CircRNA/lncRNA-miRNA-mRNA network in the blood exosomes of patients with atherosclerosis

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Abstract

Background: Atherosclerosis (AS) is a systemic, chronic and multifocal disease and is the primary pathological basis of cardiovascular diseases, such as coronary heart disease (CHD) and peripheral arterial disease (PAD). Our study attempted to identify aberrant exosome-derived circRNAs in AS and determine their potential clinical value.

Methods: The expression of mRNA, circRNA and lncRNA in the blood exosomes of CHD patients and healthy controls was obtained from the exoRBase database. The corresponding miRNAs of mRNA, circRNA and lncRNA were predicted via ENCORI and the miRcode database. The circRNA/lncRNA-miRNA-mRNA interaction network was established based on a competitive endogenous RNA regulatory mechanism. Aberrant circRNAs in the aforementioned network were validated in patients with PAD by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR).

Results: Based on the cutoff criteria of $P < 0.05$, we identified 85 differentially expressed circRNAs (4 up- and 81 downregulated), 43 differentially expressed lncRNAs (24 up- and 19 downregulated) and 312 differentially expressed genes (55 up- and 257 downregulated). Gene Ontology (GO) analysis revealed that the biological process (BP) terms of the DEGs were significantly enriched in the positive regulation of phosphoprotein phosphatase activity and the positive regulation of protein dephosphorylation. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis demonstrated that DEGs were closely related to the glucagon signaling pathway and estrogen signaling pathway. The relative expression level of hsa_circ_0001360 was significantly downregulated in blood exosomes from patients with PAD, exhibiting an area under the curve of 0.92 ($P = 0.0283$).

Conclusion: The circRNA/lncRNA-miRNA-mRNA interaction network might help to elucidate the pathogenesis of AS. Hsa_circ_0001360 was significantly downregulated in blood exosomes in patients with PAD, and this RNA might represent an important diagnostic biomarker of AS.

Background

Atherosclerosis (AS) is a systemic, chronic and multifocal disease and is the primary pathological basis of cardiovascular diseases. Coronary heart disease (CHD) and peripheral arterial disease (PAD) are common clinical manifestations of cardiovascular diseases[1, 2]. Epidemiological studies have revealed that more than 16 million adults suffered from CHD in the United States during 2016[3], and the number suffering from PAD was nearly 8 million[4]. PAD and CHD are highly correlated and responsible for adverse limb and cardiovascular events, resulting in patient disabilities and mortalities worldwide[5, 6]. Hence, both conditions place a great burden on patients' physical and psychological health, as well as society's healthcare resources[7-9]. The pathological process of AS involves lipid deposition, intimal injury, platelet activation, inflammatory cell infiltration, and vascular smooth muscle cell migration[10]. However, based on the high prevalence of AS, we believe that there is still novel insight to be gained regarding AS.

Exosomes, membrane-bound extracellular vesicles, are characterized by nanosized endocytic vesicles secreted by cells with a diameter of 30~100 nm and contain biological molecules, such as noncoding RNA, mRNA and proteins, that are derived from the corresponding cells[11, 12]. Noncoding RNA refers to RNA that does not encode proteins, including circular RNAs (circRNAs), long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and RNA with unknown functions, and it plays an important role in bioprocesses, including cell proliferation, differentiation, and migration[13, 14]. These bioactive molecules enable exosomes to function as potent communicating intermediaries that can transmit genetic information and subsequently participate in physiological and pathological processes[15, 16]. Recent studies have also determined that exosomes play a crucial role in intercellular communication in the inflammatory response, which implies a close relationship between exosomes and AS[17-19]. However, to the best of our knowledge, the exosome-related noncoding RNAs involved in the pathogenesis of AS have not fully elucidated to date.

In the present study, we constructed a circRNA/lncRNA-miRNA-mRNA network in blood exosomes derived from patients with CHD based on a biological database. Next, we validated the aberrant circRNAs in the aforementioned network in patients with PAD by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). A receiver operating characteristic (ROC) curve was plotted to analyze the clinical implications of these results. We attempted to provide novel diagnostic biomarkers for AS and contribute to our understanding of the pathogenesis of AS.

