MiR-31 Regulates Rho-Associated Kinase-Myosin Light Chain (ROCK-MLC) Pathway and Inhibits Gastric Cancer Invasion: Roles of RhoA

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BC 1 Zhuo Chen*
DF 2 Shengnan Liu*
CD 1 Yuan Xia
ADG 2 Kejian Wu

1 Xuzhou Medical College, Xuzhou, Jiangsu, P.R. China
2 Department of Gastroenterology, The Affiliated Hospital of Xuzhou Medical College, Xuzhou, Jiangsu, P.R. China

Corresponding Author:
Source of support:

* Zhuo Chen and Shengnan Liu contributed equally to this work
Kejian Wu, e-mail: kejian_wu@163.com
Departmental sources

Background: This study evaluated how the expression of miR-31 can be used to detect gastric cancer (GC) to help illuminate the role of miR-31 and RhoA in GC cells.

Material/Methods: We carried out our experiments using tissue specimens from 70 GC patients. The relative expression of miR-31 and RhoA mRNA in tissues and cells was detected by RT-PCR. The expression level of RhoA protein was detected by immunohistochemistry. GC cell line BGC-823 was transfected with six groups of vectors: blank group, NC (negative control) group, miR-31 mimics group, miR-31 mimics + RhoA group, miR-31 mimics + ROCK group, and miR-31 mimics + MLCK agonist group. AGS cells were also transfected with six groups of vectors: blank group, NC group, miR-31 inhibitor group, miR-31 inhibitor + RhoA siRNA group, miR-31 inhibitor + ROCK siRNA group, and miR-31 inhibitor + MLCK inhibitor group. Transwell assay was performed to detect the invasion and migration of cells. The protein expression in different transfected groups was detected using Western blotting.

Results: GC tissues exhibited significantly lower levels of miR-31 expression compared to pericarcinous tissues ($p<0.01$). Moreover, a significantly higher expression of RhoA in GC tissues was observed ($p<0.05$). MiR-31 inhibited RhoA expression by binding to 3'UTR of mRNA, whereas miR-31 mimics significantly decreased the number of invaded and migrated cells ($p<0.05$). The activation of RhoA, ROCK, and phosphorylation of MLC remarkably exacerbate the invasion and migration ability of GC cells ($p<0.05$).

Conclusions: We found miR-31 could downregulate the ROCK/MLC pathway by inhibiting the expression of RhoA in order to suppress the invasion and migration of GC cells.

MeSH Keywords: Cell Migration Assays • MicroRNAs • Neoplasm Invasiveness • rho-Associated Kinases • Stomach Neoplasms

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/898399
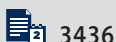
📄 3436   ▦ 2   📊 7   📚 32

Background

Gastric cancer (GC) is currently the fifth most prevalent cancer in the world. Both the incidence and mortality of GC have a decreasing trend, but approximately 951,600 new GC cases and 723,100 deaths from GC were observed in 2012 [1]. Moreover, the prognosis of GC remains poor, especially for GC patients with advanced or metastatic stage cancer; the five-year survival rate after diagnosis is only 9–10% [2]. The unexpectedly high mortality observed in GC may be attributed to delayed diagnosis due to the lack of definite symptoms during early disease stages in which rapid metastasis of malignant cells may occur. About 80% of patients with local or distant metastasis have unfavorable clinical outcomes [3]. GC is a complex disease caused by the interaction between environmental factors and genetic disorders or alterations [4]. Although previous research has indicated that various genetic alterations contribute to the development of GC, the molecular mechanism of its pathogenesis remains unclear [5]. Migration and invasion of malignant cells are critical in the course of cancer metastasis, as during this process, malignant cells proliferate, detach, circulate, transfer, arrest in organs, adhere to vascular walls, establish a microenvironment, and finally proliferate in distant organs [6].

As small non-coding RNAs, microRNAs (miRNAs) are post-transcriptional regulators that inhibit or degrade the translation of target gene messenger RNA (mRNA), further contributing to translation inhibition or targeted degradation and gene silencing [7]. Since miRNAs are differently expressed between tumor and normal tissues, they have significant diagnostic, prognostic, and potential therapeutic values for cancer [8]. For instance, Chan et al. found that miR-21 was overexpressed in 92% of GC tissues, which suggests that it may be considered as an alternative diagnostic marker for GC [9]. Another study reported miR-31 expression was significantly downregulated in GC tissues compared to normal tissues [10].

Small guanosine triphosphatase (GTPase) Rho along with its downstream effector Rho-associated kinase (ROCK) are well-established cellular attachment and migration regulators, and they are involved in both cancer cell invasion and metastasis [11]. Emerging evidence suggests that Rho GTPases (e.g., RhoA, Rac1, and Cdc42) regulate the actin cytoskeleton, which coordinates other cellular activities such as gene transcription, cell shape modification, and adhesion dynamics [12]. RhoA is activated by its effector Rho-kinase (ROCK), particularly when it stimulates the actomyosin contractility and drives the translocation of the cell body [13]. Consequently, the overactivation of the RhoA/ROCK pathway is thought to be associated with tumor invasion and metastasis properties [14].

Even though several studies separately reported that miR-31, RhoA, and ROCK-MLC pathways were linked with GC, the underlying mechanism that explains how these factors influence GC is still unknown. Therefore, this study was designed to investigate the role of RhoA and miR-31 in GC cells.

Material and Methods

Clinical samples and cell lines

Gastric cancer (GC) biopsy specimens from 70 GC patients (38 males and 32 females) were collected from Xuzhou Medical College and the Affiliated Hospital of Xuzhou Medical College between May 2013 and October 2014. The patients' diagnoses of GC were confirmed by pathology, and the patients had not received any radiotherapy or chemotherapy prior to their surgery. As suggested by the tumor node metastasis (TNM) Classification Criteria of the American Joint Committee on Cancer (AJCC), GC samples were further classified into different groups [15]. All clinical characteristics of patients with GC are presented in Table 1. GC tissue and matched adjacent normal tissue were collected during surgery and stored at –70°C. Informed consent was obtained from all patients. The research protocol were approved by the Ethics Committee of Xuzhou Medical College and the Affiliated Hospital of Xuzhou Medical College.

One normal gastric mucosal cell line (GES-1) and three GC cell lines (AGS, SGF-7901, and BGC-823) were purchased from the Xuzhou Medical College and the Affiliated Hospital of Xuzhou Medical College. Cells were cultured at 37°C in Dulbecco modified Eagle medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 5% CO_2. Cell passage was carried out until the cell attachment rate of the monolayer cells reached 80%.

RNA isolation and RT-PCR

Total RNA from tissue specimens or cells was extracted using TRIzol reagent (Invitrogen, USA). The ReverTra Ace qPCR RT Kit (Toyobo, Japan) was used to reverse transcribe total RNA into cDNA. Real-time PCR (RT-PCR) was performed using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan) along with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For detecting miR-31, the primer of miR-31 used in the reaction was 5'-AGGCAAGATGCTGGCATAGCT-3', and U6 snRNA was used as the internal control. The sequence of U6 snRNA primer was 5'-TGACACGCAAATTCGTGAAG-3'. The reaction condition was set as follows: pre-degeneration for 10 minutes at 95°C, degeneration for 10 seconds at 95°C, annealing for 20 seconds at 60°C, and extending for 34 seconds at 72°C, for 40 cycles. For detecting the mRNA of RhoA, the sense primer sequence of

Table 1. Relationship of miR-31expression levels in cancer tissues with clinicopathological factors in patients with gastric cancer.

