RNA-seq Identifies circRNAs Expression Profiles and Potential Biomarkers for Intracerebral Hemorrhage

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Abstract

Background: Circular RNAs (circRNAs) have shown promise as biomarkers because of their stability in peripheral blood, and they participate in various pathological processes of ischemic stroke; however, their expression profiles and the potential functions for intracerebral hemorrhage (ICH) stroke remain unclear.

Methods: RNA sequencing was used to investigate the expression profiles of circRNA in a discovery sample of 129 subjects (44 patients with ICH, 42 hypertension (HTN) controls, and 43 patients with cerebral infarction (CI)), and an independent validation sample of 54 subjects (20 patients with ICH, 18 HTN controls, and 16 patients with CI). Quantitative real-time polymerase chain reaction was used to validate the circRNA expression levels. Logistic regression models were conducted to assess circRNAs as potential biomarkers for ICH. Bioinformatics analysis was performed to identify potential functions of circRNAs.

Results: We found that 15 circRNAs including 5 upregulated circRNAs and 10 downregulated circRNAs were consistently altered only after ICH (fold change >1.5 and FDR < 0.05). We validated that hsa_circ_0001240 and hsa_circ_0001947 were upregulated and that hsa_circ_0001386 was downregulated in ICH compared with HTN and CI. The combination of 3 circRNAs showed an area under the curve of 0.92 with a sensitivity of 86% and a specificity of 88% for diagnosing ICH. Together with ICH risk factors, the circRNAs showed an area under the curve of 0.97 in diagnosing ICH. Functional analysis revealed circRNA target miRNA and mRNA are mainly involved in fatty acid biogenesis, lysine degradation, integrin cell surface interactions and the immune system.

Conclusion: This is the first report to study the expression profile of circRNAs in peripheral blood after ICH, and a set of 3 circRNAs could serve as potential biomarkers for predicting and diagnosing ICH.

Introduction

Stroke is the second most common cause of death worldwide after coronary heart disease, and the incidence of stroke observed in China is 354 /100,000 person-years [1, 2]. Intracerebral hemorrhage (ICH) is a deadly stroke subtype with high mortality and disability. ICH accounts for 10%-15% of stroke cases in Western countries, while it accounts for approximately 23.8% of stroke cases in China, with a mortality rate of 67.9% [3, 4]. Moreover, the highest incidence and mortality of stroke was found in northern China [5]. Hypertension (HTN) is the most prevalent stroke risk factor [2, 5], accounting for approximately 65% of all ICH cases [6, 7]. Although effective HTN management has decreased the incidence of ICH in some high-income countries, the incidence and prevalence rates in China have increased [5]. Thus, finding biomarkers for hypertensive subject early prediction of ICH is meaningful. Although many studies have examined the mechanisms of brain injury after ICH, such as brain edema and inflammation, few studies have examined the molecular mechanisms and pathology of ICH. Currently, ICH is often diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI) to exclude cerebral infarction (CI); however, it is difficult to diagnose when brain imaging lacks abnormalities or is atypical in the super acute phase [8]. Therefore, identifying potential biomarkers for the early prediction and diagnosis of ICH is essential.

Peripheral blood cells play vital roles in the brain injury caused by ICH, and previous studies have reported that noncoding RNA (ncRNA) expression profiles are altered rapidly in the blood [9–11]. CircRNAs are a large class of ncRNAs produced by back-splicing [12]. Unlike that of linear RNA, the circRNA structure is a closed loop without a free 3' or 5' end and is resistant to RNA exonuclease; thus, circRNAs are more stable and can serve as biomarkers in many diseases [13, 14]. CircRNAs serve as sponges of multiple miRNAs involved in regulating transcriptional and translational processes [15]. There is growing evidence that circRNAs could serve as potential biomarkers in peripheral blood in coronary artery disease, early pre-eclampsia and cancers [16–20]. Moreover, multiple recent studies have reported circRNA expression profiles changed in ischemic stroke (IS) [21–25], suggesting that circRNAs are potential biomarkers and therapeutic targets in IS. In addition,

two recent studies reported that circRNA expression profiles are altered after ICH in rat brain tissue [26, 27]. However, the expression profile of circRNAs in human peripheral blood cells after ICH remains unclear.

In this study, we investigated the expression profile of circRNAs in peripheral blood cells after ICH by using RNA sequencing from two independent samples. The significantly altered circRNA host genes were examined by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to characterize the potential functions. We replicated the candidate circRNAs by using quantitative reverse transcription-PCR (RT-PCR) analysis to evaluate patients with ICH, patients with CI and control subjects with HTN in all samples. Furthermore, a logistic regression model was performed to assess the diagnostic value of candidate circRNAs as potential biomarkers for ICH. Finally, we constructed a circRNA-miRNA-mRNA network and explored the potential functions of circRNAs in ICH.

