High-Throughput Data Reveals Novel Circular RNAs via Competitive Endogenous RNA Networks Associated with Human Intracranial Aneurysms

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Background: Little is known about epigenetic regulation of intracranial aneurysms (IAs). Circular non-coding RNAs (circRNAs) play crucial roles in cardiovascular diseases, but they have received scant research attention regarding their relationship with IAs. This study aimed to explore new pathological mechanisms of IA through circRNA expression profiles and to provide novel therapeutic strategies.

Material/Methods: The comprehensive circRNA and mRNA expression profiles were detected by RNA-Seq in human IA walls and superficial temporal arteries (STAs). The RNA-Seq findings were validated by qRT-PCR. GO and KEGG analyses indicated the functions of these circRNAs. A competing endogenous RNA (ceRNA) network was constructed to reveal the circRNA-miRNA-mRNA relationship. Two newly discovered circRNAs were further detected in peripheral blood of IA patients and healthy people to clarify their expression patterns in the periphery.

Results: Many differentially expressed circRNAs are closely involved in immune/inflammatory response and cell adhesion/adherens junction. The novel circRNAs (hsa_circ_0072309 and hsa_circ_0008433) regulate DDR2 and MMP2, respectively, which are associated with SMC dysfunction and vascular injury through ceRNA. Moreover, we found differential expression of these 2 circRNAs in the peripheral blood of IA patients, and the expression pattern of hsa_circ_0072309 had central and peripheral consistency.

Conclusions: To the best of our knowledge, this is the first study to perform circRNA sequencing analysis of IAs. hsa_circ_0072309 and hsa_circ_0008433 are novel and pivotal circRNAs related to IAs. This study provides new insights into therapeutic targets and biomarkers for IA patients.

MeSH Keywords: High-Throughput Nucleotide Sequencing • Intracranial Aneurysm • RNA Polymerase III

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Intracranial aneurysm (IA) is a local pathological expansion of the cerebral arteries. Its prevalence is approximately 3% of the population worldwide, with a mean age of 50 years [1]. Rupture of IAs is an acute stroke and critical condition called spontaneous subarachnoid hemorrhage (SAH), which has a mortality rate of nearly 50% [2]. There has been much recent research exploring the molecular mechanisms of IAs, but the genetic pathology of IA remains unclear.

CircRNA is a novel type of endogenous non-coding RNA without a 5’ cap and 3’ poly (A) end. It is widely present in human cells, regulating transcriptional or posttranscriptional gene expression [3]. They are widely expressed in the central nervous system (CNS) and enrich biological functions, such as the function of “miRNA sponge”, which subsequently affects mRNA expression and interacts with proteins through CeRNA [4]. Multiple studies have revealed that the ceRNA mechanism is involved in many crucial pathological processes, including inflammation, smooth muscle cell (SMC) phenotype conversion, and extracellular matrix (ECM), which play important roles in vascular diseases [5–7].

Using next-generation high-throughput sequencing technology, this study is the first to define the circRNA expression profiles of IAs. Compared with microarrays, sequencing methodology provides more comprehensive and reliable data [8], which will improve our knowledge of the epigenetic mechanisms of IAs. We found that some novel circRNA molecules are closely involved in the pathogenesis of IAs and show a certain consistency both in central and peripheral. These findings were further verified by the subsequent peripheral blood PCR experiments in the IA population with enlarged sample sizes. In summary, these results may provide novel insights into the molecular mechanisms of IAs, shed light on new treatments, and promote exploration of circRNAs as biomarkers.