Factors	Number	microRNA-31	t value	P value
Age (year)				
<60	45	0.51±0.17	0.251	0.803
≥60	25	0.50±0.14		
Gender				
Male	38	0.53±0.17	1.062	0.292
Female	32	0.49±0.14		
Lymphatic metastasis				
Positive	33	0.39±0.11	8.733	<0.05
Negative	37	0.62±0.11		
TNM stage				
I/II	41	0.58±0.13	5.389	<0.05
III/IV	29	0.41±0.13		

RhoA was 5'-CGGGAGCTAGCCAAGATGAAG-3' and the reverse primer sequence of RhoA was 5'-GCTTGCAGAGCAGCTCTCGTA-3'. GAPDH was used as the control and the sense primer sequence of GAPDH was 5'-GTGGACCTGACCTGCGTCT-3', while the reverse primer sequence was 5'-GGAGGAGTGGGTGTCGCTGT-3'. The reaction condition was set as: pre-degeneration for 2 minutes at 50°C, degeneration for 10 minutes at 95°C, annealing for 15 seconds at 95°C, and extending for 45 seconds at 56°C, for 40 cycles. All the primers were purchased from the Shanghai Sangon Company. The expression levels of target genes were normalized to those of the control gene and were calculated using the method of $2^{-\Delta\Delta CT}$. Each experiment was independently replicated three times.

In situ hybridization of miR-31 and immunohistochemistry of RhoA

In situ hybridization of miR-31 for formalin-fixed and paraffin-embedded GC and pericarcinous tissue samples was performed using a miRCURY LNA™ miR-31 detection probe via ISH optimization kit (Exiqon, Vedbaek). Scramble-miR probes were used as negative controls.

Immunohistochemical analysis of RhoA in tissue samples was carried out using the EnVision two-step method, as previously described [16]. Rabbit polyclonal anti-RhoA antibody (Zhongshan Biology Company, Beijing) was used as the first antibody and was diluted 100 times. The avidin-biotin-peroxidase anti-goat antibody was used as the second antibody (5 μg/mL, Vecor Laboratories, CA, USA). RhoA was stained a brown color and results were quantified as follows: – (stained cells <5%), + (stained cells 5–25%), ++ (stained cells 25–75%), +++ (stained cells >75%) [17]. Results from immunohistochemistry were independently assessed by two observers.

Luciferase activity assay

The wildtype 3' untranslated region (UTR) of RhoA containing miR-31 binding sites was cloned into the downstream psi-CHECK-2 luciferase vector (Promega, USA), named wt 3' UTR. The binding site was mutated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, USA) and the resultant mutant 3' UTR was cloned into the same vector, named mut 3' UTR.

The AGS cells maintained in 48-well plates were co-transfected with different groups of vectors: one group was co-transfected with 200 ng pGL3-control luciferase reporter, 10 ng pRL-TK vector, and miR-31 vector, while in the other group, miR-31 was replaced with the negative control vector. The transfected cells were analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA) 48 hours after transfection.

Transfection

BGC-823 cells were transfected with six groups of vectors: blank group (cells without any transfection), NC group (negative control, cells were transfected with scramble), miR-31 mimics group (cells were transfected with miR-31 mimics, purchased from the Shanghai GenePharma Company), miR-31 mimics + RhoA group (cells were co-transfected with miR-31 mimics and RhoA vector, Shanghai GenePharma Company), miR-31 mimics + ROCK group (cells were co-transfected with miR-31 mimics and ROCK, Shanghai GenePharma Company) and miR-31 mimics + SP group (cells were transfected with miR-31 mimics and then treated with SP which is an agonist for MLCK, Enzo). In addition, AGS cells were transfected with six groups of vectors: blank group (cells without any transfection), NC group (cells were transfected with scramble), miR-31 inhibitor group (cells were transfected with
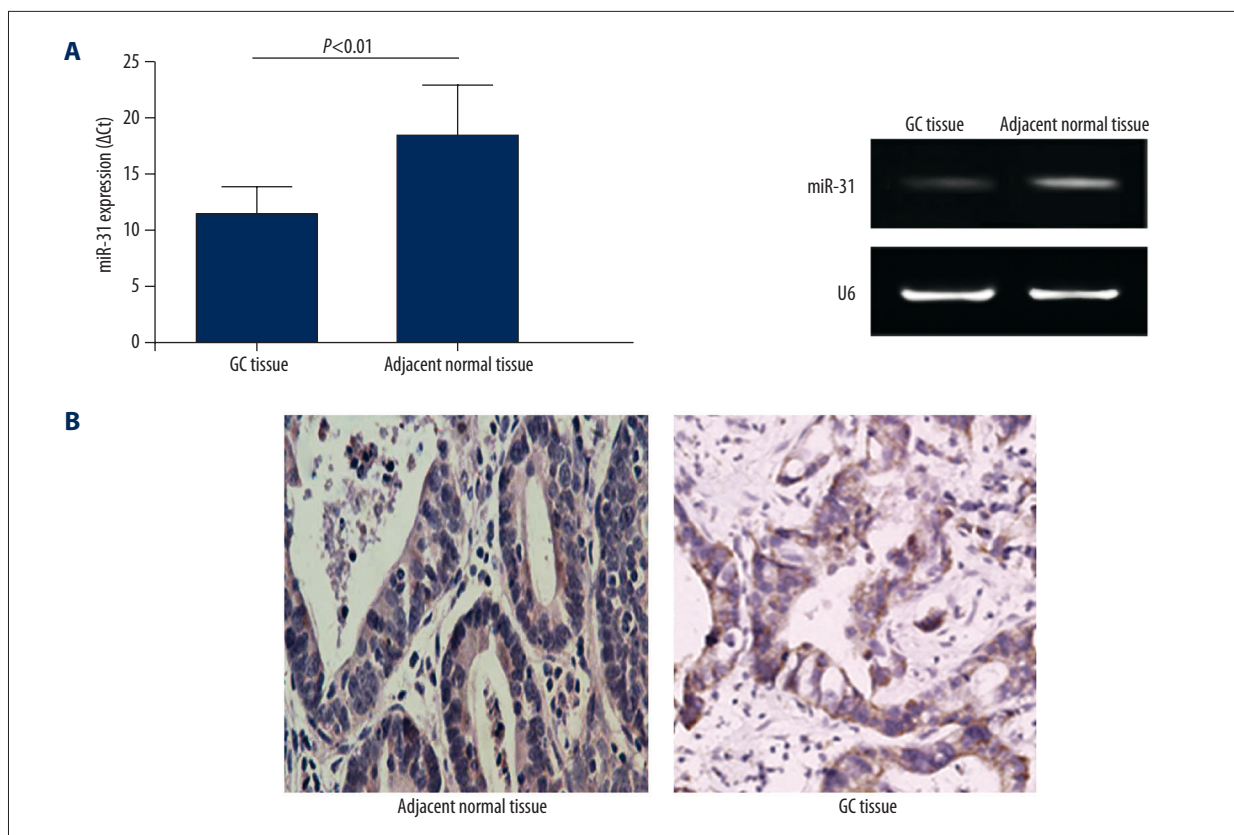
Figure 1. The expression of miRNA-31 in GC and adjacent normal tissues. (**A**) miR-31 levels were detected by RT-PCR in GC and adjacent normal tissue. (**B**) miR-31 levels were detected in GC and adjacent normal tissue by *in situ* hybridization.

miR-31 inhibitor, purchased from the Shanghai GenePharma Company), miR-31 inhibitor + RhoA siRNA group (cells were co-transfected with miR-31 inhibitor and RhoA siRNA), miR-31 inhibitor + ROCK siRNA group (cells were co-transfected with miR-31 inhibitor and ROCK siRNA) and miR-31 inhibitors + ML-7 group (cells were transfected with miR-31 inhibitors and then treated with ML-7, which is an antagonist for MLCK, purchased from Enzo). The sequences of RhoA siRNA and ROCK siRNA were obtained from the Shanghai GenePharma Company. We used lipofectamine 2000 (Invitrogen, USA) to transfect oligonucleotides into cells according to the manufacturer's instruction.

Cell apoptosis assay

An annexin V-FITC cell apoptosis detection kit was used according to the protocol for investigating the apoptosis of transfected cells. A total of 2×10^6 cells were collected into tubes and centrifuged for 5 minutes at 1000 rev/minute for solution removal. Cold PBS was used to wash the cells twice. Then the washed cells were suspended in 200 μL Binding Buffer. Additionally, 5 μL annexin V-FITC was added into the cell suspension and gently mixed with 5 μL propidium iodide (PI). The mixture was reacted in the dark for 15 minutes. FACSCalibur

flow cytometry (Becton-Dickinson, USA) was used to detect the apoptosis rate of cells.

