SHOCK, Vol. 60, No. 4, pp. 594–602, 2023

OPEN

miR-379-5p INHIBITION ENHANCES INTESTINAL EPITHELIAL PROLIFERATION AND BARRIER FUNCTION RECOVERY AFTER ISCHEMIA/REPERFUSION BY TARGETING EIF4G2

Zirui Jia,*† Yuhang Wang,*† Jiacheng Gao,*† Mingcan Zheng,*† Puxu Wang,*† and Guo Zu*

**Department of Gastrointestinal Surgery, The Dalian Municipal Central Hospital Affiliated to Dalian Medical University, Dalian, China; and †Department of Graduate School, Dalian Medical University, Dalian, China*

Received 28 Apr 2023; first review completed 16 May 2023; accepted in final form 31 Jul 2023

ABSTRACT—Gut barrier dysfunction caused by intestinal ischemia/reperfusion (I/R) injury is associated with substantial death and morbidity. In this research, the role of microRNAs (miRNAs) in regulating intestinal I/R injury was investigated. We used miRNA sequencing to analyze clinical ischemic and normal intestinal samples. Through bioinformatics analysis based on sequencing results, we found that upregulated miRNAs inhibited epithelial barrier function and cell proliferation, with miR-379-5p being the most significantly upregulated in the ischemic intestine. Further studies confirmed the role of miR-379-5p through experiments in the human ischemic intestine, the mouse I/R injury model *in vivo*, and cell hypoxia/reoxygenation models *in vitro*. Inhibiting miR-379-5p increased epithelial cell proliferation and improved barrier function after I/R injury. We also identified eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) as a downstream target gene of miR-379-5p through bioinformatics prediction and experimental verification. The findings suggest that inhibiting miR-379-5p could improve intestinal epithelial cell proliferation and barrier function by targeting EIF4G2. The goal of this study was to find a potential target for treating I/R injury in the intestine, as well as to prevent and mitigate the damage caused.

KEYWORDS—miR-379-5p; proliferation; barrier function; intestinal ischemia/reperfusion; eukaryotic translation initiation factor 4 gamma 2

INTRODUCTION

Ischemia/reperfusion (I/R) damage of the intestine is induced by a wide range of clinical diseases, including small bowel obstruction, mesenteric artery thrombosis, hemorrhagic shock, and sepsis (1–3). Intestinal I/R injury results in substantial damage to intestinal epithelial cells and mucosal barrier function. Enhanced permeability of the intestinal mucosa and bacterial translocation due to compromised intestinal mucosal barriers can contribute to fatal outcomes such as systemic inflammatory response syndrome and multiple organ dysfunction syndrome (4). Recent research has shown that increased epithelial cell proliferation can improve the function of the intestinal mucosal barrier after intestinal I/R damage, and the prognosis of intestinal I/R injury is further improved (5–7). To date, an increasing number of studies have investigated the molecular mechanism of epithelial cell proliferation. However, the precise molecular mechanism has not been fully understood. Therefore, it is important to further investigate intestinal epithelial cell proliferation and mucous barrier function postintestinal I/R injury.

MicroRNAs (miRNAs) are a type of small, endogenous noncoding RNA that suppresses the translation of target transcripts or promotes mRNA degradation (8,9). Studies have shown that miRNA regulation of target gene expression, including those related to myocardial or cerebral ischemia injury, promotes organ repair after I/R injury (10,11). Growing evidence has detected the roles of miRNAs in various disease models through sequencing results. Saddic et al. sequenced miRNAs in human left ventricular tissue from normal and ischemic samples. These researchers discovered 21 differentially expressed miRNAs, and therapeutics that target miR-139-5p may lead to new strategies for reducing the injury caused by ischemia insults to the human heart (12). Li et al. (13) conducted miRNA microarray screening in the I/R-affected intestine of mice and found 57 miRNAs to be upregulated and 74 miRNAs to be downregulated. These researchers subsequently identified miR-665-3p as a novel miRNA that is related to autophagy. To date, miRNA sequencing (miRNA-Seq) in clinical patients with intestinal I/R injuries has not been reported.

In this research, we first performed miRNA-Seq analysis in clinical ischemic and normal intestines and further screened miR-379-5p, which was upregulated most significantly in the ischemic intestine. We subsequently explored the expression and function of miR-379-5p in the human ischemic intestine, mouse intestinal I/R models, and IEC-6 hypoxia/reoxygenation (H/R) model. Furthermore, we identified that knocking down miR-379-5p enhanced intestinal epithelial cell proliferation and barrier function after intestinal I/R damage. We further explored whether eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) is a downstream target gene of miR-379-5p and found a miRNA-regulated

Address reprint requests to Guo Zu, PhD, Department of Gastrointestinal Surgery, The Dalian Municipal Central Hospital Affiliated of Dalian Medical University, No. 826 Southwest Road Shahekou District, Dalian, 116033, PR China. E-mail: zushanghai@163.com

Declarations: CRediT authorship contribution statement: Zirui Jia: conceptualization, methodology, formal analysis, investigation, writing—original draft. Yuhang Wang: methodology. Jiacheng Gao: methodology. Mingcan Zheng: investigation. Puxu Wang: investigation. Guo Zu: conceptualization, supervision, writing—review and editing.

Funding: This study was funded by the National Natural Science Foundation of China (grant no. 81700465) and the Natural Science Foundation of Liaoning Province, China (no. 2019-BS-059).

Conflicts of interest: The authors report no conflicts of interest.

Ethics approval and consent to participate: This research was authorized by the ethics committee of Dalian Municipal Central Hospital Affiliated to Dalian Medical University (grant no. YN2022-013-01).

Supplemental digital content is available for this article. Direct URL citation appears in the printed text and is provided in the HTML and PDF versions of this article on the journal's Web site (www.shockjournal.com).

DOI: 10.1097/SHK.0000000000002205

pathway that effectively reduces damage caused by intestinal I/R. Through our research, we aimed to uncover a possible therapeutic strategy for treating intestinal I/R damage, with the goal of preventing and mitigating injury.

MATERIALS AND METHODS

Patients and sample collection

We chose 9 patients (all of them were older than 18 years and were definitively identified with intestinal ischemia necrosis during the surgical process in the Department of Gastrointestinal Surgery) from Dalian Municipal Central Hospital (Dalian, China) between July 2020 and March 2021. All of the patients signed informed consent forms that complied with the Helsinki Declaration. All samples were collected in compliance with hospital procedures, and the research was authorized by the ethics committee of Dalian Municipal Central Hospital Affiliated to Dalian Medical University (grant no. YN2022-013-01). The basic information related to the patients are as follows: the age range was from 45 to 78 years, and the average age was 65.67 ± 6.32 years, 5 male patients and 4 female patients, 2 cases of upper mesenteric arterial embolism, 2 cases of upper mesenteric arterial thrombosis, 2 case of strangulated hernia, 2 case of small intestine volvulus, and 1 case of internal abdominal hernia. For follow-up research, intestinal mucosal samples were obtained from patients with intestinal ischemia injury as well as normal intestinal tissues. One section of intestinal tissue was excised, immediately transported to a liquid nitrogen storage tank, and stored until use. The other portion of the intestinal specimens was stored in 10% formalin.

