Original Article
MicroRNA-613 suppresses colon cancer proliferation via targeting neurokinin-1

Jilin Hu[1], Chen Qin[2], Yuan Gao[1], Xianxiang Zhang[1], Maoshen Zhang[1], Yun Lu[1]

[1]Department of General Surgery, The Affiliated Hospital of Qingdao University, Qingdao 266555, PR China;
[2]Department of Oncology, Qingdao Central Hospital, Qingdao 266555, PR China

Abstract: Background: MicroRNAs are widely involved in colon cancer development. However, the detailed biological functions of microRNA-613 (miR-613) in colon cancer are still unexplored. The present study aims to investigate the role of miRNA-613 and the potential mechanisms in the proliferation of colon cancer. Methods: Quantitative Real-time PCR was used to detect the expression of miR-613 expression in colon cancer tissues. Luciferase reporter assays were conducted to detect the associations between miR-613 and 3'-UTRs of neurokinin-1. CCK8 assay and transwell assay were performed to evaluate biological functions of miR-613. Results: MiR-613 was significantly down-regulated in colon cancer tissues and correlated closely with tumor differentiation. MiR-613 suppressed *in vitro* colon cancer cell proliferation and induced cell apoptosis. Neurokinin-1 was identified as direct targets of miR-613. Neurokinin-1 downregulation can inhibit colon cell proliferation and activate Caspase-3. Conclusions: This study revealed the interaction between miR-613 and neurokinin-1 in colon cancer. miR-613 functions as a tumor suppressor in colon cancer by targeting neurokinin-1. Restoration of miR-613 expression can be considered as a promising therapeutic approach for colon cancer treatment.

Keywords: MiR-613, neurokinin-1, proliferation, metastasis, prognosis

Introduction

Colon cancer is one of the commonest malignant tumors with a high incidence and mortality worldwide. Despite the use of active targeted drugs for treatment of metastatic colon cancer in the past decade, cure rates remain low [1]. Investigation on novel markers for colon cancer diagnosis and treatment is among the most active areas in colon cancer study [2].

MicroRNAs (miRNAs) are a class of small regulatory RNA molecules which are highly conserved in the regulation of a diversity of biological functions [3]. Theycan be served as novel biomarkers for colon cancer diagnosis and treatment [4]. MiRNAs can dysregulate the colon cancer development through targeting mRNA transcripts by binding to their 3'-untranslated region (UTR) [5]. Although miRNAs have been the subject of extensive research in recent years, the molecular regulatory mechanisms of miRNAs and their effects on colon cancer are not well explored. It is essential to further investigate the aberrantly expressed miRNAs in colon cancer and the underlying mechanisms.

MiR-613 has been found to be lost in several cancers, such as lung cancer, gastric cancer, ovarian cancer and prostatic cancer [6-9]. It functions as a pleiotropic modulator of cancer cell proliferation, invasion, and metastasis. The known targets of miR-613 in tumor include KRAS, CDK4, and Frizzled7 [6-9]. Till now, the biological roles and underlying mechanisms of miR-613 in colon cancer development are still unclear. In this study, we examined miR-613 expression in colon cancer tissues, and then we investigated the biological functions and identified its potential targets. We found that miR-613 was decreased in colon cancer, and miR-613 could inhibit colon cancer proliferation by directly targeting neurokinin-1.

Materials and methods

Cell lines and tissues

Human colon cell lines HCT8 and DLD1 were cultured in RPMI-1640 medium (Gibco, Grand

Island, NY USA) containing 10% fetal bovine serum (FBS, Biowest, Nuaillé, France). All the cell lines were cultured in a 5% CO_2 humidified at mosphere at 37°C. Fresh colon cancer tissues and the corresponding normal tissues were collected from 100 patients who underwent colon adenocarcinoma resection without prior radiotherapy and chemotherapy at our institution in 2010. These samples were collected immediately after resection, snap-frozen in liquid nitrogen, and then stored at -80°C until needed. The histopathological grade was assigned according to the criteria of the WHO classification. Adenocarcinomas are graded on the basis of the extent of glandular appearances, and were divided into well, moderately and poorly differentiated.

Immunohistochemistry

Four-μm sections were cut from formalin-fixed, paraffin-embedded blocks and then deparaffinized in xylene and rehydrated using a series of graded washes with ethanol. After inhibition of endogenous peroxidase and antigen retrieval (microwave irradiation in 0.01 M citrate buffer at pH 6.0), the sections were incubated with each primary antibody (neurokinin-1 was purchased from Sigma, Cat.#S8305, Sigma-Aldrich, St. Louis, MO USA) at 4°C overnight followed by incubation with horseradish peroxidase conjugated secondary antibodies (Dako, Cat.#K406511, Glostrup, Denmark). Slides were then developed for 5 minutes with the chromogen, 3,3'-diaminobenzidine (DAB), and counterstained with hematoxylin to distinguish the nucleus from the cytoplasm.