Results

Screening of differentially expressed genes, circRNAs and lncRNAs

After expression profile normalization, the circRNA and gene expression matrix derived from 32 normal and 6 CHD samples was later used to screen differentially expressed RNAs. Based on the cutoff criteria of a P-value less than 0.05, we identified 85 differentially expressed circRNAs (4 up- and 81 downregulated), 43 differentially expressed lncRNAs (24 up- and 19 downregulated) and 312 differentially expressed genes (55 up- and 257 downregulated). The expression profiles of screened RNAs are presented in volcano plots (Fig. 1A). Cluster heatmaps in Fig. 1B detail the most significant differentially expressed circRNAs (top 4 up- and 36 downregulated), lncRNAs (top 21 up- and 19 downregulated), and mRNAs (top 20 up- and 20 downregulated).

Construction of circRNA/lncRNA-miRNA-mRNA network

First, 176 miRNAs through previously identified targeted mRNAs and 171 miRNAs with binding sites on differentially expressed circRNAs were both predicted by ENCORI. Second, 166 miRNAs that included binding sites of differentially expressed lncRNAs were obtained from the miRcode database. The overlaps of predicted miRNAs were selected based on a competitive endogenous regulatory network. Finally, the circRNA/lncRNA-miRNA-mRNA regulatory network of blood exosomes involved in AS was constructed with Cytoscape 3.8.1. As a result, 72 overlapping miRNAs were selected, and the network was visualized with Cytoscape 3.8.1 (Fig. 1C).

KEGG and GO enrichment analyses of DEGs

The online tool DAVID v6.8 was used to extract and summarize functional annotations associated with DEGs (Table I). GO analysis revealed that BP terms of the DEGs were significantly enriched in positive regulation of phosphoprotein phosphatase activity, positive regulation of protein dephosphorylation, mRNA transport, intracellular transport of virus, positive regulation of cyclic nucleotide metabolic process, and positive regulation of cyclic-nucleotide phosphodiesterase activity, that CC terms were primarily enriched in nucleoplasm, cytoplasm, calcium channel complex, growth cone, microtubule cytoskeleton, and intracellular membrane-bounded organelle, and that MF terms were primarily enriched in N-terminal myristoylation domain binding, beta-adrenergic receptor kinase activity, histone methyltransferase activity (H4-K20 specific), protein binding, protein phosphatase activator activity, and G-protein coupled receptor kinase activity. KEGG pathway enrichment analysis demonstrated that DEGs were closely related to the glucagon signaling pathway and estrogen signaling pathway.

Validation of differentially expressed circRNAs in blood exosomes from patients with PAD

Five paired blood exosome samples were collected from the control group (3 men; mean age, 65.8 y; range, 56-79 y) and PAD group (4 men; mean age, 69.0 y; range, 58-78 y). The morphological characteristics and size distribution of exosomes were further assessed by transmission electron microscopy and nanoparticle tracking analysis (Fig. 2A and B). Fifteen circRNAs from the predicted network were selected for subsequent validation through RT-qPCR assays, including hsa_circ_0000038, hsa_circ_0001360, hsa_circ_0001020, hsa_circ_0000160, hsa_circ_0000639, hsa_circ_0001309, hsa_circ_0000019, hsa_circ_0001540, hsa_circ_0001648, hsa_circ_0000842, hsa_circ_0001016, hsa_circ_0000091, hsa_circ_0000043, hsa_circ_0001684, and hsa_circ_0000311. As shown in Fig. 2C, the relative expression level of hsa_circ_0001360 was significantly downregulated ($P < 0.05$) in patients with PAD, while no noticeable changes in other circRNAs were observed. Further ROC curve analysis was performed to evaluate the potential diagnostic value of hsa_circ_0001360 in AS (Fig. 2D). The area under the curve of hsa_circ_0001360 was 0.92 ($P = 0.0283$) with a 95% confidence interval of 0.7385-1.000. In addition, the optimal cutoff value was 6 with a corresponding sensitivity and specificity of 100% and 80%, respectively.

