Analyzing the interactions of circRNAs, miRNAs, and mRNAs to predict ceRNA networks in human acute type A aortic dissection

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Abstract
Background: Acute type A aortic dissection (ATAAD) is a life-threatening vascular disease. However, its underlying mechanism is still not well understood. Here, circular RNAs (circRNAs) were shown to function as competitive endogenous RNAs (ceRNAs) to regulate the effect of microRNAs (miRNAs) on their target genes may play a critical role in ATAAD. However, comprehensive identification and integrated analysis of the circRNA-miRNA-mRNA network in ATAAD have not been performed. Results: The gene expression profile of circRNAs, miRNAs, and mRNAs was performed between 6 ATAAD patients and 6 age-matched normal ascending aortic wall tissues patients were analyzed using the Arraystar human RNAs microarray. We identified that the expression of 12576 circRNAs, 1603 miRNAs, and 14596 mRNAs were found to be differentially expressed (DE). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway analyses (KEGG) were performed on these DE mRNAs and miRNA-mediated target genes of circRNAs. Furthermore, we used a multi-step computational framework and several bioinformatics methods to construct a ceRNA network containing circRNAs, miRNAs, and mRNAs based on co-expression analysis between the DE genes. The constructed ceRNA regulatory network containing 25 circRNAs, 17 miRNAs and 72 mRNAs. In the whole ceRNA network. We identified that plenty of key genes, such as hsa_circRNA_404522, hsa_circRNA_0022920, hsa_circ_0075881, hsa-miRNA1285-3p, hsa-miRNA-1285-3p, hsa-miRNA-637, hsa-miRNA-650, TINAGL1, JPH4, PLXNA2, TGFBR1, and THSD4. Furthermore, we also integrated the circRNA-miRNA-mRNA regulatory modules of the key genes. Conclusions: This study found a profile of dysregulated circRNAs, miRNA and mRNAs, and competitive circRNA-miRNA-mRNA regulatory networks were comprehensively integrated and predicted to be involved in ATAAD by GO and KEGG pathway analysis. It might be prospective clinical markers associated with ATAAD, and it is worthwhile to perform further studies to reveal the underlying link between these key genes and the molecular mechanisms of AD.

Keywords: aortic dissection; circRNAs; miRNAs; mRNAs; ceRNA

Background
ATAAD occurs when an intimal tear in the aorta creates a false lumen in the ascending aorta, it is a
highly lethal vascular disease. In many patients with ATAAD, the aorta progressively dilates and ultimately ruptures[1, 2]. Dissection formation, progression, and rupture cannot be reliably prevented pharmacologically because the molecular mechanisms of aortic wall degeneration are poorly understood. The key histopathologic feature of aortic dissection (AD) is medial degeneration, a process characterized by smooth muscle cell depletion and extracellular matrix degradation. These structural changes have a profound impact on the functional properties of the aortic wall and can result from excessive protease-mediated destruction of the extracellular matrix, altered signaling pathways, and altered gene expression [3].

Hitherto, studies are primarily focused on mutations in several categories of AD-associated protein-coding genes such as ECM genes (FBN1, LOX, COL3A1) [4-6] and SMCs genes (ACTA2, MYH11, FLNA) [7-9], or other factors involved in the TGF-β pathway (TGRBR1[10], TGFBR2[11], SMAD3[12]) which partially elucidated the general pathological processes of AD. Recently, non-coding RNAs (ncRNAs) have been reported to play a critical role in aortic aneurysmal disease[13-15]. Therefore, excluding the protein functions, efforts should be emphasized on understanding the non-protein functions in the progression of AD and investigating the functions of ncRNAs that have received extensive attention due to their differential expression in AD and normal aorta tissue[16, 17]. MiRNAs, the most studied ncRNAs, have been shown to contribute towards the altered ECM and SMCs in an aortic aneurysm and dissection[13, 18]. Compared with miRNAs, circRNAs are another new type of ncRNAs that are formed covalently closed loop structures[19] and widely expressed in human cells[20]. Most circRNAs are ceRNA and show tissue and developmental stage-specific expression[21]. It was reported that circRNAs could regulate the expression of parental gene [22, 23] or function as miRNAs sponges [24, 25]. By competing for common miRNA response elements (MREs), ceRNAs can break the balance between miRNAs and target genes to regulate the physiological and pathophysiological process[26]. These ceRNAs include various types of RNAs, such as circRNAs, miRNAs, and mRNAs.

