

Original Article

Identification of DLEU2 as biomarker based on lncRNA-Mediated ceRNA network in rupture of intracranial aneurysm

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Article Info

Abstract



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Intracranial aneurysms (IA) is a potentially devastating clinical problem that may cause lethal subarachnoid hemorrhage upon rupture, but the molecular mechanisms remain further elucidated. Our goal in this work was to build the lncRNA-mediated ceRNA network in IS and explore the associated pathways and functions. The deep transcriptome sequencing dataset profile of rupture of IA and normal tissues (SRP150595) was obtained from NCBI database. To determine which genes were differently expressed, weighted gene co-expression network analysis and other integrated bioinformatics techniques were used (DEGs). The action mechanism and associated pathways of DEGs in IA were investigated using GO annotations and KEGG analysis. The Starbase database was used to build the ceRNA network. Vascular smooth muscle cells (VSMC) were used for the transwell assay and CCK-8. A total of 248 common differentially expressed-protein coding RNA and 76 DE-lncRNAs were obtained. Functional enrichment analysis indicated that the DEGs of IA are involved in pathways of inflammation and immune response. A lncRNAs-mediated ceRNA network including lncRNAs BASP1-AS1, DLEU2, LINC02035, LINC02363, MMP25-AS1, AC008771.1 was constructed. The biological behavior of VSMC was suppressed when DLEU2 was knocked down. In conclusion, a lncRNAs-mediated ceRNA network was constructed in IA based on the integrated bioinformatics analyses, in which DLEU2 was identified to be a novel and potential biomarker of IA.

Keywords: Intracranial aneurysm (IA), Bioinformatics, lncRNAs, ceRNA network, Inflammation and immune, DLEU2

1. Introduction

Ruptured intracranial aneurysm (IA) is a vascular disease that often leads to deadly vascular rupture and subarachnoid hemorrhage, with a fatality rate of over 50%. Its frequency in the general population is 3.2% [1, 2]. Patients aged 40–60 are the high-incidence population of IA disease [3]. IA is one of the most deadly types of cerebrovascular illnesses. Its primary clinical features are cerebral vasospasm, oculomotor nerve palsy, and spontaneous cerebral bleeding [4]. Significantly, heredity has a major role in the development of IA, and a family history of IA has also been proposed as proof that cerebral aneurysms are inherited [5]. Clinicians continue to face challenges due to the unexpected nature of IA rupture and its disastrous implications [6]. Thus, it is essential to investigate the underlying genetic process of IA.

Currently, mRNAs that code for proteins have been the most thoroughly studied RNA class, first via the use of microarrays and subsequently through next-generation sequencing methods [7–10]. Long noncoding RNAs (lncRNAs, >200 nt) are naturally occurring molecules that cannot code for proteins but have a range of uses, including as gene control and epigenetics [11]. By use of miRNA response elements (MREs), which affect the development

of diseases by influencing the regulation of target genes by miRNA, lncRNA may function as competitive endogenous RNAs (ceRNAs) and compete with other RNAs for microRNAs (miRNAs) [12]. The growing body of research on epigenetics, non-coding RNAs, and vaccination has drawn interest in the molecular processes underlying IA. Nevertheless, extra investigation is required to understand the roles played by the lncRNA-mediated ceRNA network in the pathophysiology of IA, despite the fact that several studies have examined microRNAs (miRNAs) [13, 14] or circular RNA (circRNA) [15, 16] in IA.

Here, investigating the fundamental mechanism of ceRNA in IA was the goal of this investigation. The weighted gene co-expression network analysis (WGCNA), a component of integrated bioinformatics analysis, was used to find common differentially expressed genes (DEGs) in the RNA-Seq dataset (SRP150595). Additionally, the underlying mechanism and linked pathways in IA were investigated using functional enrichment analysis, which included Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). After that, the Starbase information was used to build the lncRNA-mediated ceRNA network. Last but not least, the ceRNA network revealed six lncRNAs that may be biomarkers for IA, and the asso-

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ciated signaling pathway was markedly enriched. These findings provide further support for the investigation of IA and could open up a fresh door for focused treatment.

2. Materials and Methods

2.1. Data acquisition

The National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/geo>) provided the RNA-Sequencing (RNA-Seq) dataset profile of ruptured intracranial aneurysm and normal tissues (SRP150595). Twenty controls and 19 patients in the acute period of IA rupture (RAA, first 72 hours) were chosen for follow-up study using the deep transcriptome sequencing data of IA rupture. 28 miRNAs related to IA were obtained from a research study by Rafal Morga et al. [17].

