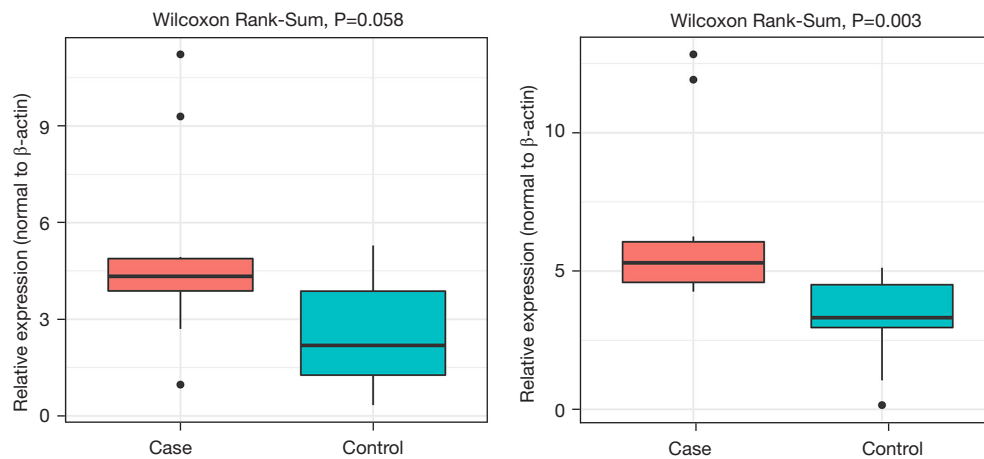
Figure 4 Relative levels of mRNAs and lncRNAs in 10 cases and 10 controls. Using qRT-PCR to calculate the relative fold-change to the house-keeping gene (β-action). From left to right are lncRNA ENST00000590604 and CD226. mRNA, messenger RNA; lncRNA, long non-coding RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.
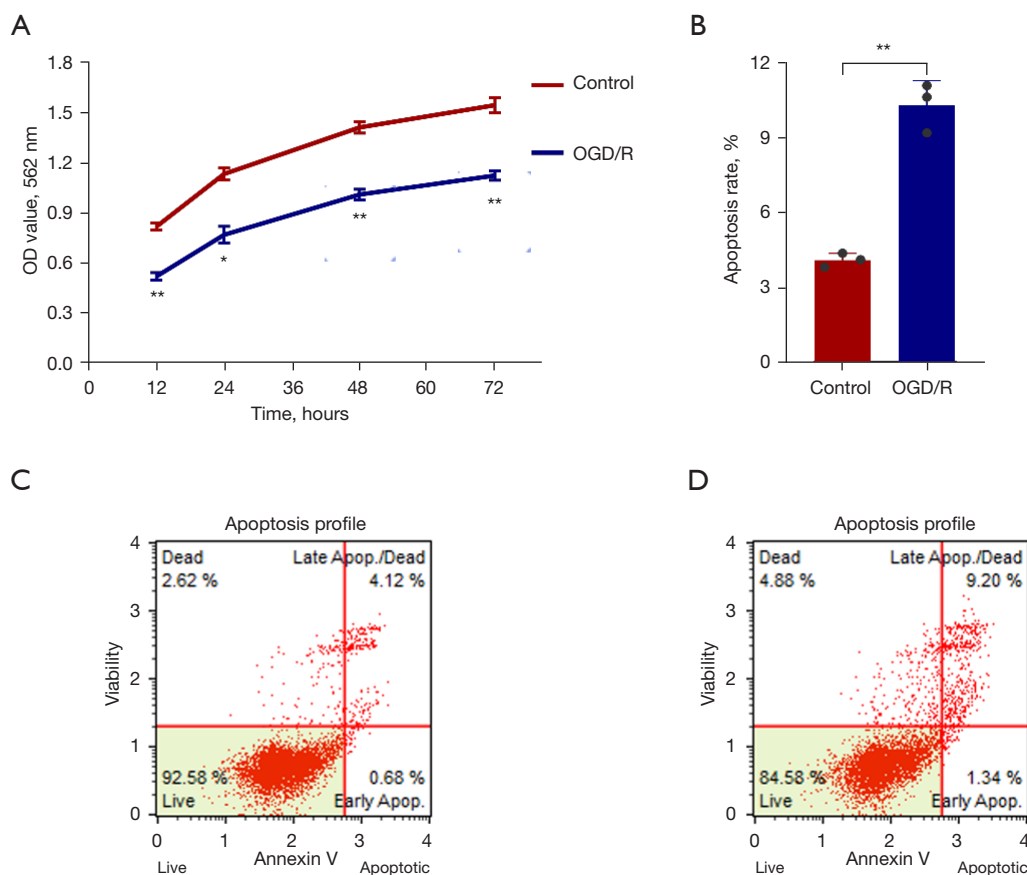
Figure 5 The effect of OGD/R on proliferation and apoptosis rate of HBMECs. (A) HBMECs' viability was declined after OGD/R compared with control group (*P<0.05, **P<0.01 *vs.* Control group). (B) The apoptosis rate of OGD/R group was increased compared with control group (**P<0.01 *vs.