Cell migration and invasion assay

The migration and invasion status of GC cells was detected using Transwell assay. Cells were starved for 24 hours in serum-free DMEM medium and then transferred into a 24-well Transwell plate with an 8-um pore membrane insert (Corning, USA). The chemoattractant, which included DMEM medium with 10% PBS, was placed in the lower chamber. Matrigel was added to the DMEM medium for detecting invading cells. After 72-hour incubation, cells that penetrated into the membrane were treated with 95% ethanol and stained with crystal violet. Finally, metastatic cells were observed and counted under the microscope.

Western blotting

Tissues and cells were harvested and lysed using radio immunoprecipitation assay (RIPA) buffer. Total protein was separated and measured using the Bradford method [18]. Then the total protein was denatured in boiled water and transferred onto polyvinylidene fluoride (PVDF) membranes for sodium dodecyl
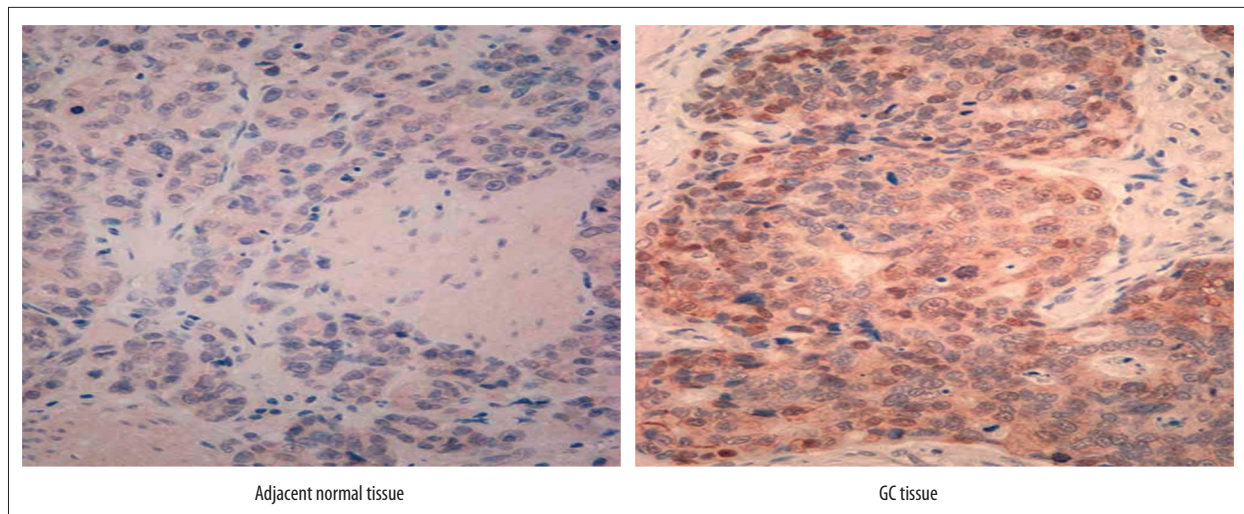
Figure 2. The expression of RhoA was detected using immunohistochemistry in GC and adjacent normal tissue (×200).

Table 2. The positive rate of RhoA expression in GC and adjacent normal tissues.

Protein expression		GC tissues	Adjacent normal tissues	χ^2 value	P value
RhoA	+	51	12	43.90	<0.05
	−	19	58		

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, the membranes were washed in Tris Buffered Saline Tween (TBST) with 5% skim milk for one hour and then treated with primary antibodies against RhoA, ROCK, and p-MLC (1:800 dilution, purchased from CST) at 4°C overnight. After membranes were washed, they were incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-goat, 1:2000 dilution, purchased from CST). Samples, along with reduced actin as the endogenous control, were processed with enhanced chemiluminescence and quantified by Lab Works 4.5 software (Mitov Software, USA).

Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (Chicago, Illinois, USA). Data are shown in the form of mean ± standard deviation (SD). Categorical data were analyzed using the chi-square test. Two-tailed Student's t-test or one-way analysis of variance (ANOVA) was used to evaluate differences in continuous variables among different groups. A value of $p<0.05$ provided evidence for statistical significance.

Results

Downregulated expressions of miR-31 in GC tissues

MiR-31 expression was significantly decreased in GC tissues compared to pericarcinous tissues ($p<0.01$, Figure 1A).

Furthermore, we analyzed the correlation between the expression of miR-31 and clinicopathological characteristics of GC patients, including age, gender, lymphovascular invasion, and pathological TNM stage. As shown in Table 1, patients with lymphovascular invasion were associated with significantly lower miR-31 expression, and GC patients at TNM III-IV stage had significantly lower miR-31 expression compared to GC patients at TNM I–II stage (all $p<0.05$). No significant relationship was found between miR-31 expression and age or gender (all $p>0.05$). In addition, in situ hybridization further confirmed that GC tissues had lower miR-31 levels than pericarcinous tissues (Figure 1B).

Upregulated expressions of RhoA in GC tissues

As suggested by Figure 2, RhoA was stained with brown color in epithelial cells of GC tissues, pericarcinous tissues, and lymph nodes. Among the 70 specimens, high RhoA expressions were observed in 51 GC tissues and 12 pericarcinous tissues. This evidence suggests that significantly higher expressions of RhoA were present in GC tissues compared to pericarcinous tissues ($p<0.05$, Table 2).

