Role of mast cell-miR-490-5p in irritable bowel syndrome

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**Abstract**

**AIM**
The functional role of miR-490-5p in mast cell proliferation and apoptosis, and in the mast cell tryptase/PAR-2 signal pathway.

**METHODS**
The 3rd generation of lentivirus vector systems containing enhanced green fluorescent protein (EGFP) (Ruisai Inc., Shanghai, China), which acts as a reporter gene was used to construct the mmu-miR-490-5p lentivirus expression vector pEGFP-antagomiR-490-5p, and the lentivirus vector pEGFP-negative was used as a negative control. The stably transfected mast cell line p815 was then constructed. GFP positive cells were successfully transfected cells. We determined the expression of miR-490-5p in p815 mast cells before and after transfection using quantitative real-time PCR (qRT-PCR). In addition, after transduction with the lentivirus vectors, the role of miR-490-5p in mast cell proliferation and apoptosis was investigated using the CCK-8 assay and flow cytometry, respectively. The mRNA levels of tryptase and PAR-2 were detected by qRT-PCR and the protein levels were detected by Western blot.

**RESULTS**
The inhibition of miR-490-5p expression promoted apoptosis and inhibited proliferation of p815 mast cells. The mRNA levels of tryptase and PAR-2 were significantly increased after transfection compared...
with the control group, tryptase ($P = 0.721$, normal vs null; $P = 0.001$, siRNA vs normal; $P = 0.002$, siRNA vs null) and PAR-2 ($P = 0.027$, siRNA vs null; $P = 0.353$, normal vs null; $P = 0.105$, siRNA vs normal). The protein levels of tryptase and PAR2 were slightly higher in the siRNA group than those in the control group, but the difference was not statistically significant ($P > 0.05$).

CONCLUSION

miR-490-5p plays a vital role in the pathogenesis of irritable bowel syndrome by affecting mast cell proliferation and apoptosis; with down-regulation of miR-490-5p, the mRNA level of mast cell tryptase and PAR-2 increased, and the protein level increased, but the difference was not statistically significant.

Key words: miR-490-5p; Mast cell tryptase; PAR-2; Irritable bowel syndrome

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INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal (GI) disorders. The cause of IBS is unknown and seems to be multifactorial[1-4]. Several mechanisms including visceral hypersensitivity, motility disorder, infection, and psychiatric factors, have been suggested as possible etiologic links to the development of IBS[5,6]. In addition, there is increasing evidence of a genetic contribution in IBS such as gene polymorphism[7-9] and dysregulated microRNA (miRNA) expression[10-14]. Mast cells and mast cell degranulation play important roles in the pathophysiology of irritable bowel syndrome. miRNA is a class of important endogenous single-stranded non-coding RNA, and plays an essential regulatory role in complex biological systems without protein translation. In the present study, we aimed to determine the role of miR-490-5p in regulating mast cell proliferation and apoptosis, the expression of mast cell tryptase and PAR-2, and thus predict the role of miR-490-5p in the pathogenesis of irritable bowel syndrome.

Core tip: Mast cells and mast cell degranulation play important roles in the pathophysiology of irritable bowel syndrome. miRNA is a class of important endogenous single-stranded non-coding RNA, and plays an essential regulatory role in complex biological systems without protein translation. In the present study, we aimed to determine the role of miR-490-5p in regulating mast cell proliferation and apoptosis, the expression of mast cell tryptase and PAR-2, and thus predict the role of miR-490-5p in the pathogenesis of irritable bowel syndrome.

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MATERIALS AND METHODS

Cell line

The P815 mast cell line was purchased from Shanghai Cell Biochemical Institute, China Academy of Science (Shanghai, China). This cell line is a suitable transfection host. P815 cells phagocytose latex beads, but not zymosan or BCG. They do not function in antibody-dependent cell-mediated cytotoxicity. Growth of these cells is not inhibited by dextran sulfate,
lipopolysaccharide (LPS) or tuberculin purified protein derivative (PPD). The cells were also found to be negative for the ectromelia virus (mousepox).