* Control group). (C) Control group apoptosis results. (D) OGD/R group apoptosis results. OGD/R, oxygen-glucose deprivation/reoxygenation; HBMECs, human brain microvascular endothelial cells.
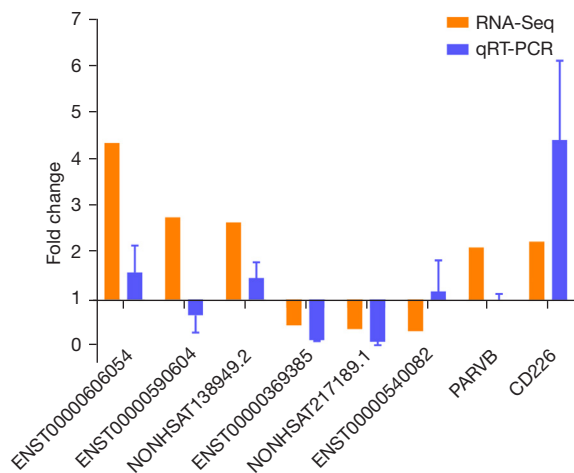
Figure 6 qRT-PCR validation (n=10) of DEGs compared to RNA-Seq (n=8). Yellow represents the expression of DELs and DE mRNAs in the ISH complicated with stroke group versus the ISH group by RNA-Seq, blue represents the expression of DELs and DE mRNAs in the Ang II and OGD/R group versus the control group by qRT-PCR. qRT-PCR, quantitative reverse transcription-polymerase chain reaction; DEGs, differentially expressed genes; DELs, differentially expressed long non-coding RNAs; DE, differentially expressed; ISH, isolated systolic hypertension; OGD/R, oxygen-glucose deprivation/reoxygenation.