Young male C57BL/6 mice 6 to 8 weeks of age were supplied by Cyagen Biosciences (Suzhou, China), Inc. The study used mice that were kept in a germ-free and temperature-controlled room at 22°C. The mice were given a week to acclimate in the same animal room before the experiment with unrestricted water and food. Under aseptic conditions, pentobarbital (50 mg/kg) was injected intraperitoneally to induce anesthetized in mice, and the mice underwent midline laparotomies. Ant-379-5p (2 mg/kg) was administered by caudal vein injection 12 h before surgery. Then, in the ant-379-5p treatment group, the superior mesenteric artery was isolated and occluded for 45 min, followed by reperfusion for 4 h; in the sham group, the artery was only occluded. Afterward, a portion of the intestinal samples were collected for various experiments. In the survival analysis, mice were subcutaneously administered buprenorphine for pain management at a dosage of 0.1 mg/kg every 6 h. When the experiments were completed, an isoflurane overdose was used to humanely kill mice. Institutional rules and guidelines for animal experimentation were strictly followed, and all animal operations were carried out in line with the *Guide for Care and Use of Laboratory Animals*.

Procedure for miRNA-Seq of human intestine

The control and ischemia groups were each assigned 3 biological replicates, and all human intestinal samples were sent to Lianchuan (Hangzhou Lianchuan Biotechnology Co, Ltd, China) for further processing. Samples for miRNA-Seq were sequenced by Lianchuan Biotechnology, following the standard protocol provided by Illumina (San Diego, California, United States). In brief, libraries for small RNA sequencing were prepared using TruSeq Small RNA Sample Prep Kits. After library preparation was completed, the generated libraries were sequenced using Illumina HiSeq2000/2500, with a single-end read length of 1*50 bp. The miRNA data analysis software used in this study was ACGT101-miR from LC Sciences (Houston, TX). miRNA-Seq data have been uploaded to the NCBI BioProject database for public access (http://www.ncbi.nlm.nih.gov/bioproject/933788) under accession number PRJNA933788.

Bioinformatics analysis for miRNA-Seq results

The expression data were normalized and transformed to a logarithm based on log2 (14). For the analysis of differentially expressed miRNAs, the R package "limma" was used (fold change = intestinal ischemia sample expression/normal intestinal sample expression, $|\log2(\text{fold change})| > 1$ and $P < 0.05$) (15). mirPath (mirPath v.3, https://dianalab.e-ce.uth.gr/) was used for KEGG analysis of the differentially expressed miRNAs (16). The results were visualized through the R programming language.

Histopathology and immunohistochemistry

We processed the intestinal tissue samples with formalin fixation and paraffin embedding, cut them into 4-μm-thick sections, and stained them with hematoxylin and eosin (HE). We observed them using a fluorescence microscope (Ts2-FL, Nikon, Tokyo, Japan). For analysis of intestinal injury after intestinal I/R, the Chiu score system was used to assess the intestinal pathological scores (17). Ki-67 staining, probed by a monoclonal anti-Ki-67 antibody (Abcam, United States), was used to quantitatively evaluate the intestinal proliferation rate.

Western blotting

We extracted the proteins from the intestinal tissue and cells by using lysis buffer containing protease inhibitors (cOmplete, Roche, Basel, Switzerland). Proteins collected by centrifugation were subjected to gel electrophoresis before being transferred to a polyvinylidene fluoride membrane. Primary antibodies against EIF4G2, ZO-1, occludin, proliferating cell nuclear antigen (PCNA), or β-actin (Proteintech, Wuhan, China) were used to incubate the membranes. Then, the sections were incubated with an anti-rabbit IgG secondary antibody (ZSGB-BIO, Beijing, China). An enhanced chemiluminescence reagent was used to visualize protein bands (MilliporeSigma, Burlington, Massachusetts, United States), and their intensity was analyzed using ImageJ software.

Model of H/R in cell assays

The IEC-6 rat intestinal epithelial cell line was purchased from ATCC (Manassas, VA). High glucose medium with 10% fetal bovine serum was used to cultivate IEC-6 cells. The IEC-6 cells were cultured under standard conditions containing 5% carbon dioxide and a high glucose Dulbecco's modified Eagle's medium fixed with 10% fetal bovine serum. The temperature was maintained at 37°C. Using a microaerophilic system (Thermo, Waltham, MA), we exposed IEC-6 cells to a gas mixture of 1% O_2, 5% CO_2, and 94% N_2 for 12 h to induce hypoxia. Subsequently, the cells were reoxygenated for an additional 6 h in a chamber with atmospheric oxygen.

Transfection of small interfering RNA and antagomirs

We used small interfering RNA (siRNA) that targets EIF4G2 to inhibit its expression. miR-379-5p expression was inhibited using a chemically modified antagomir complementary to miR-379-5p. The negative control sequence for the miR-379-5p inhibitor is nematode cel-miR-239b-5p. The oligonucleotide sequences of siRNAs, antagomirs, and corresponding negative controls are as follows: si-EIF4G2: sense, 5′-GCUUCUCGUUUCAGUGCUUTT-3′, antisense, 5′- AAGCACUGAAACGAGAAGCTT-3′; ant-379-5p: sense, 5′-UGGUAGACUAUGGAACGUAGG-3′, antisense, 5′-CCUACGUUCCAUAGUCUACCA-3′; ant-NC: sense, 5′-UUUGUACUACACAAAAGUACUG-3′, antisense, 5′-CAGUACUUUUGUGUAGUACAAA-3′. All these sequences were designed and purchased from RiboBio Co, Ltd (Guangzhou, China). Transfection of the cells with the oligonucleotides was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines.

Cell counting kit-8

The cell counting kit-8 (CCK-8) assay was performed using the CCK-8 kit (Ameresco, United States). Briefly, 1,000 cells were plated in each well along with 100 mL of medium and 10 mL of CCK-8. In a humidified incubator with 5% CO_2, cells were incubated for 12 and 24 h at 37°C to measure their proliferation ability.

Immunofluorescence

For immunofluorescence analysis, IEC-6 cells were cultured in dishes. The cells were fixed with 4% paraformaldehyde at ice-cold temperatures for 15 min. After being washed 3 times with phosphate buffered saline (PBS), the cells were permeabilized with 0.1% Triton X-100. The cells were blocked with 3% bovine serum albumin in PBS for 1 h. The nuclei were stained with DAPI after incubation with primary antibodies against PCNA. An Olympus fluorescence microscope (Tokyo, Japan) was used to observe the resultant immunofluorescence.

Suppression of miR-379-5p expression in vivo

To decrease miR-379-5p expression *in vivo*, we injected 2 mg/kg miR-379-5p antagomirs into the tail veins of mice. We procured the antagomirs from RiboBio Co, Ltd. Their sequences are detailed as follows: ant-379-5p: sense, 5′-UGGUAGACUAUGGAACGUAGG-3′, antisense, 5′-CCUACGUUCCAUAGUCUACCA-3′. The *Guide for the Care and Use of Laboratory Animals* was strictly followed for all operations involving animals.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-379-5p expression

We assessed miR-379-5p expression levels using qPCR. The small RNA fraction was isolated from cells and tissues using the mirVana miRNA isolation kit (Ambion, United States). The Transcript All-in-one SuperMix for qPCR Kit was used for reverse transcription of total RNA (TransGen, Beijing, China). Following the manufacturer's protocol, we were able to identify miR-379-5p using the Bulge-Loop miRNA qPCR Primer Set (RiboBio). The miRNA expression levels were normalized using U6 snRNA as a control. We applied the $2^{-\Delta\Delta Ct}$ method to normalize the threshold cycle (Ct) values to those of U6. Custom primers were manufactured by GenePharma (Shanghai, China) with the following sequences: miR-379-5p-FO: 5′-GACGCTGTTGGTAGACTATGGA-3′, miR-379-5p-RE: 5′- TATGGTTGTTCTGCTCTCTGTCTC-3′; U6 snRNA-FO: 5′- CGCTTCGGCAGCACATATAC-3′, U6 snRNA-RE: 5′-TTCACGAATTTGCGTGTCATC-3′.