Moreover, Meisheng Zou and her colleagues[27] predicated that the hsa_circRNA_101238 might inhibit the expression of hsa-miR-320a and increase that of MMP9 in TAD. In her research, several circRNAs, miRNAs, and mRNAs are suggested to be involved in AD, but it was only three samples, and
most of their predicted-relevant data were different from ours. These differences add to fully understand the impact of ceRNA crosstalk on ATAAD, and enrich the genetic database, it will be improtant to integrate the circRNA-miRNA-mRNA competitive regulatory networks. In this study, we developed RNA sequencing (RNA-seq) with Arraystar human RNAs microarray to comprehensively identify DE circRNAs, miRNAs, and mRNA in normal and ATAAD. Combining the identified DE RNAs, the circRNA-miRNA-mRNA regulatory network was constructed. Furthermore, analysis of the regulatory network identified some important mRNAs and their corresponding regulatory networks that were highly related to the progression of ATAAD. The flowchart for this procedure is shown in Figure 1. Our findings might provide new evidence for exploring the molecular mechanism of ATAAD, a better understanding of the molecular, cellular, and genetic mechanisms that cause this disease is necessary to developing more effective preventative and therapeutic treatment strategies.

Methods

All patients with ATAAD included in this study were diagnosed by computed tomography. The control group were obtained in the age-matched patient with normal ascending aortic wall tissues. And all operations were performed in the Second Hospital of Jilin University. Aortic wall tissues were immediately numbered, transferred into jars containing liquid nitrogen and stored for future use. The study including all experimental protocols was approved by the Second Hospital of Jilin University and all patients gave pre-operative written informed consent.

Microarray analysis and analysis of DE circRNAs, miRNAs and mRNAs

Agilent Human Microarray V2 analysis was adopted for detection of circRNAs, miRNAs, and mRNAs expression. All microarray analyses were performed in the laboratory of the KangChen bio-tech (Shanghai, China). Briefly, sample labeling and hybridization array were performed according to the manufacturer's protocol. After having washed the slides, the arrays were scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the R software limma package. DE circRNAs, miRNAs, and mRNAs between two group were identified through fold change(FC), p-value, and the false discovery rate (FDR) filtering. Moreover, DE circRNAs,
miRNAs, and mRNAs were selected with the cutoff criteria of FC\(\geq 2.0\) and p-value \(\leq 0.05\).

**Construction of the circRNA-miRNA-mRNA regulatory network**

According to the results of the DE analysis, the regulatory relationships between circRNAs and miRNAs were predicted using Circular RNA Interactome (https://circinteractome.nia.nih.gov/). Using miRWalk2.0[28] (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) to predicate the regulatory relationships between miRNAs and mRNAs. Then we selected the miRNA–mRNA pairs and circRNA-miRNA pairs with inverse expression relationships. These inverse expression relationships pairs were included for network construction. The circRNA-miRNA-mRNA regulatory network. Finally, the regulatory network was visualized using Cytoscape 3.4.0 (http://chianti.ucsd.edu/cytoscape-3.4.0/) [29]. At the same time, the Cytoscape plug-in CytoNCA[6] (Version 2.1.6, http://apps.cytoscape.org/apps/cytonca) was used to analyze the degree of node connection. If the circRNA, miRNA and mRNA with a higher node connection degree, it is more likely to be a key gene in the network.