Expression of miR-31 in GC cell lines

Based on different cell differentiation, cell lines were further divided into four groups: normal gastric mucosal cell lines (GES-1), GC cell lines with high level of cell differentiation
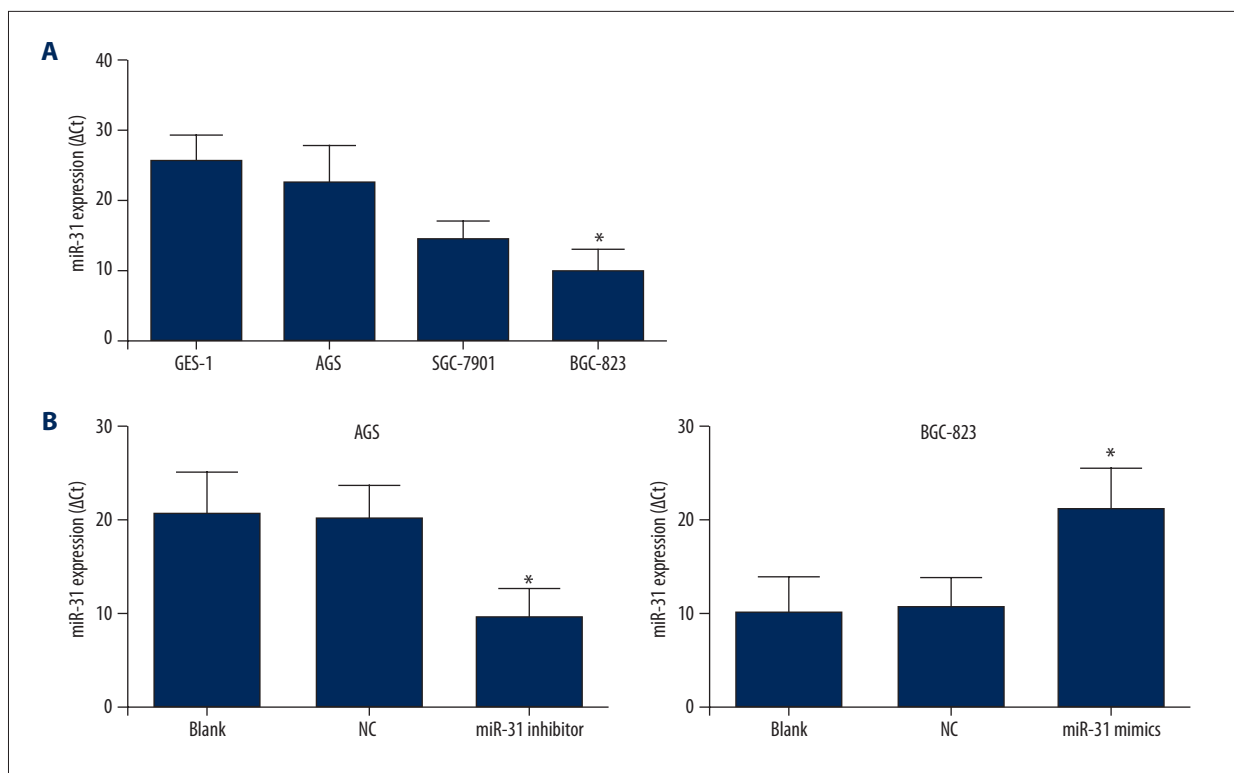
Figure 3. The expression of miRNA-31 in GC cell lines. (**A**) miR-31 was detected by RT-PCR in GC cell lines with various degrees of differentiation. (**B**) miR-31 was detected by RT-PCR in AGS and BGC-823 cell lines after transfection.
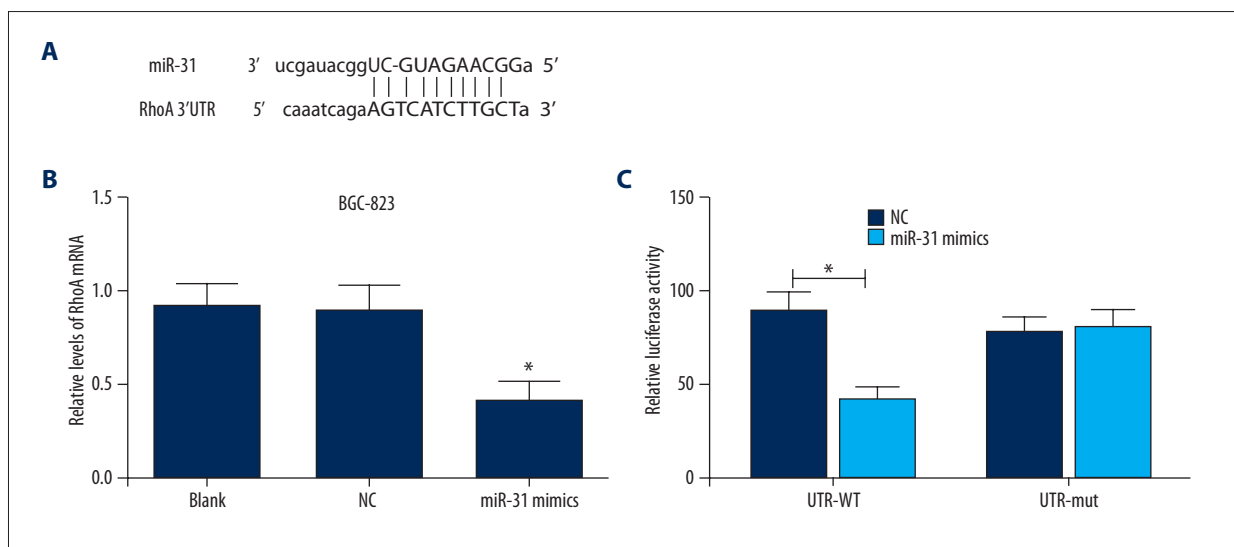

Figure 4. miR-31 targets RhoA. (**A**) Potential target sites for miR-31 in the 3'UTR of RhoA mRNA. (**B**) The mRNA levels of RhoA in BGC-823 cells, BGC-823-NC, and BGC-823-miR-31 mimics. (**C**) Luciferase activity of wild-type (wt-UTR) or mutant (mut-UTR). RhoA 3'UTR reporter genes were transfected in BGC-823-NC and BGC-823-miR-31 mimics.

(AGS), GC cell lines with moderate level of cell differentiation (SGC-7901), and GC cell lines with low level of cell differentiation (BGC-823). BGC-823 cells exhibited significantly lower miR-31 expression compared to the other three cell lines, including GES-1, AGS, and SGC-7901 ($p<0.05$, Figure 3A). Both

