MicroRNA-26b-5p Targets DAPK1 to Reduce Intestinal Ischemia/Reperfusion Injury via Inhibition of Intestinal Mucosal Cell Apoptosis

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Abstract

Background  Emerging evidence has suggested that miRNAs are important regulators of intestinal I/R injury, but their function in this context remains elusive.

Aims  To evaluate the role of miR-26b-5p in intestinal I/R injury.

Methods  We utilized in vivo murine models of intestinal I/R and in vitro Mode-K cell-based models of oxygen and glucose deprivation/reperfusion (OGD/R) to examine the function of miR-26b-5p in intestinal I/R injury. The expression of miR-26b-5p in intestinal mucosa and Mode-K cell was detected by RT-PCR. HE staining and Chiu’s score were used to evaluate intestinal mucosa injury severity. Apoptosis was detected by TUNEL stain, flow cytometry, and western blot. TargetScan and StarBase prediction algorithms were applied to predict putative target genes of miR-26b-5p and validated by luciferase reporter assays.

Results  We found that the expression of miR-26b-5p in intestinal mucosa was markedly decreased during I/R injury. We additionally found miR-26b-5p overexpression to markedly disrupt intestinal I/R- or OGD/R-induced injury in vivo and in vitro, whereas inhibiting this miRNA had an adverse impact and resulted in increased intestinal tissue injury and Mode-K cell damage. From a mechanistic perspective, miR-26b-5p was predicted to target DAPK1, which was related to cellular apoptosis. Luciferase reporter assay results confirmed that miR-26b-5p directly targets DAPK1 in Mode-K cells, thereby suppressing OGD/R-induced cell apoptosis.

Conclusion  Our findings show that miR-26b-5p may prevent intestinal I/R injury via targeting DAPK1 and inhibiting intestinal mucosal cell apoptosis, suggesting that this miRNA may be a viable target for the treatment of intestinal I/R injury.

Keywords  MiR-26b-5p · DAPK1 · Intestinal I/R · Apoptosis

Introduction

Intestinal ischemia/reperfusion (I/R) injuries are most often observed in patients suffering from serious conditions including septicemic shock, aortic aneurysm, cardiopulmonary bypass, trauma, or small bowel transplantation [1]. Hypovolemic shock is associated with the preferential shunting of blood flow to the heart and the brain, thus leaving the small intestine at a heightened risk of ischemia [2]. This ischemia and subsequent reperfusion can lead to direct epithelial cell damage and disruption of the integrity of the intestinal barrier, in turn resulting in bacterial translocation, systemic inflammation, and multiple organ failure that can cause high rates of morbidity and mortality [3].

Numerous factors govern intestinal I/R injury development, including the apoptotic death of epithelial cells as well as the excessive production of pro-inflammatory factors, nitric oxide (NO), and reactive oxygen species (ROS) [4–6]. Apoptotic cell death, in particular, is thought to be a major regulator of intestinal I/R injury, as it can directly result in the compromise of the intestinal barrier [7]. Caspase proteins are essential effectors and mediators of apoptosis, which can be induced via both the intrinsic and the extrinsic pathways. The exact mechanisms responsible for intestinal epithelial apoptotic cell death in the context of I/R injury, however, remain to be defined [8]. As such, it is
essential that additional studies be conducted with the goal of better understanding this condition and thereby identifying novel drug biomarkers that may be targeted in order to effectively prevent or treat intestinal I/R injuries.

MicroRNAs (miRNAs) are non-coding RNAs that are capable of suppressing target mRNA translation or directly mediating target mRNA degradation [9–11]. Many miRNAs have been shown to play key roles in the context of intestinal I/R injury, including miR-29b, miR-378, and miR-351 [12–14]. We have previously identified miR-26b-5p as a miRNA that is differentially expressed in intestinal mucosal tissues in response to intestinal I/R [14]. Other recent work has suggested that miR-26b-5p dysregulation is also evident in the context of cerebral and myocardial I/R [15]. To date, however, no studies have demonstrated a functional link between miR-26b-5p expression and the incidence of I/R injury.