Discussion

Extensive studies have previously demonstrated the association between blood exosomes, AS and cardiovascular diseases[15]. CircRNAs, characterized by a conserved ring structure and stronger resistance to RNase, could serve as miRNA sponges through a competitive endogenous regulatory mechanism and subsequently participate in the regulation of gene expression[13]. Recently, circRNAs derived from blood exosomes have attracted increasing attention. Based on the high prevalence of AS and cardiovascular disease, circRNAs derived from blood exosomes might also play an important role in the diagnosis and treatment of cardiovascular diseases.

Our study comprehensively constructed a circRNA/lncRNA-miRNA-mRNA regulatory network based on the exoRBase database. We identified 85 significantly differentiated circRNAs, 43 significantly differentiated lncRNAs and 312 significantly differentiated mRNAs in blood exosomes from CHD patients. The most meaningful BP, CC and MF terms were primarily enriched in positive regulation of protein dephosphorylation, cytoplasm, and histone methyltransferase activity (H4-K20 specific), respectively. These biological processes are implicated in the pathogenesis of AS[20-22].

In addition, KEGG analyses showed that significantly differentiated mRNAs were primarily involved in "Glucagon signaling pathway", "Estrogen signaling pathway", "Phototransduction", and "RNA transport". Glucagon is a peptide hormone secreted by alpha cells of the pancreas and plays a pivotal role in maintaining glucose homeostasis in humans. Disruption of the glucagon signaling pathway leads to metabolic disorders and diabetes, which is an identified risk factor for AS[23]. A previous study also demonstrated that the glucagon signaling pathway was implicated in familial hypercholesterolemia[24], suggesting a potential link between the glucagon signaling pathway and AS. In addition, the role of estrogen in AS has become a heavily researched topic in recent years. The incidence of cardiovascular disease in middle-aged women is lower than that in middle-aged men. However, the percentage of cardiovascular disease in postmenopausal women is gradually increasing, and the prevalence in women aged 65-70 y is equal to that in men[25]. Therefore, an increasing number of studies have focused on the protective effect of estrogen on AS. Extensive evidence has suggested that the resistance of estrogen to AS is mostly based on the regulation of estrogen in endothelial cells, vascular smooth muscle cells, lipid metabolism and anticoagulant effects[26-28].

Our results also revealed that both calmodulin1 and calmodulin2 were significantly downregulated in the blood exosomes of CHD patients and were involved in the glucagon signaling pathway and estrogen signaling pathway. Calmodulin is a multifunctional protein that exists in all kinds of eukaryotic cells and can bind to calcium ions. Calmodulin is involved in a variety of intracellular signal transduction pathways and plays a key role in Ca^{2+} -dependent signal transduction pathways. Known as a dynamic Ca^{2+} sensor, calmodulin can respond to a wide range of Ca^{2+} concentrations and transmit downstream signals. In addition, multiple studies have reported the interaction between calmodulin and Akt, suggesting that calmodulin may be involved in Akt activity. Long et al[29] further demonstrated that ATPase plasma membrane Ca^{2+} transporting 1 (ATP2B1) gene silencing-induced enhanced insulin sensitivity in endothelial cells was dependent on increased intracellular Ca^{2+} concentration and the resultant activation of the Ca^{2+} /calmodulin/eNOS/Akt signaling pathway. These findings might help to elucidate the pathogenesis of AS.

Owing to the considerable progress made in sequencing technologies, circRNAs have been widely identified and function as critical regulatory elements in transcription and posttranscription[13]. Enhanced stability conferred by a covalently closed loop enables endogenous transcript circRNAs to become ideal candidates for potential diagnostic biomarkers and therapeutic regimens. In our study,

based on the circRNA/lncRNA-miRNA-mRNA regulatory network concerning CHD, we identified 15 circRNAs (1 up- and 14 downregulated).