2.2. Identification of DEGs

To find DEGs, the R program DESeq2 was used. The differential expression of differentially expressed-protein coding RNAs (DE-pcRNAs) and DE-lncRNA was filtered using the criterion of adjusted P-value < 0.05 and fold change ≥ 1.5 after the data had been integrated into an expression matrix and normalized.

2.3. Co-expression network construction by WGCNA

Using samples of normal abdominal aorta and IA, a co-expression network for every gene was created using the "WGCNA" R package. The top 50% of the genes were chosen for WGCNA analysis after the rows in the expression matrix that did not match the gene symbol were removed. Samples were utilized to calculate Pearson's correlation matrices. The weighted adjacency matrix was then created using the formula $a_{mn} = |c_{mn}|^\beta$, where a_{mn} is the adjacency between gene m and gene n, c_{mn} is Pearson's correlation, and β is the soft-power threshold. The criterion for merging comparable modules was set at 0.25, and the minimum gene module size was set at 30 in order to get appropriate modules.

2.4. Function enrichment analysis

The functions of each DE-mRNA were examined using the KEGG pathway analysis and GO annotations. Using the DAVID database (<https://david.ncifcrf.gov>), GO annotations for biological process (BP), cellular component (CC), and molecular function (MF) were carried out [18, 19]. Cytoscape ClueGo was used to build the KEGG network. P-value < 0.05 indicated substantial enrichment in the pathways.

2.5. CeRNA network construction

The website StarBase (<http://starbase.sysu.edu.cn/>) was used to forecast miRNA-pcRNA and lncRNA-miRNA interaction data [20]. Next, using Cytoscape (version v3.7.2, <https://cytoscape.org/>), a ceRNA network was built utilizing the miRNA that regulates both lncRNA and mRNA.

2.6. Cell culture and knockdown

The Chinese Academy of Sciences' Institute of Biochemistry and Cell Biology is the source of human vascular smooth muscle cells (VSMC). For regular culture, DMEM medium with 10% fetal bovine serum was employed (37°C, 5% CO₂). The sequencing of DLEU2 shRNA were as following:

shRNA- DLEU2-1: 5'-CAUUGAAUACUAU-

CAAAAAGGAA-3'; shRNA- DLEU2-2: 5'-TGCTGAAACTGCACAAAAAATCG-3'; shRNA- DLEU2-3: 5'-TTGCTGAAACTGCACAAAAAATC-3'; sh-NC: 5'-UUCUCCGAACGUGUCACGUTT-3'

2.7. RT-PCR assay

Using the TRIzol reagent, the total RNA of A549 and H1299 cells was extracted (Invitrogen, Carlsbad, CA, USA). Using the ImProm-IITM Reverse Transcription System kit (Promega Corporation, Madison, WI, USA) and following the manufacturer's instructions, reverse transcribed RNA into cDNA. They then used SYBR® GREEN qPCR Super Mix to perform qPCR (Invitrogen, Carlsbad, CA, USA). To put it briefly, the reaction system was loaded with primer, cDNA, and SYBR GREEN qPCR Super Mix. After two minutes of denaturation at 95°C, the PCR thermocycling parameters were as follows: forty cycles of 15 seconds at 95°C and 32 seconds at 60°C. The internal control used to normalize BLK expression was GAPDH. BLK expression was evaluated using the 2^{-ΔΔCt} technique. The primers were: GAPDH forward, 5'-CCAGGGAAGGATGTAGCTGTG-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'; DLEU2 forward, 5'-TCCGAGAGTATAGCGCCACT-3' and reverse, 5'-ACTGCCCTTTGCTCCAAGTA-3'. Every group underwent three rounds of experiments.

2.8. CCK-8 assay

To examine the cell proliferation experiment, Beyotime Biotech's Cell Counting Kit (CCK)-8 reagent (Shanghai, China) was used. Proliferation of cells was seen at 0, 24, 48, and 72 h. In summary, following the addition of 10 μl of CCK-8 solution to each well, the plate was incubated at 37°C for 2 h. The cells' absorbance at 450 nm was measured using a Thermo Fisher Scientific, Inc. (Waltham, MA, USA) Multiscan MK3 microplate reader.

