Construction of lncRNA-miRNA-mRNA networks reveals functional lncRNAs in abdominal aortic aneurysm

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Abstract. Abdominal aortic aneurysm (AAA) is one of the most significant causes of morbidity and mortality in populations aged >65 years worldwide. However, the underlying mechanisms of AAA based on the competitive endogenous RNA (ceRNA) hypothesis have remained elusive. In the present study, differently expressed long non-coding RNA (lncRNA)-microRNA (miRNA)-mRNA networks in AAA were constructed by analyzing public datasets, including GSE7084, GSE24194 from rats and that of a previous study. A total of 1,219 mRNAs, 2,093 lncRNAs and 57 miRNAs were identified to differently express in AAA. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses were performed to explore the potential roles of differently expressed lncRNAs based on their regulating mRNAs. Based on the ceRNA hypothesis, lncRNA-miRNA-mRNA networks in AAA were, for the first time, constructed at a system-wide level. The present study identified 5 upregulated lncRNAs [nuclear paraspeckle assembly transcript 1, cyclin-dependent kinase inhibitor 2B antisense RNA 1, small Cajal body-specific RNA 10, AC005224.4 and SUMO1/sentrin/SMT3-specific peptidase 3-eukaryotic translation initiation factor 4A1] and the downregulated zinc ribbon domain containing 1 antisense RNA 1 as key lncRNAs in ceRNA networks. To the best of our knowledge, the present study was the first to screen ceRNA networks in AAA. In addition, key lncRNA-mRNA-biological processes analysis indicated that these key lncRNAs were involved in regulating signal transduction, protein amino acid phosphorylation, immune response, transcription, development and cell differentiation. The present study provides novel clues to explore the molecular mechanisms of AAA progression in terms of lncRNA implication.

Introduction

Abdominal aortic aneurysm (AAA) is defined as a maximum infrarenal abdominal aortic aneurysm with a diameter of ≥3.0 cm and is characterized by permanent, localized dilations of the abdominal aorta (1,2). AAA is one of the most significant cause of morbidity and mortality in populations aged >65 years worldwide (3,4). Acute rupture is the most dangerous clinical consequence of AAA progression and causes ~80% of associated deaths in the US (5). Pathologic features of AAA include vascular smooth muscle cell apoptosis, infiltration of inflammatory cells, loss of integrity of the arterial wall, increase of oxidative stress and significant matrix degradation (6). However, the precise molecular mechanisms underlying the progression of AAA progression still remain elusive. Therefore, it is important to elucidate the etiological mechanisms of AAA progression to develop novel targets for the diagnosis, treatment, and prognostication of AAA patients.

Long non-coding (lnc)RNAs are a class of non-coding RNAs of >200 nucleotides in length, which have little or no protein-coding function (7). It has been discovered that lncRNAs have important roles in the progression of numerous human diseases, including AAA (8). For instance, Yang et al (9) identified 3,688 differentially expressed lncRNAs between AAA and normal tissues. lncRNAs regulate the protein expression at epigenetic, transcriptional and post-translational levels (10-13). One of the most well-known mechanisms of lncRNAs is their action as competing endogenous (ce)RNAs (14). The ceRNA hypothesis, which was proposed by Tay et al (15), holds that pseudogenes, lncRNAs, circular RNAs and mRNAs may impair micro (mi)RNA activity through sequestration, thereby upregulating miRNA target gene expression. Franco-Zorrilla et al (16) reported for the first time that non-coding RNA interferon-β promoter stimulator 1 promoted phosphate metabolism (PHO)2 protein in plants by sequestering miR-399 and preventing it from inhibiting the stability and translation of PHO2 mRNA. Poliseno et al (17) also reported that certain protein-coding genes and their pseudogenes contain the same evolutionarily conserved miRNA binding sites in their 3'-untranslated

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regions, and that they regulate their respective expression levels by competing for miRNA binding. Emerging studies have indicated that ceRNAs act as important regulators in different types of disease, including cancer, cardiac fibrosis, rheumatoid arthritis and type 2 diabetes mellitus (18-20). Thus, constructing ceRNA networks provides a novel perspective to explore the function of yet uncharacterized lncRNAs involved in AAA progression.

In the present study, differentially expressed RNAs, miRNA and mRNAs in AAA were identified from data provided by the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO), and lncRNA-miRNA-mRNA networks were constructed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were also performed to explore the potential roles of differentially expressed lncRNAs in AAA. The present study aimed to provide useful information to identify novel lncRNAs as biomarkers for AAA.

Materials and methods

Microarray data. In the present study, two public datasets, GSE7084 (21) and that provided by Yang *et al* (9) were analyzed to identify differentially expressed mRNAs in AAA. The GSE7084 dataset was downloaded from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/). The dataset from Yang *et al* (9) was downloaded from the supplementary information of their publication. To further identify differentially expressed lncRNAs in AAA, the dataset by Yang *et al* (9) was analyzed, providing 896 upregulated and 1,197 downregulated lncRNAs. Differently expressed miRNAs were determined from the GSE24194 dataset from rats (22), which was downloaded from the NCBI GEO website. A t-test (23) in the Limma package (24) in R (25) was used to identify differentially expressed genes between normal and AAA samples. The threshold for the differentially expressed genes (DEGs) was set as a corrected P-value of <0.05 and |log2 fold-change (FC)|≥1.