GES-1 and AGS cell lines had relatively high expression of miR-31. AGS and BGC-823 cell lines in which miR-31 were stably expressed were selected for further study. Compared to the blank and NC group, miR-31 inhibitors significantly downregulated the expression of miR-31 in AGS cell lines, while miR-31
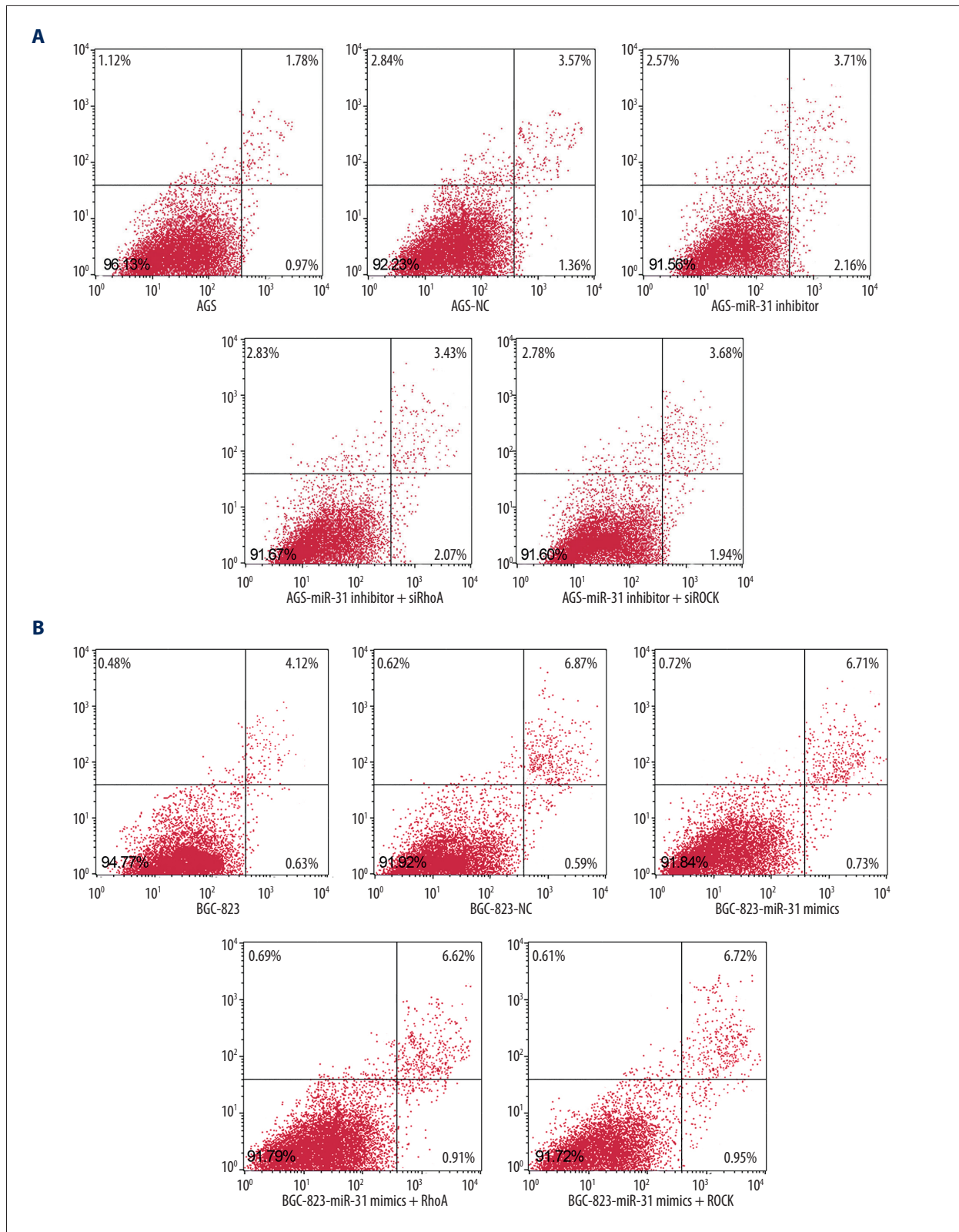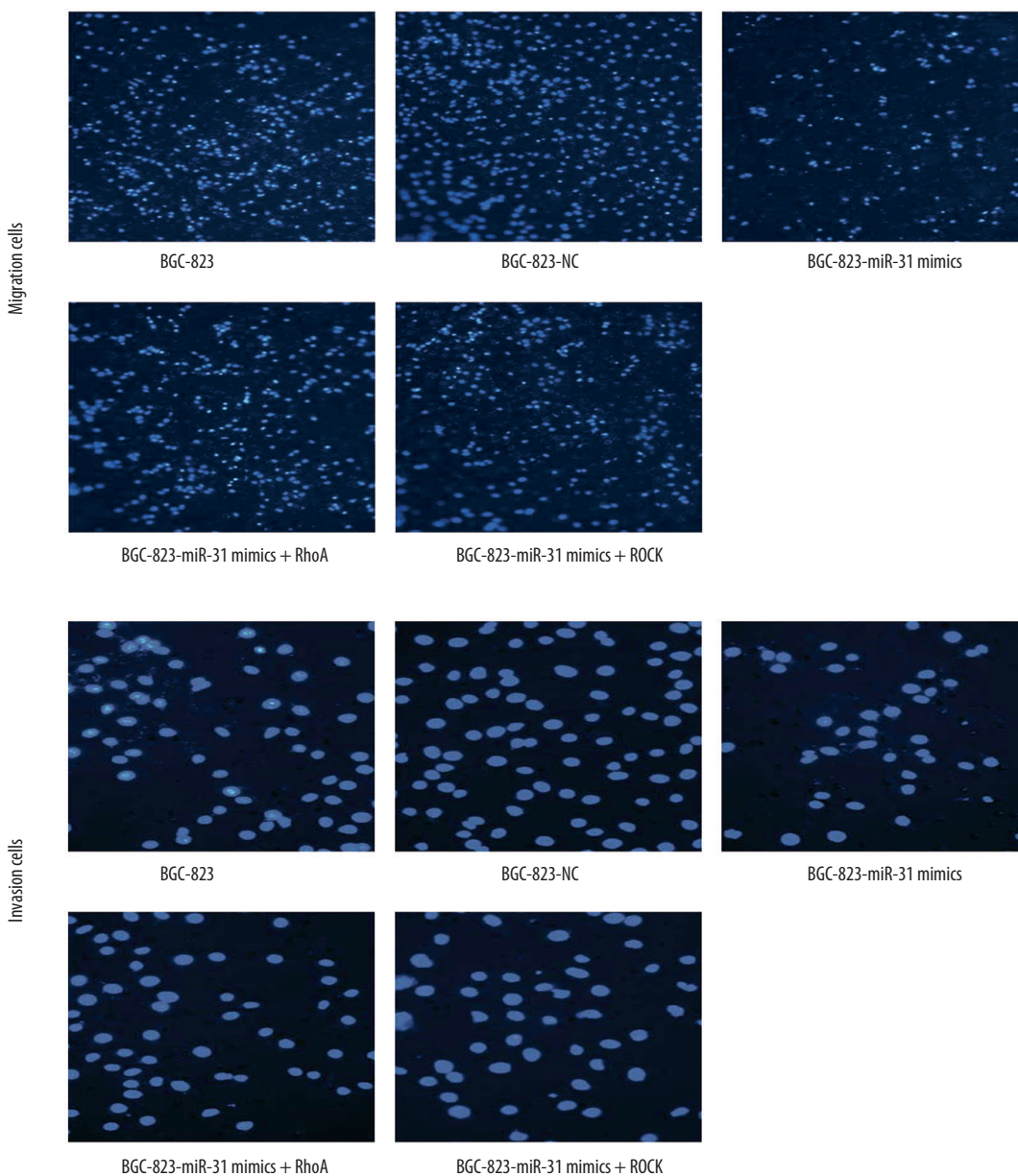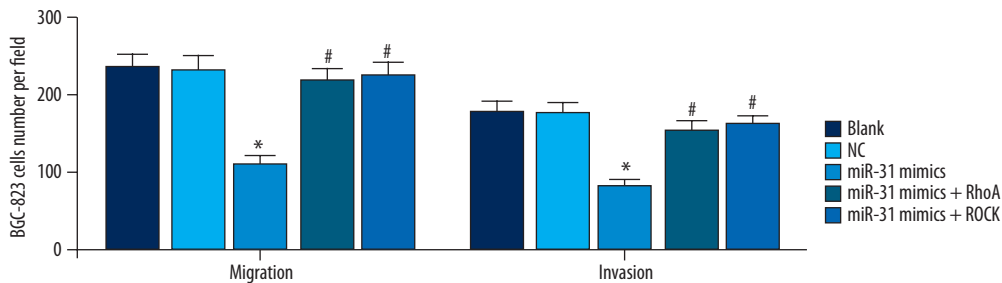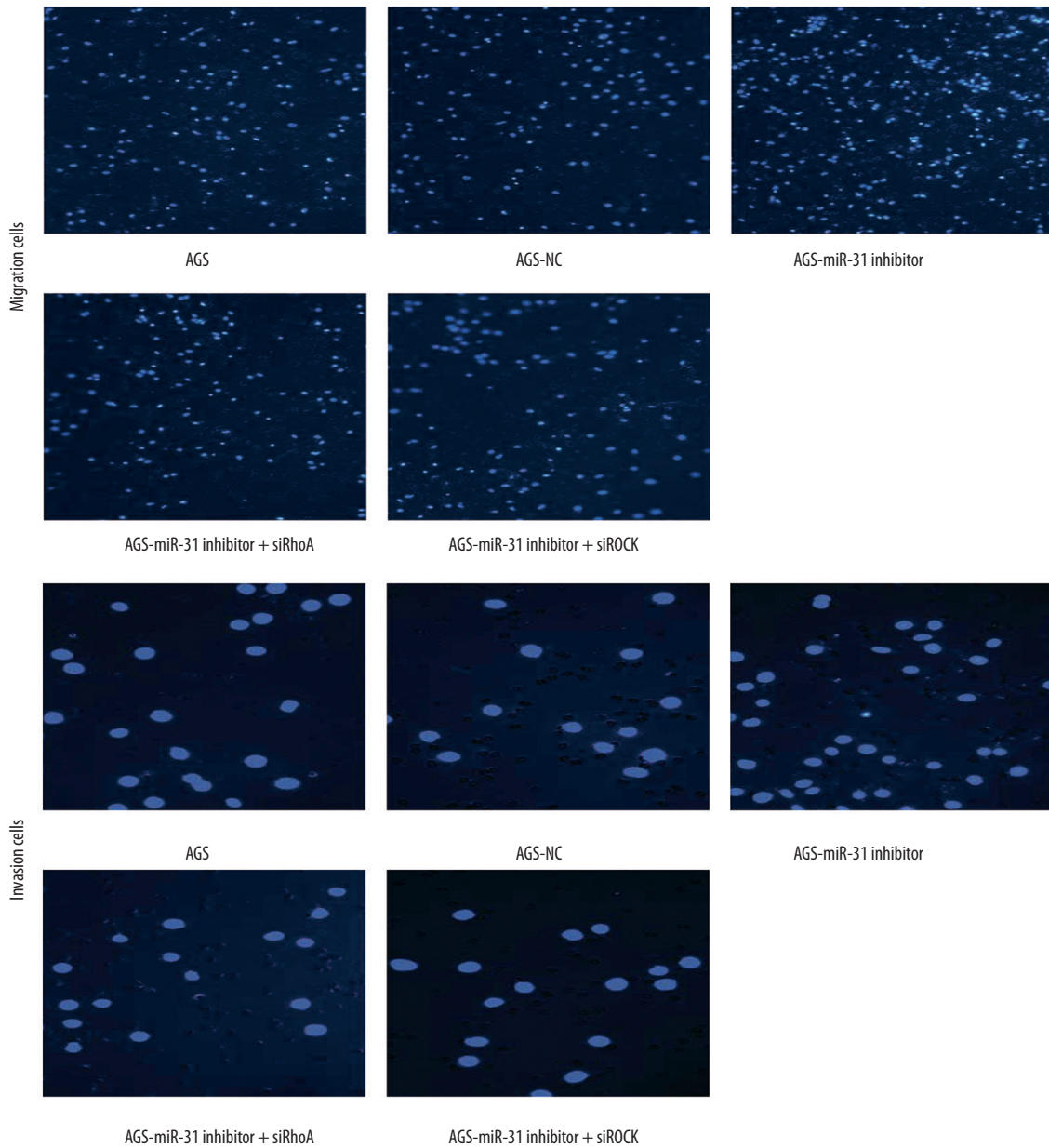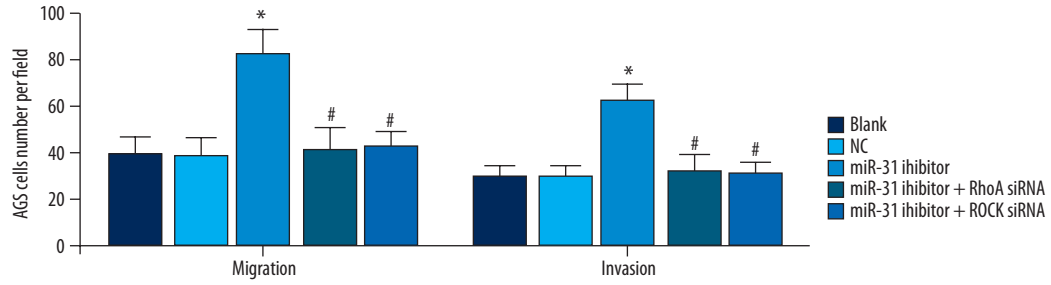
A