**Functional enrichment analysis**

GO[30] analysis and KEGG[31] pathway analysis of DE mRNAs and host gene of the DE circRNAs were carried out by Database for Annotation, Visualization and Integrated Discovery (DAVID; http://www.david.abcc.ncifcrf.gov/). In order to predicts the function of the miRNA, we performed KEGG pathway enrichment analysis on the mRNA of the target gene of the miRNA. A GO term or KEGG pathway with FDR <0.05 and enrichment count not less than 3 were considered statistically significant. The top 10 enriched GO terms and pathways of DE mRNAs were ranked by enrichment score \((-\log_{10}(P\text{-value}))\). GO analysis was performed to annotate genes and gene products with terms from three aspects: biological process (BP), cellular component (CC), and molecular function (MF). KEGG pathway analysis was conducted to predict the molecular interactions and reaction networks associated with differentially expressed genes. Data were analyzed by two-sided Fisher's exact test, and the FDR was calculated to correct the \(-\log_{10}(p\text{-value})\). A cutoff of \(-\log_{10} \leq 0.05\) was set for statistical significance.

**qRT-PCR validation and statistical analysis.**
The results of high-throughput sequencing analysis were verified by qRT-PCR. Total RNA was reverse-transcribed to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer’s instructions. Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA) was used to design primers, and a QuantStudio™ 7 Flex real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) was used. The primer sequences are listed in Table 1. The results were collected in three independent wells. All samples were normalized to GAPDH. And the relative expression levels of each gene were calculated using $2^{-\Delta\Delta Ct}$ methods. Statistical analysis was performed with SPSS software (version 19.0 SPSS Inc.). P-values < 0.05 were considered as statistically significant.

Results

Identification and general characteristics of DE circRNAs, miRNAs, and mRNAs in ATAAD

Microarray analysis were performed in ATAAD and matched normal aortic wall tissues using an Agilent Human Microarray V2. According to microarray data, a total of 881 significantly dysregulated circRNAs (FC > 2.0, p < 0.05) including 285 up-regulated and 596 down-regulated circRNAs were found to be differentially expressed(Fig. 2a,2d,and 2g), the DE circRNAs type as is shown in Fig. 3a, and 3b. A total of 185 significantly dysregulated miRNAs (fold change > 2.0, p < 0.05) including 170 up-regulated and 15 down-regulated miRNAs were found to be differentially expressed(Fig. 2b,2e,and 2h). In addition, 1928 up-regulated mRNAs and 906 downregulated mRNAs were identified (FC > 2.0, p < 0.05) (Fig. 2c,2f,and 2i). Both circRNAs, miRNAs, and mRNAs transcripts were found to be distributed on all chromosomes (Fig. 1j).

DE mRNAs, miRNAs, and circRNAs functional prediction in ATAAD

A total of 1928 up-regulated mRNAs and 906 downregulated mRNAs were performed to analyze their potential function and KEGG analysis. The top10 Enrichment score of up-regulated and down-regulated mRNAs of the BP, CC, and MF terms, and KEGG pathway are shown in Fig. 4a, and 4b respectively. According to the DE genes, and the result of co-expression analysis. A total of 7911 circRNA-miRNA pairs(Fig. 5a) and 613 miRNA-mRNA pairs(Fig. 5b). The host genes of circRNA included in the circRNA-miRNA pairs were analyzed by GO(BP) and KEGG pathway(Fig. 6), the top10 Enrichment score of the BP terms and KEGG pathway are shown in Fig. 6a. A total of 3 significant
miRNA KEGG pathway (Fig. 7a) and 21 significant miRNA GO(BP) were obtained (Fig. 7b).

**CircRNA-miRNA-mRNA network in ATAAD**

A total of 2181 circRNA-miRNA-mRNA network were generated from circRNA-miRNA pairs and miRNA-mRNA pairs. Then, combining the positive co-expression relationship between mRNA and circRNA (Pearson correlation coefficient > 0.85), A total of 105 circRNA-miRNA-mRNA network was generated including 72 mRNAs, 25 circRNAs, and 17 miRNA (Fig. 8). According to the analysis of the degree of node connection, the top 5 of the circRNAs, miRNAs, and mRNAs are shown in table 2, it is more likely to be the key genes in the network.