Methods And Materials

Study population

For the whole study, a total of 183 male (M) and female (F) patients with hypertensive ICH (n = 64, 34 M/30 F) and hypertensive CI (n = 59, 29 M/30 F), as well as six age-matched controls with simple HTN (n = 60, 28 M/32 F) were recruited between 2014 and 2019 from two individual samples. The discovery sample included 44 patients with ICH, 43 patients with CI and 42 HTN controls enrolled between 2014 and 2017 from Cangzhou Central Hospital. The validation sample included 20 patients with ICH enrolled from Affiliated Hospital of Hebei University, 16 patients with CI and 18 HTN controls enrolled from General Hospital of Ningxia Medical University between 2017 and 2019. ICH and CI were diagnosed by professional neurologists based on histories and exams and confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). Matched controls with HTN but without a previous history of stroke or cardiovascular events were selected. ICH patients, CI patients and HTN controls received antihypertensive drugs. This study was conducted in North China among the Han population. The demographic and clinical characteristics were obtained through a face-to-face survey and checking of hospital records or medical examination records (Table 1). Exclusion criteria included autoimmune diseases, cardiac disease, liver diseases, renal diseases, cancer, or a previous history of stroke and CI with hemorrhagic transformation.

RNA isolation and sequencing

Blood was drawn into PAXgene tubes through the patient antecubital vein and RNA was isolated as previously described [28]. RNA sequencing and libraries were constructed by Annoroad Gene Technology Company Limited (Beijing, China). The detailed procedure was performed as previously described [22]. Briefly, RNA integrity and concentration were assessed using 1% agarose gel electrophoresis and RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). An RNA integrity number (RIN) ≥ 7.5 and a 28S:18S rRNA ratio ≥ 1.8 were used to construct libraries. Three micrograms of total RNA per sample was used to construct libraries. Ribo-Zero™ Gold Kits (Illumina, San Diego, CA) were used to eliminate all ribosomal RNAs (rRNAs) from blood-derived total RNA. Linear RNAs were eliminated by RNase R (Epicenter) digestion to enrich circRNAs. The pure circRNAs were treated with NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, USA) according to the manufacturer's instructions. The constructed libraries were subjected to paired-end sequencing on an Illumina PE150 platform.

Differential expression analysis

Raw data were processed with Perl scripts to ensure the quality of the data. The reference genomes and the annotation file were downloaded from the ENSEMBL database (<http://www.ensembl.org/index.html>). CIRI2 [29] was used to detect paired chiasmic clipping signals according to the mapping of reads. Reads were mapped to the reference genome using the BWA-MEM method. The expression level of circRNAs was measured by junction reads, which refers to SRPBM (spliced reads per billion mapping), $SRPBM = SR \times 10^9 / N$. SR represents the number of spliced reads. N represents the total number of mapped

reads in a certain sample. Differential expression analysis was performed using the DESeq2 R package [30]. There were two sets for comparison: ICH versus HTN and CI versus HTN. A p-value was assigned to each gene and adjusted by multiple testing with the Benjamini-Hochberg method for controlling the false discovery rate (FDR). All the genes with |fold change| ≥ 1.5 and $FDR < 0.05$ were identified as differentially expressed circRNAs.

Bioinformatic analysis

Hierarchical clustering with heatmaps, volcano plots and principal component analysis (PCA) generated based on the normalized values of differentially expressed genes using R package. Venn diagrams were used to present the common differentially expressed genes in the discovery and validation samples. The circRNA host gene biological functions and pathways were annotated by GO and KEGG pathway analysis. P-values were calculated with Fisher's exact test with the hypergeometric algorithm.

Real-time polymerase chain reaction validation

The cDNA synthesis reaction was completed using 1 μg of total RNA and a Transcriptor First Stand cDNA Synthesis Kit (Roche, Takara). Quantitative real time polymerase chain reaction (RT-PCR) was performed by means of SYBR Master Mix (Yeasen, China) according to the manufacturer's instructions in the ViiA 7 Real-time PCR System (Applied Biosystems). CircRNA primers were designed to overlap the back-spliced junction by using the NCBI primer blast website. Primer sequences were listed in Additional file 2: Table S7. The relative expression level was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method normalized to GAPDH.

Constructed circRNA-miRNA-mRNA network

To further explore 3 circRNA functions in ICH, we constructed a circRNA-miRNA-mRNA network. First, miRanda [31] (<http://www.microrna.org/>) and Circular RNA Interactome [32] (<https://circinteractome.nia.nih.gov/index.html>) were used to predict circRNA target miRNAs. The overlapping miRNA functions were analyzed in the DIANA-mirPath v.3 platform [33]. Next, we predicted the miRNA target mRNAs using miRwalk [34], and the target mRNA in miRwalk that overlapped with previously differentially expressed mRNAs (fold change >1.5 and $FDR < 0.05$) [35] was selected to construct the circRNA-miRNA-mRNA network. Finally, we enriched the target mRNA functions using GO and KEGG pathway analysis. The networks and target mRNA functions were visualized by Cytoscape (version 3.8) [36]. The circRNA RNA binding proteins (RBPs) were analyzed with circular RNA interactome, and the functions of RBPs were further analyzed by GO and KEGG pathway enrichment.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 (IBM, Inc., USA). All characteristics of the sample distribution were determined by the Kolmogorov-Smirnov normality test. One-way ANOVA with the Bonferroni post hoc test was performed when more than 2 groups were evaluated. Data represent the means $\pm$ standard deviations. Statistical comparisons for percentages were performed using chi-square statistics analysis. In the RNA sequencing analysis, differentially expressed RNAs were chosen if significant differences ($FDR < 0.05$) between the ICH and HTN (CI and HTN) groups were observed using the Mann-Whitney unpaired test. Spearman's correlation analysis was constructed to investigate the correlation of ICH risk factors and circRNAs. Logistic regression models were performed to identify whether circRNAs were independent factors for ICH. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the diagnostic performance of circRNAs for ICH. $P < 0.05$ was considered statistically significant.