Since CHD and PAD share the same risk factors and a common pathogenesis of AS, there may be a potential relationship between PAD and CHD. People with a family history of CHD have a 2.5-fold risk for PAD. However, no established guidelines were available to support the screening test for potential PAD in patients with CHD. Moreover, early identification of high-risk patients with CHD and/or PAD could contribute to better prevention and treatment of AS itself and AS-related adverse cardiovascular events and subsequently a reduction in both morbidities and mortalities. Thus, these differentially expressed circRNAs were intentionally validated in blood exosomes in patients with PAD (with no concomitant CHD). Among these circRNAs, the expression level of hsa_circ_0001360 was clearly decreased in the PAD group compared with the control group ($P < 0.05$). Additionally, hsa_circ_0001360 may serve as a novel diagnostic biomarker for CHD and potential PAD according to ROC analysis. These findings demonstrated the role played by hsa_circ_0001360 in the progression of AS.

This study has several limitations. First, the circRNA/lncRNA-miRNA-mRNA network was confirmed based on online databases and lacked further validation in blood exosomes from CHD. Second, the study was limited by a small cohort size in a single center, and a large sample analysis was necessary to enhance statistical power. Finally, the role played by hsa_circ_0001360 in the pathogenesis of AS should be verified in further *in vivo/in vitro* experiments.

Conclusion

Our study demonstrated differentially expressed RNAs in CHD, and a circRNA/lncRNA-miRNA-mRNA network was established as a competitive endogenous RNA regulatory mechanism. Among the circRNAs, hsa_circ_0001360 was significantly downregulated in patients with PAD, which helped to elucidated the role played by hsa_circ_0001360 in the pathogenesis of AS.

Materials And Methods

Ethics statement

The study was approved by the ethics committee of Huadong Hospital, affiliated with Fudan University (2017K001). All procedures conformed to the principles outlined in the Declaration of Helsinki. All patients provided written informed consent before enrollment in the study.

Data source and preprocessing

ExoRBase is a repository of human blood exosome-derived long RNA species, including circRNAs, lncRNAs and mRNAs. Following detection by normalized RNA-sequencing data analysis, exoRBase collected and integrated RNA expression profiles involving healthy individuals and patients with corresponding diseases[30]. The RNA expression profiles of normal individuals (n=32) and patients with

CHD (n=6) were downloaded from the exoRBase database followed by background correction and data normalization in R software (version 4.0.2).

Screening of differentially expressed genes, circRNAs and lncRNAs

The limma and sva packages were applied to the RNA-sequencing data analysis to remove batch effects and filter differentially expressed genes, circRNAs and lncRNAs by comparing the CHD group to the control group. Long RNA species with P-values of less than 0.05 were considered to be differentially expressed genes, circRNAs and lncRNAs. The volcano plot and heatmap were created by GraphPad Prism 8.0 and TBTools, respectively.

Prediction of potential miRNAs for differentially expressed genes, circRNAs and lncRNAs

ENCORI, the encyclopedia of RNA interactomes (<http://starbase.sysu.edu.cn/>), was applied to the prediction of potential miRNAs for differentially expressed genes and circRNAs. The predicted miRNAs of genes were further validated by the TargetScan and miRanda databases. Only the intersecting miRNAs were selected for subsequent analysis. The miRcode database (<http://www.mircode.org/>), which provides transcriptome-wide microRNA target prediction, was used to study lncRNA-miRNA interactions. lncRNAs/circRNAs and mRNAs with the same targeted miRNAs were selected to construct an interaction network, and the results were visualized with Cytoscape 3.8.1.

Functional and pathway enrichment analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/home.jsp>) v6.8 was applied to function and pathway enrichment analysis, including Gene Ontology (GO) terms in the molecular function (MF), biological process (BP) and cellular component (CC) categories for target genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses.

Patient sample collection

Patients with lower limb PAD (PAD Group) were recruited between August 2020 and November 2020. Patients with PAD all satisfied the diagnostic criteria[31], and they all had an intermittent claudication of <200 m, an ABI of <0.9 and an occluded superficial femoral artery of ≥ 10 cm based on computed tomography angiography. Age-matched healthy individuals were recruited as the control group (without cardiovascular disease, diabetes or hyperlipidemia), and all participants had a normal ABI of approximately 1.2. The exclusion criteria included (1) history of tumor or connective tissue disease, (2) steroid use within 3 months, (3) recent infection, (4) failure to sign the informed consent form, and (5) history of CHD.