The serine/threonine kinase death-associated protein kinase 1 (DAPK1) is a calcium/calmodulin-regulated protein that has previously been shown to promote apoptotic cell death via both caspase-independent and -dependent mechanisms [16–18]. In the present study, we identified DAPK1 as a putative miR-26b-5p target using a microRNA database (TargetScan http://www.targetscan.org). Based on this predicted relationship, we conducted this analysis with the goal of exploring the functional relationship between miR-26b-5p, DAPK1, and the pathogenesis of intestinal I/R injury.

Materials and Methods

Murine Intestinal I/R Model

Male C57BL/6 J mice (8–10 weeks old) from the Experimental Animals Center at Southern Medical University were used for all animal studies described herein, which were approved by the animal ethics committee of NanFang Hospital based upon National Institutes of Health guidelines. Prior to surgery, animals were fasted for 12 h with free access to water. All mice were then anesthetized via the i.p. injection of 200 mg/kg ketamine and 10 mg/kg xylazine hydrochloride, after which intestinal I/R modeling was conducted as in prior studies [14]. Briefly, we initially exposed the small intestine by generating a ~1 cm ventral midline incision (~1 cm) in the abdomen. A microvessel clip was then used to occlude the superior mesenteric artery (SMA) for 1 h, after which the clip was removed and reperfusion was allowed to proceed for 1, 2, 4, or 12 h. Sham-operated control mice underwent the surgical procedures detailed above, but no occlusion of the SMA was conducted following its exposure. A subcutaneous saline solution injection (50 mL/kg) was administered to all animals after surgery to facilitate resuscitation. Intestinal segments were fixed in 10% neutral formaldehyde, paraffin-embedded for morphological analysis. The intestinal mucosa was washed with cold saline after being scraped off and preserved at –80 °C for detection [14].

Cell Culture and OGD/R Modeling

Mode-K intestinal epithelial cells from the Shanghai Institutes for Biological Sciences (Shanghai, China) were cultured using DMEM containing 10% FBS (Gibco, CA, USA) at 37 °C in a 5% CO2 and 21% O2 incubator (Thermo, CA, USA). OGD conditions were used to mimic intestinal I/R in vitro. This was accomplished by initially growing Mode-K cells to 80% confluence, transferring them to D-Hanks buffer, and culturing them in a 95% N2 and 5% CO2 incubator for 4 h at 37 °C (Thermo). After this OGD induction period, reoxygenation was simulated by restoring these cells to an oxygen-containing incubator for 0, 1, 4, 12, and 24 h as above [19]. Control cells were grown under normoxic conditions for equivalent periods of time.

Tissue Histology

After animals were euthanized, segments of the small intestine were fixed using 4% paraformaldehyde, embedded in paraffin, used to prepare 5 μm sections, and hematoxylin and eosin (H&E)-stained. Two pathologists blinded to study groups then independently evaluated these stained sections, with Chiu’s score which grades from 0 to 8 being used to evaluate injury severity as in prior studies [20]. Criteria of Chiu’s score are listed in Table S1. Cell Viability Analysis

MODE-K cells were plated in 96-well plates (1 × 104/well) overnight in sextuplicate, after which OGD/R experimentation was conducted as above. At appropriate time points, 10 μl of CCK8 solution was added to each well for 2 h at 37°C after which a POLARstar OPTIMA plate reader (Bio-Rad, CA, USA) was used to assess absorbance at 450 nm.

Flow Cytometry

Mode-K cell apoptosis following OGD/R was assessed via flow cytometry using a commercial kit [21] (KeyGEN, Nanjing, China). Briefly, cells were isolated at appropriate time points, washed with PBS, and stained using Annexin V-FITC/propidium iodide (PI) for 15 min, after which they were evaluated via flow cytometer within 1 h.
Western Blotting

Following total protein extraction from murine intestinal tissues and Mode-K cells, Western blotting was conducted as in prior studies. Briefly, we separated 30 μg of protein per sample via 6–20% SDS-PAGE, followed by transfer onto Immobilon-P membranes (Millipore, USA). Blots were blocked using 5% non-fat milk (Biological Technology, China) for 1 h, after which they were probed overnight with anti-DAPK1 (Abcam, MA, USA), anti-cleaved caspase-3 (C-caspase-3) (Abcam, MA, USA), anti-Bcl-2 (Santa Cruz Biotechnology, CA, USA), anti-Bax (Santa Cruz Biotechnology), or anti-GAPDH (Abcam) at 4 °C. Blots were next probed with HRP-linked secondary antibodies, and Pierce ECL Western Blotting Substrate (Thermo Scientific) was then used to visualize protein bands, with GAPDH being used to normalize band intensity values \( (n = 5) \).