GO and KEGG pathway analysis. To identify functions of DEGs in AAA, GO term enrichment analysis in the categories biological process, cellular component and molecular function was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/). KEGG pathway enrichment analysis was also performed to identify pathways enriched by DEGs in AAA using DAVID. The P-value was calculated by hypergeometric distribution and a pathway with P<0.05 was considered as significant.

Construction of the lncRNA-miRNA-mRNA network. The StarBase dataset was used to identify potentially dysregulated lncRNA-miRNA pairs. Next, miRcode (26), StarBase (27) and the Targetscan database (http://www.targetscan.org) were used to identify miRNA-mRNA pairs. Finally, lncRNA-miRNA-mRNA networks were constructed. In the present study, only downregulated miRNAs and upregulated mRNAs were integrated into the ceRNA network for upregulated lncRNAs, while only upregulated miRNAs and downregulated mRNAs were integrated into the ceRNA network fir downregulated lncRNAs.

Results

Identification of differentially expressed lncRNAs, mRNAs and miRNAs in AAA. In the present study, two public datasets, GSE7084 (21) and that of Yang *et al* (9), were analyzed to identify differentially expressed mRNAs (Fig. 1A and B). mRNAs with a FC of ≥2 or ≤0.5 and P<0.05 were selected as differentially expressed mRNAs. A total of 2,292 upregulated and 2,501 downregulated mRNAs were identified from the GSE7084 dataset. In the dataset from Yang *et al* (9), 1,501 upregulated and 969 downregulated mRNAs were identified. In total, the two datasets had 722 upregulated and 497 downregulated mRNAs in common (Fig. 1C and D). To identify differentially expressed lncRNAs, the dataset from Yang *et al* (9) was re-analyzed, and 896 upregulated and 1,197 downregulated lncRNAs were obtained (Fig. 1E). Furthermore, miRNAs with a FC of ≥2 or ≤0.5 and P<0.05 were selected as differentially expressed miRNAs. A total of 21 upregulated miRNAs and 36 downregulated miRNAs were selected for further ceRNA network construction by analyzing the GSE24194 dataset obtained from rats (Fig. 1F). Hierarchical clustering provided systematic variations in the expression of mRNAs, miRNAs and lncRNAs in AAA.

Functional prediction of differentially expressed mRNAs in AAA. GO and KEGG pathway analyses were performed to explore the function of the 1,219 differentially expressed mRNAs using DAVID. The results indicated that the upregulated mRNAs were enriched in GO terms including immune response, signal transduction, inflammatory response, chemotaxis, cell adhesion, cell-cell signaling, protein amino acid phosphorylation, proteolysis, cellular defense response, regulation of transcription and apoptosis (Fig. 2A), while the downregulated genes were enriched in GO terms including regulation of transcription, oxidation/reduction, cell adhesion, signal transduction, ion transport, protein amino acid phosphorylation, development, nervous system development, muscle development and cell-matrix adhesion (Fig. 2B).

KEGG pathway analysis revealed that the upregulated mRNAs were mainly involved in cytokine-cytokine receptor interaction, hematopoietic cell lineages, the B-cell receptor signaling pathway, cell adhesion molecules, leukocyte transendothelial migration, the T-cell receptor signaling pathway, graft-versus-host disease, Toll-like receptor signaling pathway and the mitogen-activated protein kinase signaling pathway (Fig. 2C), while the downregulated genes were involved in regulation of the actin cytoskeleton, calcium signaling pathway, focal adhesion, neuroactive ligand-receptor interaction, tight junctions, tryptophan metabolism, and valine, leucine and isoleucine degradation (Fig. 2D).