**DE circRNAs validated by qRT-PCR**

To verify the results of the RNA-seq experiments, eight key circRNAs with P < 0.05 and FC ≥2 were selected for qRT-PCR validation. The circRNAs were amplified with divergent primers (Table 1). The circRNA_0041871 and circRNA_0001394 were up-regulated in the ATAAD group compared to the control group, and the circRNA_404522, circRNA_022920, and circRNA_000536 were down-regulated. All the results were consistent with the normalized expression of RNA-seq are shown in Fig. 9.

**Discussion**

CircRNAs, miRNAs, and mRNAs form largescale ceRNA cross-talk networks through MREs, which has exciting implications for gene regulation at the post-transcriptional level during multiple physiological and pathophysiological processes [14, 32-34]. In recent years, studies have focused on the functions and clinical implications of ceRNAs in cancer [35]. Recently, circRNAs and miRNAs have been investigated in cardiovascular diseases and reported to play an important role in hypertension, coronary artery disease, heart failure, and myocardial infarction, which may present opportunities for new therapeutic approaches for diseases [36-40]. Despite this increase in interest of the cardiovascular diseases, in the AD disease is rarely reported [27]. To fully understand the impact of ceRNA crosstalk on AD, it will be crucial to integrate the competitive circRNA-miRNA-mRNA regulatory networks. In our study, 881 DE circRNAs, 185 DE miRNAs, and 283 DE mRNAs were identified. The DEGs in ATAAD analyzed by GO functional enrichment analysis showed that the downregulated DE mRNA mainly enriched in regulation of actin-mediated cell contraction, transmembrane receptor
protein serine/threonine kinase, regulation of calcium-mediated signaling, and actin-myosin filament sliding. While upregulated DEGs were shown to be concerned with immune response to stress, defense response, cell cycle process, and inflammatory response. This conforms to the knowledge that regulation of actin-mediated cell contraction, transmembrane receptor protein serine/threonine kinase, cell cycle process, and inflammatory responses are main mechanisms of ATAAD development and progression[3, 41-45].

Through KEGG pathway analysis, mRNAs of ceRNA networks were predicted to be involved in the DNA replication, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC), Hypertrophic cardiomyopathy (HCM), adrenergic signaling in cardiomyocytes, cell cycle, vascular smooth muscle contraction, regulation of actin cytoskeleton, and TGF-beta signaling pathway. It is well-known that the vascular smooth muscle contraction, regulation of actin cytoskeleton, and TGF-beta signaling pathway were confirmed to associated with proliferation, migration and apoptosis of vascular smooth muscle cells in aortic dissection, and focal adhesion and regulation of actincytoskelet[45-51].

In the present study, we found dysregulated circRNAs, miRNAs and mRNA in the aortic wall of the ATTAD patients and predicted their ceRNA network. A total of 105 circRNA-miRNA-mRNA network was generated. According to the analysis of the degree of node connection, the top 5 host gene of the circRNAs were INADL, FIBP, LRRC16A, ADARB1, and LOC100506499, it is may be the key circRNAs during disease development and progression. FIBP may play an important role in proteolysis, inflammation, and apoptotic processes in the disease of abdominal aortic aneurysms[52]. In addition, the top 5 miRNA was hsa-miR-1285-3p, hsa-miR-637, hsa-miR-650,hsa-miR-485-5p, and hsa-miR-509-5p, the top 5 target genes were TINAGL1, JPH4, PLXNA2, TGFBR1, and THSD4. As well as we known, only a small portion of the DE gene in the ceRNA network have been annotated. For example, inhibition of extracellular regulated protein kinase(ERK) phosphorylation or blockade of angiotensin II type I receptor (AT1R) prevented aneurysmal degeneration of TGFBR1 deficient aorta, loss of SMC-TGFBR1 triggers multiple deleterious pathways, including abnormal TGFBR2, ERK, and AngII/AT1R signals that disrupt aortic wall homeostasis to cause aortic aneurysm formation[53]. CircRNA_010567
could regulate miR-141 and its target TGF-β1 expression and may mediate fibrosis-associated protein resection[36]. TINAGL1, JPH4, PLXNA2, and TGFBR1 is the key mRNAs in our study, it could play an important role in the AD, the separate ceRNA network is shown in Fig. 10. It is worthwhile to perform further studies to reveal the underlying link between these key genes and the molecular mechanisms of AD.