Material and Methods

Patient selection and specimen collection for RNA sequencing

A total of 4 human IA walls (2 were ruptured IAs and the other 2 were unruptured IAs) and 4 normal human superficial temporal arteries (STAs) were harvested from patients in the Department of Neurosurgery, the Second Affiliated Hospital of Fujian Medical University between December 2017 and June 2018. Their respective peripheral blood samples were collected at the same time. All selected patients were confirmed to have IAs by head computed tomography angiography (CTA) or digital subtraction angiography (DSA) and they consented to microsurgical clipping. We excluded those who had cerebrovascular diseases other than IA, systemic malignant tumor, or severe complications. In case of ruptured IAs, surgeries were performed within the acute phase (24 h) after initial subarachnoid hemorrhage [9]. The aneurysmal domes were obtained during the operation without endangering patients. As controls, we collected STA tissues from patients with craniocerebral trauma if the tissues had been injured during craniotomy [10–12]. All control patients underwent head CTA to exclude IAs before surgery. The included patients’ baseline information and clinical features of IAs are shown in Table 1. Tissue samples were immediately stored in liquid nitrogen (<1 min after resection) and transferred to a −80°C freezer. All included volunteers signed informed consent and the study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University.

RNA isolation and purification

Total RNA was extracted from each tissue and peripheral blood sample using TRIzol reagent (Takala, USA) according to the kit’s instructions. The quality of the RNA samples was assessed using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and OD260/OD280 ratios between 1.8 and 2.1 were accepted. Agarose gel electrophoresis and the Nanodrop spectrophotometer were used to check the quantity of RNA before sequencing and qRT-PCR.

CircRNA and mRNA sequencing

The Ribo-Zero™ Removal Kit (Epicentre, USA) was used to ribo-consume the total RNA. The rRNA elimination was assessed using an Agilent bioanalyzer (RNA 6000 Pico chip). The ribo-consumed RNA was treated with RNaseR (20 U/L, 1 h at 37°C) and further purified with RNA Cleanup magnetic beads (Geneaid, USA). Next, circRNA and mRNA libraries were constructed according to the manufacturer’s instructions (New England BioLabs, NEBNext Ultra Directional RNA Library Prep Kit).
We used the Library Quantification Kit (Kapa Biosystems, USA) to identify and quantify sequencing using the Illumina HiSeq4000 system. Finally, circRNA sequences were predicted using CircRNA identifier (CIRI) software.

**Verification with qRT-PCR**

Reverse transcription of quantified RNA was performed using SuperScript® III Reverse Transcriptase (RT) according to the manufacturer's instructions. The expression levels of circRNAs were verified using SYBR Green assays (ArrayStar). 

- **b-actin** was used as an internal control and the experiment was independently repeated 3 times. The 2^–DD_Ct method was applied to calculate the relative expression of each circRNA.

**Gene ontology and KEGG pathway analysis**

Gene ontology enrichment analysis (http://www.geneontology.org/) was conducted to explore potential biological functions of the target genes. KEGG pathway analysis (http://www.genome.jp/kegg/) revealed the signaling pathways of these genes at the molecular level. Genes with corrected P<0.05 were considered enriched.

### CircRNA-miRNA-mRNA network construction

miRNA-binding sites of circRNA were predicted in both TargetScan and miRanda. Information on miRNA-mRNA regulatory relationships was identified using the miRTarBase database. Cytoscape was used to draw the graph of circRNA-miRNA-mRNA interaction networks.

**Statistical analysis**

We compared the expression of circRNAs between the IA group and the control group by Mann-Whitney U test. Categorical variables were assessed by the chi-square (c^2) test or two-tailed t test for normally distributed data, and the Mann-Whitney U test was used for skewed data. Pearson coefficient analysis was used to assess the CeRNA network construction. A p value <0.05 (two-tailed) was considered significant. All statistical analyses were performed using SPSS 24.0 software.

