Expression characteristics of exosomal long non-coding RNA in abdominal aortic aneurysm

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Abstract

Background and objective:

Abdominal aortic aneurysm (AAA) is one of the important causes of morbidity and mortality in middle-aged and elderly people. Although the understanding of the physiology and pathology of AAA has been improved, the potential molecular mechanism of AAA is still unclear. The existing evidence confirms that exosomal lncRNAs have a wide range of biological functions, and its regulatory disorders are related to the occurrence of diseases such as AAA, but the internal mechanism is not clear. The main purpose of this study is to screen the differentially expressed lncRNAs in exosomes between normal people and patients with AAA and to understand its internal mechanism.

Materials and methods:

The plasma of a healthy control group and patients with abdominal aortic aneurysm was collected, and the lncRNAs of exosomes were extracted and sequenced. Differential expression was assessed by DEseq using read counts as input and chosen according to the criteria of |log2(fold change)| > 1 and adjusted p-value < 0.05. Based on the Kyoto encyclopedia of genes and genomes (KEGG) and biological pathway and gene ontology (GO) functional enrichment analysis, the target genes were analyzed, and the correlation between lncRNA and target genes was analyzed.

Result:

We screened 45 species differentially expressed lncRNAs and found pathway significantly related to these genes, namely metabolic pathways, calcium signaling pathways and protein processing in endoplasmic reticulum and They play a significant and important role in the metabolic process and the cell signaling.

Conclusion:

There was significant difference in expression of exosomal lncRNAs between normal subjects and AAA patients. LncRNAs in exosomes regulate in the progress of AAA by activating metabolic pathway and calcium signaling pathway, but the specific mechanism is not clear and needs to be further explored.

Introduction

Abdominal aortic aneurysm (AAA) is defined as focal dilatation of the abdominal aorta, which is 50% larger than the normal diameter, or when the diameter of the aorta exceeds 30 mm (1, 2) and it is one of the important causes of morbidity and mortality in middle-aged and elderly people (3). The prevalence of AAA is 4–7% in men and 1–2% in women over the age of 65 (4, 5). Acute rupture is the most dangerous clinical consequence of AAA progression, and about 80% of related deaths are caused by acute rupture (6). The pathological features of AAA include vascular smooth muscle cell apoptosis, inflammatory cell infiltration, loss of arterial wall integrity, increased oxidative stress and significant
matrix degradation\textsuperscript{(7, 8)}. However, the exact molecular mechanism of the progress of AAA is still unclear. Therefore, it is very important to clarify the etiological mechanism of AAA progression and to provide new targets for the diagnosis, treatment and prognosis of AAA patients. In recent years, it has been found that exosomes play an important role in signal transduction and regulation of the internal environment. Their biological effects on receptor cells depend on the composition of exosomes and the microenvironment of receptor cells. The mechanisms involved in their formation and secretion are just beginning to be clarified\textsuperscript{(9)}. Intervention in their functions undoubtedly represents a way to understand their participation in basic cellular processes. IncRNA, which exists in exosomes, is a class of non-coding RNA, with a length of more than 200 bp with almost no protein coding function\textsuperscript{(6)}. It has been found that IncRNAs plays an important role in the development of many human diseases and may be used as a potential biomarker for disease and drug therapy\textsuperscript{(10, 11)}. Unfortunately, due to the small number of samples in this study and the lack of basic experiments, the results are relatively preliminary. The next stage is to add basic experiments to further verify our conclusions.

Materials And Methods

Sample collection:

The plasma samples of 3 patients with abdominal aortic aneurysm and 3 healthy persons were provided by the first Affiliated Hospital of Zhengzhou University. All patients with AAA were confirmed by CTA that the diameter of the abdominal aorta was larger than 50 mm. The healthy group without AAA was selected as the control group. The blood is collected into the EDTA anticoagulant tube and gently mixed to ensure exposure to the wall of the tube coated with EDTA.

Exosome Isolation And Identification:

LX-2 cells were cultured in DMEM containing 10% exosome-depleted FBS. LX-2-exosomes were extracted using Ribo\textsuperscript{™} Exosome Isolation Reagent for cell culture media (RiboBio, Guangzhou, China), according to the manufacturer\textquoteright s recommendations. To test the exosomes, exosomal markers, CD63 and CD81 were analysed with monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA) by Accuri C6 Flow cytometry (FCM) (Becton, Dickinson and Company, New Jersey, USA). The size of exosomes was tested with ZETASIZER Nano series-Nano-ZS (Malvern, England).