2.9. Transwell assay

Serum-free media was used to seed 3×10⁵ (without Matrigel) or 5×10⁵ (with Matrigel) cells in the upper chamber. To serve as a chemo-attractant, a ten percent FBS supplement was given to the medium in the bottom chamber. After being incubated for 48 hours, cells that stuck to the filter's bottom or poked through it were counted under a light microscope, stained with crystal violet, and fixed. Using a cotton swab, the cells on the filter's upper surface were eliminated.

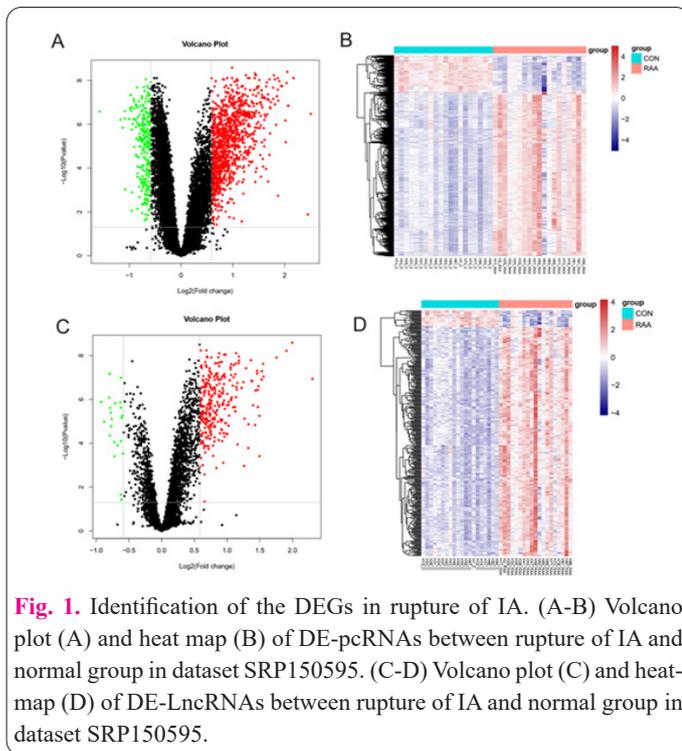
2.10. Statistical analysis

We performed correlation analysis using GraphPad Prism 8 (La Jolla, CA, USA) and the Student's t-test; a p-value of less than 0.01 was considered statistically significant. Data analysis and processing using Microsoft Excel and R (R software, version 3.5.1).

3. Results

3.1. Identification of the DEGs in rupture of IA

Using bioinformatics analysis, the deep transcriptome sequencing data of IA rupture was downloaded in order to look at the differences in molecular expression between IA rupture tissue and normal tissue. A total of 1914 DEGs were identified, 1433 DE-pcRNAs, which included 1171 up-regulated and 262 down-regulated DE-pcRNAs (Figure 1A). Besides, a total of 319 DE-lncRNAs including



297 up-regulated and 22 down-regulated one were also screened out (Figure 1B). Furthermore, heat map cluster analysis on DE-pcRNAs and DE-LncRNAs was carried out to get a deeper understanding of the expression distribution of DEGs in the rupture of IA group and the normal group, respectively (Figure 1C, D).

3.2. WGCNA analysis identified the key module in rupture of IA

The WGCNA analysis was then carried out to determine which coexpression network was most strongly associated with the rupture of the IA condition. 38 samples (H62-RAA samples with significant abnormalities were excluded.) were included in the co-expression analysis (Figure 2A). A power of $\beta=9$ (scale-free $R^2 = 0.85$) was selected as the soft-thresholding parameter to ensure a scale-free network (Figure 2B). Fifteen modules in the condition were identified. Thereafter, the turquoise module including 5731 genes was found to have the highest correlation with acute phase of IA rupture (RAA) condition (turquoise, $r=0.79$, $P=3e-09$) (Figure 2C and 2D). Thereafter, the turquoise module was selected as the module for the following investigation. In addition, 438 hub genes with strong correlation with RAA condition were identified (module membership value > 0.8 and gene significance value > 0.8 as the screening criteria) (Figure 2E).