qRT-PCR

Trizol (Takara, Japan) was used to extract RNA from Mode-K cells and intestinal samples, after which kits from Toyobo and Takara were used to prepare cDNAs for mRNA and miRNA samples, respectively. The expression of miR-26a-5p was evaluated via TaqMan miRNA assay kit (Applied Biosystems, USA) with U6 snRNA as an internal control, whereas SYBR Green Real-Time PCR Master Mix (ThermoFisher) was used for qRT-PCR. The relative expression level was analyzed using the \( 2^{-\Delta\Delta Ct} \) method.

TUNEL Staining

A TUNEL apoptosis assay kit (Solarbio, Beijing, China) was used based on provided directions to assess apoptosis \([21]\). A Nikon ECLIPSE microscope (200x) was employed to identify TUNEL-stained nuclei, with the apoptotic rate (%) being given by counting the numbers of positive cells in five randomly chosen areas of view for each sample.

Transfection

Genechem synthesized miR-26b-5p mimics (sense 5′-UUC AAGUAUUCAGGAUGGU-3′, antisense 5′-UUCAAG UAAUUCAGGAUGGU-3′), miR-26b-5p inhibitors (5′-ACCUAUCCUGAUACUUGA-3′), and appropriate negative controls (mimic-NC, sense 5′-UUUCCC GAACUGUGUCAGUTT-3′, antisense 5′-ACUGUGACAC GUUCGGAGAATT-3′ and inhibitor-NC, 5′-CAGUAC UUUUGUGAGUACAA-3′). These constructs were transfected into Mode-K cells plated in 6-well plates using Lipofectamine 3000 (Invitrogen, USA) based on provided directions \([22]\).

For in vivo overexpression and knockdown of miR-26b-5p, agomiR-26b-5p, antagomiR-26b-5p, and appropriate negative controls (agomir-NC and antagomiR-NC) were injected into mice via the tail vein (40 mg/kg, \( n = 8 \) per group) for three days. AgomiR-26b-5p and antagomiR-26b-5p were synthesized by RiboBio (Guangzhou, China).

Dual-Luciferase Reporter Assay

TargetScan (http://www.targetscan.org) was used as a means of identifying predicted sites for miR-26b-5p binding within the DAPK1 mRNA. DAPK1 cDNAs that contained either wild-type or mutated versions of this binding site (DAPK1-WT or -MUT) were synthesized and cloned into the pmirGLO dual-luciferase reporter vector (Genechem, China). Mode-K cells were plated in 24-well plates followed by transfection with appropriate mimic, inhibitor, or negative control constructs for 48 h. A dual-luciferase reporter assay system (Promega, WI, USA) was then used to assess luciferase activity in these cells, with Renilla luciferase serving as a normalization control \([23]\).

Statistical Analysis

Data are means ± SD from triplicate experiments. GraphPad Prism 6.01 (GraphPad Software, USA) was used for all statistical comparisons, with data being compared via Student’s t tests and one-way ANOVAs as appropriate. \( P < 0.05 \) was the significance threshold.

Results

I/R and OGD/R Conditions Are Associated with the Induction of Epithelial Damage

In our murine model of I/R injury, we observed apparent intestinal damage at a range of time points following a 1 h ischemia period and subsequent reperfusion. At 2 h post-reperfusion, there was clear evidence of inflammatory cell infiltration and edema within the mucosal villi, whereas injury severity decreased over time after this 2 h time point as indicated by H&E staining and Chiu’s scores (Fig. 1a, b). In line with these findings, we found that the in vitro OGD/R treatment of Mode-K cells was associated with their decreased viability, especially following 4 h of reoxygenation. (Fig. 1c) TUNEL staining also indicated an increase in the numbers of apoptotic cells in the I/R group in mice and the OGD/R group in Mode-K cells (Fig. 1d–g).
**MiR-26b-5p Overexpression Ameliorates I/R-Induced Intestinal Injury**