Library Construction And Sequencing:

Total RNA was extracted from exosomes using the Trizol (invitrogen) according to the manufacturer\textapos;s protocol. Fragmented RNA (the average length was approximately 200 bp) were subjected to first strand and second strand cDNA synthesis following by adaptor ligation and enrichment with a low-cycle according to instructions of NEBNext\textsuperscript{®}Ultra\textsuperscript{™} RNA Library Prep Kit for Illumina (NEB, USA). The purified library products were evaluated using the Agilent 2200 TapeStation and Qubit\textsuperscript{®}2.0 (Life Technologies,
USA). The libraries were paired-end sequenced (PE150, Sequencing reads were 150 bp) at Guangzhou RiboBio Co., Ltd. (Guangzhou, China) using IlluminaHiSeq 3000 platform.

**Pre-processing of sequencing reads / Quality Control:**

1) Raw fastq sequences were treated with Trimmomatic tools (v 0.36) using the following options: TRAILING:20, MINLEN:235 and CROP:235, to remove trailing sequences below a phred quality score of 20 and to achieve uniform sequence lengths for downstream clustering processes.

2) Sequencing read quality was inspected using the FastQC software. Adapter removal and read trimming were performed using Trimmomatic. Sequencing reads were trimmed from the end (base quality less than Q20) and filtered by length (less than 25).

**Quantification Of Gene Expression Level:**

Paired-end reads were aligned to the mouse reference genome mm10 with HISAT2. HTSeq v0.6.0 was used to count the reads numbers mapped to each gene. The whole samples expression levels were presented as RPKM (expected number of Reads Per Kilobase of transcript sequence per Million base pairs sequenced), which is the recommended and most common method to estimate the level of gene expression.

**Differential Expression Analysis:**

The statistically significant DE genes were obtained by an adjusted P-value threshold of < 0.05 and |log2(fold change)| > 1 using the DEGseq software. Finally, a hierarchical clustering analysis was performed using the R language package gplots according to the RPKM values of differential genes in different groups. And colors represent different clustering information, such as the similar expression pattern in the same group, including similar functions or participating in the same biological process.

**GO Terms And KEGG Pathway Enrichment Analysis:**

All differentially expressed IncRNAs were selected for GO and KEGG pathway analyses. GO was performed with KOBAS3.0 software. GO provides label classification of gene function and gene product attributes (http://www.geneontology.org). GO analysis covers three domains: cellular component (CC), molecular function (MF) and biological process (BP). The differentially expressed IncRNAs and the enrichment of different pathways were mapped using the KEGG pathways with KOBAS3.0 software (http://www.genome.jp/kegg).
Co-expression Network Of Differentially Expressed lncRNAs/mRNAs:

To investigate the potential functions of differentially expressed lncRNAs and the interactions between mRNAs and lncRNAs, we constructed a lncRNA/mRNA transcripts co-expression network. The co-expression network was constructed by calculating the Pearson correlation coefficient and P value between multiple genes. In our study, the transcripts were filtered using a COR of > 0.85 and adjust P-value of < 0.05. Then we chose 44 DE mRNA transcripts that were enriched in reproduction-related pathways and all DE lncRNAs to construct a co-expression network. The co-expression network was illustrated using Cytoscape software.

Results

We screened 45 species differentially expressed lncRNAs by DEGseq, of which 24 species were up-regulated and 21 species were down-regulated (Table 1), and NR_031720.1(Log2 (Foldchange) = 5.962683) was the most significantly upregulated and NR_003002.1(Log2(Foldchange)=-5.46614) were the most markedly downregulated. The heatmap showed the differential gene expression among the six samples(Fig. 1.a). The volcano plot revealed significantly expressed lncRNAs with|log2FC|≥ 1 and P value < 0.05 (Figure1.b).
Table 1
Differential expression lncRNAs in AAA and Control Tissues

