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ORIGINAL RESEARCH

Potentially Functional microRNA-mRNA Regulatory Networks in Intestinal Ischemia-Reperfusion Injury: A Bioinformatics Analysis

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Background: Intestinal ischemia-reperfusion (II/R) injury is a common clinical complication associated with high mortality, for which microRNA (miRNA) drives potentially its pathophysiological progression. MiRNAs regulate different messenger RNAs (mRNAs). However, the regulatory network between miRNAs and mRNAs in intestinal ischemia-reperfusion injury is elusive.

Methods: We analyzed the different expression of mRNAs and miRNAs in intestinal tissues from patients from three groups (arterial group (group A), venous group (group V), control group (group C)). Common differentially expressed (Co-DE) miRNAs and differentially expressed mRNAs were acquired via concerned analyses among the three groups. Co-DE mRNAs were shared parts of target mRNAs and differentially expression mRNAs. Cytoscape was employed to construct the regulatory network between miRNAs and mRNAs. Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway depicted the functions and potential pathway associated with Co-DE mRNAs. Using the STRING and Cytoscape, we found critical mRNAs in the protein–protein interaction (PPI) network.

Results: The miRNA-mRNA network comprised 8 Co-DE miRNAs and 140 Co-DE mRNAs. Of note, 140 Co-DE mRNAs were targets of these 8 miRNAs, and their roles were established through the functional exploration via GO analysis and KEGG analysis. PPI network and Cytoscape revealed COL1A2, THY1, IL10, MMP2, SERPINH1, COL3A1, COL14A1, and P4HA1 as the top 8 key mRNAs.

Conclusion: This study has demonstrated a miRNA-mRNA regulatory network in intestinal ischemia-reperfusion injury, and explored the key mRNAs and their potential functions. These findings could provide new insight into prognostic markers and therapeutic targets for patients with intestinal ischemia-reperfusion injury in clinical practice.

Keywords: intestinal ischemia-reperfusion injury, microRNA, prognostic marker, bioinformatics

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Introduction

Intestinal ischemia-reperfusion (II/R) injury is a severe clinical complication common in the Intensive Care Unit (ICU). It is associated with high morbidity and mortality.[1] Usually, this problem is followed by various causes, including sepsis, shock, trauma, and so on.[2] Intestinal ischemia-reperfusion injury destroys intestinal tissue and impairs the function of the intestinal barrier. These events induce multiple organ dysfunction

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syndrome (MODS) or systemic inflammatory response syndrome (SIRS).[3,4] The pathophysiological process of intestinal ischemia-reperfusion injury is complex, and different molecule mechanisms have been verified in this process.[5,6] Ischemia injury and reperfusion injury are the two stages of intestinal ischemia-reperfusion injury.[7] The former is mainly caused by hypoxemia and results in the accumulation of much hypoxanthine in cells. During reperfusion, hypoxanthine reacts with oxygen molecules re-entering the cells to generate reactive oxygen species (ROS), which could make further damage on intestine.[8] In addition, intestinal ischemia causes the dysfunction of the intestinal barrier and activates inflammatory cells that release oxygen radicals.[9,10] Compared with ischemia, the subsequent refusion usually leads to more terrible damage.[11] The severity of reperfusion injury is related to ischemia injury. Therefore, reducing ischemia injury is the key step to effective alleviation intestinal ischemia-reperfusion injury.

Various microRNAs (miRNAs), mRNAs, genes and proteins, the basic components of cells, contribute to the development of the intestinal ischemia-reperfusion injury. As a type of small non-coding RNA, miRNA regulates the expression of genes by accelerating mRNA degradation or inhibiting mRNA translation.[12,13] Studies have confirmed that miRNA mediates the progression of intestinal ischemia-reperfusion injury. Nurr1 is associated with the regeneration of the intestinal epithelial and the function of the intestinal barrier. When miR-381-3p targets the Nurr1mRNA, intestinal epithelial proliferation activity decreased and the intestinal barrier function was impaired, which could aggravate the intestinal ischemia-reperfusion injury.[14] MiR-665-3p inhibits autophagy effects by targeting ATG4B, therefore, it can downregulate the inflammation and apoptosis in intestinal ischemia-reperfusion injury.[15] Caspase-3 expression is related to cell apoptosis in intestinal ischemia-reperfusion injury, and miR-378 and miR-182 both regulate Caspase-3 to exert a protective effect against this injury.[16,17] Also, miR-21 potentially regulates the function of the intestinal barrier which is an integral part of intestinal ischemia-reperfusion injury.[18] However, further research is needed to explore the mechanism of miRNAs in II/R injury. MiRNA-mRNA interactions form a competitive endogenous RNA (ceRNA) regulatory network, widely used in pathophysiology and treatment of diseases.[19–21]

In this article, we tested the expression of mRNAs and miRNAs through transcriptome sequencing and small RNA sequencing in patients with intestinal ischemia-reperfusion injury. Then, GO analysis and KEGG analysis were applied to analyze mRNA and construct a miRNA-mRNA regulatory network. We aimed to identify biomarkers and new therapeutic targets for intestinal ischemia-reperfusion injury.