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**Table 1. Baseline data of patients for sequencing and the clinical features of IAs.**

| Items          | IAs (n=4) | STAs (n=4) | T/chi^2  | P Value |
|----------------|-----------|------------|----------|---------|
| Mean age       | 57.50±3.00| 56.75±2.754| −0.368   | 0.725   |
| Sex            |           |            | 0        | 1.000*  |
| Male           | 1 (25%)   | 1 (25%)    |          |         |
| Female         | 3 (75%)   | 3 (75%)    |          |         |
| SBP (mmHg)     | 162.75±31.01| 153.25±29.07| −0.447   | 0.671   |
| DBP (mmHg)     | 83.50±14.39| 87.75±14.39| 0.418    | 0.691   |
| TG (mmol/L)    | 0.62±0.31  | 1.55±0.94  | 1.873    | 0.110   |
| LDL (mmol/L)   | 1.73±0.58  | 2.83±1.00  | 1.903    | 0.106   |
| HDL (mmol/L)   | 1.45±0.69  | 1.66±0.44  | 0.509    | 0.629   |
| Hypertension   |           |            | 1.000 *  |         |
| Yes            | 3 (75%)   | 2 (50%)    |          |         |
| No             | 1 (25%)   | 2 (50%)    |          |         |
| Smoking        |           |            | 1.000*   |         |
| Yes            | 1 (25%)   | 2 (50%)    |          |         |
| No             | 3 (75%)   | 2 (50%)    |          |         |

**IA sample ID**

| IA 1 | Location  | Ruptured/Unruptured | Type     | Size (mm) |
|------|-----------|---------------------|----------|-----------|
| AcoA | Ruptured  | Saccular            | 7        |
| IA 2 | MCA       | Ruptured            | Irregular| 11        |
| IA 3 | PcoA      | Unruptured          | Saccular | 15        |
| IA 4 | MCA       | Unruptured          | Saccular | 9.5       |

- AcoA – anterior communicating artery aneurysm; PcoA – posterior communicating artery aneurysm; MCA – middle cerebral artery aneurysm; SBP – systolic blood pressure; DBP – diastolic blood pressure; TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; Size – Maximum Diameter; * Fisher test.
Results

CircRNA and mRNA expression profiles

Compared with the STA group, 315 circRNAs and 4159 mRNAs were found to be significantly differentially expressed in the IAs group (FC>1.5, P<0.05). Among them, 116 circRNAs were upregulated and 199 were downregulated, while 1297 mRNAs were upregulated and 831 were downregulated (Figure 1A, 1B). Heatmaps assessed and compared the expression changes of circRNAs and mRNAs between the 2 groups (Figure 1C, 1D). We found that 76.15% of circRNAs are derived from exons (Figure 1E), 83.6% of circRNAs have been identified in circBase, and 16.4% are novel (Figure 1F). Most circRNAs had a predicted spliced length of <1000 nt (Figure 1G). These dysfunctional circRNAs were widely distributed among all chromosomes, including sex chromosomes (Figure 1H). The top 5 most significantly differentially expressed circRNAs and mRNAs are listed in Table 2.

Verification of candidate circRNAs by qRT-PCR

Seven differentially expressed circRNAs were selected to confirm the sequencing data by qRT-PCR in the same tissue samples used for the sequencing, including 5 upregulated (hsa_circ_0008433, hsa_circ_0033144, hsa_circ_0005571, hsa_circ_0040809, hsa_circ_0056285) and 2 downregulated (hsa_circ_0072309, hsa_circ_0007142) circRNAs. We also explored the expression of these candidate circRNAs in the peripheral blood of sequencing patients (Figure 2A–2G). The results showed that all selected circRNAs expression was consistent with the RNA-seq results, which confirmed the accuracy of
Figure 1. The circRNA and mRNA expression profiles compared between IAs and STAs. (A, B) Volcano plots show significantly differentially expressed circRNAs (A) and mRNAs (B) between IAs and STAs (FC>1.5, P<0.05). The red and green dots represent upregulated and downregulated circRNAs and mRNAs. (C, D) Heatmaps classified the differential expressed circRNAs and mRNAs between IAs and STAs. (E) The percentage of differentially expressed circRNAs arising from genomic locus. (F) The length distribution of differentially expressed circRNAs. (G) Proportion of previously known and newly discovered circRNAs detected by RNA-Seq. (H) Chromosomal location of circRNAs.