antisense intergenic RNA) is one of the most well-studied lncRNAs that are overexpressed in a variety of cancers (21). Though the significance of lncRNAs is being increasingly found in hypertension, the expression profile and the mechanism of lncRNAs remain unclear in ISH.

In this study, our RNA-seq data revealed multiple lncRNAs dysregulated in ISH&ACI. The GO and KEGG enrichment analyses found that the intersection of the targets of DELs and DE mRNAs were mainly associated with platelet activation, ECM-receptor interaction, and focal adhesion. Platelet activation signaling pathway plays a critical role in the function of platelets in hemostasis and thrombosis (22). Activated platelets aggregate at the site of endothelial cell erosion, stimulating thrombus formation and promoting atherothrombotic disease (23). The focal adhesion signaling pathway promotes cell migration and angiogenesis (24,25).

We also found 2 upregulated hub genes in ISH&ACI (*CD226* and *PARVB*) by the lncRNA-mRNA co-expression network. The gene *CD226* is an immunoglobulin-like glycoprotein and mediates platelet and megakaryocytic

cell adhesion to vascular endothelial cells (11,26). Research has suggested that platelet-derived miR-142-3p regulating apoptosis of endothelial cells in hypertension and the dysfunction of vascular endothelial cells play crucial roles in vascular remodeling during hypertension (27,28). The *PARVB* gene has been found to affect human endothelial cell shape and motility, suggesting an important role of *PARVB* in vessel growth and vessel stability (29,30).

We further validated the results by qRT-PCR in another group of 20 patients. FCM was also performed to evaluate proliferation and apoptosis rate in HBMECs cells and real-time PCR was performed to validate the lncRNA-mRNA pairs in HBMECs cells. Here, we found lncRNAs ST00000590604 regulated the expression of *CD226*. The *CD226* gene was associated with vascular endothelial cells growth and stability through platelet activation and focal adhesion pathway. The lncRNAs ENST00000590604 and *CD226* could be the potential biomarkers for ISH&ACI and this finding provides a new idea for the clinical treatment of ISH&ACI.

Limitations

There were several limitations to this study, as follows: the cases included in this research were limited in number, thus a larger sample size is necessary for future research to generate more reliable results. Also, since no medication targeted the differentially expressed lncRNAs or mRNAs, further research on the pharmacological mechanism is still needed to unveil the essential roles of these potential biomarkers. This study did not explore the relative expression of lncRNA associated with disease risk, severity, and inflammatory cytokine levels in patients with systolic hypertension with atherosclerotic cerebral infarction. No animal experiments were performed to verify the results. This paper did not explore the relationship between polymorphisms of lncRNA gene and isolated systolic hypertension and atherosclerotic cerebral infarction. There is no condition to add co-expression module analysis.

Comparison with existing literature

Past research has mostly focused on the differential expressions of lncRNAs and mRNAs in ACI patients and healthy controls or in hypertension patients and healthy controls. The differential expressions of lncRNAs in ISH&ACI patients and ISH patients have not been investigated. We initiated the probe into the differentially

expressed lncRNAs and mRNAs in ISH&ACI patients and ISH patients at the transcriptive level in hope of demonstrating the potential mechanism for the progression of the disease.

Implications for research and practice

The findings of this study allow discussion of the physiological foundation for ISH&ACI from the level of lncRNAs via high-throughput sequencing. We drew a preliminary conclusion on the potential pathomechanism for ISH patients to lapse into ACI. Our research provides a theoretical foundation for future precise diagnoses and treatments with medication.

Conclusions

We have established a novel mRNA-lncRNA interaction network. The lncRNAs ENST00000590604 and *CD226* might be the potential biomarkers of ISH&ACI. This exploratory work generates a novel mRNA-lncRNA interaction network that was never before been carried out in ISH&ACI patients. The two lncRNAs ENST00000590604 and *CD226* verified as potential biomarkers for ISH&ACI had not been mentioned in former literature.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-5176

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-5176

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-5176). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (ethical review approval number: AF/SC-08/03.0). Written informed consent was provided by all participants or their families under the Declaration of Helsinki (as revised in 2013).

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Table S1 qRT-PCR primer sequences

LncRNA or mRNA	Forward primer (5'–3')	Reverse primer (5'–3')
ENST00000565493	CAGTCCCTTTGTGAATGTCCT	ACCCTTCCTCTTCTCCCTCTT
ENST00000606054	GTCCCTGGCATTTCCCGTGCTG	CCGGTGGATTCTTCTCCGTTCC
ENST00000590604	ATGAAGATGGGAAATTAACGC	GACAAAGAGGCTGTGCTGAAA
NONHSAT138949.2	CAGAACGGCAACACCAAGCAA	TCCCACGCATAGTCGTGACCC
ENST00000566942	CTGACATTTCTAATCTCCCTT	CTGTCCTCCACTAATCACAGA
ENST00000369385	AAAGCCCAGAAGAAAGACGGCAAGA	TGGACCCGCTTCAGCACCTTG
NONHSAT217189.1	GCACAGGTAAATGGTGGGAGT	CGCTGGATAGTAGGTAGGGAC
ENST00000540082	CGAGGAGTGAGTTGAGCTTTC	ACTTGGATTTGTCAGGTGGTC
CD226	TAAATGGCTATTGGTTGCGTAT	CAAAGCTGGAAAGTTGGAAAG
PARVB	TCAAGAAACCCAAGGCTCGTC	TGGAAAGGCACAGGGCACAGA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
β-actin	AAGGAGCCCCACGAGAAAAAT	ACCGAACTTGCATTGATTCCAG