| Upregulated lncRNA in AAA | Downregulated lncRNA in AAA |
|---------------------------|-----------------------------|
| Seqname                   | Log2(FC)        | p-value   | q-value   | Seqname                   | Log2(FC)        | p-value   | q-value   |
| NR_0317 20.1              | 5.962683       | 1.61E-09  | 2.69E-06  | NR_0030 02.1              | -5.46614       | 2.69E-06  | 2.25E-03  |
| NR_0374 45.1              | 5.544998       | 1.67E-07  | 2.13E-04  | NR_0029 53.1              | -5.15633       | 2.85E-05  | 1.81E-02  |
| NR_0034 99.1              | 5.297583       | 1.60E-06  | 1.53E-03  | NR_0068 80.1              | -4.17777       | 3.30E-185 | 2.94E-181 |
| NR_0361 55.1              | 4.771466       | 6.42E-05  | 3.65E-02  | NR_0039 25.1              | -3.35023       | 1.08E-13  | 3.20E-10  |
| NR_0396 45.1              | 4.656931       | 2.16E-08  | 3.03E-05  | NR_0030 51.3              | -3.22245       | 8.22E-140 | 5.49E-136 |
| NR_1069 87.1              | 4.37233        | 4.61E-05  | 2.74E-02  | NR_0031 37.2              | -3.15163       | 8.61E-49  | 3.83E-45  |
| NR_0362 26.1              | 4.193048       | 3.63E-06  | 2.85E-03  | NR_0028 19.4              | -3.11422       | 9.48E-13  | 1.81E-09  |
| NR_0033 10.1              | 3.932536       | 3.96E-07  | 4.60E-04  | NR_1445 67.1              | -3.11296       | 4.82E-13  | 1.07E-09  |
| NR_0399 73.1              | 3.904803       | 9.43E-06  | 6.81E-03  | NR_1454 59.1              | -3.10746       | 4.27E-13  | 1.14E-09  |
| NR_0302 84.1              | 3.552012       | 1.21E-05  | 8.54E-03  | NR_0044 04.1              | -3.09439       | 4.83E-14  | 1.61E-10  |
| NR_0034 95.1              | 2.976283       | 2.09E-06  | 1.80E-03  | NR_1445 68.1              | -3.09178       | 4.54E-13  | 1.10E-09  |
| NR_0396 48.1              | 2.929885       | 3.52E-05  | 2.19E-02  | NR_0029 07.2              | -3.03854       | 8.90E-10  | 1.59E-06  |
| NR_0296 61.1              | 2.852065       | 3.99E-05  | 2.42E-02  | NR_0397 57.1              | -3.00138       | 2.01E-07  | 2.44E-04  |
| NR_1068 33.1              | 2.705413       | 1.51E-06  | 1.49E-03  | NR_0043 87.1              | -2.99072       | 2.79E-05  | 1.82E-02  |
| NR_0498 74.1              | 2.627684       | 8.22E-05  | 4.57E-02  | NR_0030 08.2              | -2.94434       | 1.16E-08  | 1.82E-05  |
| NR_0498 14.1              | 2.591369       | 6.78E-07  | 7.24E-04  | NR_0451 17.1              | -2.77663       | 1.72E-08  | 2.56E-05  |

*FC: Fold change.
### Upregulated lncRNA in AAA

| lncRNA   | Log2 Fold Change | p-value  | q-value |
|----------|------------------|----------|---------|
| NR_0297 05.1 | 2.364047        | 1.91E-06 | 1.70E-03 |
| NR_0361 47.1 | 2.360889        | 4.98E-06 | 3.80E-03 |
| NR_0362 00.1 | 2.35511         | 3.77E-08 | 5.03E-05 |
| NR_0044 30.2 | 2.252049        | 1.74E-05 | 1.19E-02 |
| NR_1070 02.1 | 2.221228        | 2.01E-05 | 1.34E-02 |
| NR_0374 22.1 | 1.963732        | 1.69E-06 | 1.56E-03 |
| NR_0397 49.1 | 1.526416        | 4.64E-05 | 2.70E-02 |
| NR_0398 10.1 | 1.419895        | 3.33E-06 | 2.69E-03 |

### Downregulated lncRNA in AAA

| lncRNA   | Log2 Fold Change | p-value  | q-value |
|----------|------------------|----------|---------|
| NR_0030 45.1 | -2.68019         | 5.89E-07 | 6.56E-04 |
| NR_0030 23.1 | -2.44477         | 8.06E-13 | 1.66E-09 |
| NR_0360 81.1 | -2.25266         | 1.40E-06 | 1.44E-03 |
| NR_0014 45.2 | -1.69002         | 2.86E-115 | 1.53E-111 |
| NR_0396 66.1 | -1.40517         | 6.10E-06 | 4.52E-03 |

*FC: Fold change.