Real-time quantitative RT-PCR

Total RNA was extracted from the cells and tissues using Trizol reagent (Invitrogen, Carlsbad, CA USA), according to the manufacturer's instructions. The cDNA was then synthesized from total RNA using an access reverse transcription system (Promega, Madison, WI USA). All-in-One™ miRNA quantitative real-time polymerase chain reaction (qRT-PCR) Detection Kit (Gene Copoeia, Rockville, USA) was used in reverse transcription (RT). The RT reaction system was 25 μL, containing 1 μL of 2.5 U/μL poly A polymerase, 1 μL of RTase Mix, 5 μL of 5 × PAP/RT buffer, 2 μg of total RNA templates, and RNase/DNase-free ddH$_2$O. The reaction conditions were performed at 37°C for 60 min and

85°C for 5 min. The 20 μl reaction system for qPCR comprised 10 μL of 2 × qPCR mix, 2 μL of first-strand cDNA (diluted 1:5), 2 μL of universal adaptor PCR primer, 2 μL of All-in-One™ miRNA qPCR primer and 4 μL of ddH$_2$O. Amplification was performed in Bio-Rad single color real-time PCR system (Bio-Rad, Hercules, CA, USA) under the following reaction conditions: 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. The expression levels of miRNAs in tissue and serum samples were normalized to GAPDH. Relative quantification of miRNA expression was calculated with $2^{-\Delta Ct}$ method. Primer sequences of miR-613: forward: 5'-GAGTGCGT-TTCCAAGTGTG-3'; reverse: 5'-GGCAAAGAAGG-AACATTCC-3'. GAPDH, forward: 5'-GACCCCTTC-ATTGACCTCAAC-3', reverse: 5'-TTCTCCATGGTG-GTGAAGA-3'.

Western blot

Total protein was extracted from cells with RIPA lysis buffer (Cat.#sc-24948, Santa Cruz, Dallas, TX USA). Equal amounts of proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% milk in Tris-HCl buffered solution for 2 h, and then subsequently incubated overnight at 4°C with primary antibodies against neurokinin-1 (Cat.#S8305, Sigma) and secondary antibodies (Cat.#7074, Cell Signaling, Beverly, MA USA) at 37°C for 1 h. To confirm equal protein loading, the membranes were incubated with β-actin (Cat.#8457S, Cell Signaling) as internal control. The signals were visualized using an enhanced chemiluminescent substrate and detected by a FluorChem Q imaging system (Protein Simple, Santa Clara, CA USA). Images from western blot were quantified using Quantity One® software (Bio-Rad, Hercules, CA USA). The expression level was normalized with respect to internal control.

Cell transfection

MiR-613 mimics, miR-613 inhibitors and negative control oligonucleotides (NC) were purchased from Invitrogen and transfected into cells with Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nM, according to the manufacturer's instructions.
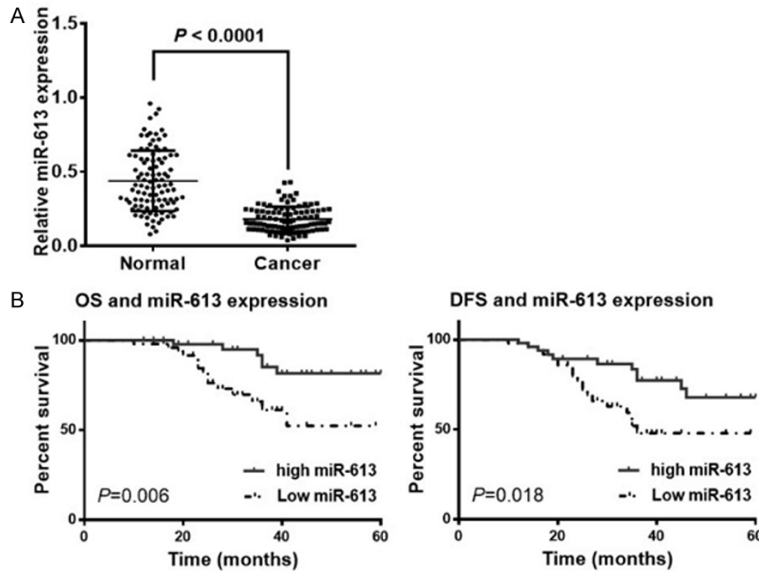
Figure 1. MiR-613 expression in breast cancer tissues. A. MiR-320a was underexpressed in breast cancer tissues. Data were analyzed by student's t test. B. Kaplan-Meier survival curves in 100 colon cancer patients with different miR-613 levels. During the follow-up period, a total of 20 patients with low miR-613 expression suffered recurrence/metastasis and 15 died of cancer related complications. In contrast, only 11 patients with high miR-613 expression suffered cancer recurrence/metastasis and 6 vanished. Further log-rank test showed that miR-613 expression level affected OS and DFS patterns. Patients with low miR-613 expression suffered a short life expectancy and a high recurrence/metastasis possibility. Data were anlyzed with log-rank test.