Patients and Methods

Patients and Sample Collection

We selected 13 patients (8 patients were with intestinal ischemia-reperfusion injury patients, and 5 patients without intestinal ischemia-reperfusion injury, all patients who underwent surgery were over 18 years old and were clearly diagnosed with intestinal injury during the surgical procedure in a medical ICU at Shanghai Zhongshan Hospital Affiliated to Fudan university) from the Intensive Care Unit (ICU) in Zhongshan Hospital (Shanghai, China), between July 2020 and March 2021. Intestinal epithelial tissue was collected from patients with intestinal ischemia-reperfusion injury (n=8) and normal control patients (group C, n=5) for follow-up tests. Patients with intestinal ischemia were categorized into the arterial group (Group A, n=5) and the venous group (Group V, n=3). Arterial group is defined as arterial ischemic intestinal injury caused by mesenteric artery embolism or thrombosis during the surgery. Venous group is defined as intestinal injury caused by venous congestion or incarceration leading to impaired blood circulation during the surgery. Intraoperatively resected intestinal specimens from patients in the enrolled arterial, venous, and control groups were excised in whole layers of approximately 1 cm, immediately transferred in a liquid nitrogen tank, and placed in a −80°C refrigerator for storage. All samples were obtained in accordance with the hospital's regulations and the Ethics Committee of Shanghai Medical College of Fudan University approved the study. All patients signed the corresponding informed consent. This study was conducted in accordance with the Declaration of Helsinki.

RNA Isolation, Library Preparation and RNA Sequencing

The mRNA which had polyA enrichment was enriched by Oligo(dT), and RNA was fragmented using related RNA fragmentation reagent. The cDNA was synthesized by using random hexamer-primer and dNTPs. The qualified double-strand DNA library was transformed into a single-stranded circular DNA library through DNA-denaturation and circularization. Small RNA was selected from the total RNA, and then the 3′ end of the small RNA fragment was ligated by the 5-adenylated and 3-blocked adaptor. Unique

molecular identifiers (UMI) labeled Primer was added into the 3′ end of the small RNA fragment and the 5′ end of the small RNA fragment was ligated. Single stranded cDNA was synthesized and amplified by PCR. The PCR products in the range of 110–130bp was isolated by PAGE electrophoresis. A highly efficient library was built. The Agilent 2100 Bioanalyzer was used to establish the quality of libraries. Libraries were sequenced on the DNBseq platform by Beijing Genomics Institute (BGI), China.

Analysis of Common Differentially Expressed (Co-DE) miRNAs

Differentially expressed (DE) miRNAs in intestinal ischemia-reperfusion injury from group C vs group A, group C vs group V were screened and analyzed: $|\log_2{}^{\text{fold-change}}(\log_2 \text{FC})| \geq 1$ and $p < 0.05$ were set as the screening criterion. Co-DE miRNAs were DE miRNAs found in both comparison groups. The ggplot2 package in R software was adopted to generate volcano maps and heatmaps of DE miRNAs. Co-DE miRNAs were listed in the two comparison groups.

Predicting Target Genes of Co-DE miRNAs

Online databases (miRDB, miRWalk and TargetScan) were used to predict the potential targets of Co-DE miRNAs. To improve the accuracy of results, each target gene was listed in the three databases.

Analysis of Common Differentially Expressed (Co-DE) mRNAs

Differentially expressed (DE) mRNAs in intestinal ischemia-reperfusion injury from group A vs group C and group V vs group C were screened and analyzed. The screening criterion was $|\log_2{}^{\text{fold-change}}(\log_2 \text{FC})| \geq 1$ and p-value < 0.05. mRNAs found in both DE mRNAs and target genes of Co-DE miRNAs were described as Co-DE mRNAs.

Constructing Co-DE miRNAs – Co-DE mRNAs Regulatory Network and Analyzing Biological Function of Co-DE mRNAs

Based on miRNAs and their target mRNAs, we constructed the network of Co-DE miRNA and Co-DE mRNA via Cytoscape (version 3.8.0). Data for Co-DE mRNAs in intestinal ischemia-reperfusion injury were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to explore the function of mRNAs using package "clusterProfiler", "AnnotationHub" and "org.Hs.eg.db". Due to the small number of screened mRNA, we chose p-value < 0.05 as the screening criterion.