Exosome isolation

Blood samples were collected from both groups at 8:00 am after fasting for at least 8 hours. Fresh blood samples were subjected to centrifugation at a speed of 3000 g for 10 min at 4°C. The supernatant was subsequently transferred to another tube, and serum exosomes were isolated using a Hieff™ Quick exosome isolation kit (for Serum/Plasma, Yeasen Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The morphological characteristics and size distribution of serum-derived exosomes were further assessed by transmission electron microscopy and nanoparticle tracking analysis as previously described[32].

RT-qPCR assays

Total RNA was extracted from serum exosomes with TRIzol reagent (Invitrogen, Carlsbad, Calif). Subsequent reverse transcription of circRNA was conducted with Random Primers N6 from Hifair® III 1st Strand cDNA Synthesis Kit (gDNA digester plus, Yeasen Biotech Co., Ltd.) following the instructions of the manufacturer. The comparison of the expression level of target circRNA was performed by RT-qPCR based on the ABI QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), with GAPDH as an internal reference. Corresponding primers were designed with circPrimer 1.2 (<http://www.bioinf.com.cn/>, Supplementary Table I).

Statistical analysis

Statistical analysis was performed with Student's t-test or the χ^2 test as appropriate using SPSS (version 24.0). A P-value of less than 0.05 was considered to be significant.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of Huadong Hospital, affiliated with Fudan University (2017K001). All participants understand the research details and provided written informed consent prior to the study.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

The raw data of this study are derived from the ExoRBase database (<http://www.exorbase.org/>), which is publicly available database. All data analyzed during this study are included in this published article [and its supplementary information file]

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author contribution

Wan Zhang and Zhenyu Guo designed the experiments.

Wan Zhang, Li Li and Zhenyu Guo performed the experiments.

Wan Zhang, Li Li, Ting Zhu and Zhenyu Guo analyzed the data and drafted the manuscript.

References

1. Frostegård J. Immunity, atherosclerosis and cardiovascular disease. *BMC Med.* 2013;11:117.
2. Iida M, Harada S, Takebayashi T. Application of Metabolomics to Epidemiological Studies of Atherosclerosis and Cardiovascular Disease. *J Atheroscler Thromb.* 2019;26(9):747-57.
3. Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A. Epidemiology of coronary heart disease and acute coronary syndrome. *Ann Transl Med.* 2016;4(13):256.
4. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation.* 2017;135(10):e146-e603.
5. Berger A, Simpson A, Leeper NJ, Murphy B, Nordstrom B, Ting W, et al. Real-World Predictors of Major Adverse Cardiovascular Events and Major Adverse Limb Events Among Patients with Chronic Coronary Artery Disease and/or Peripheral Arterial Disease. *Adv Ther.* 2020;37(1):240-52.
6. Bauersachs R, Zeymer U, Brière JB, Marre C, Bowrin K, Hulsebeck M. Burden of Coronary Artery Disease and Peripheral Artery Disease: A Literature Review. *Cardiovasc Ther.* 2019;2019:8295054.
7. Lekakis JP, Papamichael CM, Cimponeriu AT, Stamatelopoulos KS, Papaioannou TG, Kanakakis J, et al. Atherosclerotic changes of extracoronary arteries are associated with the extent of coronary atherosclerosis. *Am J Cardiol.* 2000;85(8):949-52.
8. Manfrini O, Amaduzzi PL, Cenko E, Bugiardi R. Prognostic implications of peripheral artery disease in coronary artery disease. *Curr Opin Pharmacol.* 2018;39:121-8.
9. Mirek AM, Wolińska-Welcz A. Is the lumen diameter of peripheral arteries a good marker of the extent of coronary atherosclerosis? *Kardiologia Pol.* 2013;71(8):810-7.
10. Wolf D, Ley K. Immunity and Inflammation in Atherosclerosis. *Circ Res.* 2019;124(2):315-27.
11. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science.* 2020;367(6478).