Results

CircRNA expression profiles in ICH patients in the discovery samples

To determine the expression profile of circRNAs associated with the occurrence and development of ICH, we first carried out RNA sequencing in the discovery samples (Fig. 1). The demographics and characteristics of the patients and matched controls are shown in Table 1. A total of 37,637 circRNAs were detected in the peripheral blood cells in the discovery samples. The significantly differentially expressed circRNAs were determined by fold change > 1.5 and FDR < 0.05. In total, we detected 390 circRNAs that were significantly differentially expressed between ICH patients and HTN controls, including 229 upregulated circRNAs and 161 downregulated circRNAs (Fig. 1, Additional file 2: Table S1). PCA plots were used to distinguish between the ICH and HTN groups (Fig. 2a). We constructed volcano plots to evaluate the variation and reproducibility of circRNA expression between ICH patients and HTN controls (Fig. 2b). There were 208 upregulated and 103 downregulated circRNAs that were classic exon back-splicing; a total of 20 alternate exons, 29 introns, 17 overlapping exons, and 9 antisense and 4 intergenic circRNAs were detected between ICH and HTN controls (Fig. 2c). In addition, we performed a circus plot indicating that the circRNAs were distributed in all chromosomes (Fig. 2d).

Functional analysis of circRNA host genes

The host genes of circRNAs are able to produce mRNA transcripts and are involved in diverse processes of pathological development, and their expression levels are also influenced by circRNAs. Hence, we performed functional analysis of the significantly altered circRNA host genes. GO categorized analysis indicated that the host genes were involved in biological regulation, regulation of metabolic process, cell communication, signal transduction protein binding and enzyme binding (Fig. 3a). The KEGG pathway analysis showed that these host genes were involved mainly in lysine degradation, inositol phosphate metabolism, phosphatidylinositol signaling system, endocytosis, cell cycle, base excision repair and the HIF-1 signaling pathway (Fig. 3b). These biological processes and pathways are associated with the pathology and pathogenesis of ICH.

Differentially expressed circRNAs in validation samples

Next, we validated the circRNA profile in an independent sample using RNA sequencing, the strategies and statistics used in the discovery samples. The demographics and characteristics of the patients and matched controls in the validation samples are shown in Table 1. Overall, we detected 125 circRNAs that were significantly differentially expressed between ICH patients and HTN controls, including 56 upregulated circRNAs and 69 downregulated circRNAs (Fig. 1, Additional file 2: Table S2). PCA plots were used to distinguish between the ICH and HTN groups (Fig. 4a). In the volcano plots, the variation in circRNA expression was evaluated between ICH patients and HTN controls (Fig. 4b). There were 45 upregulated and 51 downregulated circRNAs that were classic exon back-splicing, and a total of 3 alternate exons, 10 introns, 13 overlapping exons, and 2 antisense circRNAs and 1 intergenic circRNA were detected between ICH and HTN controls (Fig. 4c). In addition, we performed a circus plot indicating that the circRNAs were distributed in all chromosomes, similar to the discovery sample (Fig. 4d).

For CI versus HTN, we detected 110 and 66 circRNAs in the discovery and validation samples, respectively (Additional file 1: Fig. S1a, 1b, Additional file 2: Table S3 and S4). Furthermore, we compared ICH to HTN and CI to HTN and obtained ICH-specific circRNAs for further analysis. All 16 ICH-specific circRNAs overlapped in two samples, among which 15 circRNAs were consistently altered, including 5 upregulated circRNAs and 10 downregulated circRNAs (Additional file 1: Fig. S2a-e). Among which 3 circRNAs (1 up and 2 down) were not found in the circRNA database. We designated novel circRNAs with host gene symbols. The 5 upregulated circRNAs in ICH included hsa_circ_0084615, hsa_circ_0001240, hsa_circ_0091669, hsa_circ_0001947 and novel_circ_PLXNC1, whose host gene was PLXNC1 (Table 2). The 10 downregulated circRNAs in ICH were hsa_circ_0008983, hsa_circ_0001306, hsa_circ_0001386, hsa_circ_0033144, hsa_circ_0005838, hsa_circ_0005044, hsa_circ_0004096, hsa_circ_0006491, novel_circ_ERBB2 (host gene ERBB2) and novel_circ_11364 (no host gene) (Table 2). The 15 circRNA expression variations were shown with hierarchical clustering heatmaps in the discovery and validation samples (Fig. 4e, f), indicating that circRNA expression profiles in ICH patients were distinctly different from those in HTN controls.