Luciferase activity assay

We purchased plasmids from GenePharma that included either the miR-379-5p wild-type 3′-untranslated region (3′-UTR-WT) or a mutant form (3′-UTR-mut). In 24-well plates of cultured IEC-6 cells, plasmid DNA was cotransfected with ago-379-5p or ago-NC. The cells were tested 36 h after transfection using the Dual-Luciferase Reporter Assay Kit (Promega, 8, Wisconsin, United States), and the luciferase activity was assessed with a Dual-Light Chemiluminescent Reporter Gene Assay System (Berthold, Germany) and normalized to the Renilla luciferase activity.

Barrier measurement

To evaluate intestinal barrier permeability *in vivo*, we measured the fluorescence intensity of fluorescein isothiocyanate (FITC)–labeled 4.4-kDa dextran (FD4, Sigma-Aldrich, United States). During ischemia, we administered 200 μL of PBS containing 25 mg/mL FD-4 to the mice *via* oral gavage. After the reperfusion period, a 100-μL blood sample was obtained. FITC-dextran in plasma was measured by the Enspire2300 microplate reader. The wavelengths of excitation (470 nm) and emission (520 nm) were used accordingly.

Since IEC-6 cells seldom form a tight monolayer *in vitro*, we used Caco-2 cells to conduct a transepithelial electrical resistance (TEER) experiment to evaluate intestinal barrier function *in vitro* (18,19). In addition, 24-well Costar Transwell (0.4 μm) plates were used to perform the TEER experiment (Corning Incorporated, Corning, NY). Simply put, we seeded 0.3 mL of Caco-2 cells and placed them in an environment of 0.7-mL medium in the basal chamber for 21 days. In a steady monolayer state, antagomirs, negative controls, or siRNA were transfected into Caco-2 cells before being subjected to H/R conditions for 12/6 h. An epithelial volt ohmmeter (WPI, Sarasota, FL) was used to measure the TEER value on a daily basis. The TEER value was calculated using the following formula: TEER (Ω cm^2) = [TEER total − TEER blank] × membrane area.

Statistical analysis

The statistical analysis was performed using R and RStudio software (version 4.13). Briefly, the measurement data conforming to the normal distribution in this study are reported as the mean ± standard deviation (SD). Student *t* test and 1-way analysis of variance were used to analyze normally distributed data. Kaplan-Meier analysis was applied to evaluate the prognostic results. The relationship between proteins and miR-379-5p expression levels was analyzed using Pearson correlation analysis. The accuracy of all biological experiments was ensured by repeating them independently at least 3 times to validate the results.

RESULTS

Decreased intestinal epithelial proliferation and mucosal barrier function in clinical ischemic samples

HE staining was used to assess clinical intestinal damage caused by ischemia. The extent of intestinal histological injury was assessed using the Chiu pathologic score, a scoring system that evaluates the degree of mucosal injury (Fig. 1, A and B). Immunohistochemistry for Ki-67 (Fig. 1, C and D) and Western blotting for PCNA (Fig. 1, E and F) were used to analyze intestinal epithelial proliferation. Furthermore, Western blotting for occludin and ZO-1 (Fig. 1, E, G, and H) was used to analyze intestinal barrier function. Our results showed that in clinical ischemic tissues, injury to the intestinal mucosa was much worse, and the Chiu score was higher for those ischemia intestinal tissues (0.556 ± 0.527) than that in normal intestinal tissues (3.889 ± 0.601). However, the expression levels of Ki-67 and PCNA were much lower in ischemic tissue than in normal intestinal tissues, and ischemic tissue had lower expression levels of occludin and ZO-1 than normal intestinal tissue. Therefore, clinical ischemic samples showed decreased intestinal epithelial cell proliferation and impaired mucosal barrier function.

MicroRNA expression profile in intestines from ischemic patients

To investigate the miRNAs that are differentially expressed in the clinically ischemic and normal intestine, we performed miRNA-Seq on 3 ischemia patients and 3 healthy controls. Of all the detected miRNAs, 11 were upregulated and 7 were downregulated after intestinal ischemia (log2(fold change) ≥ 1, $P < 0.05$). The volcano plot filtering approach was used to identify the miRNAs that showed statistically significant differential expression between the 2 groups (Fig. 2A). The total distribution of differentially expressed miRNAs is presented using a heat map (Fig. 2B). The results of differential miRNA expression analysis for the top 500 are demonstrated in Supplemental Digital Content 1, http://links.lww.com/SHK/B751. The results of the analysis of KEGG signaling pathways in genes targeted by upregulated and downregulated miRNAs are shown as bubble diagrams in Figure 2, C and D ($P < 0.05$). The analysis of KEGG signaling pathways revealed that the upregulated miRNAs were significantly associated with the inhibition of epithelial barrier function and cell proliferation.

miR-379-5p is significantly upregulated in different models

We selected miR-379-5p for further research because it is involved in the inhibition of epithelial barrier function and cell proliferation, as suggested by KEGG pathway analysis. More importantly, miR-379-5p was upregulated in ischemia samples, as shown by miRNA-Seq, and the differences were most significant in upregulated miRNAs (Fig. 3A). To demonstrate the clinical importance of miR-379-5p in intestinal I/R damage, we assessed its expression in ischemic and normal intestinal tissues from clinical patients. Ischemic injury to the intestines increased the expression of miR-379-5p (Fig. 3B). In addition, the expression level of miR-379-5p was strongly increased under ischemic and I/R conditions in mouse models (Fig. 3C). Furthermore, the expression level of miR-379-5p was strongly increased under H/R conditions in IEC-6 cell culture models (Fig. 3D).

Knocking down miR-379-5p improved intestinal epithelial proliferation, barrier function, and survival rate after I/R injury in mice

We used ant-379-5p to knock down miR-379-5p expression in mice to investigate its role in intestinal I/R injury. Ant-379-5p significantly suppressed the upregulation of miR-379-5p caused by intestinal I/R damage (Fig. 4A). Intestinal histological injury was significantly decreased when miR-379-5p was silenced (Fig. 4, B and C). As a consequence, occludin, ZO-1, and PCNA protein expression was elevated after ant-379-5p treatment (Fig. 4, D–G). In addition, we observed that ant-379-5p attenuated the increase in FITC-dextran paracellular permeability induced by I/R injury (Fig. 4H). To evaluate the protective impact of ant-379-5p against I/R damage, we also explored the 24-h survival rates of mice. The I/R + ant-379-5p group exhibited mean survival time (17.5 ± 7.10 h), which was much higher than the I/R group (12.5 ± 6.74 h) ($P = 0.020$) (Fig. 4I). miR-379-5p inhibition resulted in enhanced epithelial cell proliferation, restored barrier function, and an improved survival rate after intestinal damage, according to these results.

miR-379-5p inhibition improves intestinal epithelial cell proliferation and barrier function after H/R injury

The role of miR-379-5p in epithelial cells under H/R damage was also explored. As shown in Figure 5A, ant-379-5p significantly