Table 2. The top 5 up- and downregulated circRNAs in the 2 groups.

| CircRNA ID | Regulation | Log2 FC | P     | Host gene | CircBase ID       | Length |
|------------|------------|---------|-------|-----------|-------------------|--------|
| chr17: 74801278-7480270: + | Up | 5.9628048 | <0.001 | chr17 | SNORD10 | 142 |
| chr14: 23371395-23371591: – | Down | –8.5141271 | 0.020 | chr11 | hsa_circ_0000348 | 3309 |
| chr1: 66378927-66384518: + | Up | 5.7986801 | 0.002 | chr14 | RBM23 | 196 |
| chr17: 80992910-81006661: – | Down | –6.9305180 | <0.001 | chr2 | TTN | 84 |
| chr15: 38523520-38530768: – | Down | –6.5674489 | 0.004 | chr19 | hsa_circ_0005571 | 165 |
| chr11: 92085261-92088570: + | Up | 5.5674489 | 0.004 | chr19 | hsa_circ_0005571 | 165 |
| chr5: 38523520-38530768: – | Down | –6.5674489 | 0.004 | chr5 | LIFR | 580 |
Figure 2. PCR validation of circRNAs in tissue and peripheral blood. (A–G) The PCR results of the 7 selected circRNAs in IA tissues and peripheral blood. (H) Relative fold changes of circRNAs by RNA-seq and qRT-PCR. (I) The expression trends of hsa_circ_0072309 in each IA and STA sample from tissues and peripheral blood. * P<0.05
sequencing (Figure 2H). Hsa_circ_0072309 showed the same expression patterns in IA tissues and peripheral blood (P < 0.05) (Figure 2G), while the expression patterns of hsa_circ_0008433 and hsa_circ_0033144 in blood were different from those in tissues (P < 0.05) (Figure 2A, 2B). Interestingly, the expression trend of hsa_circ_0072309 in each sample showed some similarity (Figure 2I).

GO and pathway analysis

GO analysis indicated that most of the up- and downregulated functional terms were related to immune/inflammatory response and cell adhesion/adherence junction (Table 3). The top 5 upregulated biological processes were also involved in defense response to virus and T cell/TNF receptor binding, while the top 5 downregulated mainly included extracellular matrix, cytoskeleton, and actin filament binding. KEGG analysis consistently revealed that most upregulated target genes were enriched in inflammatory pathway, including classical ones such as chemokine and NF-kB. Among them, the most relevant pathways were NK cell-mediated cytotoxicity (Figure 3). As shown in the figure, many genes involved in this pathway [13] were significantly differentially expressed in our RNA-Seq and targeted by circRNAs. For downregulated genes, the most affected pathways were cell adhesion/adherence junction. In addition, it was also involved in regulation of lipolysis and vascular smooth muscle contraction, which is related to arterial wall strength and integrity (Table 4).

Prediction of circRNA-miRNA-mRNA network

TargetScan and miRanda were utilized to predict miRNAs targeted by the differentially expressed circRNAs. The first 5 miRNAs combined with the 7 selected circRNAs were predicted using Tot Energy detection, in which lower Tot Energy is associated with a closer combination between circRNAs and miRNAs (Table 5). The miRNA-mRNA regulatory relationships were further identified using miRTarBase. The miRNAs were found to be significantly differentially expressed in RNA-Seq, and those predicted by the bioinformatics analyses were further selected for ceRNA construction. In this network, hsa_circ_0008433 can act as a ceRNA to affect SMC regulators DDR2 and SAT2 through hsa-miR-519e-5p and hsa-miR-516b-5p, respectively (Figure 4).