Constructing the Protein–Protein Interaction (PPI) Network

We acquired data of Co-DE mRNAs and constructed the PPI network via STRING database (https://string-db.org/). This allowed us to further understand interactions among Co-DE mRNAs. The following criterion was used: (1) Homo sapiens; (2) medium confidence 0.400. After that, the top eight Co-DE mRNAs were selected.

Results

Raw Sequence Reads and Work Flow

In RNA sequence, total clean reads were 118.99, 119.04, 47 million and clean reads ratio was over 99.15% from group A, C, and V, respectively (Table S1). In small RNA sequence, the proportion of clean tag was over 93.7% (Table S2). All data from intestinal ischemia-reperfusion injury were disposed and analyzed according to the work flow (Figure 1).

Analysis of Differentially Expressed mRNA and miRNA in Intestinal Ischemia-Reperfusion Injury

A total of 24,237 mRNAs and 3280 miRNAs were identified in three groups. The boxplots (Figure 2A and B) revealed no statistical difference in the mRNA and miRNA distributions in the samples. Furthermore, 13 differentially expressed (DE) miRNAs were identified from group C vs group V, whereas 27 DE miRNAs were identified from group C vs group A (Figure 2C and D, Tables S3 and S4). According to the screening criterion ($|\log_2 \text{FC}| \geq 1$, p-value < 0.05), there were 2902 DE mRNAs (1309 upregulated and 1593 downregulated, Table S5) and 931 DE mRNAs (489 upregulated and 442 downregulated, Table S6) in group C vs group A and group C vs group V, respectively.

Construction of ceRNA Regulatory Network

We found 8 common differentially expressed miRNAs (Co-DE miRNAs) (Figure 3A), including 7 consistent Co-DE miRNAs (2 upregulated and 5 downregulated) and 1