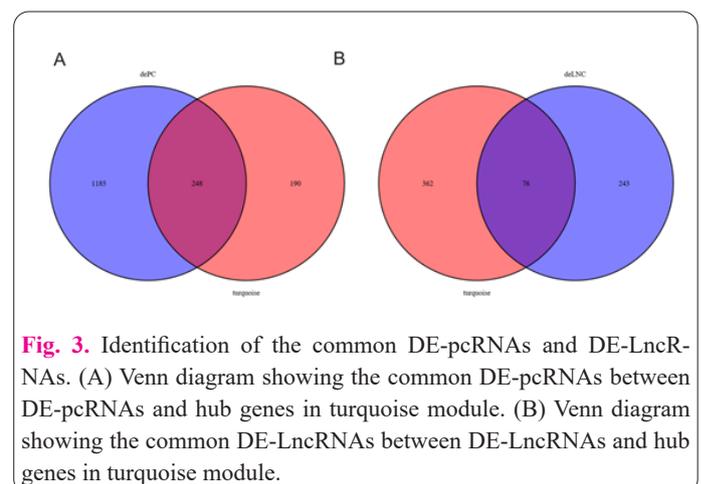
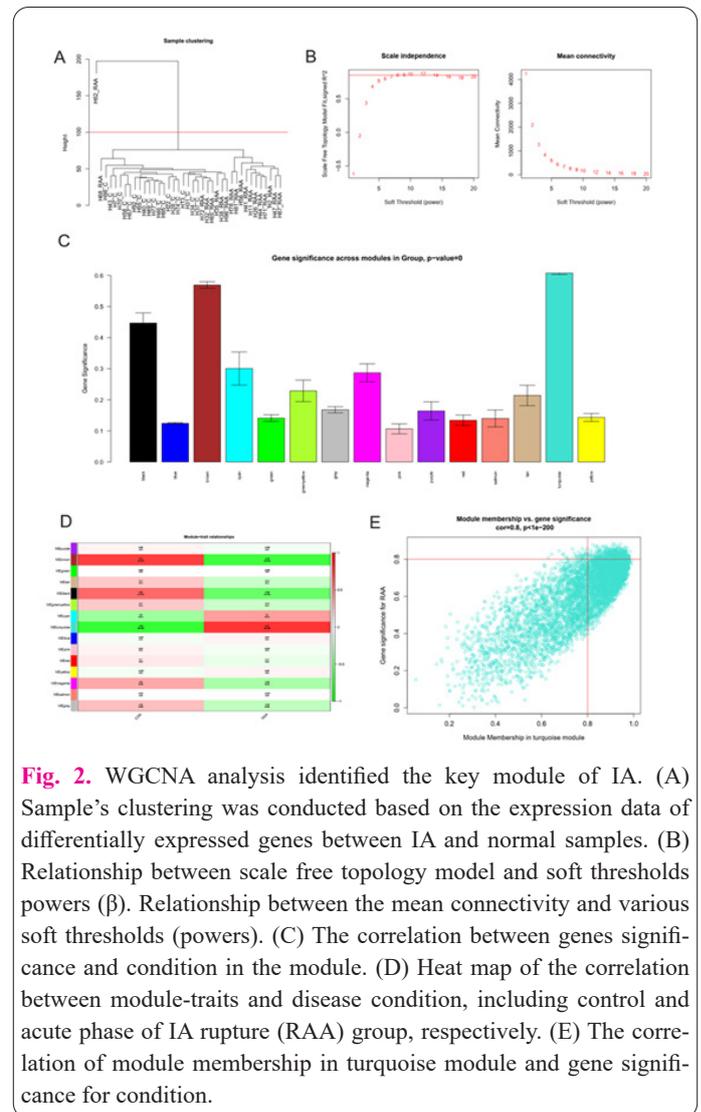
3.3. Functional enrichment analysis of common DEGs