In order to explore the potential biological functions and pathways of lncRNA, the enrichment of KEGG pathway and the biological processes of GO were analyzed by using predicted target genes. KEGG analysis presented that pathway significantly related to these genes, namely metabolic pathways, calcium signaling pathways and protein processing in endoplasmic reticulum (Fig. 2a). Specially, metabolic pathways was the most significant (Fig. 2b). GO analysis consists of three independent ontologies: biological processes, molecular functions and cellular components. Through the classification of different functional genes, gene annotation and functional enrichment analysis were carried out. The results of biological processes show that target genes are involved in the process of biological regulation and metabolism. The results of cell component enrichment showed that the target genes were significantly enriched in both intracellular organelles and membrane-bound organelles (Fig. 3). Furthermore, They play a significant and important role in the metabolic process and the cell signaling (Fig. 4a, 4b). The correlation between mRNA and lncRNA is co-expressed by network analysis (Fig. 5).

## Discussion

Abdominal aortic aneurysm (AAA) is defined as focal dilatation of the abdominal aorta, which is either 50% larger than the normal diameter, or characterized by permanent localized dilatation of the abdominal aorta when the diameter of the aorta exceeds 30 mm (1, 2, 12). AAA is one of the most important causes of morbidity and death in middle-aged and elderly people (3). Risk factors for AAA include advanced age,
male, Caucasian race, disease, atherosclerotic disease and smoking, which are considered to be the main modifiable risk factor (5, 13, 14). Other potential risk factors include diabetes (DM), which has been shown to be negatively associated with AAA (15), higher height (16), and low fruits and vegetables intake (17). Acute rupture is the most dangerous clinical consequence of AAA progression. About 80% of related deaths in the United States are caused by acute rupture (6), and AAA pathological features include vascular smooth muscle cell apoptosis, inflammatory cell infiltration, loss of arterial wall integrity, increased oxidative stress and significant matrix degradation (7, 8), and aortic wall thinning caused by vascular smooth muscle cell (VSMCs) loss (18–20). There are many potential mechanisms for the pathogenesis of AAA, including inflammation, VSMCs apoptosis, ECM degradation and oxidative stress (8, 21, 22). Pro-inflammatory cytokines such as IL-1 β, IL-10, IL-6, IL-13, TNF-α and interferon-γ in diseased aortic walls promote the development of abdominal aortic aneurysms by regulating the secretion of matrix metalloproteinases (23). The prevalence of AAA is 4 to 7 per cent in men and 1 to 2 per cent in women over the age of 65, which may only be due to the protection of estrogen, as women are no longer protected against AAA after menopause (24). However, women had a higher incidence of ruptured aneurysms after surgical repair (25, 26). AAA is usually asymptomatic and does not seem to pass routine screening, which is accidentally found in imaging tests used to assess other health problems (27–29). At a later stage, ruptured abdominal aorta can lead to high mortality. Therefore, it is particularly urgent to explore the molecular mechanism of AAA and improve the treatment level of AAA patients.

Exosomes are extracellular vesicles with diameters between 30 and 100 nanometers (30). These nanosized vesicles are produced in poly cystic bodies and are released by cells through the fusion of these compartments with the plasma membrane. They are important components of intercellular communication through the transmission of intracellular components, such as DNA, RNA and proteins (31). Exosomes can be released by various types of cells, including immune cells and tumor cells. Tumor cells secrete more exosomes than normal cells (32). The exosome secreted can be ingested by nearby or distant cells. In the past decade, exosomes have become the focus of research. It is worth noting that exosomes from tumors play an important role in the occurrence and development of tumors. For example, gastric cancer (GC)-derived exosomes promote tumor cell proliferation by activating phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) pathways (33). (HCC)-derived exosomes of primary hepatocellular carcinoma regulate the adhesion of HCC cells in circulation by transmitting SMAD family member 3 (SMAD3), thus promoting tumor metastasis (34). Exoskeletons derived from (HNSCC) in head and neck squamous cell carcinoma can promote angiogenesis by reprogramming receptor endothelial cells (34). In addition, exosomes were found in various body fluids such as saliva, blood and urine (35, 36). The accessibility of exosomes in almost all body fluids shows their potential as potential non-invasive biomarkers for different types of cancer. Most importantly, what is closely related to this study is the involvement of macrophages in the pathogenesis of AAA.

