Roles of miR-128 in the pathogenesis of constipation and the related mechanism

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Abstract

The aim of this study is to explore the relationship between miR-128 and interstitial cells of Cajal (ICCs) in the pathogenesis of constipation and the related mechanism. We observed that the expression of miR-128 and c-kit were significantly down-regulated in constipated rats compared with the control rats; moreover, transient knockdown of miR-128 induced significant increase in the apoptosis and marked decrease in the proliferation of ICCs; finally, transfection of miR-128 inhibitors also lead to increased expression of Bax and Caspase-3, as well as reduced expression of Bcl-2 in ICCs. Taken together, our results proved for the first time that miR-128 can regulate the proliferation and apoptosis of ICCs via Bcl-2/Bax/Caspase-3 signaling pathway, suggesting that miR-128 has the potential to become a novel therapeutic target for the treatment of constipation.

Keywords: miR-128, ICCs, constipation, proliferation, apoptosis

Introduction

Constipation is a common gastrointestinal disease. It is characterized by difficulties in defecation as well as hard and dry stool (1,2). Constipation affects about 2–28 % of the population in Western countries and approximately 11.6–14.3 % of the population in Asian, which seriously affect the quality of life of the patients (3). Unfortunately, the pathogenesis of constipation is still unclear, and there is no golden standard for treating constipation. As a result, to deeper explore the underlying mechanism of constipation and identify novel therapeutic target is in a great need.

In recent years, the studies of MicroRNAs (miRNAs) have become an area of focus. miRNAs are a group of non-coding small RNAs with the length of about 21–23 nucleotides. MiRNAs exert their function through binding to the 3′-untranslated region (UTR) of their target mRNAs, and consequentially inhibit the translation of their target genes. MiRNAs have been proved to participate in many different biologic processes e.g. cell proliferation, migration, apoptosis, differentiation, tumorgenesis (4-6). In the field of gastrointestinal diseases, for example irritable bowel syndrome the roles of miRNAs have also been discussed previously (7-10).

As a member of the miRNA family, the roles of miR-128 in different diseases have been investigated in many previous studies(5,11-13). In a very recent study, it has been observed that miR-128 was significantly down-regulated in colonic specimens of patients with constipation (3); however, the underlying mechanism still requires further investigation. In the present study, we will explore roles of miR-128 in the pathogenesis of constipation using rat constipation models; moreover, the effect of miR-128 on the proliferation and apoptosis of interstitial cells of Cajal (ICCs) will also be explored. Our study may provide theoretical basis for the management of constipation.

Material and methods

Establishing of the constipation animal model

A number of 20 male adult SD rats were purchased from the animal center of Nanjing Medical University (Nanjing, China). Rats were raised at 23℃ with 50% humidity under a strict 12/12 h of light and dark cycle, and provided with ad libitum and standard diet and water. Rats were randomly divided into two groups: non-constipation group (control, n=10), constipation group (constipation, n=10). Constipation model was established by subcutaneous injection of Loperamide (4 mg/kg) twice a day for continuous 3 days. Rats in the control group was injected with the same amount of saline. On day 4, rats in both two groups were sacrificed and the colons were collected and stored in liquid nitrogen until future analysis. This study has been proved by the Animal Ethics Committee of The First People's Hospital of Lianyungang.

Real-time quantitative PCR

Total RNA was isolated from the colonic specimens by TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The levels of expression of miR-128 was evaluated by Hairpin-it^{TM} miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China) on ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols. U6 (RNU6B; GenePharma) has been used for normalization. The relative expressions of Bcl-2, Bax and Caspase-3 in cells were detected by SYBR® Fast qPCR Mix (Takara, Dalian, China), GAPDH has been applied for normalization. RT-qPCR was conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The thermocycling profiles were: 95℃ for 30 sec, followed by 40 cycles of 95 ℃ for 5 sec and 60 ℃ for 30 sec.

Western blot analysis

Cells and tissue samples were lysed and the protein concentration was measured using BCA Protein Assay Kit (Beyotime, Shanghai, China). Then protein was separated by electrophoresis, and transferred to PVDF membranes; the membranes were blocked with 5% non-fat milk and incubated with primary antibodies (anti-rat

Bcl-2, anti-rat Bax, anti-rat Caspase-3 and anti-rat GAPDH, all purchased from Santa Cruz, CA, USA) at 4℃ overnight; in day 2, the membranes were incubated with the secondary antibodies (Santa Cruz, CA, USA), and then incubated with enhanced chemiluminescent reagent (Beyotime, Shanghai, China). Finally, the signals were visulized using ChemiDoc™XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

Cell culture

Murine interstitial cells of Cajal were purchased from the Biofavor Biotech (Wuhan, China). Cells were cultured in muscle growth medium (SMGM) (Clonetics, San Diego, CA, United States) supplemented with 5 ng/mL of murine stem cell factor (SCF) (Sigma-Aldrich, St. Louis, MO, United States) and 100 U penicillin/mL, 100 mg streptomycin/mL, in a humidified incubator at 37 ℃ with 5% CO_2.