To further investigate the function of miR-26b-5p in the context of intestinal I/R injury in vivo, we overexpressed this miRNA in C57BL/6 mice via the tail vein injection of miR-26b-5p agomir and confirmed successful overexpression by qRT-PCR (Fig. 4a). The intestinal injury induced by I/R in these mice was significantly attenuated after miR-26b-5p agomir administration as indicated by H&E staining and Chiu's scores (Fig. 4e). Moreover, fewer apoptotic cells were observed in the intestinal mucosa from the agomir group mice compared to the negative control group (NC) after I/R (Fig. 4e). Consistent with this, pretreatment with miR-26b-5p agomir resulted in a significant reduction in apoptotic marker expression (Fig. 4g).

**MiR-26b-5p Targets DAPK1**

We next utilized the StarBase (http://starbase.sysu.edu.cn/mirMrna.php) and TargetScan (http://www.targetscan.org) databases to identify a predicted miR-26b-5p binding site within the DAPK1 3'-UTR. We additionally found that miR-26b-5p overexpression in Mode-K cells was associated with reduced DAPK1 expression, whereas the opposite was observed when this miRNA was knocked down (Fig. 5a). Our in vivo experiments showed similar results as obtained in vitro (Figure S1A and S1B). To confirm that miR-26b-5p targets DAPK1 directly, we transfected Mode-K cells with luciferase reporter constructs containing wild-type or mutated versions of this putative 3'-UTR miRNA binding sequence (DAPK1 3'-UTR-WT or DAPK1 3'-UTR-MUT) (Fig. 5b). We found that miR-26a-5p mimic transfection suppressed and miR-26a-5p inhibitor transfection increased the luciferase activity of WT but not the MUT DANK1 3'-UTR reporter constructs (Fig. 5c, d), suggesting that miR-26a-5p can directly bind to the DAPK1 3'-UTR within Mode-K cells. We next determined...
whether DAPK1 was responsible for the protective effects induced by miR-26b-5p overexpression. As shown in Fig. 6a, b, miR-26b-5p mimic improved cell viability and reduced apoptotic marker expression under OGD/R conditions, as described above. However, it did not confer such protection after the restoration of the expression of DAPK1, despite the upregulation of miR-26b-5p. Taken together, these data reveal that DAPK1 is required for the regulation of miR-26b-5p-mediated protection against OGD/R injury.
Discussion

Intestinal I/R injuries can arise following shock, trauma, intestinal infarction, and other serious conditions [24]. These injuries can, in turn, result in a breakdown of the intestinal barrier, thereby facilitating bacterial translocation, septicemia, and multi-organ failure leading to death [25]. Herein, we presented two major findings. First, we found that miR-26b-5p was downregulated in the intestinal mucosa during I/R and we confirmed the protective effect...
of miR-26b-5p against intestinal I/R. Second, we uncovered a novel mechanism by which miR-26b-5p inhibits cellular apoptosis through targeting DAPK1 during intestinal I/R.

I/R-induced epithelial cell apoptosis is one primary mechanism driving I/R injury [26]. Previous work has demonstrated clear roles for miRNAs in the regulation of I/R-associated epithelial cell apoptosis [27]. For example, Wei et al. observed the dysregulation of miR-467, miR-362, and miR-668 in a murine model of ischemic acute kidney injury (AKI), and they further found that increasing miR-668 expression was associated with reduced AKI owing to miR-668-mediated targeting of MTP18 and a consequent reduction in mitochondrial fragmentation and apoptotic cell death [28]. Previous work suggests that miR-26b is, along with miR-26a, a member of the miR-26 family of miRNAs [29]. There is clear evidence that miR-26b is dysregulated in a range of disease states, with reductions in circulating miR-26b levels having been observed in myocardial infarction patients, suggesting that this miRNA may protect against adverse cardiomyocyte hypertrophy [30]. Interestingly, Danielsson et al. found that miR-26b inhibited apoptotic cell death in the context of oral lichen planus [31], while other studies found that miR-26b instead promoted apoptotic death in the context of multiple myeloma and hepatocellular carcinoma [32, 33]. As such, the effect