Table S2 Go enrichment

GO_ID	GO_term	TYPE	target_gene_in_this_GO	all_target_gene_in_all_GO	all_gene_in_this_GO	all_gene_in_all_GO	gene_list	Pvalue	Qvalue
GO:0050839	cell adhesion molecule binding	molecular_function	4	43	198	21445	ENSG00000150637 (CD226), ENSG00000135124 (P2RX4), ENSG00000259207 (ITGB3), ENSG00000150093 (ITGB1),	0.000126273	0.192061381
GO:0019901	protein kinase binding	molecular_function	4	43	545	21445	ENSG00000150637 (CD226), ENSG00000114302 (PRKAR2A), ENSG00000169756 (LIMS1), ENSG00000150093 (ITGB1),	0.010777719	0.745132323
GO:0019900	kinase binding	molecular_function	4	43	610	21445	ENSG00000114302 (PRKAR2A), ENSG00000169756 (LIMS1), ENSG00000150093 (ITGB1), ENSG00000150637 (CD226),	0.016991975	0.923028374
GO:0042802	identical protein binding	molecular_function	7	43	1268	21445	ENSG00000150637 (CD226), ENSG00000101457 (DNTTIP1), ENSG00000259207 (ITGB3), ENSG00000066427 (ATXN3), ENSG00000177119 (ANO6), ENSG00000135124 (P2RX4), ENSG00000119917 (IFIT3),	0.009510651	0.688842876
GO:0042803	protein homodimerization activity	molecular_function	4	43	749	21445	ENSG00000150637 (CD226), ENSG00000177119 (ANO6), ENSG00000101457 (DNTTIP1), ENSG00000135124 (P2RX4),	0.037752239	1
GO:0046983	protein dimerization activity	molecular_function	6	43	1207	21445	ENSG00000177119 (ANO6), ENSG00000135124 (P2RX4), ENSG00000150093 (ITGB1), ENSG00000150637 (CD226), ENSG00000101457 (DNTTIP1), ENSG00000061918 (GUCY1B3),	0.024453622	1
GO:0019899	enzyme binding	molecular_function	7	43	1792	21445	ENSG00000114302 (PRKAR2A), ENSG00000169756 (LIMS1), ENSG00000150093 (ITGB1), ENSG00000147475 (ERLIN2), ENSG00000150637 (CD226), ENSG00000259207 (ITGB3), ENSG00000066427 (ATXN3),	0.061460033	1
GO:0005102	receptor binding	molecular_function	6	43	1628	21445	ENSG00000150093 (ITGB1), ENSG00000117899 (MESDC2), ENSG00000135124 (P2RX4), ENSG00000198721 (ECI2), ENSG00000259207 (ITGB3), ENSG00000150637 (CD226),	0.097893242	1
GO:0046914	transition metal ion binding	molecular_function	5	43	1579	21445	ENSG00000256671 (LIMS3L), ENSG00000278129 (ZNF8), ENSG00000169756 (LIMS1), ENSG00000001630 (CYP51A1), ENSG00000135124 (P2RX4),	0.204021337	1
GO:0008270	zinc ion binding	molecular_function	4	43	1316	21445	ENSG00000278129 (ZNF8), ENSG00000256671 (LIMS3L), ENSG00000135124 (P2RX4), ENSG00000169756 (LIMS1),	0.257199114	1