12. Zhang Y, Hu YW, Zheng L, Wang Q. Characteristics and Roles of Exosomes in Cardiovascular Disease. *DNA Cell Biol.* 2017;36(3):202-11.
13. Altesha MA, Ni T, Khan A, Liu K, Zheng X. Circular RNA in cardiovascular disease. *J Cell Physiol.* 2019;234(5):5588-600.
14. Lu D, Thum T. RNA-based diagnostic and therapeutic strategies for cardiovascular disease. *Nat Rev Cardiol.* 2019;16(11):661-74.
15. Ibrahim A, Marbán E. Exosomes: Fundamental Biology and Roles in Cardiovascular Physiology. *Annu Rev Physiol.* 2016;78:67-83.
16. Lawson C, Vicencio JM, Yellon DM, Davidson SM. Microvesicles and exosomes: new players in metabolic and cardiovascular disease. *J Endocrinol.* 2016;228(2):R57-71.
17. Deng W, Tang T, Hou Y, Zeng Q, Wang Y, Fan W, et al. Extracellular vesicles in atherosclerosis. *Clin Chim Acta.* 2019;495:109-17.
18. Chen L, Yang W, Guo Y, Chen W, Zheng P, Zeng J, et al. Exosomal lncRNA GAS5 regulates the apoptosis of macrophages and vascular endothelial cells in atherosclerosis. *PLoS One.* 2017;12(9):e0185406.
19. Wang Y, Xie Y, Zhang A, Wang M, Fang Z, Zhang J. Exosomes: An emerging factor in atherosclerosis. *Biomed Pharmacother.* 2019;115:108951.
20. Zhang N, Zhang Y, You S, Tian Y, Lu S, Cao L, et al. Septin4 Prevents PDGF-BB-induced HAVSMC Phenotypic Transformation, Proliferation and Migration by Promoting SIRT1-STAT3 Deacetylation and Dephosphorylation. *Int J Biol Sci.* 2020;16(4):708-18.
21. Lu Y, Gao X, Wang R, Sun J, Guo B, Wei R, et al. Adiponectin inhibits proliferation of vascular endothelial cells induced by Ox-LDL by promoting dephosphorylation of Caveolin-1 and depolymerization of eNOS and up-regulating release of NO. *Int Immunopharmacol.* 2019;73:424-34.
22. Huang J, Cai C, Zheng T, Wu X, Wang D, Zhang K, et al. Endothelial Scaffolding Protein ENH (Enigma Homolog Protein) Promotes PHLPP2 (Pleckstrin Homology Domain and Leucine-Rich Repeat Protein Phosphatase 2)-Mediated Dephosphorylation of AKT1 and eNOS (Endothelial NO Synthase) Promoting Vascular Remodeling. *Arterioscler Thromb Vasc Biol.* 2020;40(7):1705-21.
23. Hædersdal S, Lund A, Knop FK, Vilsbøll T. The Role of Glucagon in the Pathophysiology and Treatment of Type 2 Diabetes. *Mayo Clin Proc.* 2018;93(2):217-39.
24. Wang D, Liu B, Xiong T, Yu W, She Q. Investigation of the underlying genes and mechanism of familial hypercholesterolemia through bioinformatics analysis. *BMC Cardiovasc Disord.* 2020;20(1):419.
25. Rosano GM, Vitale C, Silvestri A, Fini M. Hormone replacement therapy and cardioprotection: the end of the tale? *Ann N Y Acad Sci.* 2003;997:351-7.
26. Li H, Mani S, Wu L, Fu M, Shuang T, Xu C, et al. The interaction of estrogen and CSE/H(2)S pathway in the development of atherosclerosis. *Am J Physiol Heart Circ Physiol.* 2017;312(3):H406-h14.