Cell transfection

The miR-128 inhibitors and miR-128 inhibitors negative control (NC) oligonucleotides were purchased from GenePharma (Shanghai, China). ICCs cells were seeded onto six-well plates with the density of 100,000 cells/well, and transfection of miR-128 inhibitors or NC using Lipofectamine RNAi Max (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were harvested 48 hours after transfection and stored at -20 ℃ for future analysis.

Cell proliferation analysis

MTT assay was performed at 48h after transfection to determine the cell viability using MTT proliferation assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instruction.

Cell apoptosis analysis

For the apoptosis analysis, cells were stained with PI/Annexin V-FITC apoptosis detection kit(BD Biosciences, San Jose, California, USA), and the apoptosis rate was analyzed on BD FACSVerse flow cytometer (BD Biosciences, San Jose, California, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data were

presented as the means ± standard deviation, and the two-independent sample T-test was performed to draw a comparison between groups. Kaplan-Meier analysis was performed to compare the OS curves in the different groups, and $P<0.05$ indicated statistically significant difference.

Results

Decreased expression of miR-128 and c-kit in colonic specimens of constipated rats

First of all, we established rat constipation models and compared the expression miR-128 in the colonic samples of constipated rats and normal rats. As shown in Fig.1A, the expression of miR-128 was significantly decreased in colonic samples of constipated rats compared with normal rats ($p<0.01$); moreover, results of WB indicated that the expression of c-kit, which is a biomarker of ICCs, was also significantly decreased in constipated rats (Fig.1B and C).

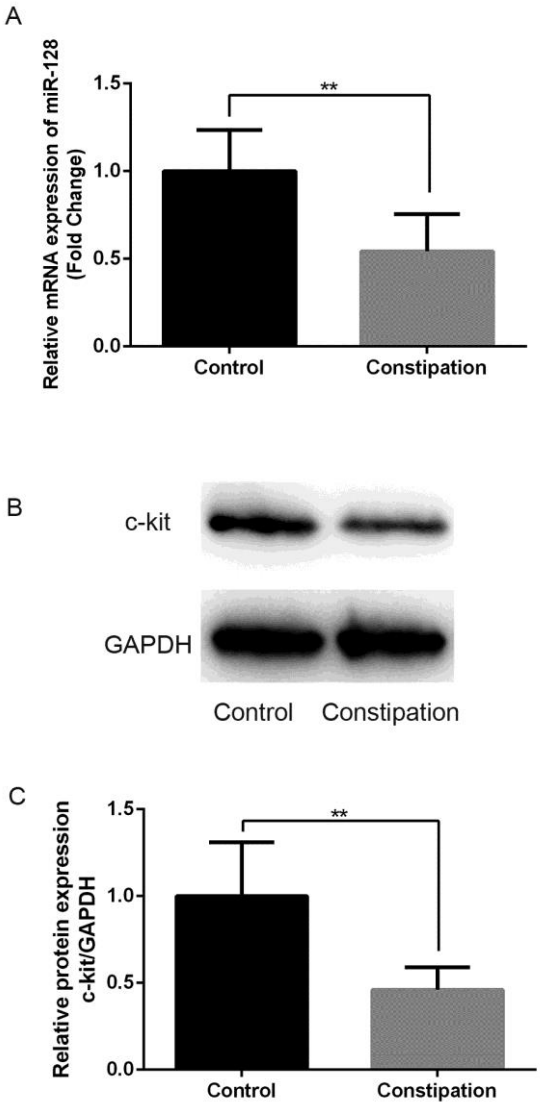

Figure 1. Comparison of the expressions of miR-128 and c-kit in colonic tissue sample of constipated rats and normal rats. (A) Relative mRNA expressions of miR-128 in colonic tissue sample of constipated rats and normal rats by qRT-PCR methods; (B) Relative protein expressions of c-kit in colonic tissue sample of constipated rats and normal rats by WB method. (C) Quantified results of (B); **P<0.01 vs. the normal group. miR-128, microRNA-128.

Effect of miR-128 on the proliferation and apoptosis of ICCs in vitro

Next, we further explored the effect of miR-128 on the proliferation and apoptosis of ICCs *in vitro*. ICCs were transfected with either miR-128 inhibitors or NC, and the effect of miR-128 on the proliferation and apoptosis of ICCs were examined using MTT and flow cytometry methods. As shown in Fig. 2A, transfection

of miR-128 inhibitors induced significant decrease cell proliferation at different time point; while on the other hand, transfection of miR-128 inhibitors also induced significant increase in the apoptosis of ICCs *in vitro* (Fig.2B).