The limitation of this study is that we detected circRNAs, circRNAs and miRNA changes, only selected eight DE circRNAs for qRT-PCR experiments. What excites us is that the results of qRT-PCR experiments were consistent with the normalized expression of RNA-seq. However, we did not validate the miRNA and mRNA, and further research is urgently needed. Maybe these circRNAs can interact with miRNAs and alter the expression of miRNAs and/or its target genes may have great significance in the pathogenesis research for AD and may point out a potential direction of future treatment as well.

Conclusions
We found a profile of dysregulated circRNAs, miRNAs and mRNAs, and competitive circRNA-miRNA-mRNA regulatory networks were comprehensively integrated and predicted to be involved in ATAAD by GO and KEGG pathway analysis. It might be prospective clinical markers associated with the development of ATAAD, and it is worthwhile to perform further studies to reveal the underlying link between these key genes and the molecular mechanisms of AD.

Abbreviations
ATAAD: Acute Type A Aortic Dissection; CircRNAs: Circular RNAs; CeRNAs: Competitive Eendogenous RNAs; miRNAs: microRNAs; mRNA: messenger RNA; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes pathway analyses; TINAGL1: Tubulointerstitial nephritis antigen-like 1; JPH4: junctophilin 4; PLXNA2: plexin A2; TGFBR1: Transforming Growth Factor Beta Receptor 1; THSD4: Thrombospondin type-1 domain-containing protein 4; AD: aortic dissection; ECM: extracellular matrix; FBN1: Fibrillin-1; LOX: lactate oxidase; COL3A1: Collagen, type III, alpha 1; SMCs: smooth muscle cells; ACTA2: alpha-actin-2; MYH11: Myosin-11; FLNA: Filamin-A; ncRNAs: non-coding RNAs; MREs: miRNA response elements; MMP9: matrix metalloprotein 9; TAD: thoracic aortic dissection; RNA-seq:
RNA sequencing; qRT-PCR: quantitative real-time polymerase chain reaction; DE: differentially expressed; FDR: false discovery rate; FC: fold change; DAVID: Database for Annotation, Visualization and Integrated Discovery; BP: biological process; CC: cellular component; MF: molecular function; ARVC: arrhythmogenic right ventricular cardiomyopathy; HCM: Hypertrophic cardiomyopathy; TGF-beta: transforming growth factor-beta; ERK: extracellular regulated protein kinase; AT1R: angiotensin II type I receptor.

Declarations

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Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files.

Authors’ contributions

MXH and KXL designed the study. MXH, HLP, RHX, DL, and TCW curated data, implemented the pipeline and conducted computational analyses. MXH, ZZC, YW, BL, and HLP analyzed the results. MXH drafted the manuscript, all authors revised and approved the final manuscript.

Ethics approval

The study including all experimental protocols was approved by the Second Hospital of Jilin University and all patients gave pre-operative written informed consent.