Expression of candidate circRNAs in peripheral blood of IA population

Based on the findings of sequencing, we selected 3 circRNAs (hsa_circ_0072309, hsa_circ_0008433, and hsa_circ_0033144) with significant difference in expression (P < 0.05) in tissues and blood for further verification. In 30 pairs of IA patients and healthy people, the expression level of hsa_circ_0033144 in peripheral blood was not significantly different between the 2 groups (Figure 5A). Hsa_circ_0072309 and hsa_circ_0008433 still showed significant differences in expression between the 2 groups. The expression pattern of hsa_circ_0072309 in blood was consistent with that in tissues, while the expression trend of hsa_circ_0008433 in blood differed from that in tissues. This was consistent with the small samples test results (Figure 5B, 5C). Areas under the ROC curve of hsa_circ_0008433 and hsa_circ_0072309 were 0.8022 (95% CI: 0.6913–0.9132; P < 0.001) and 0.7544 (95% CI: 0.6337–0.8752; P < 0.001), respectively (Figure 5D, 5E).

| GO term | Gene count | Fold enrichment | P value* |
|---------|------------|----------------|----------|
| Upregulated target genes | | | |
| GO: 0006954: Inflammatory response | 75 | 3.4 | 1.4E-21 |
| GO: 0051607: Defense response to virus | 52 | 5.1 | 1.1E-23 |
| GO: 00060337: Type I interferon | 31 | 8.4 | 6.9E-22 |
| GO: 0005164: TNF receptor binding | 14 | 7.7 | 5.2E-10 |
| GO: 0042608: T cell receptor binding | 6 | 15.2 | 2.0E-07 |
| Downregulated target genes | | | |
| GO: 0007155: Cell adhesion | 44 | 2.4 | 6.7E-08 |
| GO: 0031012: Extracellular matrix | 42 | 3.5 | 1.0E-12 |
| GO: 0005856: Cytoskeleton | 41 | 2.8 | 2.8E-09 |
| GO: 0033735: Ribosomal structure | 23 | 3.3 | 3.0E-07 |
| GO: 0051015: Actin filament binding | 22 | 3.1 | 1.9E-06 |

* IAs vs. STA.
Discussion

Recent research has shown that circRNAs are involved in various neurological diseases [14]. In this study, we are the first to present expression profiles of circRNAs in IA, and revealed that most dysfunctional circRNAs are mainly involved in immunity/inflammation and cell adhesion/adherens junction processes that are known to be critical for the pathogenesis of IA [15,16].

Inflammatory response and degeneration of vascular media layer are the pathological basis for the development of IAs [17]. CircRNAs have been verified to exert significant roles in inflammation, including regulating inflammation genes, recruiting macrophages, and adjusting crucial signal pathways [18]. Our results suggest that several inflammation-related biological processes or pathways are potentially regulated by circRNAs. The most affected pathway was NK/T cell-mediated cytotoxicity. Infiltration of inflammatory cells is the primary pathological change in early stages of IAs [19]. Related studies also confirmed that NK cells intensively infiltrate in abdominal aortic aneurysm (AAA) and damage the vessel by producing cytotoxicity to arterial SMCs [20]. However, inflammatory cells release cytokines and promote the overexpression of MMPs, which degrades the extracellular matrix (ECM) and weakens vessels, and MMP2 and MMP9 are closely associated with formation of aneurysms [21,22]. According to our ceRNA network analysis, hsa_circ_0008433 targeted hsa-miR-181c-5p to regulate MMP2, which has been found to be highly expressed in IA walls, and these result in arterial elastic fiber destruction and pathological remodeling of vessels [23].

The necrosis and apoptosis of SMCs contribute to the formation, progression, and rupture of IAs [24]. In our study, the necroptosis pathway enriched 34 genes targeted by several circRNAs. Among them, hsa_circ_0072309 regulates discoid domain receptor 2 (DDR2) and systemic amino acid transporter 2 (SAT2) through competitively sponging hsa-miR-519e-5p and hsa-miR-516b-5p, respectively. DDR2 is a critical factor in

![Figure 3. Upregulated target genes in the NK cell-mediated cytotoxicity pathway. Target genes are marked with a green star. Image produced using DAVID.](image-url)
SMC-mediated regulation of collagen turnover in atherosclerosis, which is the pathological basis of IAs [25]. SAT2 regulates vascular SMCs growth and collagen synthesis by mediating l-valine transport and promotes pathological remodeling of vessels, which is one of the molecular mechanisms involved in vascular diseases [26]. Moreover, studies have shown that hsa_circ_0072309 is directly involved in the regulation of cell proliferation and migration by sponging miR-492 [27]. These findings elucidate the regulation of SMCs by circRNA in IAs.