B

Figure 5. The apoptosis of GC cells detected by flow cytometry. (**A**) Groups of AGS cell lines: AGS, AGS-NC, AGS-miR-31 inhibitor, AGS-miR-31 inhibitor + RhoA siRNA, AGS-miR-31 inhibitor + ROCK siRNA. (**B**) Groups of BGC-823 cell lines: BGC-823, BGC-823-NC, BGC-823-miR-31 mimics, BGC-823-miR-31 mimics + RhoA, BGC-823-miR-31 mimics + ROCK.

This work is licensed under Creative Common Attribution-
NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)

4686

Indexed in: [Current Contents/Clinical Medicine]   [SCI Expanded]   [ISI Alerting System]
[ISI Journals Master List]   [Index Medicus/MEDLINE]   [EMBASE/Excerpta Medica]
[Chemical Abstracts/CAS]   [Index Copernicus]

B

Migration cells

AGS

AGS-NC

AGS-miR-31 inhibitor

AGS-miR-31 inhibitor + siRhoA

AGS-miR-31 inhibitor + siROCK

Invasion cells

AGS

AGS-NC

AGS-miR-31 inhibitor

AGS-miR-31 inhibitor + siRhoA
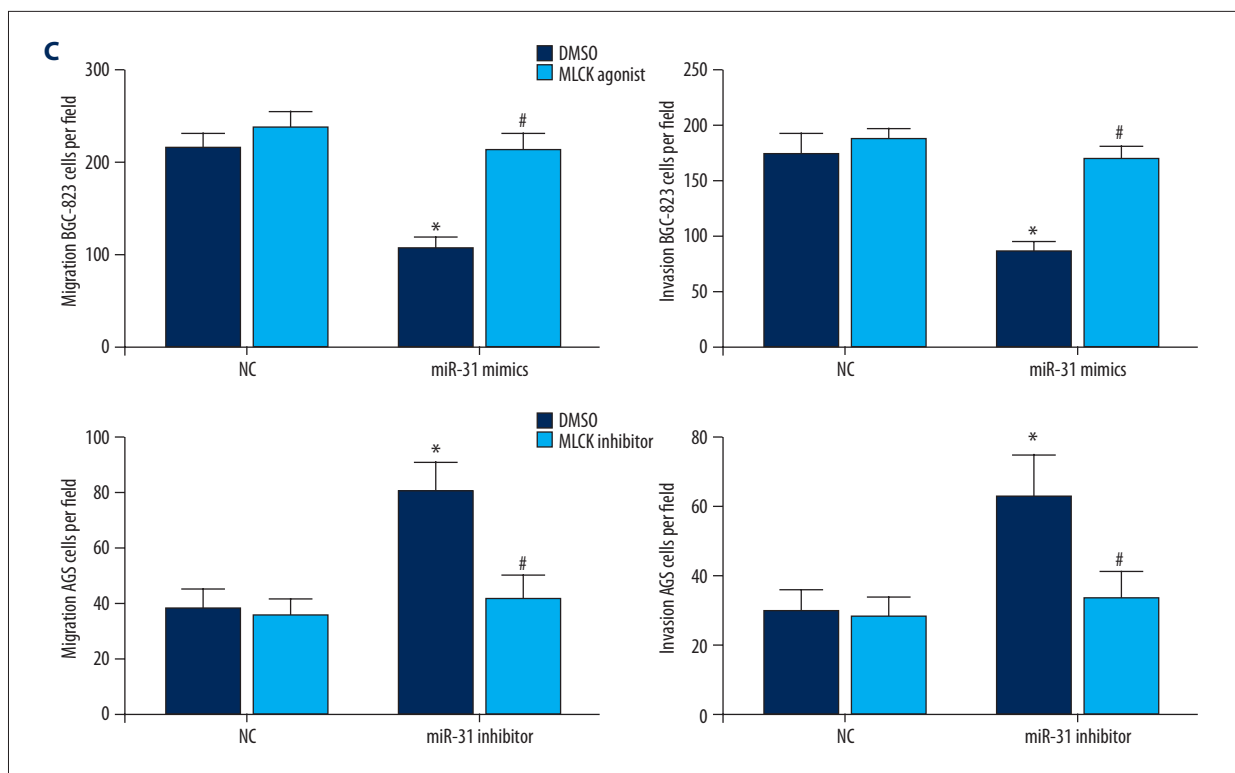
AGS-miR-31 inhibitor + siROCK