Consent for publication
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables
Table 1 The primers of GAPDH 5 and circRNAs.
| Gene name          | Forward primer          | Reverse primer          |
|--------------------|-------------------------|-------------------------|
| GAPDH              | TGACTTCAACAGCGACACCCA   | CACCCTGTGTCGTAGGCC      |
| hsa_circ_0001394   | GACGATGACCCACAGTTTACC   | AATAACCATGAAAGGCACAA    |
| hsa_circ_0041871   | ATTTCCAAGAGGCAAGACT     | CACTAGATTCAAGGCAGA      |
| hsa_circ_0022920   | GAGTCTGCTCACAAGCCACC    | ACGAGGCAACACAAAGGC      |
| hsa_circ_0000536   | TCATTTAAGGAGGAGGCC      | CTTTCAACAAAGAAGCAGA     |
| hsa_circRNA_404522 | AAAGGCAAGCTGTGTTTTCATCAGT | GCCACGCAAACACCTACTG     |

Table 2  The top 5 of the circRNAs, miRNAs, and mRNAs

| Gene name          | Host_gene | Degree | Up/Down-regulation |
|--------------------|-----------|--------|--------------------|
| hsa-miR-1285-3p    |           | 21     | Up                 |
| hsa-miR-637        |           | 16     | Up                 |
| hsa-miR-650        |           | 14     | Up                 |
| hsa-miR-485-5p     |           | 12     | Up                 |
| hsa-miR-509-5p     |           | 12     | Up                 |
| hsa_circRNA_404522 | INADL     | 15     | Down               |
| hsa_circRNA_022920 | FIBP      | 12     | Down               |
| hsa_circRNA_075881 | LRRC16A   | 10     | Down               |
| hsa_circRNA_103147 | ADARB1    | 10     | Down               |
| hsa_circRNA_000536 | LOC100506499 | 8   | Down               |
| TINAGL1            |           | 9      | Down               |
| JPH4               |           | 8      | Down               |
| PLXNA2             |           | 8      | Down               |
| TGFRB1             |           | 8      | Down               |
| THSD4              |           | 7      | Down               |

Figures
Figure 1

Flow-chart of data analysis.
Figure 2

DE circRNAs, miRNA, and mRNAs between ATAAD group and control group. a-c: Heat maps. d-f: Scatter plots. g-i: Volcano plots. j: Circos plot: The outermost layer of the circos plot is a chromosome map of the human hg19 genome. There are 6 circles of line plot, which represent significantly regulation of ATAAD group to control group and the exact fold change of circRNAs, miRNAs, and mRNAs respectively.
Figure 3

The DE circRNAs type. a: histogram; b: Pie chart.
The top10 Enrichment score of the BP, CC, and MF terms, and KEGG pathway analysis. a: Up-regulated mRNA; b: Down-regulated mRNA.
Figure 5

Co-expression network. a: circRNA-miRNA pairs (Red nodes, miRNA, blue nodes, circRNA); b: miRNA-mRNA pairs (Red nodes, miRNA, green nodes, mRNA).
The top10 Enrichment score of the host genes of circRNA of the BP terms and KEGG pathway (Red, GO BP; yellow, KEGG pathway; X-length indicates the number of enriched genes, and black polyline indicates the p-value).

7a
Figure 7

GO annotations and KEGG pathway analysis of miRNAs. a: GO annotations; b: KEGG pathway analysis (Top 5).
circRNA-miRNA-mRNA network (Green circles, down-regulated mRNA, red circles, up-regulated mRNA; purple squares, down-regulation circRNAs, blue squares, up-regulation circRNAs; pink inverted triangles, down-regulation miRNAs, yellow inverted triangles, up-regulation miRNAs. Yellow arrows, miRNA-mRNA pairs, black T-shaped line, circRNA-miRNA pairs, and the node size indicates the degree of connectivity).
qRT-PCR validation of dysregulated DE circRNAs in ATAAD compared with age-matched patient with normal ascending aortic wall tissues[* represents $P < 0.05$; ** represents $P < 0.01$].
The separated circRNA-miRNA-mRNA network of TINAGL1, JPH4, PLXNA2, and TGFBR1. a: TINAGL1; b: JPH4; c: PLXNA2; d: TGFBR1.