GO:0005215	transporter activity	molecular_function	4	43	1341	21445	ENSG00000177119 (ANO6), ENSG00000250021 (C15orf38-AP3S2), ENSG00000135124 (P2RX4), ENSG00000170144 (HNRNPA3),	0.271865206	1
GO:0046872	metal ion binding	molecular_function	13	43	4408	21445	ENSG00000135482 (ZC3H10), ENSG00000150093 (ITGB1), ENSG00000163590 (PPM1L), ENSG00000061918 (GUCY1B3), ENSG00000278129 (ZNF8), ENSG00000169756 (LIMS1), ENSG00000135124 (P2RX4), ENSG00000001630 (CYP51A1), ENSG00000167625 (ZNF526), ENSG00000256671 (LIMS3L), ENSG00000198482 (ZNF808), ENSG00000172183 (ISG20), ENSG00000241404 (EGFL8),	0.132599619	1
GO:0003723	RNA binding	molecular_function	5	43	1715	21445	ENSG00000172183 (ISG20), ENSG00000119917 (IFIT3), ENSG00000049883 (PTCD2), ENSG00000135482 (ZC3H10), ENSG00000170144 (HNRNPA3),	0.269727149	1
GO:0043169	cation binding	molecular_function	13	43	4496	21445	ENSG00000278129 (ZNF8), ENSG00000061918 (GUCY1B3), ENSG00000135124 (P2RX4), ENSG00000169756 (LIMS1), ENSG00000135482 (ZC3H10), ENSG00000150093 (ITGB1), ENSG00000163590 (PPM1L), ENSG00000198482 (ZNF808), ENSG00000167625 (ZNF526), ENSG00000256671 (LIMS3L), ENSG00000241404 (EGFL8), ENSG00000172183 (ISG20), ENSG00000001630 (CYP51A1),	0.149832621	1
GO:0043167	ion binding	molecular_function	18	43	6572	21445	ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000278129 (ZNF8), ENSG00000169756 (LIMS1), ENSG00000135124 (P2RX4), ENSG00000135482 (ZC3H10), ENSG00000150093 (ITGB1), ENSG00000163590 (PPM1L), ENSG00000198721 (ECI2), ENSG00000107679 (PLEKHA1), ENSG00000074935 (TUBE1), ENSG00000167625 (ZNF526), ENSG00000256671 (LIMS3L), ENSG00000198482 (ZNF808), ENSG00000172183 (ISG20), ENSG00000241404 (EGFL8), ENSG00000114302 (PRKAR2A), ENSG00000001630 (CYP51A1),	0.15484274	1
GO:0097159	organic cyclic compound binding	molecular_function	17	43	6392	21445	ENSG00000135124 (P2RX4), ENSG00000278129 (ZNF8), ENSG00000066427 (ATXN3), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000135482 (ZC3H10), ENSG00000172183 (ISG20), ENSG00000119917 (IFIT3), ENSG00000147475 (ERLIN2), ENSG00000101457 (DNTTIP1), ENSG00000198482 (ZNF808), ENSG00000167625 (ZNF526), ENSG00000074935 (TUBE1), ENSG00000049883 (PTCD2), ENSG00000001630 (CYP51A1), ENSG00000114302 (PRKAR2A), ENSG00000170144 (HNRNPA3),	0.200137941	1
GO:0016787	hydrolase activity	molecular_function	7	43	2701	21445	ENSG00000163590 (PPM1L), ENSG00000155099 (TMEM55A), ENSG00000074935 (TUBE1), ENSG00000066427 (ATXN3), ENSG00000147003 (TMEM27), ENSG00000041353 (RAB27B), ENSG00000172183 (ISG20),	0.361361838	1