Figure 6. The migration and invasion results of Transwell assay (×200). (**A**) The migrated and invaded cells in BGC-823 groups: blank (BGC-823), BGC-823-NC, BGC-823-miR-31 mimics, BGC-823-miR-31 mimics + RhoA, BGC-823-miR-31 mimics + ROCK. * Represents the comparison to NC group, * $p<0.05$. # Represents the comparison to miR-31 mimics group, # $p<0.05$. (**B**) The migrated and invaded cells of AGS groups: blank (AGS), AGS-NC, AGS-miR-31 inhibitor, AGS-miR-31 inhibitor + RhoA siRNA (siRhoA), AGS-miR-31 inhibitor + ROCK siRNA (siROCK). * Represents the comparison to NC group, * $p<0.05$. # Represents the comparison to miR-31 inhibitor group, # $p<0.05$. (**C**) BGC-823 cells treated with or without MLCK agonist SP; AGS cells treated with or without MLCK inhibitor ML-7. * Represents the comparison to NC group, * $p<0.05$. # represents the comparison to miR-31 mimics or inhibitor group, # $p<0.05$.
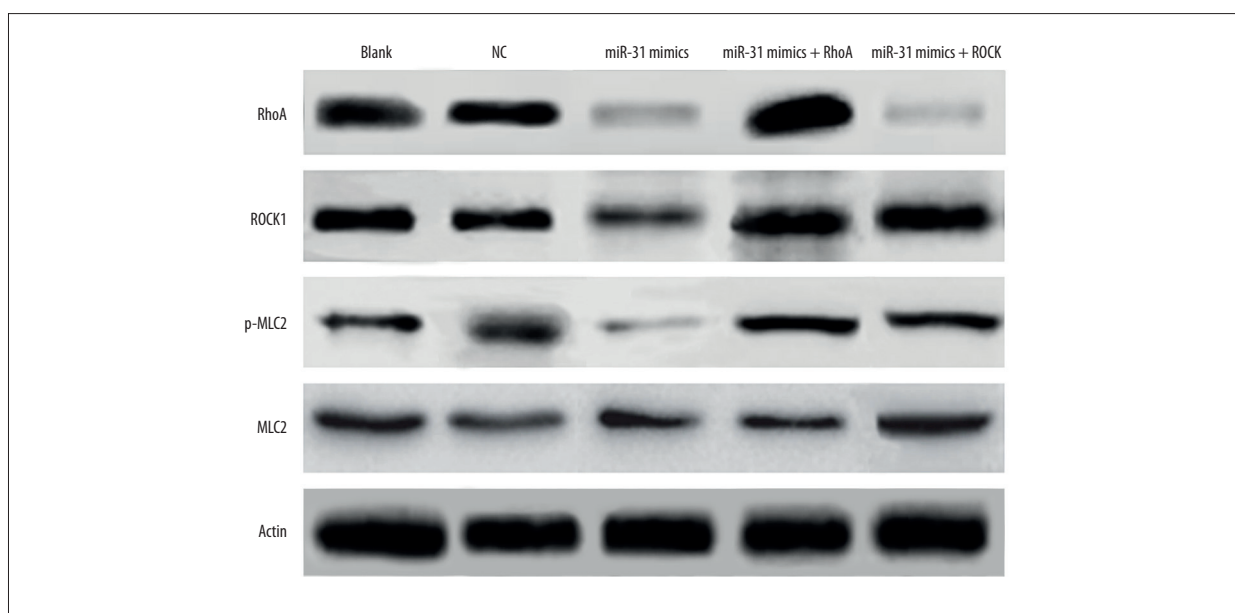

Figure 7. The expression levels of various protein detected by Western blotting.

mimics significantly upregulated the expression of miR-31 in BGC-823 cell lines (all $p<0.05$, Figure 3B).

Suppressed RhoA expression induced by miR-31 binding to 3' UTR of RhoA

As suggested by an online database (*www.microrna.org*), one highly conserved miR-31 binding site was predicted in 3'-UTR of RhoA (Figure 4A). The mRNA expression of RhoA in BGC-823 cells transfected with miR-31 mimics was significantly lower than the blank or NC group, suggesting a negative association between miR-31 and the mRNA expression of RhoA (Figure 4B). The mRNA expression of RhoA in cells co-transfected with wt 3'-UTR of RhoA and miR-31 mimics was significantly lower than cells co-transfected with wt 3'-UTR of RhoA and scramble ($p<0.05$). On the other hand, there was no remarkable difference in the mRNA expression of RhoA between cells co-transfected with mut 3'-UTR of RhoA and miR-31 mimics or scramble (Figure 4C).

Effects of miR-31, RhoA and ROCK on GC cell apoptosis

As shown in the flow cytometry experiment, no significant difference in the apoptosis rate was observed among all groups. Therefore, treatment of miR-31 mimics, RhoA, ROCK, miR-31 inhibitor, RhoA siRNA, and ROCK siRNA had no significant effect on GC cell apoptosis (all $p>0.05$, Figure 5A, 5B).

Effects of miR-31, RhoA, and ROCK on GC cell invasion and migration

As shown in Figure 6A, transfection with miR-31 mimics significantly restricted the migration and invasion rates in the BGC-823 cell lines compared to the blank and NC groups (all $p<0.05$). In addition, the number of metastatic cells in the miR-31 mimics + RhoA and miR-31 mimics + ROCK groups was significantly increased compared to the miR-31 mimics group (all $p<0.05$).

In addition, transfection with miR-31 inhibitor significantly increased the migration and invasion rates in the AGS cell lines compared to the blank and NC groups (all $p<0.05$, Figure 6B). The number of migrated and invaded cells in the miR-31 inhibitor + RhoA siRNA and miR-31 mimics + ROCK siRNA groups was significantly less compared to the miR-31 inhibitor group (all $p<0.05$, Figure 6B).

Compared to the control group with DMSO treatment, MLCK agonist SP significantly exacerbated the invasion and migration of BGC-823 cells, while MLCK inhibitor ML-9 substantially suppressed the migration and invasion of AGS cells (all $p<0.05$) (Figure 6C).

Effects of miR-31, RhoA, and ROCK on RhoA, ROCK, MLC, and p-MLC expressions

As suggested by Western blotting, no significant difference in protein expressions of RhoA, ROCK, p-MLC, and MLC was detected between the blank and NC groups (all $p>0.05$). Compared to the blank and NC groups, overexpression of miR-31 significantly decreased the expressions of RhoA, ROCK, and p-MLC (all $p<0.05$). Compared to the miR-31 mimics group, the miR-31 mimics + RhoA group exhibited significantly higher expressions of RhoA, ROCK, and p-MLC, while the miR-31 mimics + ROCK group exhibited significantly higher expressions of ROCK and p-MLC (all $p<0.05$). Results from Western blotting are displayed in Figure 7.

Discussion

Gastric cancer (GC), with an estimated five-year survival rate of 5–15%, is the second most common cause of cancer-related death in the world, which could be partially attributed to delayed diagnosis [19–21]. Hence, discovering the molecular mechanisms of GC cells with respect to disease formation is critical to improve the overall survival status of GC patients.

Our research revealed that miR-31 expression was significantly reduced in both GC tissues and GC cell lines compared to pericarcinous tissues or normal cell lines. Other research groups have reported similar results to our findings [10,22–24], indicating a close connection between miR-31 and GC. We further studied the relationship between the decline in miR-31 and lymphovascular invasion or pathological TNM stage. GC patients with lymphovascular invasions or advanced TNM stages were associated with significantly lower miR-31 expressions. Moreover, lower levels of miR-31 expression were associated with lower levels of cell differentiation. However, Zhang et al. did not find an association between the expression levels of miR-31 and lymphovascular invasion or cell differentiation [10]. This inconsistence may be attributed to different patient samples that were used in the two studies. For instance, patients who had been treated with radiotherapy or chemotherapy may have been included in the study conducted by Zhang et al., which may have a large effect on the expression of miR-31. However, Zhang et al. [10] confirmed that miR-31 was a better biomarker in diagnosing GC compared to serum CEA. Our experiments, *in vitro,* also showed that miR-31 can significantly decrease the migration and invasion rates in GC cells, indicating its important anti-metastasis role in GC. In order to investigate how miR-31 suppressed GC, RhoA (whose 3' untranslated region contains miR-31 binding sites) were studied.

RhoA is one of the members of the Rho family and it acts as a GTPase that intermediates and facilitates interactions between

cell surface receptors and downstream signaling molecules [25]. The main functions of RhoA, including changing the structure of the cytoskeleton, degrading the matrix, and influencing tumor cell adhesion and movement, have been reported to enhance tumor invasion or metastasis [26,27]. As suggested by our immunohistochemical analysis, RhoA was overexpressed in GC tissues, which could impede or weaken the anti-metastasis function of miR-31. Moreover, the binding of miR-31 to 3' UTR of RhoA could suppress the expression of RhoA. These results indicated that miR-31, which functions as a GC suppressor by targeting the 3' UTR of RhoA, could inhibit the invasion of GC cells. Other researchers, including Duan et al. [28], Matsuoka et al. [11], and Lin et al. [29] also reported a similar role for RhoA in the invasion of GC cells. However, none of these other studies discovered that miR-31 played a key role in modulating the function of RhoA, which is critical to the invasion of GC cells.