Construction of lncRNA-miRNA-mRNA networks in AAA. To explore the functions of lncRNAs acting as miRNA sponges in AAA, two lncRNA-mediated ceRNA networks were first constructed by using bioinformatics analysis. First, the StarBase dataset was used to identify potentially dysregulated lncRNA-miRNA pairs. Next, miRcode (26), StarBase (27) and the Targetscan database were used to identify miRNA-mRNA pairs. Finally, lncRNA-miRNA-mRNA networks were constructed. In the present study, only downregulated miRNAs
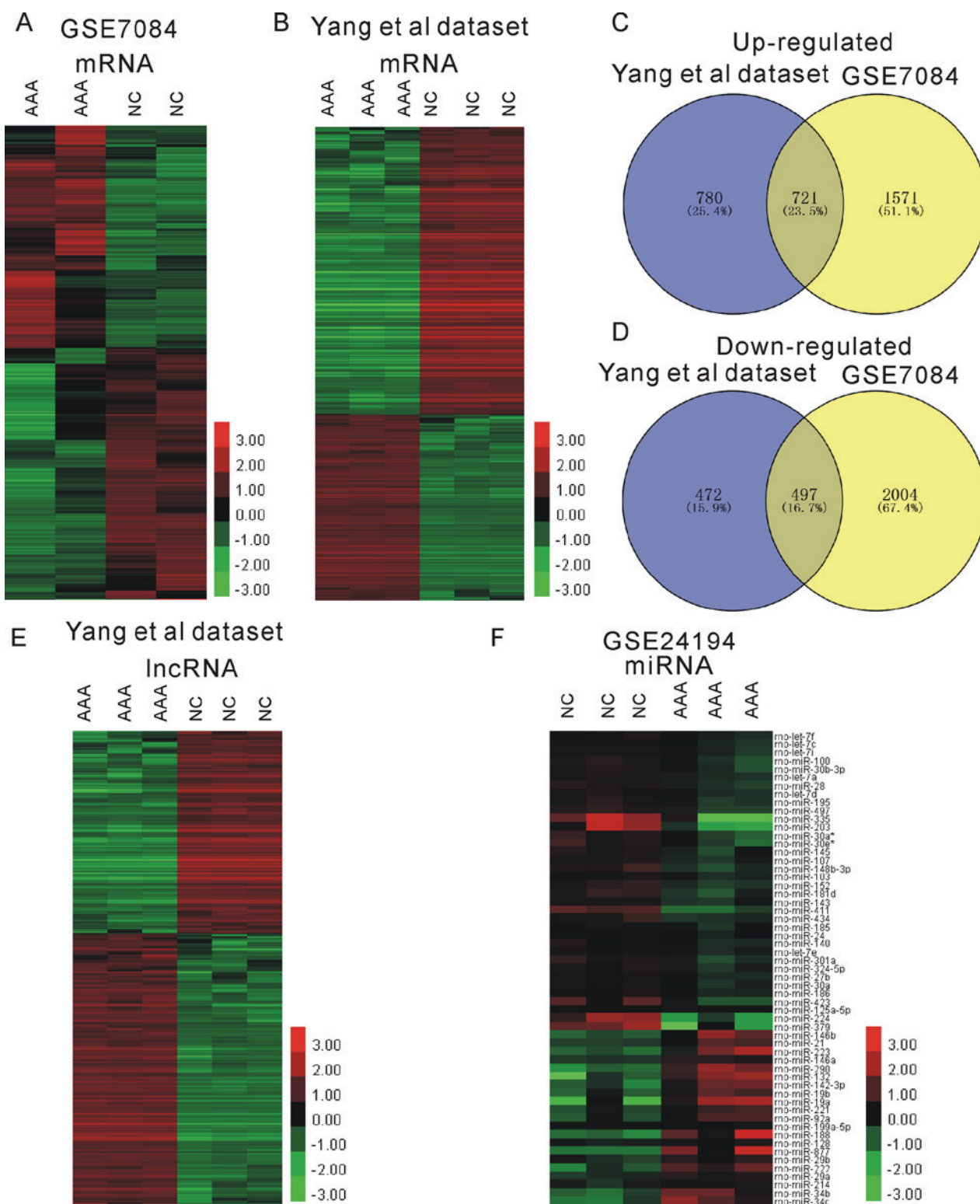
Figure 1. Identification of differentially expressed lncRNAs, mRNAs and miRNAs in AAA. (A and B) Heat map displaying differential mRNA expression in AAA based on (A) the GSE7084 dataset and (B) that of Yang *et al* (9). (C) Heat map presenting differential lncRNA expression in AAA within the Yang *et al* (9) dataset. (D and E) Venn diagrams displaying the overlap of up- and downregulated mRNAs in AAA using the (D) GSE7084 and (E) Yang *et al* (9) datasets. (F) Heat map displaying differential miRNA expression in AAA within the GSE24194 dataset. Red indicates high relative expression and green indicates low relative expression in AAA. AAA, abdominal aortic aneurysm; NC, negative control; lncRNA, long non-coding RNA; miRNA, microRNA; rno, *Rattus norvegicus*.

and upregulated mRNAs were integrated into the ceRNA network mediated by upregulated lncRNAs, while, only upregulated miRNAs and downregulated mRNAs were integrated into the ceRNA network mediated by downregulated lncRNAs.