Reagents
Mast cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin, penicillin/streptomycin combination, miR-490-5p-siRNA vector, siRNA negative lentiviral vector; blasticidin, RIPA lysate [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100 (v/v)], protease inhibitor cocktail, 1 mmol/L sodium vanadate and 10 mmol/L NaF. DNA marker, agarose, EB substitute, Trizol and cDNA reverse transcription kit were obtained from Takara. Polymerase chain reaction (PCR) primers were synthesized by Sangon Biotechnology (Shanghai, China). SuperReal PreMix Plus was purchased from Tiangen Biotechnology (Beijing, China). Mouse anti-GAPDH monoclonal antibody for western blot was obtained from Cell Signaling Technology (Danvers, MA, United States). Anti-PAR-2 monoclonal antibody for western blot was obtained from Abcam (Shanghai, China). Antitryptase clonal antibody for western blot was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Screening for target miRNA
Based on the results of a previous high-throughput microarray and bioinformatics analysis, TargetScan analysis (http://www.targetscan.org) was used to predict the target gene of the miRNA. A GO enrichment analysis and a KEGG pathway analysis of the target gene were subsequently performed. Based on the results of the bioinformatics analysis, we searched miRDB and miRBase to query the specific features of target genes of interest. Our selection criteria for the target miRNA were as follows: (1) it is abnormally expressed in IBS-D patients\(^\text{[22]}\); (2) the biological function of the target gene should be related to mast cell degranulation, proliferation and apoptosis or the signal transduction pathway or visceral sensitivity or neurotransmitter-release associated protein or intracellular material transportation-associated protein, as the products of these target genes may correlate highly with the pathogenesis of IBS\(^\text{[25]}\); and (3) it exists both in human and mouse mast cells. Finally, we choose miR-490-5p as our target miRNA.

miR-490-5p RNA interference in mast cells
The 3rd generation of a lentivirus vector system containing an expression vector and 3 packaging auxiliary plasmids was used to construct the miR-490-5p recombinant silencing vector. The expression vector contained the basic components of HIV 5 ‘LTR and 3’ LTR as well as other auxiliary components, CMV promoter, blasticidin markers and enhanced green fluorescent protein (EGFP). Packaging auxiliary plasmid contained pGag-Pol, PreV and pVSVG. According to the mouse mmu-miR-490-5p (MIMAT0017261) gene sequence in the miBase database (5’-CAAUGGAUCCAGGUGGUGG-3’), a pair of oligonucleotide chains containing the miR-490-5p sequence and the reverse complementary miR-145 sequence, which can form an shRNA precursor sequence containing a stem-loop structure after annealing, were designed. (Top strand: 5’-TCTGACCCACCTGGAGATCCATGGGTTTTGGCCACTGACTGACCCATGGATCCCATGGGTGGGTTGTCAGTCACTGAGCCAAAACCATCGATCTCCAGGTGTCGC-3’). The shRNA was then connected with the linearized miRNA vector, the product was used to transform Escherichia coli DH5\(\alpha\), and the recombinant plasmids were extracted and analyzed by sequencing, and named pcDNA6.2-EGFP-mmu-490-5p, which was then used as a template to amplify the following primers: (Lenti-Asc1-F: 5’-TACTGGCGCGCCGCAACCTGTGATGAGAAGAAAGGAGCGAAGGGGAGGA-3’; Lenti-Pme1-R: 5’-ACTAGTTTAACTGCGGCCAGATTTCGCGCGCGTAAGAAAATGTCttgggtgctc-3’). The plLV-shRNA lentiviral expression vector was generated via T4 DNA ligase, and an unrelated negative control sequence was established in the same manner. The above protocol was completed by Shanghai RS Biotechnology Co., Ltd (Shanghai, China).

HEK-293T cells (5 \times 10^4; ATCC, Maryland, United States) were seeded in a 6-well cell culture plate in DMEM and incubated at 37 °C with 5% CO\(_2\) for 24 h. When the cells reached 50%-70% confluence, plLV-shRNA plasmids and auxiliary plasmids were co-transfected into them using Lipo3000. After 48 h of transfection, the cell supernatant was collected for titer determination. Gradient dilution of the supernatant was performed using PBS at ratios of 10\(^{-1}\)-10\(^{-6}\). Three wells were used for each gradient, and 50 \(\mu\)L of lentiviral diluent was placed in each well for infection. After 48 h of infection, we recorded the number of infected fluorescent cells at the dilution gradient where the ratio of green fluorescent protein (GFP)\(^*\) cells was approximately 20%, and mean values were calculated. Lentiviral titers were calculated according to the following formula (BT = TU/mL): TU/mL = (P × N/100 × V) × 1/DF (P = number of GFP\(^*\) cells, N = 10\(^6\), V = volume of lentiviral diluent = 50 \(\mu\)L, DF = dilution factor).