GO:0004872	receptor activity	molecular_function	5	43	1978	21445	ENSG00000135124 (P2RX4), ENSG00000150093 (ITGB1), ENSG00000150637 (CD226), ENSG00000259207 (ITGB3), ENSG00000061918 (GUCY1B3),	0.420143897	1
GO:0043168	anion binding	molecular_function	7	43	2837	21445	ENSG00000135124 (P2RX4), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000107679 (PLEKHA1), ENSG00000074935 (TUBE1), ENSG00000114302 (PRKAR2A), ENSG00000198721 (ECI2),	0.429593941	1
GO:0017076	purine nucleotide binding	molecular_function	5	43	2036	21445	ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000074935 (TUBE1), ENSG00000135124 (P2RX4), ENSG00000114302 (PRKAR2A),	0.456699693	1
GO:0003824	catalytic activity	molecular_function	15	43	6133	21445	ENSG00000066427 (ATXN3), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000164181 (ELOVL7), ENSG00000198721 (ECI2), ENSG00000163590 (PPM1L), ENSG00000074935 (TUBE1), ENSG00000155099 (TMEM55A), ENSG00000147003 (TMEM27), ENSG00000172183 (ISG20), ENSG00000123427 (METTL21B), ENSG00000133997 (MED6), ENSG00000137364 (TPMT), ENSG00000259207 (ITGB3), ENSG00000001630 (CYP51A1),	0.356539494	1
GO:0036094	small molecule binding	molecular_function	7	43	2866	21445	ENSG00000135124 (P2RX4), ENSG00000147475 (ERLIN2), ENSG00000074935 (TUBE1), ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000114302 (PRKAR2A), ENSG00000170144 (HNRNPA3),	0.4447432	1
GO:0000166	nucleotide binding	molecular_function	6	43	2555	21445	ENSG00000135124 (P2RX4), ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000074935 (TUBE1), ENSG00000114302 (PRKAR2A), ENSG00000170144 (HNRNPA3),	0.512921749	1
GO:0003676	nucleic acid binding	molecular_function	10	43	4265	21445	ENSG00000119917 (IFIT3), ENSG00000172183 (ISG20), ENSG00000167625 (ZNF526), ENSG00000198482 (ZNF808), ENSG00000101457 (DNTTIP1), ENSG00000049883 (PTCD2), ENSG00000170144 (HNRNPA3), ENSG00000066427 (ATXN3), ENSG00000278129 (ZNF8), ENSG00000135482 (ZC3H10),	0.48555041	1
GO:0005515	protein binding	molecular_function	29	43	14009	21445	ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000066427 (ATXN3), ENSG00000169756 (LIMS1), ENSG00000177119 (ANO6), ENSG00000161911 (TREML1), ENSG00000117899 (MESDC2), ENSG00000150093 (ITGB1), ENSG00000198721 (ECI2), ENSG00000147003 (TMEM27), ENSG00000101457 (DNTTIP1), ENSG00000130311 (DDA1), ENSG00000170144 (HNRNPA3), ENSG00000259207 (ITGB3), ENSG00000114302 (PRKAR2A), ENSG00000278129 (ZNF8), ENSG00000135124 (P2RX4), ENSG00000135482 (ZC3H10), ENSG00000163590 (PPM1L), ENSG00000164181 (ELOVL7), ENSG00000256671 (LIMS3L), ENSG00000107679 (PLEKHA1), ENSG00000188677 (PARVB), ENSG00000133997 (MED6), ENSG00000147475 (ERLIN2), ENSG00000119917 (IFIT3), ENSG00000241404 (EGFL8), ENSG00000150637 (CD226), ENSG00000187699 (C2orf88),	0.768267171	1