Evaluation of circRNAs as potential diagnostic biomarkers in ICH patients

To explore the potential diagnostic value of the 15 circRNAs, we performed ROC analysis and calculated the AUC of ROC using RNA sequencing data SRPBM in all samples. The top 3 AUCs in all samples were hsa_circ_0001240 (AUC = 0.8078), hsa_circ_0001386 (AUC = 0.8058) and hsa_circ_0001947 (AUC = 0.7981) (Additional file 2: Table S5). We selected these 3 candidate circRNAs to further validate the expression levels by RT-PCR in all samples. All 3 circRNAs were significantly altered in patients with ICH compared with HTN controls (hsa_circ_0001240, $P < 0.001$; hsa_circ_0001947, $P < 0.001$; hsa_circ_0001386, $P < 0.001$) (Fig. 5a). There was also significant differential expression in patients with ICH compared with patients with CI (hsa_circ_0001240, $P = 0.006$; hsa_circ_0001947, $P = 0.002$; hsa_circ_0001386, $P = 0.009$) (Fig. 5b). There was no significant differential expression in patients with CI compared to those with HTN (hsa_circ_0001240, $P = 0.64$; hsa_circ_0001947, $P = 0.29$; hsa_circ_0001386, $P = 0.64$) (Fig. 5c). These results are consistent with the RNA sequencing results.

We furtherly constructed a logistic regression model with the combination of 3 circRNAs to identify the potential diagnostic value. The signatures of 3 circRNAs for differentiating between patients with ICH and HTN controls with the AUC was 0.92 (95% CI: 0.869-0.966), the sensitivity was 86%, and the specificity was 88% (Fig. 5d). Furthermore, 3 circRNA combinations of the risk factors (age, sex, BMI, SBP, DBP, TG, TC, HDL-C, LDL-C, smoking and drinking) showed that the AUC was increased to 0.97 (95% CI: 0.94-0.99), the sensitivity was 94.5% and the specificity was 85.7% (Fig. 5e). The AUC for differentiating between ICH and CI patients was 0.77 (95% CI: 0.688-0.855), the sensitivity was 61% and the specificity was 86% (Fig. 5f). These results showed that the 3 circRNAs could as biomarkers for diagnosis of ICH.

Identification of 3 circRNA as independent predictors of ICH

To further explore the potential value of hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 as ICH biomarkers, we performed Spearman's correlation analysis to test the correlation of these 3 circRNA expression levels with ICH patient clinical characteristics. The results show that hsa_circ_0001240 expression levels correlated with SBP, HDL-C, TC, TG and uric acid (UA) in ICH patients ($p < 0.05$); the hsa_circ_0001947 expression levels correlated with LDL-C, UA and TBIL ($p < 0.05$); and the hsa_circ_0001386 expression levels correlated with TBIL ($p < 0.05$). All 3 circRNA expression levels correlated with white blood cells (WBCs) ($p < 0.05$) (Additional file 2: Table S6). These results suggested that hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 may be involved in the pathogenesis of ICH.

In addition, logistic regression models were performed to identify whether hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 could be predictors of ICH occurrence. As shown in Table 3, with a unit of increase of hsa_circ_0001240, the odds ratio for ICH occurrence was 2.088 (95% CI: 1.418-3.075, $p < 0.001$) after adjusting for age, sex, smoking, drinking, SBP, DBP, TG, TC, HDL-C, LDL-C and glucose. The adjusted OR was 4.382 (95% CI: 2.087-9.204, $p < 0.001$) with a 0.5 unit increase in hsa_circ_0001947. In addition, the adjusted OR was 0.062 (95% CI: 0.009-0.415, $p = 0.004$) with a unit increase of hsa_circ_0001386. These results imply that hsa_circ_0001240 and hsa_circ_0001947 might increase the risk of ICH and that hsa_circ_0001386 might protect the occurrence of ICH. The upregulated hsa_circ_0001240 and hsa_circ_0001947 and downregulated hsa_circ_0001386 might increase the risk of ICH.

ceRNA network and target miRNA and mRNA function analysis

CircRNAs are known to serve as miRNA sponges, which are expected to influence downstream miRNA function, further regulating target mRNA expression. The circRNA-miRNA-mRNA regulation network is thought to play important roles in many disorders. We further investigated hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 target miRNAs with Circular RNA Interactome and miRanda. We found that hsa_circ_0001240 could target 3 miRNAs: hsa-miR-663b, hsa-miR-1270 and hsa-miR1184. Hsa_circ_0001947 has 3 miRNA target sites: hsa-miR-671-5p, hsa-miR-647, and hsa-miR-892b. Hsa_circ_0001386 has 5 miRNA target sites: hsa-miR-1265, hsa-miR-885-3p, hsa-miR-658, hsa-miR-296-5p and hsa-miR-490-5p. Next, we predicted the miRNA target mRNAs using miRwalk, selected the overlapping mRNAs among both miRwalk

and previously differentially expressed mRNAs (fold change > 1.5 and FDR < 0.05), and selected the upregulated (downregulated) circRNAs corresponding to upregulated (downregulated) mRNAs to construct a network. Overall, we obtained 107 target mRNAs that were up/downregulated, consistent with circRNAs. Finally, there were 3 circRNAs, 11 miRNAs and 107 mRNAs network shown in Cytoscape (Fig. 6).