Long non-coding RNA (LncRNAs) is a transcript whose length is larger than 200nt and does not have the ability to encode protein. LncRNAs in vitro is not affected by ribonuclease-mediated degradation and stably exists in body fluids. LncRNAs regulates protein expression by epigenetic, transcriptional and post-
translational levels (37–39). One of the most well-known mechanisms of IncRNAs is their role as competitive endogenous (Ce) RNA(40). To identify the possible function of IncRNAs adjacent genes are to explore the function of IncRNAs. It has been found that a large number of lincRNAs and IncRNAs, with enhancer-like functions are related to trans-transcriptional regulation, translation control, splicing regulation and other post-transcriptional regulation. There is more and more evidence that IncRNA is related to the occurrence and development of cancer by directly or indirectly interfering with gene expression. A new IncRNA long gene non-protein coding RNA473 (LINC00473) can promote cell proliferation and inhibit apoptosis in a variety of cancers, such as breast cancer (41), lung cancer(42), nephroblastoma(43), cervical cancer(44) and so on. In other words, LINC00473 seems to play a general inhibitory role in cancer cell apoptosis. LncRNAs has been identified as a new regulator of cancer cell proliferation and apoptosis (45–47). However, the role of most IncRNAs in AAA is not clear, and apoptosis occurs in VSMCs during this process. LINC00473 has been shown to promote cell proliferation but inhibit apoptosis in many cancers (41, 42). Therefore, more and more attention has been paid to the field of IncRNAs exocrine research. So far, a variety of IncRNAs have been reported to be involved in regulating the development of AAA. For example, IncRNAH19 promotes AAA formation and vascular inflammation by regulating the expression of IL-6 (48). In addition, LINC00265 is thought to promote AAA inflammation by isolating let-7a. H19 is considered to be an important inducer of AAA (49). In mouse AAA model, silencing PVT1 resulted in inhibition of VSMCs apoptosis. In addition, growth inhibition specificity 5 (Gas5) was found to promote apoptosis in VSMC and promote the formation of AAA(50). Hypoxia inducible factor 1 (HIF-1) and α-AS1 are the first reported LncRNA(7) associated with thoracic aortic aneurysms(51) identified 3688 differentially expressed IncRNA,AC005224.4 and SENP3EIF4A1 involved in signal transduction, protein amino acid phosphorylation and immune response, and ZNRD1-AS1 involved in transcription, development and cell differentiation. It is reported that CDKN2B-AS1 interacts with cytokinesis regulatory proteins 1 and-2, resulting in epigenetic silencing of CDKN2B(52). The Cerna hypothesis proposed by Tay et al. (53) suggests that pseudogenes, IncRNAs, circular RNAs and mRNAs may weaken the activity of tiny (MI) RNA through isolation, thus up-regulating the expression of miRNA target genes. Franco-Zorrilla et al(53). Reported for the first time that non-coding RNA interferon-β promoter stimulator 1 promotes phosphate metabolism of (Pho)-2 protein in plants by isolating miR-399 and preventing it from inhibiting PHO2mRNA stability and translation. In this study, we generally understand that exocrine IncRNAs plays an important role in vivo and plays an important role in the occurrence and development of AAA, but because this study is not in-depth, and the number of samples is small, the result is single, the current stage of work is to further in-depth and specific study of the mechanism of action of a single IncRNA, in order to achieve the purpose of clinical application. Unfortunately, due to the small number of samples in this study and the lack of basic experiments, the results are relatively preliminary. The next stage is to add basic experiments to further verify our conclusions.

Conclusion
Our study shows that there is a difference in the expression of IncRNA between normal subjects and patients with AAA. Some known exosome forms of IncRNAs have been confirmed to act on target genes in peripheral blood, such as LINC00265, IncRNAH19, AC005224.4, SENP3-EIF4A1 and so on. These IncRNAs may be regulated, leading to vascular smooth muscle dysfunction and vascular structural abnormalities, leading to AAA.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All methods were carried out in accordance with the relevant regulations. All studies obtained the informed consent of the donor.

**Competing interests**

All authors declared that they have no conflicts of interests.

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**Figures**

**Figure 1**

a: Heatmap showing 45 IncRNAs with significantly different expression (Q < 0.05, P < 0.05, | log 2 (FoldChange) | ≥ 1) between AAA plasma EVs and the control. b: Volcanic diagram of IncRNA differential expression analysis among samples.
Figure 2

a: Pathways results of the predicted genes in the KEGG  
b: Enrichment results of the predicted genes in the KEGG

Figure 3

Enrichment results of the predicted genes in the GO
Figure 4

a: GO-Biological_Process of the predicted genes  
b: GO-Molecular_Function of the predicted genes
Figure 5

Network between IncRNAs and Predicted genes