GO:0005488	binding	molecular_function	35	43	17182	21445	ENSG00000169756 (LIMS1), ENSG00000066427 (ATXN3), ENSG00000198721 (ECI2), ENSG00000150093 (ITGB1), ENSG00000117899 (MESDC2), ENSG00000172183 (ISG20), ENSG00000198482 (ZNF808), ENSG00000074935 (TUBE1), ENSG00000001630 (CYP51A1), ENSG00000259207 (ITGB3), ENSG00000170144 (HNRNPA3), ENSG00000135124 (P2RX4), ENSG00000135482 (ZC3H10), ENSG00000241404 (EGFL8), ENSG00000119917 (IFIT3), ENSG00000147475 (ERLIN2), ENSG00000133997 (MED6), ENSG00000107679 (PLEKHA1), ENSG00000150637 (CD226), ENSG00000177119 (ANO6), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000161911 (TREML1), ENSG00000101457 (DNTTIP1), ENSG00000167625 (ZNF526), ENSG00000147003 (TMEM27), ENSG00000049883 (PTCD2), ENSG00000114302 (PRKAR2A), ENSG00000130311 (DDA1), ENSG00000278129 (ZNF8), ENSG00000164181 (ELOVL7), ENSG00000163590 (PPM1L), ENSG00000188677 (PARVB), ENSG00000256671 (LIMS3L), ENSG00000187699 (C2orf88),	0.837150357	1
GO:0035639	purine ribonucleoside triphosphate binding	molecular_function	4	43	1985	21445	ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000074935 (TUBE1), ENSG00000135124 (P2RX4),	0.735372916	1
GO:0001883	purine nucleoside binding	molecular_function	4	43	1998	21445	ENSG00000135124 (P2RX4), ENSG00000061918 (GUCY1B3), ENSG00000041353 (RAB27B), ENSG00000074935 (TUBE1),	1	1
GO:0032549	ribonucleoside binding	molecular_function	4	43	1998	21445	ENSG00000074935 (TUBE1), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3), ENSG00000135124 (P2RX4),	1	1
GO:0001882	nucleoside binding	molecular_function	4	43	2005	21445	ENSG00000135124 (P2RX4), ENSG00000074935 (TUBE1), ENSG00000041353 (RAB27B), ENSG00000061918 (GUCY1B3),	1	1
GO:0003677	DNA binding	molecular_function	5	43	2569	21445	ENSG00000066427 (ATXN3), ENSG00000167625 (ZNF526), ENSG00000198482 (ZNF808), ENSG00000278129 (ZNF8), ENSG00000101457 (DNTTIP1),	1	1
GO:0016740	transferase activity	molecular_function	4	43	2361	21445	ENSG00000164181 (ELOVL7), ENSG00000137364 (TPMT), ENSG00000133997 (MED6), ENSG00000123427 (METTL21B)	1	1