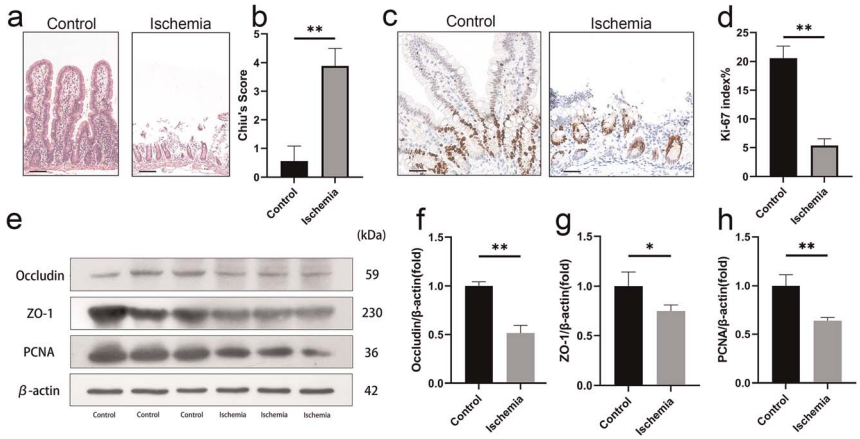

FIG. 1. **Intestinal epithelial proliferation and the mucosal barrier were decreased in clinical ischemic samples.** A, HE staining of intestinal tissue (n = 9, scale bar = 100 μm). B, Chiu's score of intestinal tissues (n = 9). C and D, Immunohistochemical staining for Ki-67 antibody in intestinal tissues for proliferation analysis (scale bar = 50 μm, n = 9). E–H, Western blot showing occludin, ZO-1, and PCNA protein expression in intestinal tissue (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$. The error bars describe the SD. HE, hematoxylin and eosin.

attenuated the miR-379-5p upregulation caused by H/R injury in IEC-6 cells. Using immunofluorescence methods to detect PCNA expression, we concluded that under H/R conditions, the suppression of miR-379-5p led to an increase in cell proliferation (Fig. 5, B and C). Consistently, miR-379-5p inhibition also restored cell proliferation, as shown by the CCK-8 value induced by H/R (Fig. 5D). In addition, we used Western blotting to explore the significance of miR-379-5p inhibition on PCNA expression under H/R conditions. The results showed that miR-379-5p inhibition prevented PCNA downregulation caused by H/R (Fig. 5, E and F). The miR-379-5p inhibition group showed significantly higher expression of occludin and ZO-1 than the negative control group under H/R circumstances (Fig. 5, E, G, and H). Furthermore, miR-379-5p inhibition also restored the TEER value (Fig. 5I). These findings indicate that inhibiting miR-379-5p in cell models can promote intestinal epithelial cell proliferation and barrier function.

EIF4G2 expression is regulated by miR-379-5p

To identify downstream target genes of miR-379-5p, we performed intersection prediction with 8 databases (miRTarBase, TargetScan, miRDB, RNAInter, ENOCRI, TargetMiner, miRWalk, and RNA22) (Fig. 6A). Through the intersection of 8 databases, we pinpointed EIF4G2 as a potential target protein of significance. Previous studies show that EIF4G2 might play a pivotal role in mediating cellular proliferation (20,21) (Fig. 6B). The dual-luciferase assay confirmed that miR-379-5p targets EIF4G2, with a significant decrease in luciferase activity observed upon miR-379-5p transfection and use of wild-type EIF4G2 3′-UTR. However, the inhibitory impact of miR-379-5p was eliminated by the use of mutant-type EIF4G2 3′-UTR (Fig. 6C). In addition, miR-379-5p silencing upregulated EIF4G2 protein levels in IEC-6 cells (Fig. 6, D and E). These results indicate that miR-379-5p specifically binds to the 3′-UTR of EIF4G2

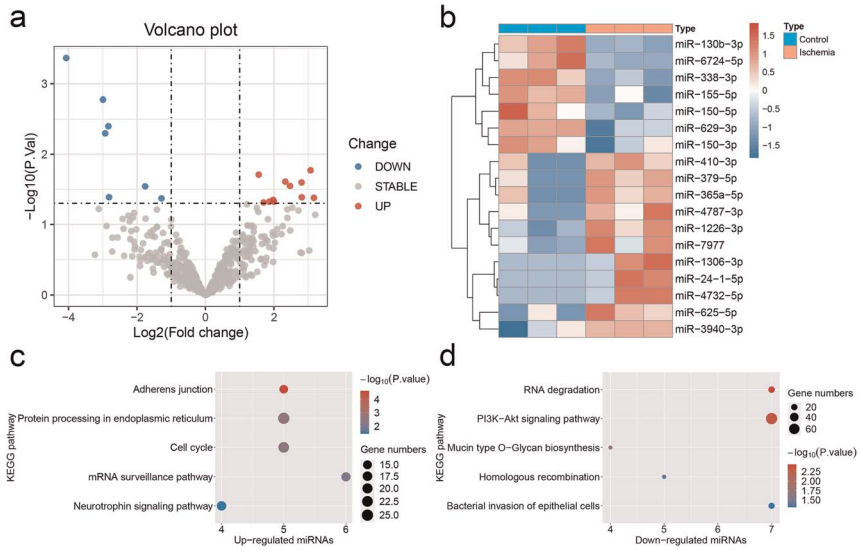

FIG. 2. **Identification of differentially expressed miRNAs in intestinal tissue from ischemia patient samples.** A, Volcano plots comparing miRNA expression between ischemic patients and controls. The red dots represent the significantly upregulated miRNAs, and blue dots represent downregulated miRNAs (|log2[fold change]| > 1 and $P < 0.05$). B, Heat map showing the overall distribution of differentially expressed miRNAs. C, KEGG pathway enrichment terms in genes targeted by upregulated miRNAs ($P < 0.05$). D, KEGG pathway enrichment terms in genes targeted by downregulated miRNAs ($P < 0.05$). miRNAs, microRNAs.

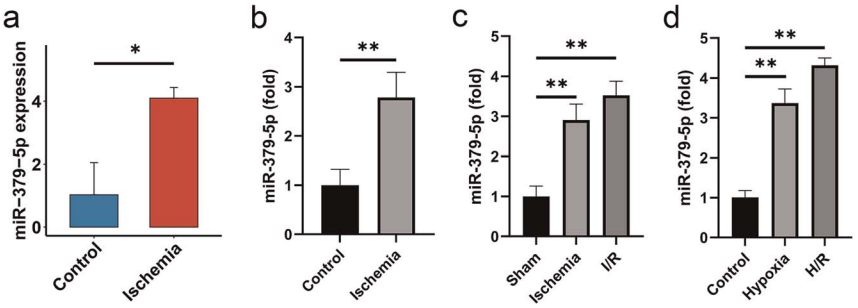

FIG. 3. **miR-379-5p is significantly upregulated in human, mouse, and rat IEC-6 cells.** A, miR-379-5p is significantly upregulated in ischemic tissue, as shown by miRNA-Seq. B, qRT-PCR showing miR-379-5p expression in normal and ischemic human intestines, n = 3. C, qRT-PCR showing miR-379-5p expression in sham, ischemic, and I/R mouse intestines, n = 3. D, qRT-PCR showing miR-379-5p expression in control, hypoxia, and H/R rat IEC-6 cells, n = 3. **P* < 0.05, ***P* < 0.01. The error bars describe the SD. I/R, ischemia/reperfusion; miRNA-Seq, miRNA sequencing; SD, standard deviation.