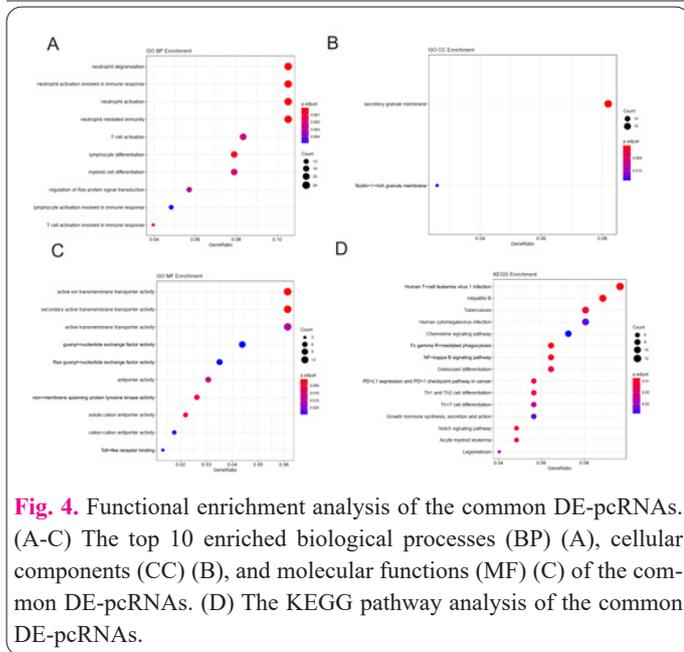
Then, Venn analysis was carried out to identify more credible common DE-pcRNAs and DE-LncRNAs. As seen in Figure 3, a total of 248 common DE-pcRNAs (Figure 3A) and 76 DE-LncRNAs (Figure 3B) were obtained. GO and KEGG analysis was carried out to investigate the related function and pathways in the biological processes in rupture of IA further. Functional analysis of the common DE-pcRNAs indicated that the most overrepresented biological processes are those related to neutrophil activation involved in immune response (GO: 0002283), T cell activation (GO: 0042110), lymphocyte differentiation (GO: 0002285) (Figure 4A). Toll-like receptor bin-

ding (GO: 0035325) was enriched in molecular function (MF) (Figure 4B). The chemokine pathway, notch, Th17 cell differentiation, Th1 and Th2 cell differentiation signaling pathway were also enriched in KEGG analysis (Figure 4D). These findings indicated that the rupture of IA is associated with immune response, immune cell plays an important role in IA rupture, which is consistent with previously studies [21, 22].

3.4. Construction of lncRNAs-mediated ceRNA network in rupture of IA

To further investigate the interaction between DE-





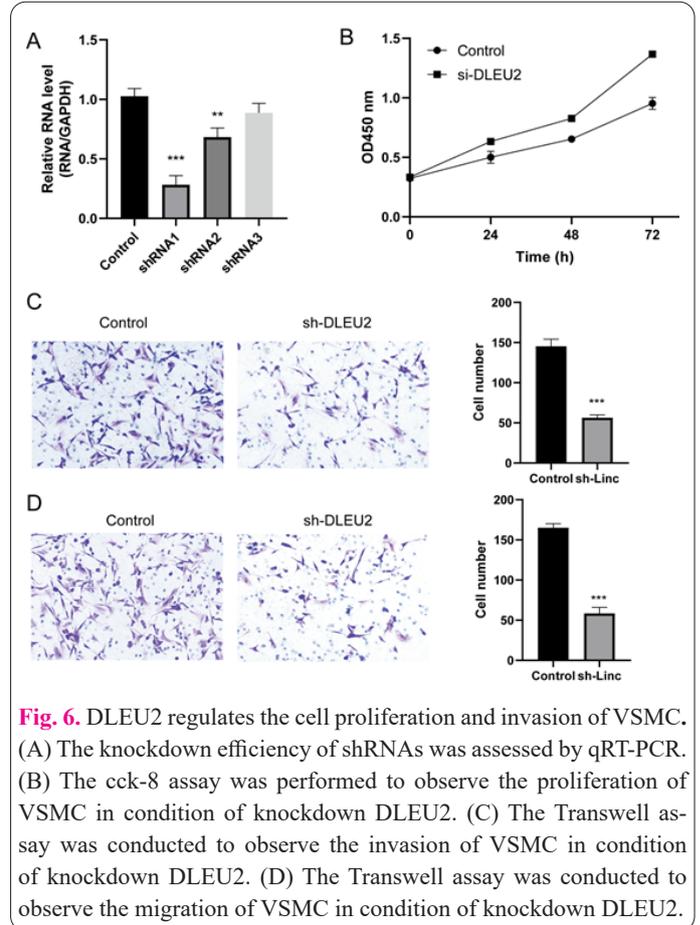
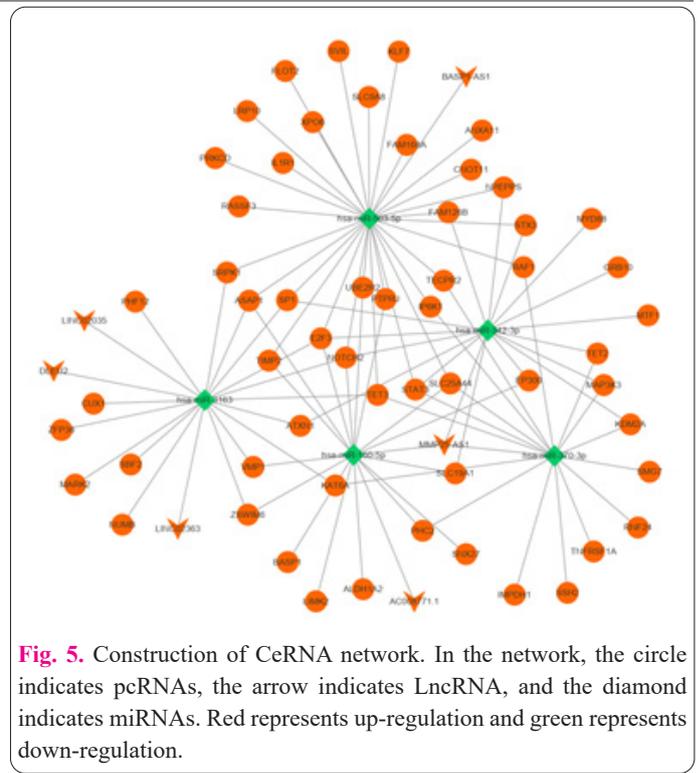
pcRNAs and DE-LncRNAs, Using Starbase, a ceRNA network containing pcRNAs, LncRNAs, and miRNAs was created [20]. A total of 248 common DE-pcRNAs and 76 common DE-LncRNAs were acquired from previous results. In addition, 43 miRNAs which related to IA were obtained from research of Morga et al. [17] As demonstrated in Figure 5, 56 DE-pcRNAs, 5 DE-miRNAs (miR-503-5p, miR-342-3p, miR-3163, miR-150-5p, miR-370-3p), and 6 DE-LncRNAs (BASP1-AS1, DLEU2, LINC02035, LINC02363, MMP25-AS1, AC008771.1.) in the ceRNA networks. Leukemia, esophageal cancer, gliomas, and other malignant tumors exhibit aberrant expression of DLEU2, a new lncRNA linked to cancer [23]. And the other has not been reported yet. Hence, we selected the DLEU2 for further investigation and validation.