As a downstream effector of RhoA, ROCK has been reported to be associated with GC progression and invasion [25,29–31]. Moreover, ROCK was shown to play an important role in MLC phosphorylation, which exacerbates cell migration [31,32]. Hence, we further studied the association between the ROCK-MLC pathway and GC cell migration. Our results suggested that ROCK could impede or weaken the anti-metastasis function of miR-31, contributing to a larger number of migrated and invaded GC cells. Furthermore, a MLCK agonist (an enzyme functioning as phosphorylating MLC) could significantly exacerbate the invasion and migration of GC cells, while a MLCK inhibitor could suppress such a trend. Western blot analysis enabled us to confirm that ROCK contributes to significant increases in the amount of p-MLC (phosphorylated MLC). Therefore, ROCK could facilitate MLC phosphorylation and eventually induce GC invasion.

Finally, we used Western blot analysis to demonstrate how miR-31 regulated the ROCK-MLC pathway via RhoA. An overexpression of miR-31 could significantly inhibit the expressions of RhoA, ROCK, and p-MLC; RhoA could upregulate both ROCK and p-MLC expressions; and ROCK could increase the expression of p-MLC. Therefore, we concluded that miR-31 regulated the ROCK-MLC pathway through RhoA, contributing to the inhibition of GC invasion.

Conclusions

This is the first study that reveals the exact molecular mechanism of miR-31 in inhibiting GC invasion. More importantly, we successfully proved that miR-31 could bind to the 3'UTR of RhoA, resulting in a decrease in the expression of RhoA. Furthermore, our research indicated potential targets for developing novel GC diagnostic tools and treatments. For example, miR-31 could be used as a promising diagnostic marker for GC in its early stages. Besides that, miR-31 could be considered as a prognostic marker for GC, since its expression significantly differed among different TNM stages and cell differentiations. Both RhoA inhibitor and MLCK inhibitor may be considered as novel therapeutic drugs for GC due to their suppressive effects on the invasion of GC cells. However, there were some limitations in our study. First, experiments *in vivo* were not carried out to confirm the *in vitro* functions of these molecules. As a result, further studies should be designed to assess the clinical value of these molecules. In conclusion, miR-31 suppressed both the invasion and migration of GC cells by targeting RhoA through the ROCK-MLC signaling pathway.

References:

1. Torre LA, Bray F, Siegel RL et al: Global cancer statistics, 2012. Cancer J Clin, 2015; 65: 87–108

2. Gomes LL, Moreira FC, Hamoy IG et al: Identification of miRNAs expression profile in gastric cancer using self-organizing maps (SOM). Bioinformation, 2014; 10: 246–50

3. Chen Z, Saad R, Jia P et al: Gastric adenocarcinoma has a unique microRNA signature not present in esophageal adenocarcinoma. Cancer, 2013; 119: 1985–93

4. Lyu X, Huang J, Liu J et al: [Detection and significance of epidermal growth factor receptor mutation in esophageal, esophagogastric junction and gastric cancers.] Zhonghua Zhong Liu Za Zhi, 2014;36: 346–50 [in Chinese]

5. Peng N, Zhao X: Comparison of mutations in lung, colorectal and gastric cancer. Oncol Lett, 2014; 8: 561–65

6. Smyth EC, Sclafani F, Cunningham D: Emerging molecular targets in oncology: Clinical potential of MET/hepatocyte growth-factor inhibitors. Onco Targets Ther, 2014; 7: 1001–14

7. Bartel DP: MicroRNAs: Target recognition and regulatory functions. Cell, 2009; 136: 215–33

8. Ueda T, Volinia S, Okumura H et al: Relation between microRNA expression and progression and prognosis of gastric cancer: A microRNA expression analysis. Lancet Oncol, 2010; 11: 136–46

9. Chan SH, Wu CW, Li AF et al: miR-21 microRNA expression in human gastric carcinomas and its clinical association. Anticancer Res, 2008; 28: 907–11

10. Zhang Y, Guo J, Li D et al: Down-regulation of miR-31 expression in gastric cancer tissues and its clinical significance. Med Oncol, 2010; 27: 685–89

11. Matsuoka T, Yashiro M, Kato Y et al: RhoA/ROCK signaling mediates plasticity of scirrhous gastric carcinoma motility. Clin Exp Metastasis, 2011; 28: 627–36

12. Nobes CD, Hall A: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell, 1995; 81: 53–62

13. Leung T, Chen XQ, Manser E, Lim L: The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol, 1996; 16: 5313–27

14. Narumiya S, Tanji M, Ishizaki T: Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. Cancer Metastasis Rev, 2009; 28: 65–76

15. Washington K: 7th edition of the AJCC cancer staging manual: Stomach. Ann Surg Oncol, 2010; 17: 3077–79

16. Ou DL, Chen CL, Lin SB et al: Chemokine receptor expression profiles in nasopharyngeal carcinoma and their association with metastasis and radiotherapy. J Pathol, 2006; 210: 363–73

17. Huang KH, Lan YT, Chen MH et al: The correlation between RhoA expression and clinicopathological characteristics in gastric cancer patients after curative surgery. World J Surg, 2015; 39: 2289–99

18. Qian X, Dong H, Hu X et al: Analysis of the interferences in quantitation of a site-specifically PEGylated exendin-4 analog by the Bradford method. Anal Biochem, 2014; 465: 50–52

19. Jemal A, Bray F, Center MM et al: Global cancer statistics. Cancer J Clin, 2011; 61: 69–90

20. Zheng L, Tan W, Zhang J et al: Combining trastuzumab and cetuximab combats trastuzumab-resistant gastric cancer by effective inhibition of EGFR/ErbB2 heterodimerization and signaling. Cancer Immunol Immunother, 2014; 63: 581–86

21. Wei J, Zhao ZX, Li Y et al: Cortactin expression confers a more malignant phenotype to gastric cancer SGC-7901 cells. World J Gastroenterol, 2014; 20: 3287–300

22. Ye B, Jiang LL, Xu HT et al: Expression of PI3K/AKT pathway in gastric cancer and its blockade suppresses tumor growth and metastasis. Int J Immunopathol Pharmacol, 2012; 25: 627–36

23. Zhang J, Cao J, Li J et al: A phase I study of AST1306, a novel irreversible EGFR and HER2 kinase inhibitor, in patients with advanced solid tumors. J Hematol Oncol, 2014; 7: 22

24. Ruoming W, Zhen Y, Tengteng Z, Jisheng H: Tumor suppressor microRNA-31 inhibits gastric carcinogenesis by targeting Smad4 and SGPP2. Cancer Gene Ther, 2015; 22: 564–72

25. Zhou J, Hayakawa Y, Wang TC, Bass AJ: RhoA mutations identified in diffuse gastric cancer. Cancer Cell, 2014; 26: 9–11

26. Liu XP, Wang HB, Yang K et al: Inhibitory effects of adenovirus mediated tandem expression of RhoA and RhoC shRNAs in HCT116 cells. J Exp Clin Cancer Res, 2009; 28: 52

27. Zhao X, Lu L, Pokhriyal N et al: Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Cancer Res, 2009; 69: 483–91

28. Duan JT, Wang XM, Zhang SQ, Zhao GJ: Effect of RhoA gene silencing on proliferation and migration of gastric MGC-803 cells. Int J Clin Exp Med, 2015; 8: 14410–15

29. Lin MT, Lin BR, Chang CC et al: IL-6 induces AGS gastric cancer cell invasion via activation of the c-Src/RhoA/ROCK signaling pathway. Int J Cancer, 2007; 120: 2600–8

30. Xu XT, Song QB, Yao Y et al: Inhibition of RhoA/ROCK signaling pathway promotes the apoptosis of gastric cancer cells. Hepatogastroenterology, 2012; 59: 2523–26

31. Eitaki M, Yamamori T, Meike S et al: Vincristine enhances amoeboid-like motility via GEF-H1/RhoA/ROCK/Myosin light chain signaling in MKN45 cells. BMC Cancer, 2012; 12: 469

32. Somlyo AP, Somlyo AV: Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol, 2000; 522 Pt 2: 177–85