As presented in Fig. 3, the upregulated lncRNA-miRNA-mRNA network contained 38 lncRNA nodes, 374 mRNA nodes, 27 miRNA nodes and 2,021 edges. In addition, the downregulated lncRNA-miRNA-mRNA network
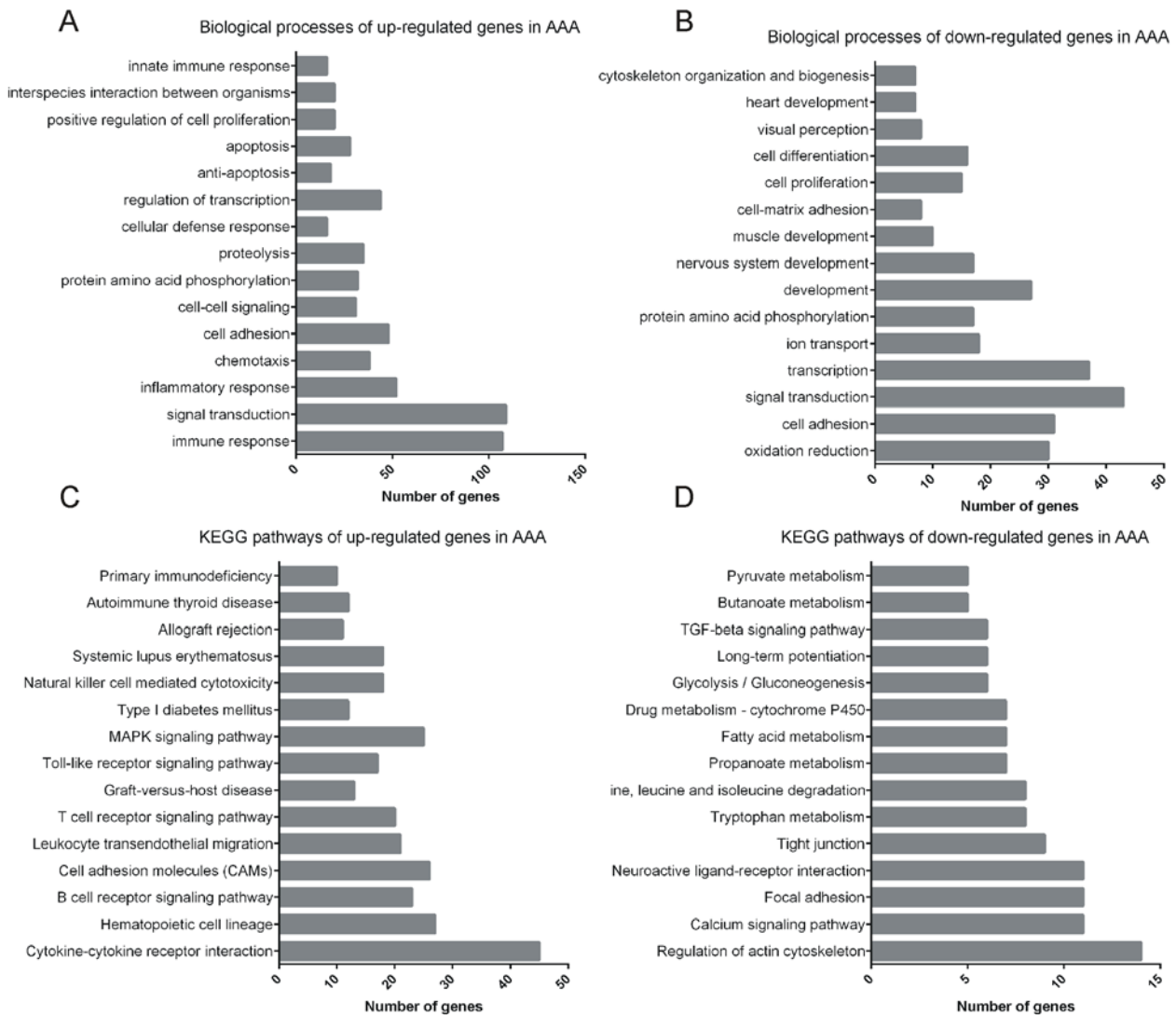
Figure 2. Functional prediction of differentially expressed mRNAs in AAA. (A) GO and (B) KEGG pathway analysis of upregulated mRNAs in AAA. (C) GO and (D) KEGG pathway analysis of downregulated mRNAs in AAA. AAA, abdominal aortic aneurysm; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, gene ontology; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor.

comprised 28 lncRNA nodes, 238 mRNA nodes, 12 miRNA nodes and 729 edges.

Identification of key lncRNA-mRNA-biological processes sub-network. According to the ceRNA network analysis, all node degrees were calculated to identify hub lncRNAs, which have critical roles in biological networks. Five upregulated lncRNAs [nuclear paraspeckle assembly transcript 1 (NEAT1), cyclin-dependent kinase inhibitor 2B (CDKN2B)-antisense RNA 1 (AS1), small Cajal body-specific RNA 10 (SCARNA10), AC005224.4 and SUMO1/sentrin/SMT3-specific peptidase 3 (*SENP3*)-eukaryotic translation initiation factor 4A1 (*EIF4A1*)] and the downregulated zinc ribbon domain containing 1 (ZNRD1-AS1) were identified as key lncRNAs in the ceRNA networks and interacted with >5 different miRNAs (Fig. 4).

Furthermore, key lncRNA-mRNA-biological processes analysis indicated that NEAT1, CDKN2B-AS1, SCARNA10, AC005224.4 and SENP3-EIF4A1 were involved in regulating signal transduction, protein amino acid phosphorylation and

immune response (Fig. 5A). ZNRD1-AS1 was associated with the regulation of transcription, development, and cell differentiation (Fig. 5B).