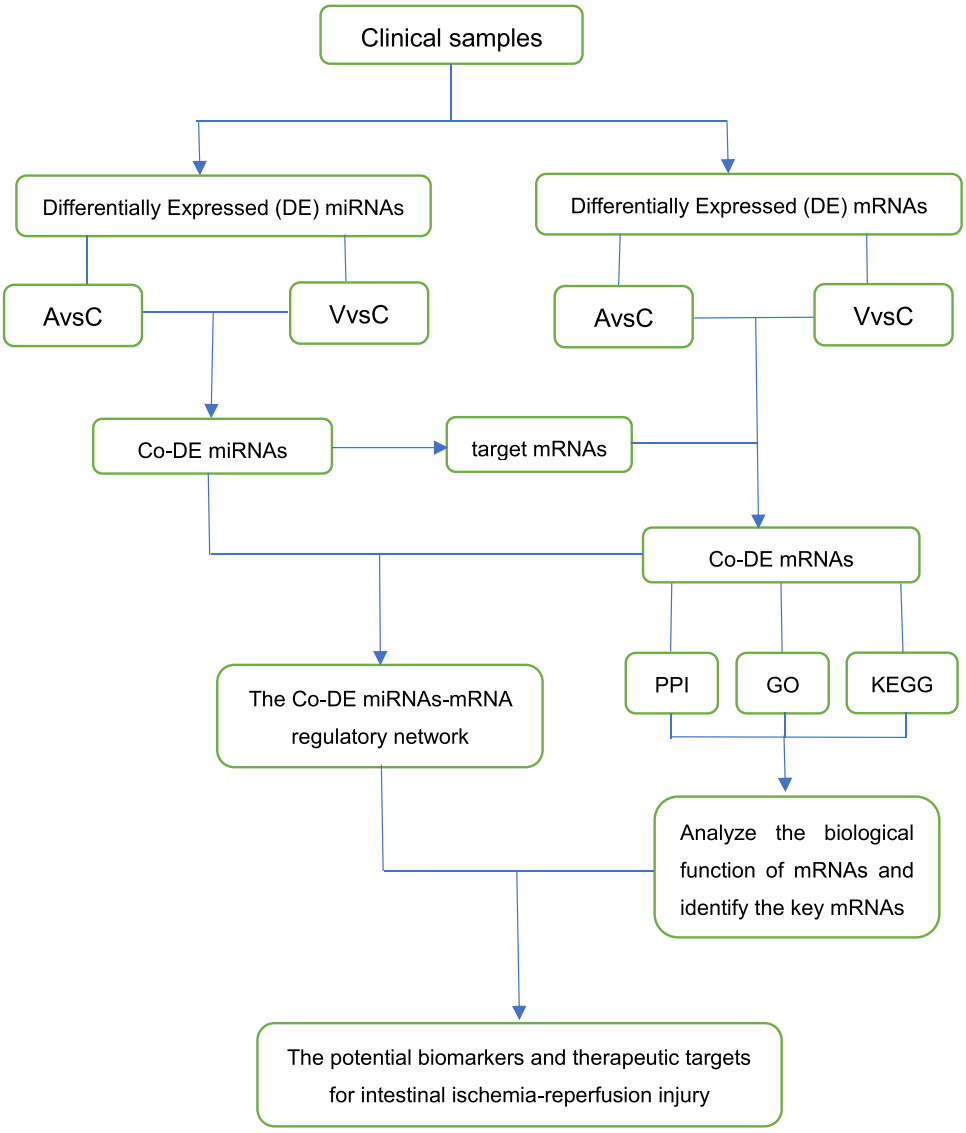


Figure 1 The workflow of study.
Abbreviations: Co-DE, common differentially expressed; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein–protein interaction.

inconsistent Co-DE miRNAs (miR-122-5p, upregulated in group C vs group V and downregulated in group C vs group A). Total target mRNAs of Co-DE miRNAs were identified via online databases (Figure 3B). We selected 140 Co-DE mRNAs through the interaction analysis with target mRNAs (Table S7) and DE mRNAs. Finally, the regulatory network of Co-DE miRNAs and Co-DE mRNAs was constructed (Figure 3C).

GO and KEGG Analysis of Co-DE mRNAs

Following GO and KEGG analysis, 140 Co-DE mRNAs were used to establish the potential function of Co-DE miRNAs. The top 10 results from the GO biological process, GO cellular component and GO molecular function were presented in bubble charts (Figure 4A, C and E), and the top 20 results were shown in bar charts (Figure 4B, D and F). The top 20 results from KEGG analysis were presented in both bubble chart and bar chart (Figure 4G and H).

Construction of PPI Network and Identification of Hub mRNAs

A total of 79 mRNAs from 140 Co-DE mRNAs were identified in the PPI network with 95 edges connected to each other (Figure 5A). The top 8 important mRNAs were identified via the cytoHubba in Cytoscape (Figure 5B and C). They included Thy-1 cell surface antigen (THY1), collagen type I alpha 2 chain (COL1A2), collagen type XIV alpha 1 chain