Construction of the stably transfected cell line
Growing mast cells were seeded in 24-well plates (30000 cells per well). After adherence and the cell density reached 50%, the cells were transduced with recombinant lentivirus vectors at a multiplicity of infection (MOI) of 60. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO\(_2\) for 24 h, and the medium was then changed. After 48 h of
transfection, the transfection efficiency was assessed by inverted fluorescence microscopy. Thereafter, positively transfected cells were selected by adding blasticidin to the medium at the minimal effective dose of 6 μg/mL (the minimal effective dose was defined as the dose at which most of the cells died within 7 d). The efficiency of GFP expression was detected by flow cytometry. When the expression efficiency of GFP was more than 85%, the dose of blasticidin was replaced by half of the minimal effective dose.

Detection of the miR-490-5p level after silencing
Total RNA was extracted both from normal cells and stably transfected cells using Trizol (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Complementary DNA (cDNA) templates were synthesized using the PrimeScript™ RT reagent Kit (Takara, Dalian, China) after the concentration and purity of total RNA were measured by NanoDrop. The expression level of miR-490-5p was measured by qRT-PCR. The primers used in RT-PCR are listed in Table 1.

Cell proliferation assay
The cell counting kit-8 (CCK-8) assay was used to examine cell proliferation. A standard curve was constructed, and the cells were then washed three times with PBS, resuspended in PBS, counted, and diluted 1:2 geometrically. The cells were seeded in 96-well plates at different final concentrations (3 × 10^4, 6 × 10^4, 12 × 10^4, 24 × 10^4, 48 × 10^4 per mL) in assay medium and incubated at 37 °C in a 5% CO₂ incubator for approximately 2-4 h. Next, 10 μL of CCK-8 was added to each well, and the cells were incubated at 37 °C. At different time points (1 h, 2 h, 3 h, 4 h), the absorbance at 490 nm was read on a microplate reader.

Table 1 Primers for mmu-miR-490-5p and U6

| Gene          | Forward/reverse primer | PCR product, bp | Gene bank No. |
|---------------|------------------------|-----------------|---------------|
| mmu-miR-490-5p| Forward: TGGCGGCCATGGATCTCCAG | 145             | NM_0012042741 |
|               | RT: GTCGATATCGAGCCAGGGTCCAGAGGTTACCTG | 139             | NM_010781.3   |
|               | Reverse: ATCCAGTCAGGTCGCAGG | 106             | NM_007974.4   |
| U6            | Forward: CGCTTCAGAATTTGGGTTTTCTTCTTCGTTCG | 106             | NM_0012042741 |
|               | Reverse: GCTTCGCCAGCAGTTACATATACTAAAAT |                |               |

Annexin V-PE/7-AAD double-staining flow cytometry for the detection of apoptosis
Cell apoptosis was detected by flow cytometry (FCM) and analyzed by CellQuest software (Becton Dickinson, Bedford, MA, United States). The transfected and normal cells were collected, washed three times with cool PBS, and then resuspended in PBS. 1 × 10^6 cells were then processed for labeling with Annexin V/7AAD according to the PE Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, United States).

Real-time polymerase chain reaction
Real-time PCR was used to detect the mRNA level of tryptase and PAR-2 in p815 mast cells after transfection. Total RNA was extracted from both normal cells and stably transfected cells using Trizol. Total RNA was transcribed using the reverse transcription kit after measuring the concentration of total RNA with Nano drop. Total RNA was quantified to 1 μg. Information regarding the primers of tryptase, PAR-2 and the endogenous control U6 are listed in Table 2. The PCR samples were prepared in a volume of 20 μL, containing 2 μL of cDNA diluted 1:5 with PCR grade water, 10.4 μL of SYBR Green Supermix, and 300 nmol/L each of the forward and reverse primers. The PCR conditions consisted of preliminary denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 32 s. Melting curve analysis was performed to confirm the specificity and the integrity of the PCR products by the presence of a single peak. Products were subjected to agarose
gel electrophoresis. Expression levels of mRNA were quantified by calculating threshold cycle values compared with the U6 endogenous control using 2-ΔΔCt.