The DIANA-mirPath analysis indicated that the miRNAs were mainly associated with the immune response, lysine degradation, fatty acid metabolism and fatty acid metabolism, cell cycle, Wnt signaling pathway and TGF- β pathway (Fig. 7a, b, Additional file 1: Fig. S3a-d). Furthermore, we found 8 RNA binding proteins (RBPs) matched to hsa_circ_0001368 by CircInteractome: AGO2 with 5 tags, DGCR8 with 1 tag, EIF4A3 with 19 tags, FMRP with 3 tags, FUS with 2 tags, HuR with 1 tag, PTB with 4 tags and U2AF65 with 1 tag. Functional analysis of the RBPs involved in RNA processing, mRNA metabolic processes, and signaling RNA binding (Additional file 1: Fig. S4a, b). A diagram combining the RBPs and miRNAs with KEGG pathway analysis is shown in Additional file 1: Fig. S4c. Furthermore, the target mRNA pathways were enriched, and the results indicated that these genes were involved in cell communication, signal transduction, integrin cell surface interactions, the PDGF receptor signaling pathway, the EGF receptor signaling pathway and the immune system (Fig. 7c, d). All these biological processes are associated with the pathology of ICH. Therefore, we conjectured that circRNAs may act through target miRNAs and further regulate integrin cell surface interactions and vascular injury, thereby being involved in the pathogenesis and pathology of ICH.

Discussion

Given the high incidence and mortality of ICH, investigating potential biomarkers for predicting and diagnosing ICH is essential. In this study, we first investigated the circRNA expression profile in the peripheral blood of ICH patients in two independent samples by using RNA sequencing. We found that 15 circRNAs (5 up and 10 down) were consistently significantly altered simply after ICH. To further explore the altered circRNA functions and diagnostic value, we found that 3 circRNAs (hsa_circ_0001240, hsa_circ_0001947, hsa_circ_0001386) were significantly altered after ICH compared to HTN in both RNA sequencing and RT-PCR. The combination of 3 circRNAs showed an AUC of 0.92, with a sensitivity of 86% and a specificity of 88%, in diagnosing ICH. Together with ICH risk factors, the 3 circRNAs showed better performance in diagnosing ICH. Spearman's correlation analysis suggested that the hsa_circ_0001240 expression level correlated with SBP, HDL-C, TC, TG and uric acid (UA) in ICH patients ($p < 0.05$); the hsa_circ_0001947 expression level correlated with LDL-C and UA, implying that hsa_circ_0001240 and hsa_circ_0001947 might be involved in the pathogenesis of ICH. Logistic regression model analysis suggested that hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 might be independent predictors of ICH. Furthermore, we constructed a circRNA-miRNA-mRNA network and analyzed the target miRNA functions associated with stroke-related pathways, including lysine degradation, fatty acid metabolism and fatty acid biosynthesis. In addition, we predicted the function of target mRNAs by GO and KEGG analysis, and the results indicated that these genes are involved in cell communication, signal transduction, integrin cell surface interactions, and the immune system. These findings showed that these circRNAs were involved in many pathophysiologic processes of ICH, and a set of 3 circRNAs could serve as novel biomarkers for predicting and diagnosing ICH.

CircRNAs are a group of ncRNAs produced by parent gene back-splicing due to closed-loop structures without a free 3' or 5' end and resistance to RNA exonuclease, making circRNAs more stable than line RNAs [13]. Furthermore, circRNAs are highly expressed in mammalian tissues, especially in brain and human blood [37–39]. These characteristics make circRNAs promising biomarkers in many human diseases [14]. In the stroke field, circRNA expression profile changes in IS have been described, supporting circRNAs as potential biomarkers and therapeutic targets in IS [21–25]. Additionally, two recent studies revealed that the circRNA expression profiles significantly changed after ICH in rat brain tissues [26, 27]. Hence, we inferred that circRNAs may play vital roles in the pathogenesis and pathology of ICH. However, the expression profile of circRNAs in human peripheral blood cells after ICH has not been reported.

In our study, we first investigated the expression profile of circRNAs after ICH and found that a set of circRNAs could serve as potential biomarkers for ICH. Previous studies reported blood biomarkers, such as S100 and IL6, with AUCs of 0.65 and 0.59 [40], glial fibrillary acidic protein (GFAP) with a sensitivity of 78% and a specificity of 95% between ICH and IS by meta-analysis [41]. In our study, the sensitivity and specificity of the set of 3 circRNAs (hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386) were 86% and 88%, respectively, which differ from the sensitivity of 61% and specificity of 86% between ICH and CI. In addition, the combination of 3 circRNAs with ICH risk factors increased the value for the identification of ICH. Previous studies revealed that lowering blood lipids was associated with an increased risk of ICH [42, 43], and our previous study also demonstrated that lower serum UA levels promote ICH [44]. In this study, we found that the expression levels of hsa_circ_0001947 and hsa_circ_0001240 were negatively associated with TG and UA and that the expression levels of hsa_circ_0001386 was positively associated with UA; therefore, the upregulation of hsa_circ_0001947 and hsa_circ_0001240 and downregulation of hsa_circ_0001386 may increase the risk of ICH. Hsa_circ_0001947 was reported to be associated with rheumatoid arthritis [45] and acute myeloid leukemia progression [46]. The pathology of rheumatoid arthritis is related to the immune response. The immune response is involved in the vascular atherosclerosis and is closely related to the pathogenesis of ICH. The risk factors for ICH triggered the activation of circular immune cells adhered to the vascular endothelium, and promoted the phenotype of smooth muscle cells transforming from a contractile to a synthetic phenotype [47, 48]. This process plays essential roles in the onset of brain vascular pathological progression.