to downregulate its expression. In summary, miR-379-5p regulates EIF4G2 expression in the intestine.

miR-379-5p inhibition improves epithelial cell proliferation and barrier function after H/R injury by targeting EIF4G2

Because we found that inhibiting miR-379-5p improves intestinal epithelial restoration and increases EIF4G2 expression, next we explored the role of EIF4G2 in the protective effects of miR-379-5p inhibition on the intestinal epithelium. Ant-379-5p significantly suppressed the upregulation of miR-379-5p caused by H/R damage in IEC-6 cells (Fig. 7A). We found that ant-379-5p increased CCK-8–labeled cell proliferation, but the elevated effect was abolished by the knockdown of EIF4G2 after H/R (Fig. 7B). Moreover, knockdown of EIF4G2 also abolished the restored TEER value caused by miR-379-5p inhibition (Fig. 7C). Through Western blot experiments, the findings indicated an increase in EIF4G2 expression with ant-379-5p transfection (Fig. 7, D and F). In addition, when EIF4G2 was silenced, the impact of ant-379-5p on increasing proliferation-related protein expression under H/R conditions was abolished (Fig. 7, D and E). Furthermore, the EIF4G2 knockdown group had a significant suppression impact on occludin and ZO-1 expression that increased with ant-379-5p (Fig. 7, D, G, and H). These results show that the protective effects of miR-379-5p inhibition

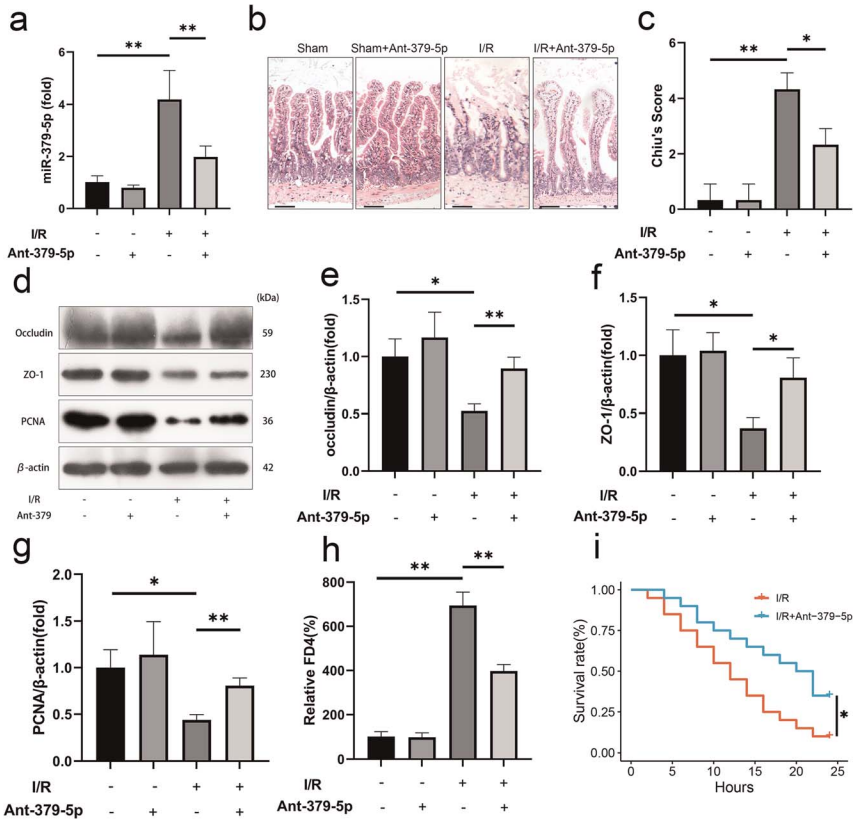

FIG. 4. **miR-379-5p inhibition improves intestinal epithelial proliferation, barrier function, and survival after intestinal I/R injury in mice.** The mice were divided into the following 4 groups: sham, sham + ant-379-5p, I/R, and I/R + ant-379-5p. A, qRT-PCR showing miR-379-5p expression in intestinal tissues (n = 8). B and C, HE-stained intestinal sections and Chiu's score of intestinal tissue (scale bar = 100 μm, n = 8). D–G, Representative Western blot showing PCNA, occludin, and ZO-1 protein expression in intestinal tissue (n = 3). H, Ant-379-5p induced changes in intestinal epithelial permeability, as measured by FITC-dextran permeability, n = 5. I, The survival rate of the mice (n = 20). **P* < 0.05, ***P* < 0.01. The error bars describe the SD. FITC, fluorescein isothiocyanate; HE, hematoxylin and eosin; I/R, ischemia/reperfusion; SD, standard deviation.

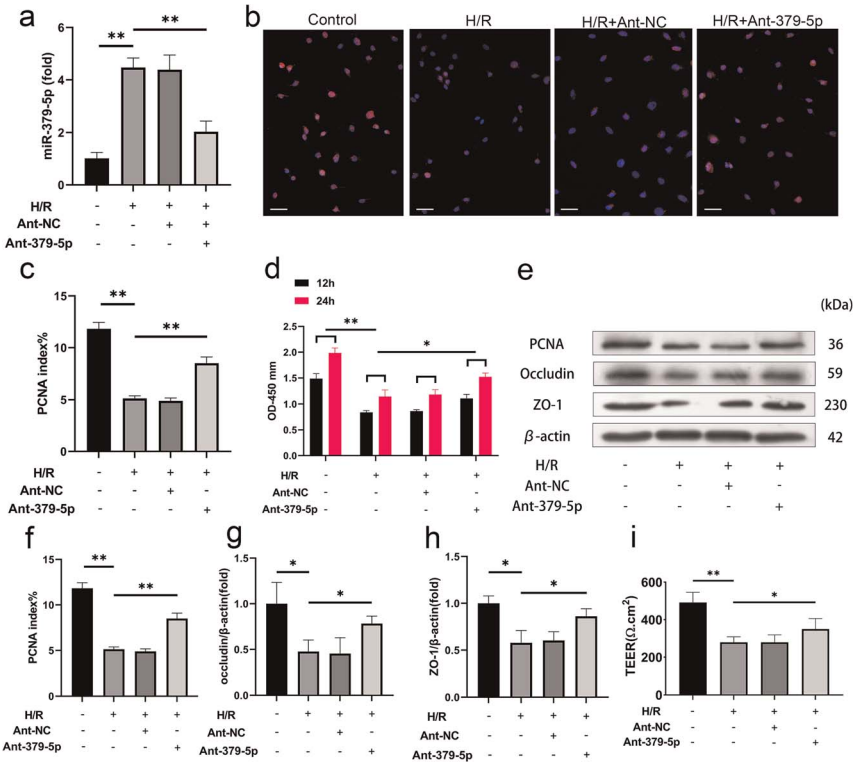

FIG. 5. **miR-379-5p inhibition promotes intestinal epithelial restoration after H/R injury.** IEC-6 cells were infected with ant-379-5p or ant-NC for 36 h and then incubated under H/R conditions for 6/6 h. A, qRT-PCR showing miR-379-5p expression in IEC-6 cells, n = 8. B and C, Immunofluorescence staining for the PCNA antibody in IEC-6 cells for proliferation analysis. Scale bar = 100 μm, n = 6. D, Cell proliferation index was detected by CCK-8 assays. E–H, Representative Western blot showing PCNA, occludin, and ZO-1 protein expression in IEC-6 cells, n = 3. I, The effect of ant-379-5p on Caco-2 cells was evaluated by TEER, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$. The error bars describe the SD. H/R, hypoxia/reoxygenation; SD, standard deviation; TEER, transepithelial electrical resistance.

on intestinal I/R injury are mediated through modulation of EIF4G2 expression.