**Western blot**

Total protein was extracted from p815 mast cells and lysed in RIPA lysate [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100 (v/v), 1 x protease inhibitor cocktail, 1 mmol/L sodium vanadate, and 10 mmol/L NaF]. Equal amounts of total protein were separated by SDS-PAGE with a 12% resolving layer and a 4% stacking layer and the proteins were transferred to PVDF membranes at 300 mA for 1.5 h. After blocking in 5% nonfat milk diluted with TBS-T for 1 h at room temperature with shaking, the membranes were incubated with primary antibodies (working dilution of tryptase 1:300; PAR-2 1:400) with gentle shaking overnight at 4 °C, washed with TBS-T (3 times for 5 min each time) and then incubated with species-appropriate secondary antibodies for 2 h at 37 °C. After washing in TBS-T (3 times for 5 min each time), the membranes were incubated using the ECL Developer Kit in a dark room. The efficiency of protein loading and transfer was assessed by reprobing the membranes with an anti-GAPDH antibody. The density of each band was compared with the corresponding control band.

**Statistical analysis**

The experimental data are represented by the mean ± SD. SPSS 20.0 was used for data analysis. One-way analysis of variance was used for analysis. Spearman analysis was used for correlation analysis. A P value less than 0.05 was considered statistically significant.

**RESULTS**

**Transfection efficiency and miR-490-5p level after silencing**

The transfection rate was measured by flow cytometry and the results showed that the efficiency was more than 85% in both the siRNA group and null group. We performed qRT-PCR to confirm the expression of miR-490-5p in p815 mast cells after silencing. As shown in Figure 1, the level of miR-490-5p in the siRNA group was significantly decreased compared with the empty vector group (P < 0.05).

**Proliferation and apoptosis**

From the results of the standard curve, we found that when the number of cells varied between 3000 and 48000, there was a linear correlation between the number of cells and OD value. Therefore, CCK-8 was suitable for determining the level of proliferation of mast cells. In the proliferation test, the results showed that inhibition of miR-490-5p expression significantly reduced cell viability compared with the control group (P < 0.05). In the apoptosis test, the rate of apoptosis was significantly higher following the inhibition of miR-490-5p expression than in the control group (P < 0.05), as shown in Figure 2.

**mRNA levels of tryptase and PAR-2**

The mRNA levels of tryptase (P = 0.721 normal vs null, P = 0.001 siRNA vs normal, P = 0.002 siRNA vs null) and PAR-2 (P = 0.027 siRNA vs null, P = 0.353 normal vs null, P = 0.105 siRNA vs normal) were higher in the siRNA group than in the normal and null groups. In addition, the difference between the groups was statistically significant, and all the real-time PCR products from the three groups migrated as expected (Figures 3 and 4).

**Protein levels of tryptase and PAR-2**

The expression results of the siRNA group were compared with normal and null controls. The density of each band was compared with the corresponding control band and normalized to the GAPDH gene. Elevated expression of tryptase and PAR-2 was found in the siRNA group compared with both the normal and null group, but there were no significant differences between the siRNA group and the controls (P > 0.05) (Figure 5).

**DISCUSSION**

In this study, we found that miR-490-5p promotes mast cell proliferation and resistance to apoptosis. Inhibition of miR-490-5p expression significantly increased the mRNA level of mast cell tryptase and PAR-2, but the protein level of tryptase and PAR-2 increased slightly, and a statistically significant difference between the siRNA and the control groups was not observed.

miRNAs are an abundant class of 20-22-nt non-coding single-stranded RNA and play significant roles in various physiological and pathological processes[24,25]. Many miRNAs have been shown to be associated with IBS. We previously detected an elevation of miR-490-5p in IBS-D patients. miR-490-5p is a member of the miR-490 family. The expression and functional role of miR-490-5p has also been reported in other diseases. It is involved in cell proliferation, apoptosis and the regulation of signaling pathways in different ways. In the study by Shiqi Li, miR-490-5p was found to be a novel tumor suppressor of bladder cancer cell proliferation by targeting c-Fos[26,27]. In renal cancer, miR-490-5p was confirmed to directly bind to the 3’UTR of PIK3CA mRNA and reduce the expression of PIK3CA at both the mRNA and protein level, which further inhibited the PIK3/Akt signaling pathway[28]. While, the role of miRNA-490-5p in the development and progression of IBS has not been
reported. Its target genes include \textit{CABP5}: encoding calcium binding protein 5; \textit{CAMK1D}: encoding calcium/calmodulin-dependent protein kinase ID; \textit{CASP3}: encoding caspase 3, apoptosis-related cysteine peptidase; \textit{TNFSF18}: encoding tumor necrosis factor (ligand) superfamily, member 18; \textit{BOP1}: encoding proliferation-associated protein. Of these, both calcium and calmodulin-dependent protein kinase were highly correlated with mast cell degranulation\cite{17,18}. Caspase, apoptosis-related cysteine peptidase and proliferation associated protein have been reported to be involved in cell proliferation and apoptosis. In the present study, we found that inhibition of miR-490-5p significantly reduced mast cell proliferation and promoted apoptosis. Furthermore, the level of miR-490-5p was highly correlated with the mRNA level of mast cell tryptase and PAR-2. However, why the increase in PAR-2 and tryptase mRNA did not lead to