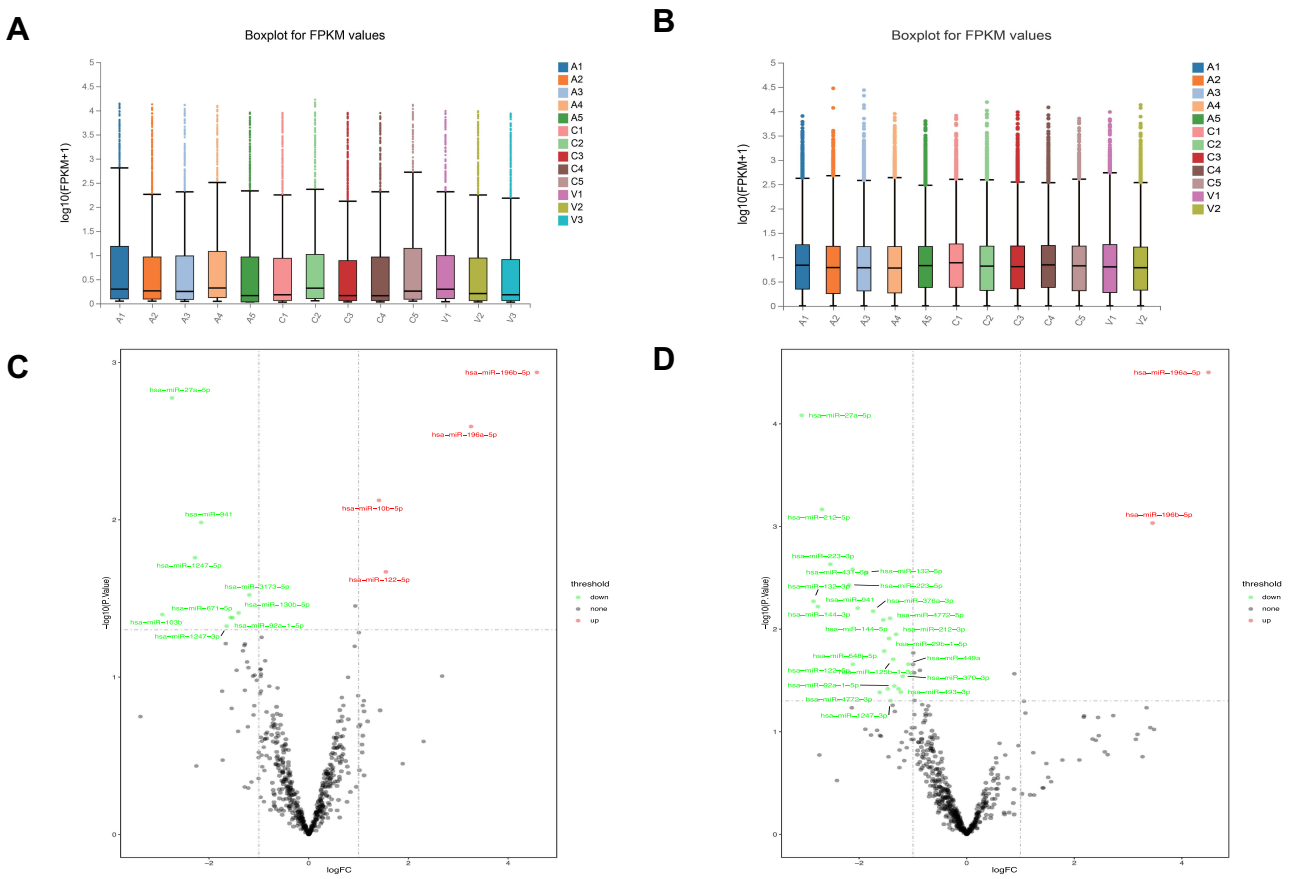


Figure 2 Differentially expressed mRNAs and miRNAs in intestinal tissues in three groups. (**A**) A box plot of miRNAs in all samples. (**B**) A box plot of mRNAs in all samples. (**C**) A volcano plot of differentially expressed miRNAs from group C vs group V. (**D**) A volcano plot of differentially expressed miRNAs from group C vs group A. **Abbreviations**: mRNA, messenger RNA; miRNA, microRNA.

(COL14A1), prolyl 4-hydroxylase subunit alpha 1 (P4HA1), interleukin 10 (IL 10), collagen type III alpha 1 chain (COL3A1), matrix metalloproteinase-2 (MMP2) and serpin peptidase inhibitor, clade H, member 1 (SERPINH1).

Discussion

As a predominant high-risk disease, intestinal ischemia-reperfusion injury is associated with high mortality and a complex pathophysiological process in the Intensive Care Unit (ICU).[22] Several severe conditions, including shock, thrombus and others, are the predisposing factors for this disease.[23] Because of the damage to the intestinal barrier, patients are more prone to the translation of bacterial, septicemia, and multi-organ failure.[24] Previously, there were several studies related to the role of miRNA in intestinal ischemia-reperfusion injury.[14–16] However, the pathophysiological mechanism of this disease remains unclear.

Through transcriptome sequencing, we explored the expression profiles of mRNAs and miRNAs in the intestinal tissues of group A, group C, and group V. Moreover, the role of Co-DE miRNAs and the biological function of Co-DE mRNAs were established via GO and KEGG analyses. Our study aimed to uncover biomarkers and potential therapeutic targets for intestinal ischemia-reperfusion injury.

With the technological advancement, non-coding RNAs (ncRNAs), such as microRNA (miRNA), long non-coding RNA (lncRNA), and circRNA, have been discovered. More studies on the regulation of genes are geared towards ncRNAs.[25] According to previous research, all eight Co-DE miRNAs could be speculated as the potential regulatory targets in intestinal ischemia-reperfusion injury. As an inconsistent Co-DE miRNA, the expression of miR-122-5p varied in intestinal ischemia-reperfusion injury and ischemia-reperfusion induced arrhythmias.[26] MiR-27a-5p had been associated with a decreased level of Bach1 mRNA, which reduced apoptosis in liver ischemia-reperfusion injury.[27] Elsewhere, miR-1247-3p alleviated the damage of myocardial