27. Chen Q, Chao Y, Zhang W, Zhang Y, Bi Y, Fu Y, et al. Activation of estrogen receptor α (ER α) is required for Alisol B23-acetate to prevent post-menopausal atherosclerosis and reduced lipid accumulation. *Life Sci.* 2020;258:118030.
28. Meng Q, Li Y, Ji T, Chao Y, Li J, Fu Y, et al. Estrogen prevent atherosclerosis by attenuating endothelial cell pyroptosis via activation of estrogen receptor α -mediated autophagy. *J Adv Res.* 2021;28:149-64.
29. Long Y, Xia JY, Chen SW, Gao CL, Liang GN, He XM, et al. ATP2B1 gene Silencing Increases Insulin Sensitivity through Facilitating Akt Activation via the Ca(2+)/calmodulin Signaling Pathway and Ca(2+)-associated eNOS Activation in Endothelial Cells. *Int J Biol Sci.* 2017;13(9):1203-12.
30. Li S, Li Y, Chen B, Zhao J, Yu S, Tang Y, et al. exoRBase: a database of circRNA, lncRNA and mRNA in human blood exosomes. *Nucleic Acids Res.* 2018;46(D1):D106-d12.
31. Aboyans V, Ricco JB, Bartelink MEL, Björck M, Brodmann M, Cohnert T, et al. 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteries Endorsed by: the European Stroke Organization (ESO) The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur Heart J.* 2018;39(9):763-816.
32. Qu Z, Feng J, Pan H, Jiang Y, Duan Y, Fa Z. Exosomes derived from HCC cells with different invasion characteristics mediated EMT through TGF-beta/Smad signaling pathway. *Onco Targets Ther.* 2019;12:6897-905.

Table

Table I Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis

Category	Term	Count	P-Value*
GOTERM_BP_DIRECT	GO:0032516~positive regulation of phosphoprotein phosphatase activity	3	0.000
GOTERM_BP_DIRECT	GO:0035307~positive regulation of protein dephosphorylation	3	0.001
GOTERM_BP_DIRECT	GO:0051028~mRNA transport	3	0.006
GOTERM_BP_DIRECT	GO:0075733~intracellular transport of virus	3	0.007
GOTERM_BP_DIRECT	GO:0030801~positive regulation of cyclic nucleotide metabolic process	2	0.007
GOTERM_BP_DIRECT	GO:0051343~positive regulation of cyclic-nucleotide phosphodiesterase activity	2	0.012
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	18	0.000
GOTERM_CC_DIRECT	GO:0005737~cytoplasm	24	0.001
GOTERM_CC_DIRECT	GO:0034704~calcium channel complex	3	0.002
GOTERM_CC_DIRECT	GO:0030426~growth cone	3	0.033
GOTERM_CC_DIRECT	GO:0015630~microtubule cytoskeleton	3	0.045
GOTERM_CC_DIRECT	GO:0043231~intracellular membrane-bounded organelle	5	0.048
GOTERM_MF_DIRECT	GO:0031997~N-terminal myristoylation domain binding	2	0.007
GOTERM_MF_DIRECT	GO:0047696~beta-adrenergic receptor kinase activity	2	0.009
GOTERM_MF_DIRECT	GO:0042799~histone methyltransferase activity (H4-K20 specific)	2	0.011
GOTERM_MF_DIRECT	GO:0005515~protein binding	28	0.013
GOTERM_MF_DIRECT	GO:0072542~protein phosphatase activator activity	2	0.013
GOTERM_MF_DIRECT	GO:0004703~G-protein coupled receptor kinase activity	2	0.016
KEGG_PATHWAY	hsa04922: Glucagon signaling pathway	4	0.003
KEGG_PATHWAY	hsa04915: Estrogen signaling pathway	3	0.036
KEGG_PATHWAY	hsa04744: Phototransduction	2	0.079
KEGG_PATHWAY	hsa03013: RNA transport	3	0.096

*A P value of < 0.05 was considered statistically significant.

Figures

Figure 1

circRNA/lncRNA-miRNA-mRNA network construction. A, differentially expressed circRNA, lncRNA and mRNA in normal and atherosclerotic samples shown in the volcano plot. B, differentially expressed circRNA, lncRNA and mRNA in normal and atherosclerotic samples shown in the heatmap. C, construction of circRNA/lncRNA-miRNA-mRNA network.

Figure 2

Exosome identification and key circRNA validation. A, the morphological characteristics of exosome was assessed by transmission electron microscopy. B, the size distribution of exosome was measured by nanoparticle tracking analysis. C, validation of the key circRNA in normal and atherosclerotic samples. D, ROC curves analysis.

Supplementary Files

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- [SupplementaryTable1.docx](#)