CircRNAs have been suggested to be involved in the translational and transcriptional regulation of the pathological mechanisms of many disorders [49, 50]. Commonly, circRNAs act as miRNA sponges and RBPs and thus influence the target mRNA expression level [15]. In our study, we predicted that circRNAs interacted with multiple miRNAs. Of these, miR-663b was reported to be increased in acute myocardial infarction [51, 52] and ischemic heart disease [53], and the miR-671 expression level was reported to affect brain function [54]. MiR-885-3p is downregulated in monocular cells and regulates the inflammatory response by targeting TLR4/NF- κ B signaling [55]. MiR296-5p is involved in NRG1/ERBB2/ERBB3 signaling and suppresses EMT in hepatocellular carcinoma [56]. TLR4/NF- κ B signaling and ERBB signaling are closely related to the pathology of ICH. The circRNA target miRNAs functional analysis showed that they were associated with immune response, metabolic process and lysine degradation. Our previous study suggested that lysine is essential for maintaining basement membrane integrity in ICH [57]. Lysine participates in the crosslinking of collagen and plays essential roles in the metabolism of fatty acids [58]. Lysine degradation and fatty acid metabolism were considered as independent predictors of ischemic stroke events [21, 59]. Similar roles might also exist in ICH.

Previous studies revealed that circRNAs can also interact with RBPs involved in gene transcription and translation [60]. In hsa_circ_0001386, there are 8 RBPs, including AGO2, HuR, FUS and EIF4A3. AGO2 is associated with the miRNA profile in the brain of a rat stroke model [61]. AGO2, FUS, HuR and EIF4A3 were reported to have potential roles in IS [21], but their roles in ICH have never been reported. FMR1 and U2AF2 are involved in human neural cells and regulates the development of neural cells [62], DGCR8 is involved in the development of neuropsychiatric disorders and glioma cells [63, 64]. However, there have been no previous reports on the roles of these RBPs in ICH. The circRNA target mRNAs function analysis indicated that mainly involved in ECM receptor interaction, lysine degradation, and the TGF- β signaling pathway. Therefore, we conjectured hsa_circ_0001386 may bind RBPs or target miRNAs involved in the pathophysiological processes of ICH, this might be a promising biomarker and worth exploring in future studies.

Our study also had some limitations. First, our study sample was too small, and we validated the candidate circRNAs using discovery and validation samples. The sample size should be enlarged to externally validate candidate biomarkers in the future. Second, blood cell types should be isolated to ensure the derivation of significantly altered circRNAs in future studies. Third, the potential functions of the 3 circRNAs that we predicted need to be further explored with cell or animal-based experiments. Moreover, our study lacked of follow-up information for ICH patients, and the prognostic value of these circRNAs should be assessed in subsequent studies.

In conclusion, we demonstrated for the first time that circRNAs are differentially expressed in the peripheral blood of patients with ICH, and identified hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 as novel promising biomarkers for the diagnosis of ICH. These circRNAs target miRNAs, and mRNAs functions are mainly involved in lysine degradation, integrin cell surface interactions and the immune system. We will focus on the mechanisms of the circRNA network in the pathogenesis of ICH.

Declarations

Supplemental material

Supplemental material to this paper can be found on the online website.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Human Ethics Committee, Fuwai Hospital (Approval No. 2016-732), and the study was conducted in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki.

Availability of Data and materials

The data that support the findings in this study are available from corresponding author on reasonable request.

Consent for publication

Written informed consent was obtained from all study participants or their legal proxies.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Congxia Bai wrote this manuscript. Hao Li, Jiedan Ping, Haochen Xu and Jingzhou Chen revised this manuscript. Congxia Bai, Meijun Zhang and Yanjie Feng analyzed the data. Congxia Bai and Li Song performed the experiment of circRNA expression levels. Tingting Liu, Jing Ge, Xuliang Wang enrolled the samples and investigated the clinical data. Hao Li and Ning Xiao checked the programs of statistical analysis. Yingying Sun attributed to record the sample and data analysis. Jingzhou Chen conceived and designed the study and supervised all the sample selection, data analysis and interpretation. All authors read and approved the final manuscript.

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None

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Tables

Table 1.