Table S3 KEGG enrichment

pathway_ID	PATHWAY_DES	target_gene_in_this_ pathway	target_gene_in_all_ pathway	all_gene_in_this_ pathway	all_gene_in_all_ pathway	all_gene_list	Pvalue	Qvalue
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	2	15	74	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.001720314	0.041287533
hsa04611	Platelet activation	3	15	122	6914	ENSG00000150093 (ITGB1), ENSG00000061918 (GUCY1B3), ENSG00000259207 (ITGB3),	0.000568765	0.027300739
hsa04512	ECM-receptor interaction	2	15	82	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.002303211	0.036851376
hsa05410	Hypertrophic cardiomyopathy (HCM)	2	15	83	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.002383691	0.028604288
hsa05414	Dilated cardiomyopathy	2	15	89	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.002903562	0.027874194
hsa04510	Focal adhesion	3	15	202	6914	ENSG00000150093 (ITGB1), ENSG00000188677 (PARVB), ENSG00000259207 (ITGB3),	0.003575027	0.028600218
hsa04514	Cell adhesion molecules (CAMs)	2	15	142	6914	ENSG00000150093 (ITGB1), ENSG00000150637 (CD226),	0.010606171	0.07272803
hsa04145	Phagosome	2	15	153	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.012980299	0.077881792
hsa05205	Proteoglycans in cancer	2	15	203	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.027474421	0.146530247
hsa04015	Rap1 signaling pathway	2	15	211	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.030373543	0.145793004
hsa04810	Regulation of actin cytoskeleton	2	15	214	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.031502562	0.137465724
hsa04151	PI3K-Akt signaling pathway	2	15	340	6914	ENSG00000150093 (ITGB1), ENSG00000259207 (ITGB3),	0.099191119	0.396764476
hsa05130	Pathogenic Escherichia coli infection	1	15	55	6914	ENSG00000150093 (ITGB1),	1	1
hsa04910	Insulin signaling pathway	1	15	139	6914	ENSG00000114302 (PRKAR2A),	1	1
hsa05145	Toxoplasmosis	1	15	118	6914	ENSG00000150093 (ITGB1),	1	1
hsa04380	Osteoclast differentiation	1	15	132	6914	ENSG00000259207 (ITGB3),	1	1
hsa04146	Peroxisome	1	15	83	6914	ENSG00000198721 (ECI2),	1	1
hsa05200	Pathways in cancer	1	15	397	6914	ENSG00000150093 (ITGB1),	1	1
hsa05100	Bacterial invasion of epithelial cells	1	15	78	6914	ENSG00000150093 (ITGB1),	1	1
hsa04730	Long-term depression	1	15	60	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04713	Circadian entrainment	1	15	95	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa05222	Small cell lung cancer	1	15	86	6914	ENSG00000150093 (ITGB1),	1	1
hsa04670	Leukocyte transendothelial migration	1	15	118	6914	ENSG00000150093 (ITGB1),	1	1
hsa00062	Fatty acid elongation	1	15	25	6914	ENSG00000164181 (ELOVL7),	1	1
hsa03040	Spliceosome	1	15	134	6914	ENSG00000170144 (HNRNPA3),	1	1
hsa04020	Calcium signaling pathway	1	15	179	6914	ENSG00000135124 (P2RX4),	1	1
hsa01100	Metabolic pathways	1	15	1238	6914	ENSG00000001630 (CYP51A1),	1	1
hsa04022	cGMP-PKG signaling pathway	1	15	167	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04919	Thyroid hormone signaling pathway	1	15	118	6914	ENSG00000259207 (ITGB3),	1	1
hsa04972	Pancreatic secretion	1	15	96	6914	ENSG00000041353 (RAB27B),	1	1
hsa00100	Steroid biosynthesis	1	15	20	6914	ENSG00000001630 (CYP51A1),	1	1
hsa05133	Pertussis	1	15	75	6914	ENSG00000150093 (ITGB1),	1	1
hsa00230	Purine metabolism	1	15	175	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa00071	Fatty acid degradation	1	15	44	6914	ENSG00000198721 (ECI2),	1	1
hsa04970	Salivary secretion	1	15	89	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04540	Gap junction	1	15	88	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa05206	MicroRNAs in cancer	1	15	256	6914	ENSG00000259207 (ITGB3),	1	1
hsa04924	Renin secretion	1	15	64	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04640	Hematopoietic cell lineage	1	15	87	6914	ENSG00000259207 (ITGB3),	1	1
hsa04360	Axon guidance	1	15	173	6914	ENSG00000150093 (ITGB1),	1	1
hsa00983	Drug metabolism - other enzymes	1	15	46	6914	ENSG00000137364 (TPMT),	1	1
hsa05140	Leishmaniasis	1	15	72	6914	ENSG00000150093 (ITGB1),	1	1
hsa05131	Shigellosis	1	15	65	6914	ENSG00000150093 (ITGB1),	1	1
hsa04141	Protein processing in endoplasmic reticulum	1	15	169	6914	ENSG00000066427 (ATXN3),	1	1
hsa04270	Vascular smooth muscle contraction	1	15	120	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04080	Neuroactive ligand-receptor interaction	1	15	277	6914	ENSG00000135124 (P2RX4),	1	1
hsa04921	Oxytocin signaling pathway	1	15	158	6914	ENSG00000061918 (GUCY1B3),	1	1
hsa04070	Phosphatidylinositol signaling system	1	15	98	6914	ENSG00000155099 (TMEM55A),	1	1