3.5. DLEU2 regulates the cell proliferation and invasion of VSMC

Studies suggest that the proliferation and migration of VSMC is the key to the pathogenesis of intracranial aneurysms [24]. Finally, the cell experiments were conducted to investigate the function of DLEU2. 3 kinds of shRNAs were designed and the knockdown efficiency of shRNA1 was most remarkable (Figure 6A). Thus, the shRNA1 was selected for the subsequent experiments. The cck-8 assay indicated that the proliferation was suppressed when DLEU2 was knocked down (Figure 6B). And the invasion as well as migration of VSMC was also decreased when DLEU2 was knocked down (Figure 6C and Figure 6D). These results indicated that DLEU2 regulates the biological behavior of VSMC.

4. Discussion

Being one of the brain's most difficult vascular lesions for physicians to treat, IA is affecting 1%-6% of the world's population and become an increasing social and public health burden [25, 26]. IA could cause lethal subarachnoid hemorrhage upon rupture, but the molecular mechanisms remain elucidated. The pathophysiology of lesions has received increasing interest because of the catastrophic problems that may arise from them. The cellular and molecular pathophysiological mechanisms in IA have been prioritized, according to mounting data [27, 28]. In this



study, Our goal was to look at the molecular process in IA rupture conditions by bioinformatics analysis based on a public dataset. The DE-LncRNAs and DE-pcRNAs expression profiles in IAs were identified. Besides, WGCNA was conducted, and 15 modules were established, in which the turquoise modules including 438 hub genes were found to have the highest correlation with acute phase of IA rupture. 248 common DE-pcRNAs and 76 DE-LncRNAs were obtained by the Venn analysis for the construction of

ceRNA network.