Negative correlation between miR-379-5p and its related protein expression in the ischemic intestine of clinical patients

To evaluate the clinical importance of miR-379-5p and related genes in intestinal I/R injury, we first analyzed the expression of miR-379-5p, EIF4G2, PCNA, occludin, and ZO-1 in normal and ischemic intestinal tissue samples from patients. Our results showed that EIF4G2, occludin, ZO-1, and PCNA expression was decreased in ischemic intestinal tissue (Fig. 8, A–E). In addition, miR-379-5p expression was elevated in ischemic intestinal tissue (Fig. 8F). Furthermore, we found a negative correlation between miR-379-5p and related gene expression in ischemic intestinal tissue, including EIF4G2, PCNA, occludin, and ZO-1 (Fig. 8, G–J).

DISCUSSION

The disruption or death of intestinal barrier cells can lead to serious complications in the case of intestinal I/R injury, including intestinal bacterial translocation, multiple organ damage, and even

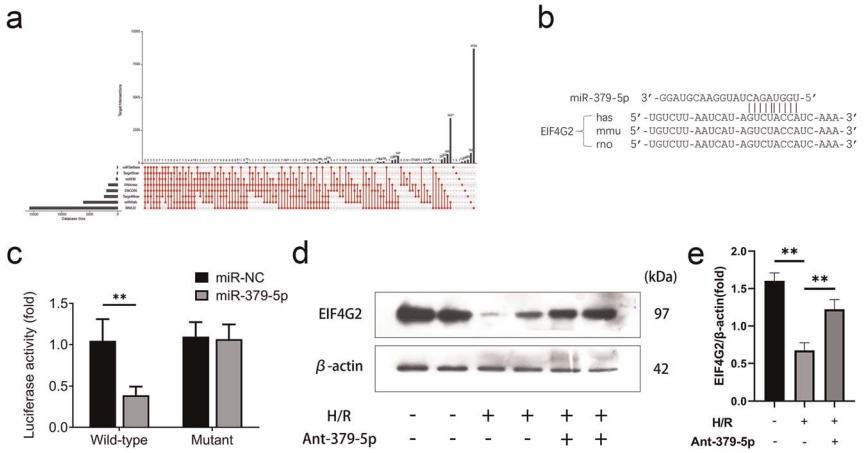

FIG. 6. **miR-379-5p regulates the expression of EIF4G2.** A, UpSet plot of interactions in miR-379-5p target genes between 8 databases. B, The miR-379-5p target sequence in the EIF4G2 3′-UTR is conserved across various species. C, IEC-6 cells were transfected with miR-379-5p or miR-NC and WT EIF4G2 3′-UTR or MT EIF4G2 3′-UTR, n = 3. D and E, IEC-6 cells were transfected with ant-379-5p, Western blot showing EIF4G2 protein expression in IEC-6 cells, n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$. The error bars describe the SD. SD, standard deviation; UTR, untranslated region; WT, wild-type.

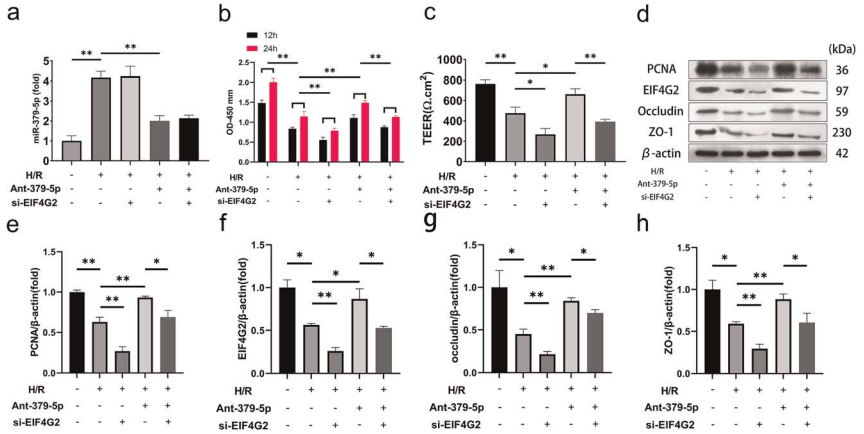

FIG. 7. **miR-379-5p inhibition provides protective effects by targeting EIF4G2.** IEC-6 cells were cotransfected with ant-379-5p, si-EIF4G2, and then incubated in H/R for 6/6 h. A, qRT-PCR showing miR-379-5p expression in IEC-6 cells, n = 8. B, The cell proliferation index was measured by the CCK-8 assay. C, The effect of ant-379-5p and si-EIF4G2 on Caco-2 cells was evaluated by TEER, n = 6. D–H, Representative Western blot showing PCNA, EIF4G2, occludin, and ZO-1 protein expression, n = 3. **P* < 0.05, ***P* < 0.01. The error bars describe the SD. CCK-8, cell counting kit-8; H/R, hypoxia/reoxygenation; SD, standard deviation; TEER, transepithelial electrical resistance.

mortality (22). Coordination of cell proliferation, migration, and differentiation is essential for intestinal barrier restoration (23). Rapid cell proliferation in the crypt is the primary driving force for cellular migration along the villi (24). During the maturation of the intestinal epithelium, enterocytes migrate from the crypt base to the villi, in which they differentiate into goblet cells and enteroendocrine cells (25,26). Thus, improved epithelial cell proliferation helps to restore intestinal barrier function (6). Recent studies have shown that miRNAs are crucial in regulating key pathological processes and impacting intestinal I/R injury (27), but overall miRNA expression in clinical intestinal ischemic tissue remains severely limited and defective. Through miRNA-Seq in the clinic with normal and intestinal ischemic injury samples, we found the following: in the intestinal I/R injury model, high expression of