Discussion

AAA is one of the most significant causes of morbidity and mortality in populations aged >65 years worldwide (3,4). Previous studies reported that various proteins, including mitochondrial uncoupling protein-2, c-Jun N-terminal kinase and matrix metalloproteinase-9 and miRNAs, including miRNA-103a and miRNA-516a-5p, were associated with AAA progression (28). Of note, the underlying mechanisms of AAA have remained to be fully elucidated. lncRNAs are a class of non-coding RNAs with no protein-coding function (7). Several studies have focused on exploring the roles of lncRNAs in aortic aneurysm disease. Hypoxia-inducible factor 1α-AS1 was the first lncRNA reported to be involved in the pathogenesis of thoracic aortic aneurysm (8). Yang *et al* (9) identified 3,688 differentially expressed lncRNAs between
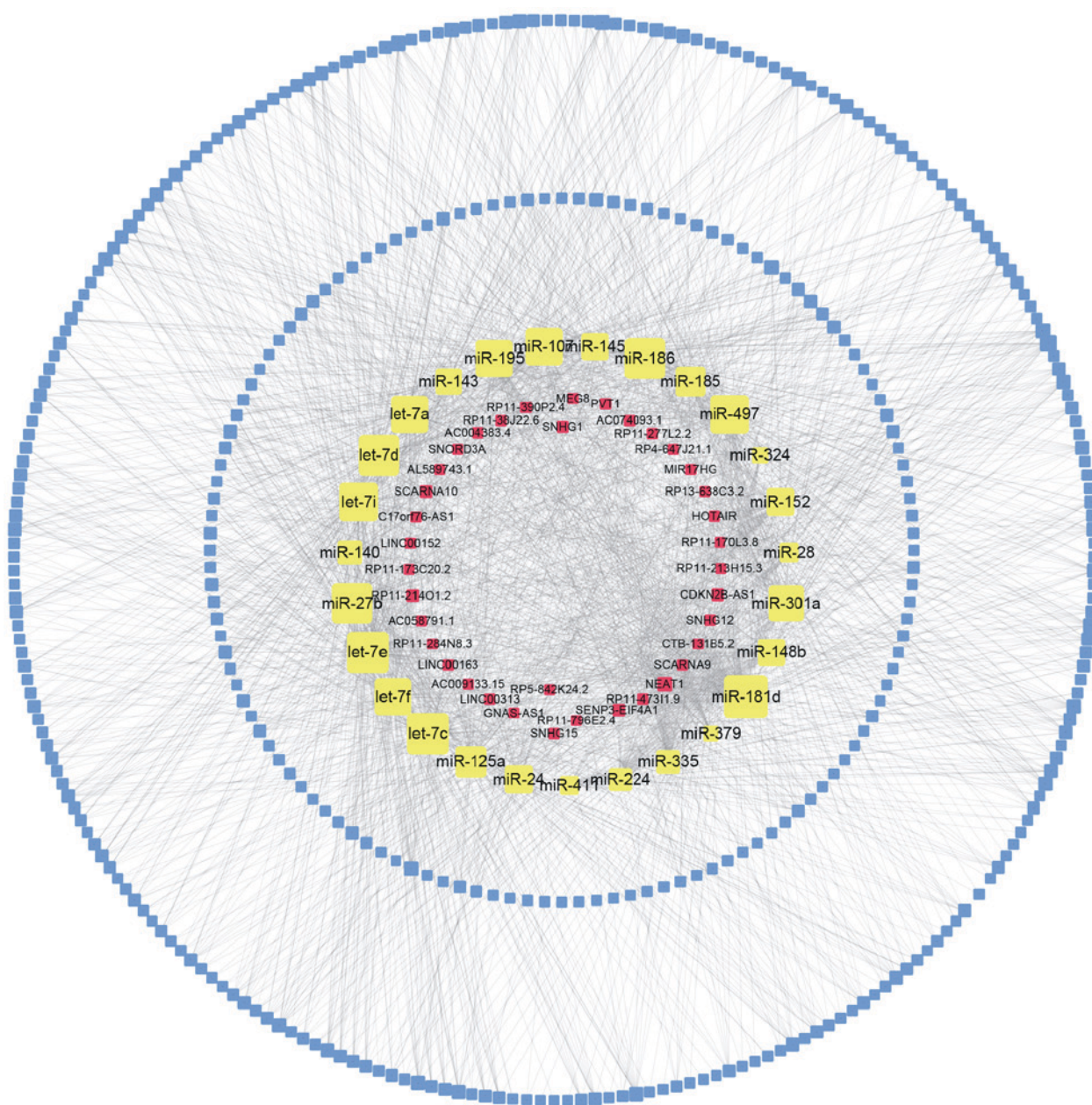
Figure 3. Construction of ceRNA network for upregulated lncRNAs. Red nodes, downregulated lncRNAs; yellow nodes, downregulated miRNAs; blue nodes, upregulated mRNAs; lncRNA, long non-coding RNA; miR, microRNA.