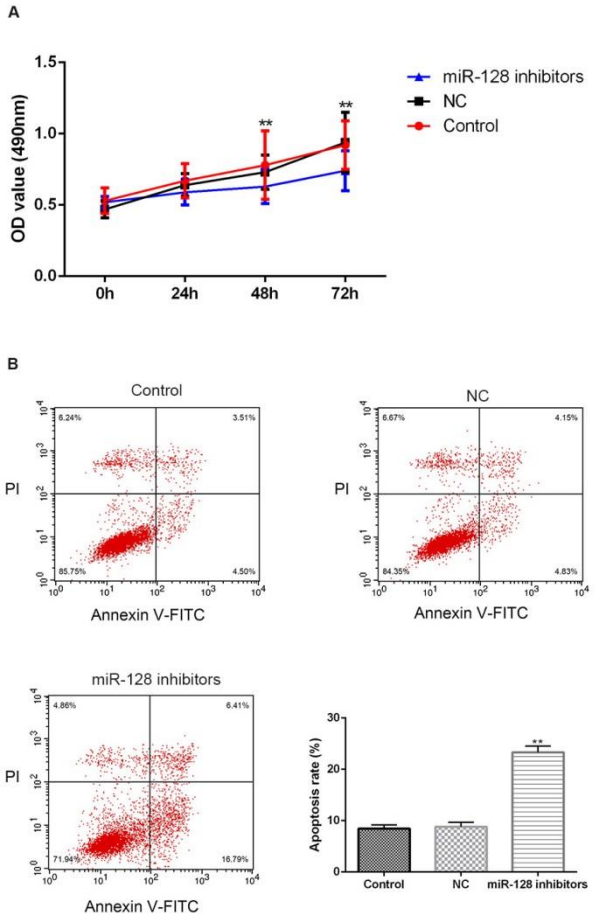

Figure 2. Effect of miR-128 on the proliferation and apoptosis of ICCs in vitro. (A) Effect of miR-128 on the proliferation of ICCs in vitro; (B) Effect of miR-128 on the apoptosis of ICCs in vitro. **P<0.01 vs. the control group. miR-128, microRNA-128; Control, un-transfected cells; NC, miR-128 inhibitors negative control transfected cells; miR-128 inhibitors, miR-128 inhibitors transfected cells.

miR-128 may regulate the proliferation and apoptosis of ICCs cells via regulating the expressions of Bcl-2, Bax and Caspase-3

Finally, we investigated the underlying molecule mechanisms of the anti-apoptotic effects of miR-128 on ICCs. The expressions of pro-apoptotic factors Bax and Caspase-3 as well as anti-apoptotic factor Bcl-2 in cells of different

treatments were examined. It was observed that compared with the control group, the expressions of both Bax and Caspase-3 were significantly increased, while the expression of Bcl-2 was markedly decreased in the miR-128 inhibitors transfected ICCs on both mRNA and protein level (Fig.3).