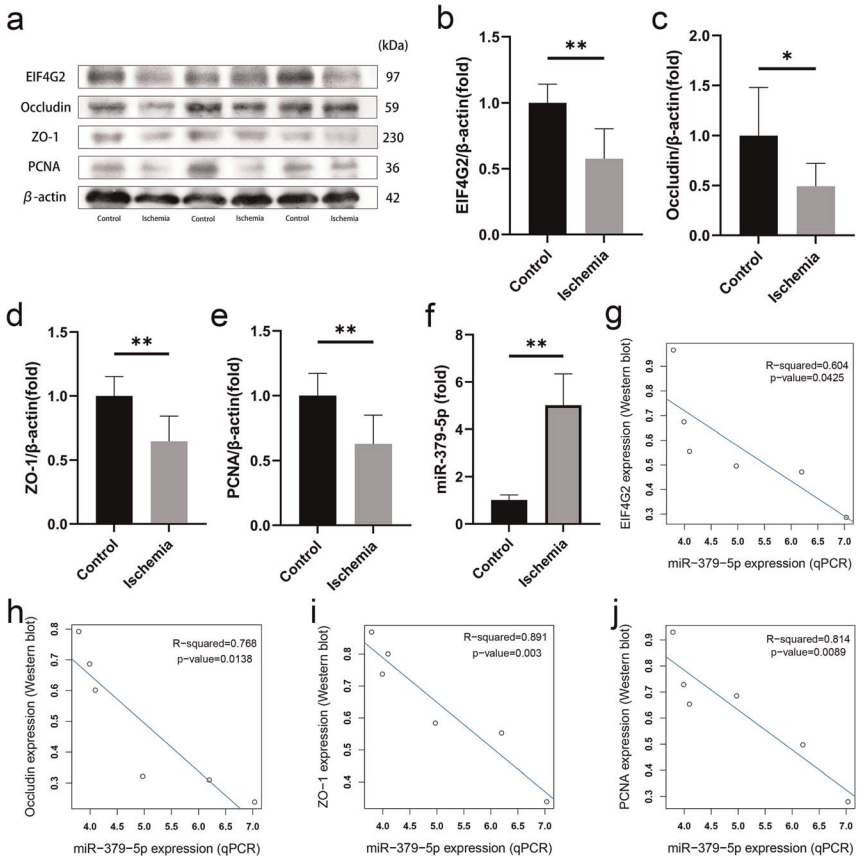

FIG. 8. **miR-379-5p, EIF4G2, and mucosal barrier protein expression levels in the ischemic intestine of clinical patients.** A–E, Representative Western blot showing EIF4G2, occludin, ZO-1, or PCNA protein expression in the intestine, n = 3. F, qRT-PCR showing miR-379-5p expression in normal and ischemic human intestines, n = 6. G, The association between miR-379-5p expression and EIF4G2 protein expression, n = 6 (r^2 = 0.604, P = 0.043). H, The association between miR-379-5p expression and occludin protein expression, n = 6 (r^2 = 0.768, P = 0.014). I, The association between miR-379-5p expression and ZO-1 protein expression, n = 6 (r^2 = 0.891, P = 0.003). J, The association between miR-379-5p expression and PCNA protein expression, n = 6 (r^2 = 0.814, P = 0.009). **P* < 0.05, ***P* < 0.01. The error bars describe the SD. SD, standard deviation.

miR-379-5p could inhibit intestinal epithelial cell proliferation and barrier function; miR-379-5p inhibition improved intestinal epithelial cell proliferation and barrier function; and miR-379-5p inhibition improved intestinal epithelial cell proliferation and barrier function by targeting EIF4G2.

Increasing numbers of studies have indicated the importance of miRNAs in regulating and impacting intestinal I/R injury (27). Recently, several studies have examined the importance of miRNAs in restoring epithelial cell proliferation and barrier function after intestinal I/R injury. However, most studies were mostly focused on miRNA expression in animal experiments, and these studies explored miRNA effects without knowledge of overall miRNA expression (27–29). Therefore, the conclusions on practical translational therapeutic applications are limited. We focused on intestinal ischemic patients and performed comprehensive high-throughput miRNA-Seq to identify differentially expressed miRNAs between intestinal ischemic and normal tissues. In our results, we discovered that 11 miRNAs were notably upregulated and 7 miRNAs were notably downregulated after ischemic conditions. Further enriched GO term analysis revealed that all differentially upregulated miRNAs were involved in the biological process of negative regulation of epithelial cell proliferation, and the fold change in miR-379-5p was the most significant. Our results show that the upregulation of miR-379-5p is negatively related to intestinal epithelial cell proliferation and barrier function. These findings identify miR-379-5p as a possible therapeutic target for repairing intestinal mucosal barrier function after injury.

The use of antagomirs, also known as anti-miRNAs, as a therapeutic approach has been gaining attention in recent years. These chemically engineered oligonucleotides have been shown to be effective in inhibiting disease-associated miRNAs, such as miR-21 inhibiting fibrosis in the heart and lungs (30–34). In this study, we used ant-379-5p to specifically inhibit miR-379-5p expression *in vivo* in C57 mice without causing obvious adverse effects. Our findings highlight an important role for miR-379-5p in modulating intestinal epithelial cell proliferation and barrier function after I/R. *In vitro* studies demonstrated that inhibiting miR-379-5p improved proliferation and barrier function after H/R injury. In addition, *in vivo* experiments revealed that knocking down miR-379-5p through the use of ant-379-5p prevented injury to the intestine by enhancing proliferation and mucosal barrier function. These results demonstrate that targeting miR-379-5p is a promising treatment approach for intestinal I/R injury because of its ability to promote epithelial proliferation and improve barrier function.

Studies have shown that miRNAs are involved in pathophysiological processes by binding and modulating target gene expression. Our findings on the impact of miR-379-5p on intestinal proliferation and barrier function prompted us to use bioinformatics analysis to identify the potential target of miR-379-5p as EIF4G2 through a cross-referencing method using 8 databases. The 3′-UTR of EIF4G2 that miR-379-5p targets is a highly conserved binding sequence in various species. Gain-of-function research further showed that miR-379-5p directly inhibited EIF4G2 translation. EIF4G2 is a critical modulator of translation initiation, acting during mitosis (35). Cell cycle research revealed that EIF4G2 silencing led to a rise in cells in the G0/G1 phase and a drop in cells in the G2/M phase (36). EIF4G2 promotes cell proliferation in a variety of tissues, such as chondrocytes and ovarian cancer (37–39). EIF4G2 has also been proven to have a strong proliferative effect on human colon cancer cells, and its expression is increased in skeletal muscle after I/R (36,40). Previous studies have demonstrated that targeting EIF4G2 may be a viable approach for treating patients with proliferation disorders related to tumors or osteoarthritis (37,41). In our results, we found that miR-379-5p inhibition increased intestinal epithelial cell proliferation and barrier function by upregulating EIF4G2, and the effects of miR-379-5p inhibition could be reversed by knockdown of EIF4G2 in mouse intestinal I/R and IEC-6 H/R models. Our results show that miR-379-5p suppression promotes intestinal epithelial proliferation and barrier function by suppressing EIF4G2 expression. We initially compared the expression of miR-379-5p, EIF4G2, PCNA, occludin, and ZO-1 in normal and ischemic intestinal tissue samples from patients to evaluate the clinical value of miR-379-5p and associated genes in intestinal I/R damage. In addition, analysis of the ischemic intestinal tissue samples confirmed the inverse relationship between miR-379-5p and related genes, including EIF4G2, PCNA, occludin, and ZO-1. Furthermore, the negative correlation between miR-379-5p and related gene expression in ischemic intestinal tissue suggests that by targeting EIF4G2, miR-379-5p contributes significantly to protecting intestinal barrier function and promoting epithelial proliferation in patients with intestinal ischemia.

Our study also has some potential limitations. First, intestinal I/R injury is an acute and severe injury. In our study, we only observed the survival in 24 h. It would be much better if it could be observed for a longer time. Furthermore, the off-target effects of inhibition of miR-379-5p are not fully understood; we will further explore this in future research. In addition, in our results, we demonstrated that inhibition of miR-379-5p reduced intestinal injury by targeting EIF4G2 after I/R injury. However, we did not further detect the inhibition of miR-379-5p in intestinal injury after ischemia. We will further explore this in future research.

In summary, through sequencing and experimental results, we found that miR-379-5p has a negative regulatory impact on epithelial cell proliferation and barrier function after I/R injury. In addition, our study has shown that miR-379-5p inhibition can improve epithelial proliferation and barrier function by targeting EIF4G2, as evidenced by bioinformatics analysis and experimental results. This discovery presents a potential new miRNA-based therapeutic strategy for treating patients with I/R injury.