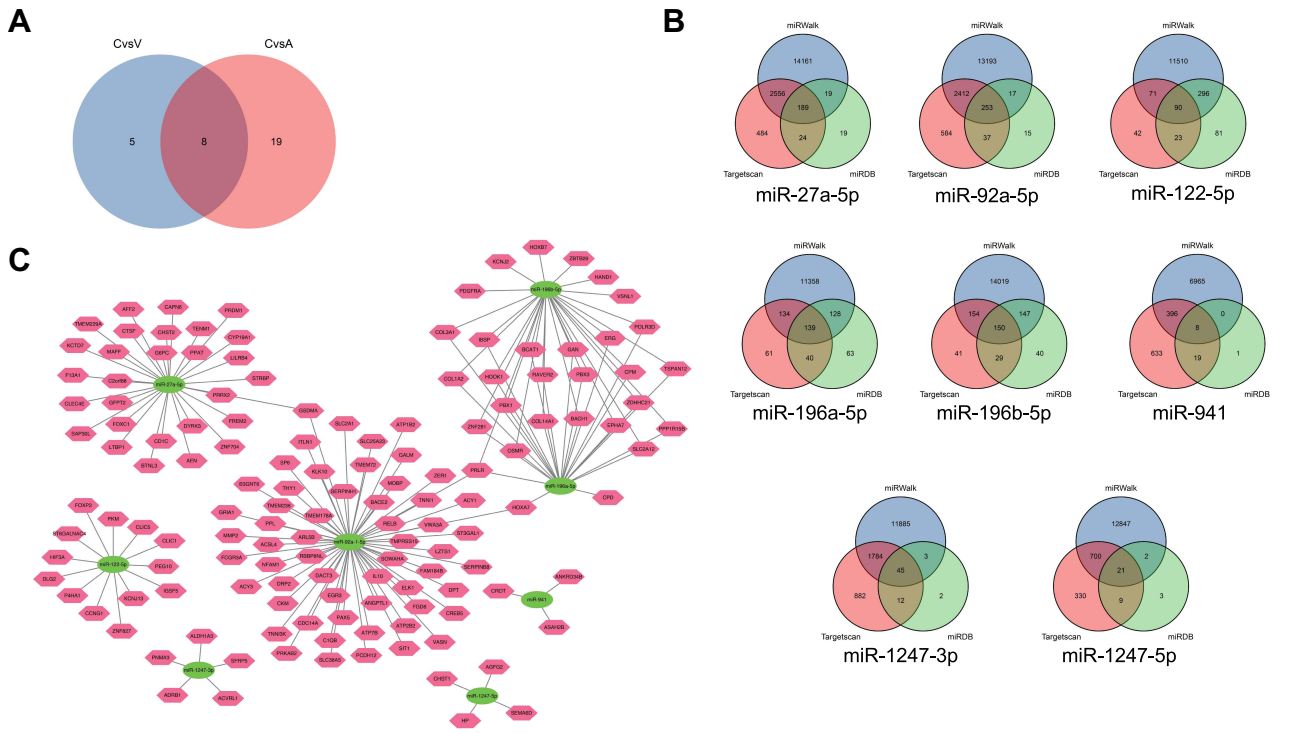


Figure 3 The Co-DE miRNA-mRNA regulatory network. (**A**) A Venn plot of common differentially expressed miRNAs. (**B**) Venn plots of Co-DE mRNAs. (**C**) The constructed miRNA-mRNA regulatory network (the green ovals and pink polygons represent miRNAs and mRNAs respectively.).
Abbreviations: Co-DE, common differentially expressed; miRNA, microRNA; mRNA, messenger RNA.

ischemia-reperfusion by targeting both BCL2L11 and caspase-2.[28] In addition, miR-1247-3p reduced apoptosis of cerebral neurons and could exert protective effects against brain stroke.[29] Although available research indicates that other Co-DE miRNAs are not directly related to ischemia-reperfusion injury, they all are associated with apoptosis, a key player in intestinal ischemia-reperfusion injury.[30–33] These Co-DE miRNAs may be associated with the occurrence and progression of intestinal ischemia-reperfusion injury, and are potential therapeutic targets.

Through GO and KEGG analysis, identified the main biological function of Co-DE mRNAs. There are several signaling pathways involved in ischemia-reperfusion injury, including Wnt signaling pathway,[34] PI3K/Akt signaling pathway,[35] JAK/STAT signaling pathway,[32] and cAMP signaling pathway[36] among others. The result of KEGG analysis verified the association of Co-DE mRNAs with the above signaling pathways, such as cAMP pathway and PI3K/Akt pathway. Using the cytoHubba, we revealed the top eight key mRNAs, including Thy-1 cell surface antigen (THY1), collagen type I alpha 2 chain (COL1A2), collagen type XIV alpha 1 chain (COL14A1), prolyl 4-hydroxylase subunit alpha 1 (P4HA1), interleukin 10 (IL 10), collagen type III alpha 1 chain (COL3A1), matrix metalloproteinase-2 (MMP2) and serpin peptidase inhibitor, clade H, member 1 (SERPINH1). The activity of Wnt signaling pathway was related to whether the cells expressed THY1 or not.[37] Existing reports show that MAPK pathway exerts a negative regulatory effect on the expression of COL1A2. Of note, MAPK pathway plays a key role in the critical phase of ischemia/reperfusion injury,[38,39] and regulates the expression of P4HA1.[40] Studies have revealed that IL10 mediates the activity of MAPK pathway, AMPK pathway, and JAK/STAT pathway,[41–43] and COL3A1 was identified as the regulatory factor of MAPK pathway.[44] MMP2, as a kind of downstream gene, was found to be regulated by MAPK pathway, Wnt pathway, and JAK/STAT pathway, which were involved in the pathophysiological process of ischemia-reperfusion injury.[45–47] Thus, all the top 8 mRNAs may play important roles in the prediction and treatment of this disease, an area that warrants further research.