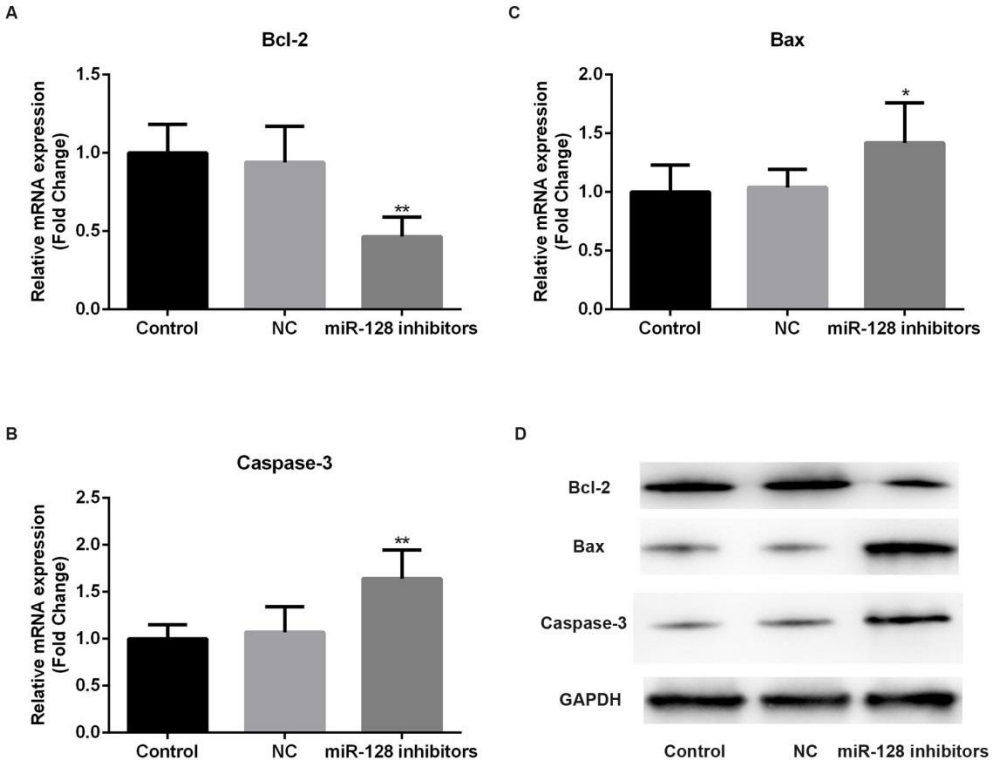

Figure 3. Effect of miR-128 on the expressions of Bcl-2, Bax, Caspase-3 in ICCs *in vitro*. (A) Relative mRNA expressions of Bcl-2 in ICCs *in vitro*; (B) Relative mRNA expressions of Bax in ICCs *in vitro*; (C) Relative mRNA expressions of Caspase-3 in ICCs in vitro; (D) Relative protein expression of of Bcl-2, Bax, Caspase-3 in ICCs *in vitro*.

Discussion

The roles of miRNAs in the pathogenesis of gastrointestinal diseases have been discussed in many previous studies. Hou *et al.* proved that miR-144 can promote the intestinal hyperpermeability and impair the protective effects of the epithelial barrier via regulating the expression of OCLN and ZO1, suggesting that miR-144 is a potential therapeutic target for the treatment of irritable bowel syndrome with diarrhea (IBS-D) ; Zhou et al observed that miR-29 can reduce the expression of CLDN1 and

NKRF and increase intestinal permeability, indicating that blockage of miR-29 might be a novel methods to recover that intestinal permeability of patients with IBS-D (14). In a recent study, Liu et al proved that miR-128 was down-regulated in constipation, and the levels of miR-128 were negatively correlated with the number of the macrophages number and microRNA-128 in colonic specimens of patients with constipation (3). In the present study, we further explored the roles of miR-128 in constipation by establishing the rat constipation models. It has been observed that the expressions of miR-128 was significantly decreased in the colonic specimens of constipated rats compared with the control group, which was consistent with the results of Liu et al, suggesting that miR-128 may be involved in the pathogenesis of constipation.

Results of previous studies indicated that decrease in the number of colonic interstitial cells of Cajal (ICCs) may contribute to the progress of constipation (15-17). ICCs represent a group of interstitial cells that can control the motility of the gastrointestinal tract. ICCs were able to induce electrical slow waves that can control the contractile activity of the smooth muscle cells. In recent years, studies towards the mechanism of constipation demonstrated that the decline of numbers of gastrointestinal ICCs may be a key reason to induce constipation (18-20). Thus, to further explore whether decreased expression of miR-128 was correlated with decreased number of ICCs in constipation, a series of *in vivo* and *in vitro* analysis have been performed. First, results of WB analysis indicated that the expression of c-kit, a biomarker that can represent the number of ICCs, was significantly down-regulated in constipated rats, which was consistent with the results of previous findings; moreover, transfection of miR-128 inhibitors has led to significant decrease in the proliferation and increase in the apoptosis of ICCs, suggesting that miR-128 can regulate the proliferation and apoptosis of ICCs *in vitro*.

The Bcl-2 family of intracellular proteins is the key regulator of caspase activation, and its opposing factions of anti- and pro-apoptotic members arbitrate the cell apoptosis and survival decision. Bcl-2 is an oncogenic protein that acts by

inhibiting programmed cell death (21,22). Bax, a pro-apoptotic member of the Bcl-2 family, will move into the mitochondrial when cells in response to a wide variety of stimuli to cell apoptosis, which can urge Smac/DIABLO and Cytochrome c translocate from mitochondrial to cytoplasm (23). Cytochrome c will combine with Procaspase-9, ATP/dATP, Apaf-1. Subsequently, Caspase-9, Caspase-3 and other Caspases will be activated promoting cell apoptosis (23,24). To further validate the underlying mechanism of miR-128 on the proliferation and apoptosis of ICCs, the expressions of Bax, Caspase-3 and Bcl-2 detected by PT-PCR and western blot method. We proved that transient knockdown of miR-128 induced significant increase in the expression of Bax and Caspase-3 and marked decrease in the expression of Bcl-2 in ICCs. Taken together, these results indicated that miR-128 can regulate the proliferation and apoptosis of ICCs through regulating the Bcl-2/Bax/Caspase-3 signaling pathway.

In conclusion, our data proved for the first time that miR-128 was down-regulated in constipation, and miR-128 may affect the proliferation and apoptosis of ICCs via Bcl-2/Bax/Caspase-3 signaling pathway. Our results have provided novel evidence for the potential application of miR-128 as a novel therapeutic target for the management of constipation.

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