REFERENCES

1. Acosta S, Björck M. Modern treatment of acute mesenteric ischaemia. *Br J Surg*. 2014;101(1):e100–e108.
2. Kong SE, Blennerhassett LR, Heel KA, et al. Ischaemia-reperfusion injury to the intestine. *Aust N Z J Surg*. 1998;68(8):554–561.
3. Lendzion RJ, Frahm-Jensen G, Keck J. Acute mesenteric ischemia. *Clin Colon Rectal Surg*. 2022;35(3):227–236.
4. Kannan L, Kis-Toth K, Yoshiya K, et al. R-spondin3 prevents mesenteric ischemia/reperfusion-induced tissue damage by tightening endothelium and preventing vascular leakage. *Proc Natl Acad Sci U S A*. 2013;110(35):14348–14353.
5. Liu L, Yao J, Li Z, et al. miR-381-3p knockdown improves intestinal epithelial proliferation and barrier function after intestinal ischemia/reperfusion injury by targeting nurr1. *Cell Death Dis*. 2018;9(3):411.
6. Nan X, Qin S, Yuan Z, et al. Hsa-miRNA-31 regulates epithelial cell barrier function by inhibiting TNFSF15 expression. *Cell Mol Biol (Noisy-le-Grand)*. 2016; 62(4):104–110.

7. Shim S, Jang HS, Myung HW, et al. Rebamipide ameliorates radiation-induced intestinal injury in a mouse model. *Toxicol Appl Pharmacol*. 2017;329:40–47.
8. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009; 136(2):215–233.
9. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003;425(6956):415–419.
10. Lesizza P, Prosdocimo G, Martinelli V, et al. Single-dose intracardiac injection of pro-regenerative MicroRNAs improves cardiac function after myocardial infarction. *Circ Res*. 2017;120(8):1298–1304.
11. Yang J, Zhang X, Chen X, et al. Exosome mediated delivery of miR-124 promotes neurogenesis after ischemia. *Mol Ther Nucleic Acids*. 2017;7:278–287.
12. Saddic LA, Chang TW, Sigurdsson MI, et al. Integrated microRNA and mRNA responses to acute human left ventricular ischemia. *Physiol Genomics*. 2015;47(10):455–462.
13. Li Z, Wang G, Feng D, et al. Targeting the miR-665-3p-ATG4B-autophagy axis relieves inflammation and apoptosis in intestinal ischemia/reperfusion. *Cell Death Dis*. 2018;9(5):483.
14. Cer RZ, Herrera-Galeano JE, Anderson JJ, et al. miRNA Temporal Analyzer (mirnaTA): a bioinformatics tool for identifying differentially expressed microRNAs in temporal studies using normal quantile transformation. *GigaScience*. 2014;3:20.
15. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
16. Vlachos IS, Zagganas K, Paraskevopoulou MD, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res*. 2015;43(W1):W460–W466.
17. Chiu CJ, McArdle AH, Brown R, et al. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg*. 1970; 101(4):478–483.
18. Li Y, Xu B, Xu M, et al. 6-Gingerol protects intestinal barrier from ischemia/reperfusion-induced damage via inhibition of p38 MAPK to NF-κB signalling. *Pharmacol Res*. 2017;119:137–148.
19. Wolf T, Baier SR, Zempleni J. The intestinal transport of bovine milk exosomes is mediated by endocytosis in human colon carcinoma Caco-2 cells and rat small intestinal IEC-6 cells. *J Nutr*. 2015;145(10):2201–2206.
20. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev*. 2004;18(5):504–511.
21. Grimson A, Farh KK, Johnston WK, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27(1):91–105.
22. Kannan KB, Colorado I, Reino D, et al. Hypoxia-inducible factor plays a gut-injurious role in intestinal ischemia reperfusion injury. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5):G853–G861.
23. Iizuka M, Konno S. Wound healing of intestinal epithelial cells. *World J Gastroenterol*. 2011;17(17):2161–2171.
24. Parker A, Maclaren OJ, Fletcher AG, et al. Cell proliferation within small intestinal crypts is the principal driving force for cell migration on villi. *FASEB J*. 2017;31(2):636–649.
25. Clevers H, Batlle E. SnapShot: the intestinal crypt. *Cell*. 2013;152(5):1198–e2.
26. Sun X, Yang Q, Rogers CJ, et al. AMPK improves gut epithelial differentiation and barrier function via regulating Cdx2 expression. *Cell Death Differ*. 2017;24(5): 819–831.
27. Liu Z, Jiang J, Yang Q, et al. MicroRNA-682-mediated downregulation of PTEN in intestinal epithelial cells ameliorates intestinal ischemia-reperfusion injury. *Cell Death Dis*. 2016;7:e2210.
28. Li Y, Wen S, Yao X, et al. MicroRNA-378 protects against intestinal ischemia/reperfusion injury via a mechanism involving the inhibition of intestinal mucosal cell apoptosis. *Cell Death Dis*. 2017;8(10):e3127.
29. Wang G, Yao J, Li Z, et al. miR-34a-5p inhibition alleviates intestinal ischemia/reperfusion-induced reactive oxygen species accumulation and apoptosis via activation of SIRT1 signaling. *Antioxid Redox Signal*. 2016;24(17):961–973.
30. Czech MP. MicroRNAs as therapeutic targets. *N Engl J Med*. 2006;354(11): 1194–1195.
31. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. *Nature*. 2005;438(7068):685–689.
32. Yue J. miRNA and vascular cell movement. *Adv Drug Deliv Rev*. 2011;63(8): 616–622.
33. Pandit KV, Corcoran D, Yousef H, et al. Inhibition and role of let-7d in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2010;182(2):220–229.
34. Adam O, Löhfelm B, Thum T, et al. Role of miR-21 in the pathogenesis of atrial fibrosis. *Basic Res Cardiol*. 2012;107(5):278.
35. Lee SH, McCormick F. p97/DAP5 is a ribosome-associated factor that facilitates protein synthesis and cell proliferation by modulating the synthesis of cell cycle proteins. *EMBO J*. 2006;25(17):4008–4019.
36. Ali SR, Orang A, Marri S, et al. Integrative transcriptomic network analysis of butyrate treated colorectal cancer cells. *Cancers (Basel)*. 2021;13(4):636.
37. Gao S, Liu L, Zhu S, et al. MicroRNA-197 regulates chondrocyte proliferation, migration, and inflammation in pathogenesis of osteoarthritis by targeting EIF4G2. *Biosci Rep*. 2020;40(9):BSR20192095.
38. Zhang J, Zhang R, Ye Y. Long non-coding RNA (LncRNA) SNHG7/ eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) involves in the malignant events of ovarian cancer cells with paclitaxel resistant. *Bioengineered*. 2021;12(2):10541–10552.
39. Zhao H, Wang A, Zhang Z. LncRNA SDHAP1 confers paclitaxel resistance of ovarian cancer by regulating EIF4G2 expression via miR-4465. *J Biochem*. 2020; 168(2):171–181.
40. Ratchford SM, Bailey AN, Senesac HA, et al. Proteins regulating cap-dependent translation are downregulated during total knee arthroplasty. *Am J Physiol Regul Integr Comp Physiol*. 2012;302(6):R702–R711.
41. Li S, Shao J, Lou G, et al. MiR-144-3p-mediated dysregulation of EIF4G2 contributes to the development of hepatocellular carcinoma through the ERK pathway. *J Exp Clin Cancer Res*. 2021;40(1):53.