There are a few limitations in our study. Firstly, because of the small number of patients, the link between the clinical information of samples and the ceRNA regulatory network

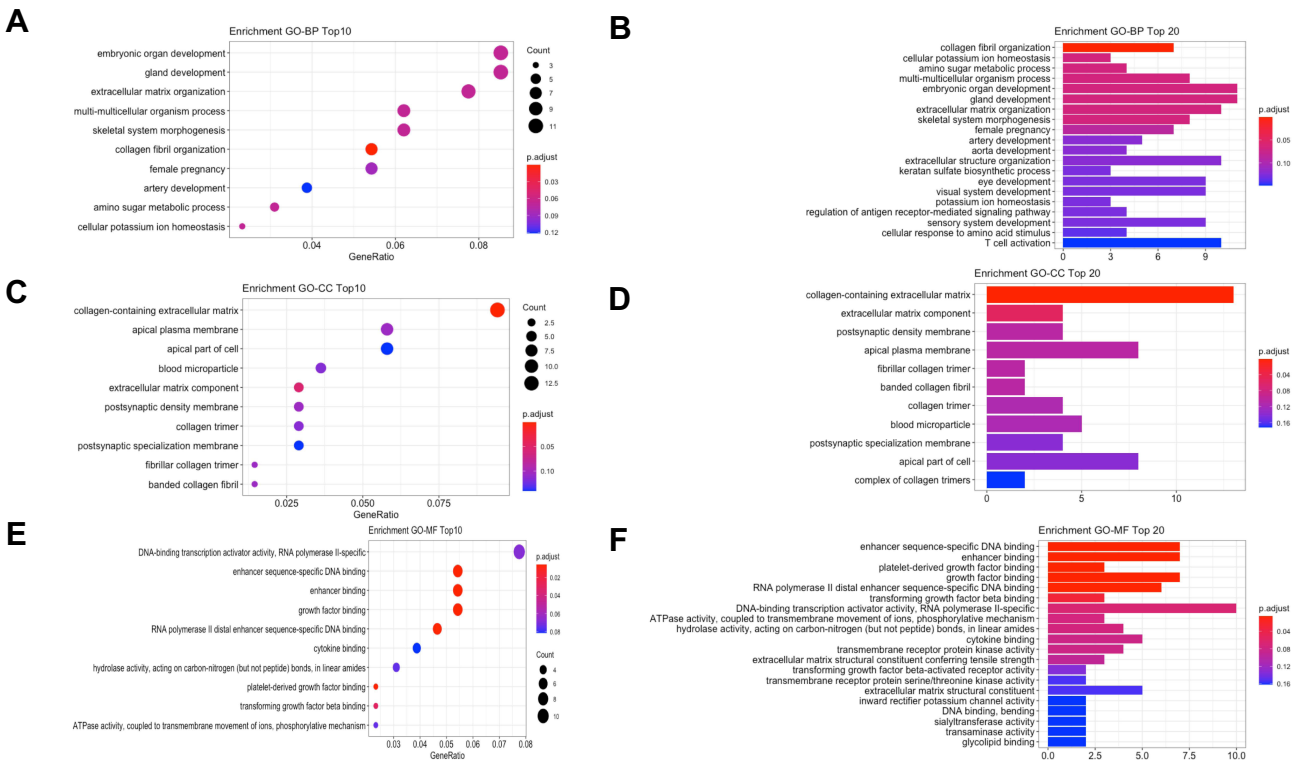
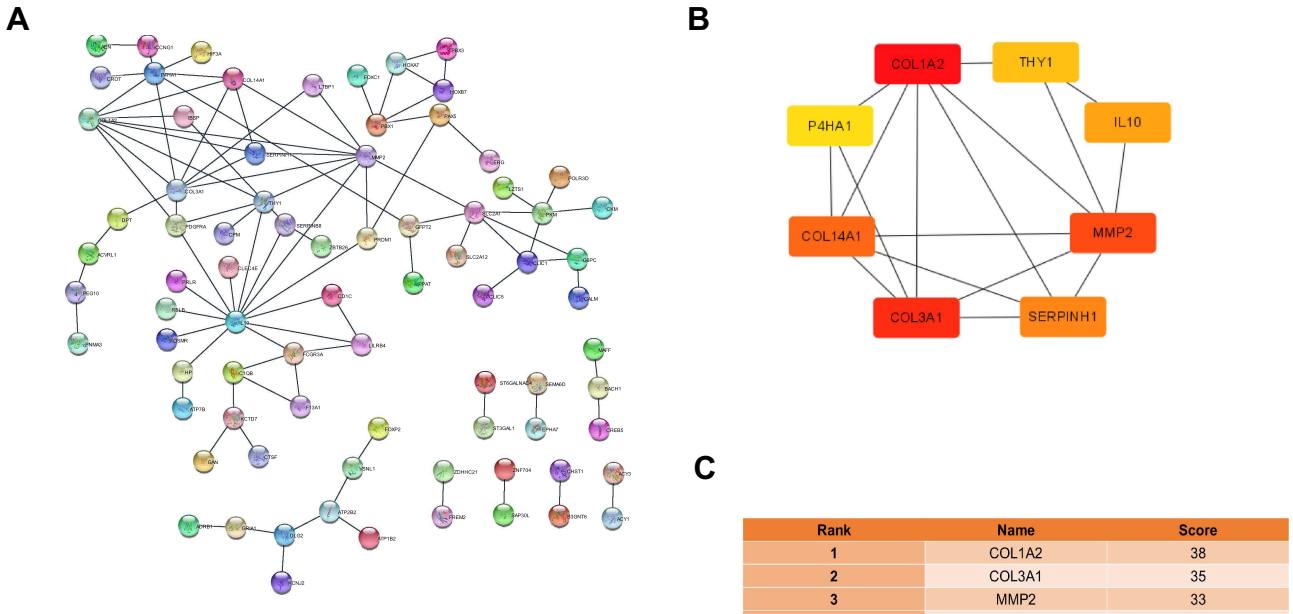