AAA and normal tissues. However, the molecular functions and detailed mechanisms of lncRNAs in AAA remain largely elusive. In the present study, DEGs in AAA were explored by analyzing a series of public datasets, including GSE7084, the dataset from Yang *et al* (9) and GSE24194 obtained from rats. A total of 1,219 mRNAs, 2,093 lncRNAs and 57 miRNAs were identified to differently express in AAA.

The ceRNA hypothesis was first proposed by Salmena *et al* (18) in 2011. This hypothesis holds that lncRNAs act as miRNA 'sponges' to promote the expression of target genes of miRNAs, which provided a novel basis for exploring the molecular functions of lncRNAs. Emerging studies have reported that ceRNAs act as important regulators in different types of disease, including cancer, cardiac fibrosis and rheumatoid arthritis (20). For instance, Karreth (29) reported that the B-Raf pseudogene functions as a competitive endogenous

RNA and induces lymphoma *in vivo*. In the present study, deregulated lncRNA-miRNA-mRNA networks in AAA were constructed. The results indicated that several key lncRNAs served important roles in regulating AAA progression by regulating a series of miRNAs and mRNAs. To the best of our knowledge, the present study was the first to screen ceRNA networks in AAA.

To date, only few studies have focused on exploring the roles of lncRNAs in AAA. In the present study, hub lncRNAs with critical roles in biological networks were identified. The upregulated lncRNAs NEAT1, CDKN2B-AS1, SCARNA10, AC005224.4 and SENP3-EIF4A1, and the downregulated ZNRD1-AS1 were identified as key lncRNAs in the ceRNA networks and interacted with >5 different miRNAs. Furthermore, key lncRNA-mRNA-biological processes analysis revealed that NEAT1, CDKN2B-AS1, SCARNA10,
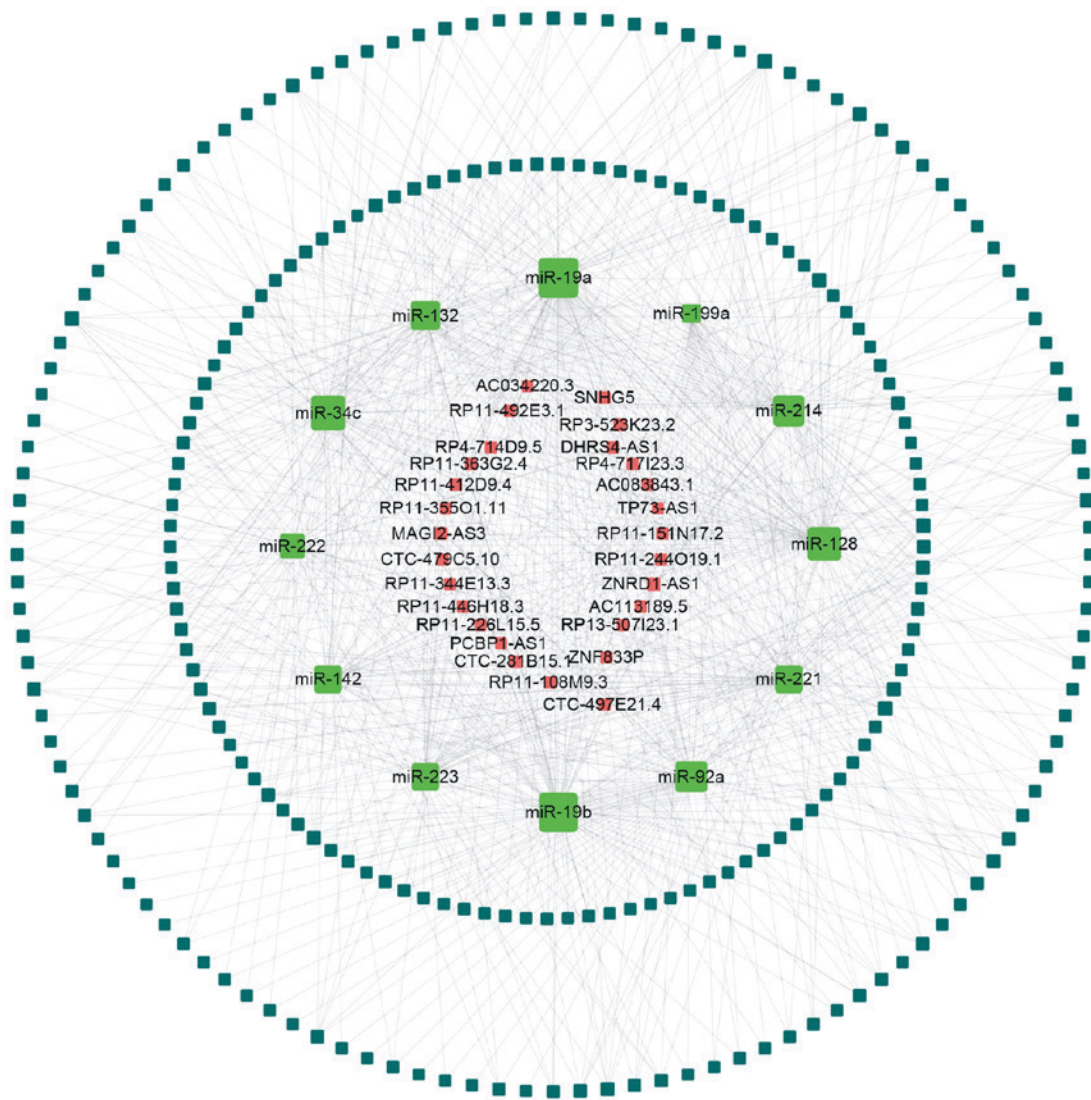
Figure 4. Construction of downregulated lncRNAs mediated competitive endogenous RNA network. Red nodes, downregulated lncRNAs; Green nodes, upregulated miRNAs; blue nodes, downregulated mRNAs; lncRNA, long non-coding RNA; miR, microRNA.
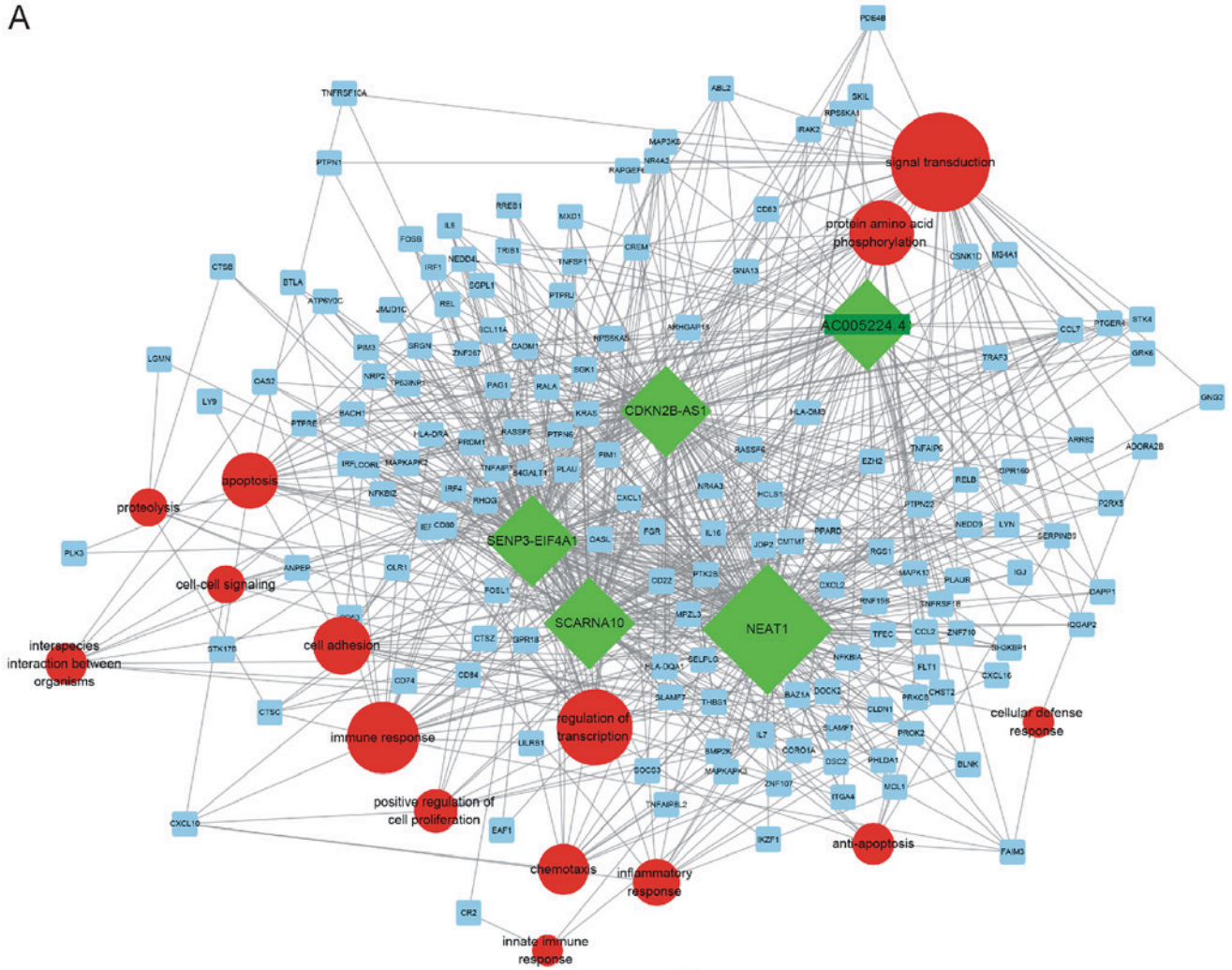
AC005224.4 and SENP3-EIF4A1 were involved in regulating signal transduction, protein amino acid phosphorylation and immune response, while ZNRD1-AS1 was associated with regulation of transcription, development and cell differentiation. Of these key lncRNAs, NEAT1 was reported to be overexpressed in different types of solid tumor, including lung cancer, oesophageal cancer, colorectal cancer and hepatocellular carcinoma (30-33). CDKN2B-AS1 was reported to interact with protein regulator of cytokinesis 1 and -2, leading to epigenetic silencing of CDKN2B (34). CDKN2B-AS1 has key roles in the progression of several cancer types (35-37), intracranial aneurysm (38), type-2 diabetes (39,40), periodontitis (41,42), Alzheimer's disease (43,44), endometriosis (45), frailty in the elderly (46) and glaucoma (36,47).