**Fig. 4** miR-26b-5p inhibited intestinal I/R injury and cellular apoptosis in vivo. **a, b** miR-26b-5p expression levels after the administration of agomir, antagonir, and NC constructs. (C and D) H&E staining and histopathological scores (Chiu’s score) for mouse intestinal mucosa samples (n=8). C57BL/6 mice were injected with agomiR-26b-5p, antagoniR-26b-5p, or corresponding NCs (100 μl) via the tail vein (40 mg/kg body weight) for 3 consecutive days and then subjected to intestinal I/R (1 h of ischemia followed by reperfusion for 2 h). Scale bar = 100 μm. (E and F) Representative TUNEL staining and apoptotic index values for mice that underwent I/R (n=8). Scale bar = 100 μm. **g, h** The protein levels of C-caspase3 (cleaved-caspase3), Bcl2, and Bax in the intestinal mucosa of mice following I/R (n=3). All data are expressed as the mean ± SD. *P<0.05, **P<0.01 versus control group.

**Fig. 5** DAPK1 is a miR-26b-5p target gene. **a** The expression levels of DAPK1 protein after OGD/R injury in Mode-K cells transfected with miR-26b-5p mimic and miR-26b-5p inhibitor constructs (n=3). **b** A diagram of the interaction between miR-26b-5p and the 3′-UTR of DAPK1. **c, d** Relative luciferase expression associated with the DAPK1 3′-UTR in Mode-K cells. Mode-K cells were co-transfected with miR-26b-5p mimic, miR-26b-5p inhibitor, or NC constructs as well as DAPK1-WT or DAPK1-MUT reporters (n=5). All data are expressed as the mean ± SD. *P<0.05, versus the mimic-NC or inhibitor-NC groups.
of miR-26b-5p on apoptosis can vary in different disease states. To date there have been no prior studies of miR-26b in the context of intestinal I/R. As such, we evaluated the impact of this miRNA on epithelial cell apoptosis in the context of intestinal I/R injury. We determined that miR-26b-5p expression was reduced in murine intestinal epithelial cells in the context of I/R injury, and that overexpressing this miRNA in vitro or in vivo was sufficient to reduce OGD/R or intestinal I/R-induced apoptotic death, whereas miR-26b-5p inhibition exacerbated this apoptotic death. As such, our findings indicate that miR-26b-5p protects against intestinal I/R-induced apoptosis.

Given that miRNAs function at the post-transcriptional level to control target mRNA translation within cells via binding to specific 3′-UTR sequences [34], we utilized a predictive miRNA database (TargetScan) to identify miR-26b-5p targets. DAPK1, which is a positive regulator of apoptotic cell death, was found to be one such putative miR-26b-5p target gene. Activation of DAPK1 in the context of cerebral I/R has been shown to be a key step in the I/R-induced death of neurons. As such, our findings indicate that miR-26b-5p protects against intestinal I/R-induced apoptosis.

There are some limitations to the present study. First, downregulation of miR-26b-5p was found in the intestinal mucosa after intestinal I/R, but the detailed mechanisms underlying miR-26b-5p dysregulation were not explored. Second, future studies are needed to investigate whether other targets for miR-26b-5p contribute to the protective effect of miR-26b-5p in intestinal I/R, such as, TCF-4 [35], Gata4 [36], and smad1 [37] which have been reported to involve in cardiac hypertrophy, adipocytic differentiation, and cerebral I/R injury. Third, miR-26b-5p has been reported to be involved in other types of cell death such as autophagy, and as such, more mechanistic studies are needed to fully understand the mechanisms by which miR-26b-5p exert its protective role in the context of intestinal I/R injury.

In conclusion, our findings offer novel evidence suggesting that the miR-26b-5p/DAPK1 signaling pathway is an important regulator of intestinal I/R injury, providing new insights into the pathogenesis of such injuries and suggesting that components of this pathway may be viable therapeutic targets for the prevention or treatment thereof.

**Supplementary Information** The online version contains supplementary material available at [https://doi.org/10.1007/s10620-021-06975-7](https://doi.org/10.1007/s10620-021-06975-7).

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**Disclosure** The authors declare that they have no conflict of interest.
Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of NanFang Hospital.

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