Figure 4 Functional enrichment analysis of common differentially expressed mRNAs. (**A**) A dotplot of GO biological process (top 10 results). (**B**) A barplot of GO biological process (top 20 results). (**C**) A dotplot of GO cellular component (top 10 results). (**D**) A barplot of GO cellular component (top 20 results). (**E**) A dotplot of GO molecular function (top 10 results). (**F**) A barplot of GO molecular function (top 20 results). (**G**) A dotplot of KEGG analysis (top 20 results). (**H**) A barplot of KEGG analysis (top 20 results).
Abbreviations: GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA.



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Rank	Name	Score
1	COL1A2	38
2	COL3A1	35
3	MMP2	33
4	COL14A1	30
5	SERPINH1	24
6	IL10	17
7	THY1	13
8	P4HA1	10

Figure 5 PPI Network and Hub mRNAs. (**A**) PPI network of 79 mRNAs from all Common differentially expressed mRNAs. (**B**) The network of the top 8 key mRNAs. (**C**) The score and rank of key mRNAs in the PPI network.
Abbreviations: PPI, protein–protein interaction; mRNA, messenger RNA; THY1, Thy-1 cell surface antigen; COL1A2, collagen type I alpha 2 chain; COL14A1, collagen type XIV alpha 1 chain; P4HA1, prolyl 4-hydroxylase subunit alpha 1; IL 10, interleukin 10; COL3A1, collagen type III alpha 1 chain; MMP2, matrix metalloproteinase-2; SERPINH1, serpin peptidase inhibitor, clade H, member 1.

could not be analyzed. Secondly, our findings are mainly based on bioinformatics analysis, further mechanistic analysis of cells and animals did not be covered. In future studies, the collection of clinical samples should be increased. Besides, we will further identify and confirm specific functional miRNA and mRNA through research on mechanism.

In conclusion, this work explored the expression profiles of mRNAs and miRNAs, which has allowed for the construction of a ceRNA regulatory network for intestinal ischemia-reperfusion injury. Further, the potential function of Co-DE miRNAs and mRNAs has been established, through functional enrichment analysis on these mRNAs via GO and KEGG analysis. The findings provide a new view on identifying biomarkers and therapeutic targets for intestinal ischemia-reperfusion injury.

Data Available

The data for this study has been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB45362 (https://www.ebi.ac.uk/ena/browser/view/PRJEB45362).

Ethics Approval and Informed Consent

All research studies on humans (individuals, samples or data) include a statement on ethics approval and consent.

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Disclosure

The authors report no conflicts of interest in this work.

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