Figure 1 Transduction efficiency and the expression of miR-490-5p after transduction. A: Renderings of mast cells after lentivirus transfection, the transduction rate in the siRNA group was almost 96%; the transduction rate in the null group was almost 86%; B: The relative expression of miR-490-5p after transduction, which was significantly decreased in the siRNA group compared with the null group; C: Mast cell transfection efficiency detected by flow cytometry, the efficiency in each group was more than 85%.
a significant elevation in PAR-protein and tryptase-protein remains unknown. Possible reasons are as follows: Firstly, some other factors may be involved in the post-transcriptional regulation or the protein translation process. Secondly, the role of miR-490-5p is mainly up-regulating the function of tryptase/PAR-2 rather than affecting their expression. Thirdly, mast cells are merely normal cells in the resting state, and lack activating factors; however, in IBS-D patients, mast cells are activated. This is the first time that the role of miR-490-5p in IBS has been demonstrated, and information on the biological role of miRNA in IBS...
which could be used for reference was limited. Previous studies have reported that the number of intestinal mucosal mast cells increased in IBS patients and the mast cell tryptase/PAR-2 signal pathway played an important role in the pathogenesis of IBS. Thus, any factors associated with the above process are important. In summary, although problems occurred in our study, which require further investigation, we can conclude that mast cell miR-490-5p may participate in the occurrence and development of IBS by regulating the proliferation and apoptosis of mast cells; however, its effect on the mast cell tryptase/PAR-2 signal pathway is complex. The limitations in this study were that miR-490-5p was highly correlated with mast cell proliferation and apoptosis, but the specific target gene through which miR-490-5p plays a crucial role in IBS remains unknown. Furthermore, the elevation of tryptase and PAR-2 mRNA did not lead to an increase in protein level, which should be explored. The next step is to screen out the target gene of miR-490-5p involved in the regulation of the above biological process using bioinformatics analysis, selectively knockout the target gene, and then determine the level of mast cell tryptase and PAR-2. This may provide a new target for the treatment of IBS.

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our project.

**COMMENTS**

**Background**

Previous studies have reported that mast cells and mast cell degranulation are involved in the pathogenesis of irritable bowel syndrome (IBS). Tryptase is an important component of mast cell degranulation which leads to visceral hypersensitivity in IBS patients by activation of PAR-2. The authors previously showed that IBS patients demonstrated dysregulation of miR-490-5p whose target gene was highly correlated with visceral sensitivity or neurotransmitter release associated protein, or intracellular material transportation associated protein. Thus, we predicted that miR-490-5p may be indirectly involved in the regulation of the mast cell-tryptase-PAR-2 signaling pathway.

**Research frontiers**

A growing number of studies have reported that different miRNAs are involved in the regulation of proliferation and differentiation of digestive tract epithelial cells and smooth muscle cells. Recent research reported that miRNA plays an important role in the differentiation, proliferation and functional regulation of bone marrow mast cells in the inflammatory response. Another study reported that miR-29a was highly correlated with increased intestinal permeability in IBS-D patients. Thus, the authors can conclude that the regulation of miRNA may be closely related to the pathogenesis of IBS.

**Innovations and breakthroughs**

The authors proved, for the first time, that miR-490-5p may be directly or indirectly involved in mast cell proliferation and apoptosis and the mast cell/tryptase/PAR-2 signaling pathway may play an important role in IBS-D.

**Applications**

miRNA-490-5p may be a new biomarker for the diagnosis of IBS and a new target for therapy.

**Terminology**

RNA interference (RNAi), a fundamental biological process by which cells regulate gene expression, acts through complementary base-pairing with target mRNA and retrieves cellular RNases which in turn degrade mRNA transcripts. RNAi is now routinely used to evaluate gene function both at cellular and organismic levels. It is a versatile tool for not only assessing gene function but also investigating potential new drug targets.

**Peer-review**

The author supported that miR-490-5p may directly or indirectly involved in the pathogenesis of IBS. It was the first research focusing on the miR-490-5p and IBS and it really could improve our understanding of IBS to some extent.

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