Demographics and Characteristics of the Discovery and Validation

	Discovery				Validation			
	HTN (n = 42)	ICH (n = 44)	CI (n = 43)	P- value	HTN (n = 18)	ICH (n = 20)	CI (n = 16)	P- value
Age, y	57.5±6.2	55.9±7.2	57.4±5.5	0.23	56.2±7.2	56.7±7.1	57.2±7.7	0.98
Men, %	18 (42.8)	24 (54.5)	21 (48.8)	0.59	11 (60)	10 (50)	8 (50)	0.93
BMI, kg/m ²	24.5±3.6	26.1±6.6	27.6±6.9	0.08	24.9±2.4	25.8±6.8	25.0±2.6	0.72
SBP, mmHg	138.3±12.7	137.4±18.2	136.4±13.2	0.86	133.1±18.9	171.2±25.7	150.6±19.4	0.001*
DBP, mmHg	91.7±19.1	87.7±11.0	92.2±17.9	0.39	91.7±12.4	103.7±13.3	89.3±13.9	0.003*
HDL-C, mmol/L	1.4±0.3	1.2±0.3	1.1±0.2	0.001*	1.2±0.3	1.0±0.3	0.9±0.5	0.09
LDL-C, mmol/L	2.9±0.7	2.5±0.6	2.4±0.8	0.001*	2.9±0.94	2.6±1.0	2.7±0.8	0.81
TC	5.6±1.0	4.8±0.9	4.6±1.0	0.001*	4.8±2.0	4.3±0.9	4.9±1.2	0.17
TG, mmol/L	1.7±1.0	1.6±1.0	1.6±0.6	0.84	1.9±0.8	1.3±0.6	1.9±0.7	0.31
Glucose, mmol/L	6.0±1.8	6.3±1.6	5.9±1.3	0.59	5.2±0.5	5.6±1.7	6.1±1.7	0.14
Smoking, %	0.92				0.99			
Never	25 (59.5%)	28 (63.7)	27 (62.8)		12 (66.7)	14 (70)	11 (68.7)	
Former	5 (11.9)	6 (13.6)	7 (16.3)		2 (11.1)	2 (10)	2 (12.5)	
Current	12 (28.6)	10 (22.7)	9 (20.9)		4 (22.2)	4 (20)	3 (18.8)	
Drinking, %	0.98				0.91			
Nondrinker	26 (61.9)	28 (63.6)	27 (62.8)		12 (66.7)	12 (60)	10 (62.5)	
Drinker	16 (38.1)	16 (36.4)	16 (37.2)		6 (33.3)	8 (40)	6 (37.5)	

Data is expressed as mean ± standard deviation or n (%). BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TC: Total cholesterol; TG: Triacylglycerol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-

density lipoprotein cholesterol; GLU, Glucose; ICH, Intracerebral hemorrhage. Statistical comparisons for percentages were performed using χ^2 analysis. Comparisons between means or medians were performed using a One-way ANOVA.

Table 2.

The consistently altered circRNAs in ICH patients compared HTN controls

Location	circRNA ID	Discovery		Validation		Up/Down	Host Gene
		FC	FDR	FC	FDR		
chr8:62593527-62596747:-	hsa_circ_0084615	3.92	0.00	2.65	0.03	up	ASPH
chr22:42807413-42807742:-	hsa_circ_0001240	2.95	0.04	2.41	0.02	up	NFAM1
chrX:147733520-147744289:+	hsa_circ_0091669	1.71	0.00	1.92	0.01	up	AFF2
chr12:94562929-94580249:+	novel_circ_PLXNC1	2.15	0.00	1.77	0.01	up	PLXNC1
chrX:147743429-147744289:+	hsa_circ_0001947	1.75	0.00	1.57	0.04	up	AFF2
chrX:51638149-51640367:+	hsa_circ_0008983	0.62	0.02	0.52	0.00	down	MAGED1
chr3:51575514-51586079:+	hsa_circ_0001306	0.50	0.03	0.46	0.03	down	Null
chr4:1219148-1235307:-	hsa_circ_0001386	0.37	0.00	0.46	0.04	down	CTBP1
chr14:99723808-99724176:-	hsa_circ_0033144	0.50	0.03	0.38	0.00	down	BCL11B
chr16:396148-397106:-	hsa_circ_0005838	0.39	0.03	0.31	0.02	down	AXIN1
chr11:36415396-36424928:+	hsa_circ_0005044	0.50	0.04	0.25	0.00	down	Null
chr17:37866066-37872192:+	novel_circ_ERBB2	0.34	0.00	0.21	0.00	down	ERBB2
chr13:114806476-114839312:-	hsa_circ_0004096	0.30	0.00	0.20	0.00	down	RASA3
chr11:36415396-36440853:+	novel_circ_11364	0.35	0.01	0.20	0.01	down	Null
chr18:42281140-42281797:+	hsa_circ_0006491	0.35	0.00	0.08	0.00	down	SETBP1

FC: fold change; FDR: false discovery rate.

Table 3.

Logistic regression analysis to identify circRNAs as independent predictor factors of ICH