The downregulated genes targeted by circRNAs in our research were most enriched in cell adhesion/adherens biological pathways, which are closely related to vascular strength and integrity. Numerous cell adhesion molecules, such as VCAM-1, were upregulated, which may contribute to the remodeling of vascular walls and the progression of atherosclerosis.

Table 4. Top 10 pathways of up- and downregulated target genes in IAs and STAs.

| KEGG term                          | Gene count | Fold enrichment | P value*   |
|------------------------------------|------------|-----------------|-----------|
| **Uregulated target genes**        |            |                 |           |
| hsa04650: NK cell-mediated cytotoxicity | 33         | 10.1            | 6.6E-11   |
| hsa04660: T cell receptor receptor interaction | 48         | 9.0             |           |
| hsa04621: NOD-like receptor         | 35         | 8.5             | 2.9E-09   |
| hsa04217: Necrosis                 | 34         | 8.3             | 4.7E-09   |
| hsa05340: Primary immunodeficiency  | 37         | 7.1             | 7.5E-08   |
| hsa05168: Chemokine                | 35         | 7.6             | 2.3E-08   |
| hsa05167: NF-kappa B signaling pathway | 34         | 8.2             | 5.6E-09   |
| hsa04380: TNF signaling pathway    | 26         | 7.0             | 9.0E-08   |
| hsa05340: Cell adhesion molecules  | 14         | 7.3             | 4.0E-08   |
| **Downregulated target genes**     |            |                 |           |
| hsa03010: Ribosome                 | 21         | 6.0             | 9.2E-07   |
| hsa04520: Adherens junction        | 12         | 4.5             | 2.9E-05   |
| hsa04923: Regulation of lipolysis in adipocytes | 10         | 4.2             | 5.4E-05   |
| hsa04360: Axon guidance            | 19         | 4.0             | 8.9E-05   |
| hsa05012: Parkinson's disease      | 16         | 3.6             | <0.001    |
| hsa04360: Rap1 signaling pathway   | 19         | 4.0             | <0.001    |
| hsa05120: Vascular smooth muscle contraction | 16         | 3.6             | <0.001    |
| hsa05412: Oxidative phosphorylation | 11         | 3.8             | <0.001    |
| hsa05410: cGMP-PKG signaling pathway | 11         | 3.2             | <0.001    |
| hsa00280: Focal adhesion           | 8          | 3.1             | <0.001    |

* IAs vs. STAs.

Table 5. Predicted miRNAs for the 7 up- or downregulated circRNAs in IAs.