Immune system activation has been shown to be an inherent element in the development of IA [6]. There is growing evidence that some lncRNAs contribute to immunity via a variety of intricate processes. Functional enrichment studies suggested that DEGs could play a role in the pathophysiology of IA and regulate a number of important biological processes, such as immunological response and immune system activation. The molecular role of the toll-like receptor binding was enriched. Toll-like receptor 4 (TLR4) is involved in inflammatory processes linked to vascular disease and Kazuha in addition to the innate immune responses. Mitsui et al indicated that TLR4 mediates the development of IA rupture [29]. Also, the chemokine pathway, notch, Th17 cell differentiation, Th1 and Th2 cell differentiation pathways were enriched. Inflammation lingers as a critical contributor to the pathogenesis of IA, in which cytokines play a major role in regulating inflammation [30]. Aneurysm development and rupture may be directly or indirectly promoted by inflammatory responses that trigger the production and aggregation of inflammatory cytokines, including as MMPs, MCP-1, TNF- α , and ZO-1 [31]. The notch signaling system is essential for the growth of blood vessels [32]. Zhang et al. reported that IA patients exhibited overrepresented Th1 and Th17 activities and underrepresented Th2 activities [33]. The Toll-like receptor binding was enriched in molecular function. TLR4 is involved in the inflammatory processes linked to vascular disease in addition to the innate immune responses, and Kazuha et al indicated that TLR4 mediates the development of IA rupture [29]. To sum, these signaling pathways all are crucial in IA pathophysiology. Although our results were consistent with these previous and recent studies [34-36], further research about their underlying mechanism is needed.

CeRNA may compete with mRNAs for miRNA that have MREs, hence influencing the cell's posttranscriptional regulatory network [37]. We could more thoroughly investigate a number of putative triplets that comprise lncRNAs with potential regulators of IA pathogenesis by using the chosen known IA-associated pcRNAs. As more research has come to light, ceRNA has been linked to a growing number of physiological and pathological processes, including cardiac hypertrophy, the hypoxia-induced microvascular response [38, 39], and others. Specifically, ceRNAs may also influence cell inflammation [40, 41], which is regarded to be crucial step in the creation of an IA [21, 42, 43]. Consequently, our data suggested that ceRNAs could have a role in the etiology of IA. For instance, by comparing IAs with corresponding control arteries, Li et al. discovered DEGs and proposed ceRNA involvement in the etiology of IA [44]. Still, extra investigation is required to understand the functions that the lncRNA-mediated ceRNA network plays in the pathophysiology of IA. Using 248 common DE-pcRNAs, 76 DE-lncRNAs, and associated 43 miRNAs that were received from Morga et al. for further research, we created a lncRNA-mediated ceRNA network in the current study.

In the ceRNA network, we obtained 6 lncRNA that could be crucial in the regulation of IA, including lncRNAs BASP1-AS1, DLEU2, LINC02035, LINC02363, MMP25-AS1, AC008771.1. lncRNAs are regulatory molecules that affect epigenetic control, genomic integrity preservation, transcription, and translation of genes [45].

Knockdown of BASP1-AS1 impaired the neuronal differentiation of human neural progenitor cells [46]. A new lncRNA linked to cancer, DLEU2 is aberrantly expressed in a variety of malignant tumors, such as gliomas, esophageal cancer, and leukemia [23]. It was the first time to report the function of DLEU2 in IA. The proliferation and migration of VSMC is the key to the pathogenesis of intracranial aneurysms [24]. And the result of cck-8 and transwell assay, we learned that the biological behavior of VSMC was suppressed when DLEU2 was knockdown. DLEU2 regulates the biological behavior of VSMC, indicating DLEU2 might be a potential biomarker in IA and further validation *in vivo* or *in vitro* is needed.

In conclusion, this present study sought to screen out the potential DEGs in IA and constructed a lncRNAs-mediated ceRNA network for further investigation. The IA dataset was obtained from a publicly accessible source, and the DEGs were screened using integrated bioinformatics techniques. In order to identify hub gene modules in IA and build a free-scale gene co-expression network, the WGCNA was used. Functional enrichment analysis indicated that the DEGs of IA are involved in inflammation and immune response. Six lncRNAs were identified as ceRNA, in which DLEU2 regulates the biological behavior of VSMC. More research on the roles and processes of these lncRNAs in diseased biological systems may open up new avenues for the discovery of IA therapeutic targets.

Declarations

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

Yanlei Gao and Tao Yu participated in the design of the present study and performed the experiments and data acquisition. Yanlei Gao drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable. This is a bioinformatics study. The Yantai Yuhuangding Hospital Research Ethics Committee has confirmed that no ethical approval is required.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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