Location	circRNA ID	Adjusted risk factors			Up/Down	Host Gene
		OR	95% CI	P-value		
chrX:147743429-147744289:+	hsa_circ_0001947	4.382	2.087-9.204	<0.001	up	AFF2
chr22:42807413-42807742:-	hsa_circ_0001240	2.088	1.418-3.075	<0.001	up	NFAM1
chr8:62593527-62596747:-	hsa_circ_0084615	1.537	1.114-2.060	0.004	up	ASPH
chrX:147733520-147744289:+	hsa_circ_0091669	1.581	1.278-1.957	<0.001	up	AFF2
chr12:94562929-94580249:+	novel_circ_PLXNC1	1.435	1.172-1.757	<0.001	up	PLXNC1
chr11:36415396-36440853:+	novel_circ_11364	0.633	0.472-0.850	0.002	down	Null
chr16:396148-397106:-	hsa_circ_0005838	0.305	0.176-0.530	<0.001	down	AXIN1
chr11:36415396-36424928:+	hsa_circ_0005044	0.463	0.284-0.757	0.002	down	Null
chr13:114806476-114839312:-	hsa_circ_0004096	0.242	0.134-0.439	<0.001	down	RASA3
chr18:42281140-42281797:+	hsa_circ_0006491	0.426	0.267-0.679	<0.001	down	SETBP1
chr14:99723808-99724176:-	hsa_circ_0033144	0.207	0.102-0.420	<0.001	down	BCL11B
chrX:51638149-51640367:+	hsa_circ_0008983	0.284	0.119-0.679	0.005	down	MAGED1
chr3:51575514-51586079:+	hsa_circ_0001306	0.132	0.051-0.344	<0.001	down	Null
chr17:37866066-37872192:+	novel_circ_ERBB2	0.112	0.044-0.285	<0.001	down	ERBB2
chr4:1219148-1235307:-	hsa_circ_0001386	0.062	0.009-0.415	0.004	down	CTBP1

Risk factors include age, sex, BMI, smoking, drinking, SBP, DBP, TG, TC, LDL-C, HDL-C, GLU

ICH: intracerebral hemorrhage; OR: odds ratio; CI: confidence interval

Figures

Fig.1

Figure 1

Work flow. ICH, Intracerebral hemorrhage; HTN, Hypertension; CI, Cerebral infraction; FC, Fold change; FDR, False discovery rate.

Fig.2

Figure 2

CircRNA expression profiles of ICH patients versus hypertension controls in the discovery sample. (a) PCA plot for sample clustering between ICH patients and hypertension controls. (b) The volcano plot of circRNA expression profiles in ICH and hypertension controls (fold change ≥ 1.5 and $FDR < 0.05$). Red dots represent upregulated genes, and blue dots represent downregulated genes. (c) The bar diagram shows the differentially expressed circRNA distribution in the chromosome region (exonic, intron, intergenic, alternate exon, overlapping exon and antisense) for ICH compared with HTN. (d) The Circos plot shows the circRNA distribution in the chromosomes after ICH. The red columns represent upregulated circRNAs, while green columns represent downregulated circRNAs. The height of the column represents the fold changes in the circRNAs. PCA: principal component analysis; DEGs, differentially expressed genes; HTN: hypertension, ICH: intracerebral hemorrhage, CI: cerebral infarction.

Fig.3

Figure 3

The GO and KEGG pathway analyses of significantly altered circRNA host genes. (a) The top 10 terms of biological process, cellular component, and molecular function from GO enrichment analysis. (b) The KEGG pathway analysis of differentially expressed circRNA host genes. The count represents the number of gene enrichments, and the height of the column represents $-\log_{10}(P\text{-value})$. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Fig.4

Figure 4

Validation of circRNA expression profiles in ICH patients versus hypertension controls. (a) PCA plot for sample clustering between ICH patients and hypertension controls. (b) The volcano plot of circRNA expression profiles of ICH and hypertension controls (fold change ≥ 1.5 and FDR < 0.05). Red dots represent upregulated genes, and blue dots represent downregulated genes. (c) The bar diagram shows the distribution of differentially expressed circRNAs in the chromosome region (exonic, intron, intergenic, alternate exon, overlapping exon and antisense). (d) The Circos plot shows the circRNA distribution in the chromosomes after ICH. The red columns represent upregulated circRNAs, while green columns represent downregulated circRNAs. The height of the column represents the fold changes in the circRNAs. Hierarchical clustering of consistently differentially expressed circRNAs between ICH and HTN in the discovery sample (e) and validation sample (f). Blue represents downregulated circRNAs, red represents upregulated circRNAs, and white represents no changes in circRNA expression. The column represents a sample, and each row represents a single circRNA. PCA: principal component analysis; DEGs, differentially expressed genes; HTN: hypertension, ICH: intracerebral hemorrhage, CI: cerebral infarction.

Fig. 5

Figure 5

Validation of circRNAs expression levels and evaluation of circRNAs diagnostic value in ICH patients. (a) RT-PCR validated expression levels of 3 circRNAs, namely, hsa_circ_0001240, hsa_circ_0001947 and hsa_circ_0001386 between ICH and HTN. (b) RT-PCR validated expression levels of 3 circRNAs between ICH and CI. (c) RT-PCR validated expression levels of 3 circRNAs between CI and HTN. (d) ROC curves were calculated using the expression levels of circRNAs for differentiating patients with ICH and HTN controls in all samples. (e) ROC of 3 circRNA sets combining ICH risk factors for differentiating patients with ICH and HTN controls in all samples. (f) ROC of 3 circRNA sets for differentiating patients with ICH and patients with CI in all samples. ROC: receiver operating characteristics; RT-PCR: real time polymerase chain reaction. ICH: intracerebral hemorrhage; CI cerebral infarction; HTN: hypertension.

Fig. 6

Figure 6

The ceRNA network. The network includes three circRNAs, 11 miRNAs and 107 mRNAs. Orange spheres represent upregulated circRNAs and mRNAs, while green spheres represent downregulated circRNAs and mRNAs. Hexagon, V and ellipse represent circRNAs, miRNAs and mRNAs, respectively.