| circRNA ID      | miRNA1          | miRNA2          | miRNA3          | miRNA4          | miRNA5          |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| hsa_circ_0008433 | hsa-miR-181c-5p | hsa-miR-499b-5p | hsa-miR-34c-5p  | hsa-miR-499a    | hsa-miR-181b-5p |
| hsa_circ_0033144 | hsa-miR-588      | hsa-miR-193a-3p | hsa-miR-361-3p  | hsa-miR-125a-3p | hsa-miR-516a-3p |
| hsa_circ_0000571 | hsa-miR-483-3p   | hsa-miR-532-3p  | hsa-miR-33b-3p  | hsa-miR-33b-5p  | hsa-miR-516a-3p |
| hsa_circ_0040809 | hsa-miR-519d-5p  | hsa-miR-525-5p  | hsa-miR-520a-5p | hsa-miR-515-5p  | hsa-miR-519e-5p |
| hsa_circ_0056285 | hsa-miR-708-5p   | hsa-miR-28-5p   | hsa-miR-539-3p  | hsa-miR-485-3p  | hsa-miR-32b-3p  |
| hsa_circ_0072309 | hsa-miR-519e-5p  | hsa-miR-515-5p  | hsa-miR-516b-5p | hsa-miR-603     | hsa-miR-584-3p  |
| hsa_circ_0007142 | hsa-miR-128-3p   | hsa-miR-216a-3p | hsa-miR-576-3p  | hsa-miR-376a-3p | hsa-miR-376b-3p |
and ICAM-1, participate in aneurysm production through recruiting NK/T cells and basophils [28]. Adherence junctions (AJs) are critical in maintaining the morphology and function of endothelial cells (ECs). Early hemodynamic changes result in abnormal expression of VE-cadherin/α-catenin mediated by p120-catenin, which promotes the development of IAs [29]. We found that lymphoid enhancer factor 1 (LEF1) and transforming growth factor beta induced TGFBI, which was targeted by several circRNAs in the ceRNA network. Binding sites of LEF1 act as promoter regions of fibronectin and α5/β1 integrin, which affects adherence junctions and is related to the completeness of cerebral vascular and blood-brain barriers. TGFBI acts on arterial ECM through cell adhesion to affect vascular stability [30]. These are the critical mechanisms for IAs and subarachnoid hemorrhage [31,32].

Finally, circRNA has superior structural stability and is released into extracellular spaces through exosomes, which can be detected in saliva, milk, and plasma [33]. These characteristics make it a good candidate for use as a disease biomarker. In this study, we verified the sequencing results in multiple samples (tissue and blood) and revealed the expression patterns of several critical IA-related circRNAs in center and periphery. Hsa_circ_0008433 and hsa_circ_0072309 were significantly differentially expressed in tissues and peripheral blood. The expression pattern of hsa_circ_0072309 in blood was highly consistent with that in tissues, which revealed its expression characteristics of central and peripheral homogeneity, and this could be very helpful for the noninvasive diagnosis of IA in the future. It remains unclear why hsa_circ_0008433 is expressed in blood as opposed to tissues, and this may be related to the heterogeneous expression of circRNA and the chemotaxis of central inflammation [34]. Further functional experiments are needed to confirm this speculation. Nevertheless, we still believe that these 2 circRNAs have broad research prospects and importance, and might provide crucial clues for the study of IA diagnostic and therapeutic markers.

**Figure 4.** Simplified sub-network containing high-score interactions of circRNA-miRNA-mRNA. The brown, red, and blue points represent circRNAs, miRNAs, and protein-coding RNAs, respectively.
However, due to the particularity of human IA samples and difficulty in collecting, the tissue sample sizes were relatively small. For this reason, we specifically collected tissue samples from the commonly preferred position of IA for RNA-Seq and included ruptured and unruptured patients to maximize objectivity and representativeness of the experiment. In addition, we also referred to other sequencing experiments of human and animal tissues that were the same or smaller than this sample size [35,36]. STAs as control materials are yet another limitation, although there are certain differences between STAs and intracranial arteries. The method of using STAs for similar experiments has been established previously, and is still the best choice [37]. Moreover, research is currently based on bioinformatics, and further in vitro and in vivo functional experiments are needed.

Conclusions

To the best of our knowledge, this is the first study to identify the differential expression profiles of circRNAs in IAs and reveal their expression patterns in center and periphery. Hsa_circ_0072309 and hsa_circ_0008433 are 2 novel and pivotal IA-related circRNAs that might be valuable for therapy and noninvasive diagnosis of IA in the future.

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Figure 5. qRT-PCR validation of specific circRNAs expression in peripheral blood of IA patients and healthy people. (A–C) The expression of hsa_circ_0033144, hsa_circ_0072309, and hsa_circ_0008433 in peripheral blood in IA vs. control groups. * P<0.05. IA group (n=30) vs. control group (n=30). (D, E) The ROC curve of hsa_circ_0008433 and hsa_circ_0072309.
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