Several limitations of the present study should be noted. First, further validation of key lncRNA expression levels in AAA samples is required. Although the datasets used in the present study have been reported and validated, further study to confirm the differences in lncRNA expression between AAA and normal samples is required. Another limitation is that the public datasets for analyzing AAA-associated

lncRNAs were limited. An urgent requirement remains to identify differently expressed lncRNAs in AAA by using high-throughput methods, including lncRNA expression array and RNA sequencing. Finally, additional functional investigations of these lncRNAs on AAA progression are still required.

Based on the ceRNA hypothesis, the present study was the first to constructed lncRNA-miRNA-mRNA networks in AAA, to the best of our knowledge. In the present study, a total of 1,219 mRNAs, 2,093 lncRNAs and 57 miRNAs were identified to be differently expressed in AAA based on analyzing public datasets including GSE7084, the dataset by Yang *et al* (9) and GSE24194. The present study further identified 5 upregulated lncRNAs (NEAT1, CDKN2B-AS1, SCARNA10, AC005224.4 and SENP3-EIF4A1) and the downregulated ZNRD1-AS1 as key lncRNAs in the ceRNA networks. Key lncRNA-mRNA-biological processes analysis indicated that these key lncRNAs are involved in regulating signal transduction, protein amino acid phosphorylation, immune response, transcription, development and cell differentiation. The present study provides novel clues for exploring the mechanistic involvement of lncRNA in AAA progression.
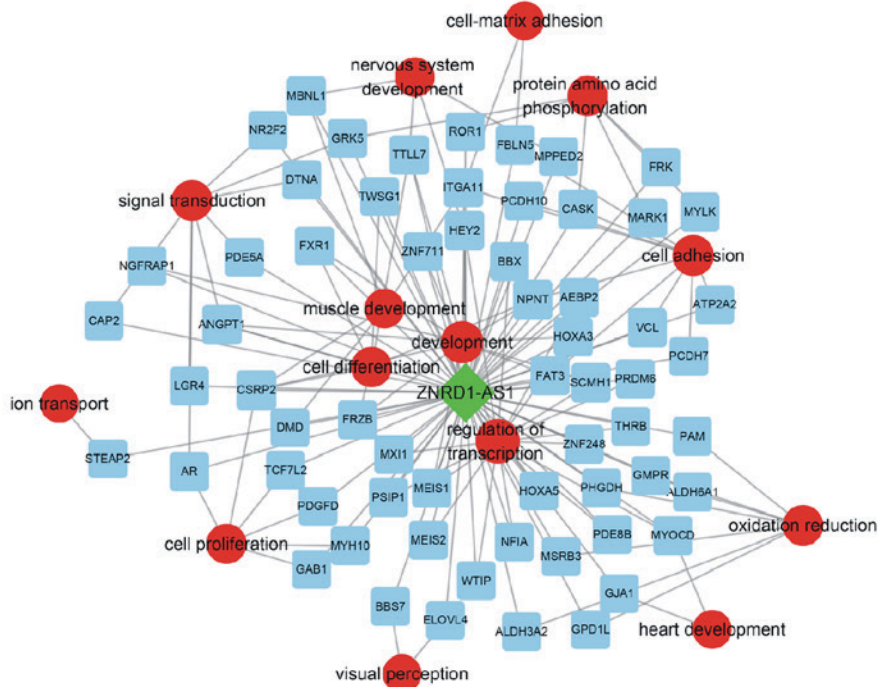
Figure 5. Identification of key lncRNA-mRNA-biological processes subnetwork. (A) Key upregulated lncRNA-mRNA-biological processes analysis indicated that NEAT1, CDKN2B-AS1, SCARNA10, AC005224.4 and SENP3-EIF4A1 are involved in regulating signal transduction, protein amino acid phosphorylation and immune response. (B) The downregulated gene ZNRD1-AS1 was associated with regulation of transcription, development, and cell differentiation. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; CDKN2B, cyclin-dependent kinase inhibitor 2B; AS1, antisense RNA 1; SCARNA10, small Cajal body-specific RNA 10; SENP3, SUMO1/sentrin/SMT3-specific peptidase 3; EIF4A1, eukaryotic translation initiation factor 4A1; ZNRD1-AS1, zinc ribbon domain containing 1.

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Availability of data and materials

High-resolution versions of Figs. 3 and 4 are available on request. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

HZ, LT and DL contributed to the conception and design of the study. LT and XH developed the methodology. YH collected the samples. ZW analyzed and interpreted the data. LT, DL and HZ wrote, reviewed and revised the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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