Cell proliferation assay

Cells were cultured in a 96-well plate at a density of 1×10^5 cells/mL. Each well contained 1×10^5 cells in a total volume of 100 μL. The plate included control wells and experimental wells. CCK-8 assay was performed after cells were seed for 24 h. Briefly, 10 μL CCK-8 was added in each well, and the optical density (OD) value was detected in a MULTISCAN GO-1510 microplate reader at 450 nm (Thermo Scientific, Shanghai, China) after incubating at 37°C for 2 h. Each experimental condition was assayed in triplicate and all experiments were performed for three times.

Cell invasion assay

The invasion capability of prostate cancer cells was determined using the Matrigel invasion assay. Briefly, Transwell membrane filter inserts (Corning, Toledo, NY USA) were precoated with Matrigel (BD Bioscience, San Jose, CA USA). The cells were transfected with the miR-613 mimics for 24 h and then resuspended into

200 μL of serum-free medium (1×10^5 cells), which was then added to the top chamber. Meanwhile, 500 μL of growth medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, non-invasive cells in the upper chamber were gently removed by a cotton swab. The invasive cells on the lower surface were then fixed, stained and observed under a microscope. The mean number of invaded cells in five random fields per membrane was counted and averaged.

Luciferase reporter constructs

The 3'-UTR fragment of neurokinin-1 (TACR1, NM_001058.3) containing the putative miR-613 binding sequence (ACAUUCCA, corresponding to 140-147nt of neurokinin-1 3'-UTR) was amplified using the primers 5'-ACCTGACCTC-CAACTGCTC-3' (forward) and 5'-CAGCATGAGGGTGGCAAAG-3' (reverse). The resulting PCR product was cloned into a firefly luciferase reporter vector (pGL3; Promega Corporation, Madison, WI, USA), and termed pGL3-neurokinin1-3'UTR. A plasmid carrying mutations in the complementary sites for the seed region of miR-613 was generated based on the pGL3-neurokinin1 3'UTR plasmid using a MutanBEST Kit (Takara Bio Inc., Shiga, Japan), and termed pGL3-neurokinin1-3'UTR-mut. The correctness of the plasmids was confirmed by sequence analysis.

Statistical analysis

Data are shown as mean ± standard deviation (SD). SPSS version 17.0 software (SPSS Inc., Chicago, IL USA) was used to perform the statistical analyses. Statistical differences were processed using Student's t-test or one-way analysis of variance. Overall and disease-free survival were calculated using the Kaplan-Meier method and compared by log-rank test. Spearman correlation was applied to assess

Table 1. Relationship between miR-613 expressions in colon cancer tissues and clinicopathologic parameters (n = 100)

	n	MiR-613 expression*		P value
		High	Low	
Age (years)				0.500
≥60	51	26	25	
<60	49	24	25	
Tumor size (cm)				0.000**
≥3	58	19	39	
<3	42	31	11	
Lymph node metastasis				0.000**
Yes	40	12	28	
No	60	38	22	
Dukesstaging				
Stage A	27	18	9	0.000**
Stage B	33	18	15	
Stage C, D	40	14	26	

*The cutoff value is the mean expression level of miR-613 (the value ≥0.1805 is considered high expression). **Data were analyzed with Chi-squre test. Difference is statistically significant.

the correlation between miR-320a and MTDH expression. A P-value less than 0.05 was considered as significance.

Results

MiR-613 expression is decreased in colon cancer tissues

We firstly examined the expression of miR-613 in 100 paired samples (cancer tissue and the corresponding non-cancerous tissue) using qRT-PCR. We found that miR-613 expression in cancer tissues was significantly lower than that in adjacent normal tissues (**Figure 1A**). Further clinicopathologic analysis showed that the expression level of miR-613 was inversely related to tumor size, lymph node metastasis, and Duke's stage. There was no significant relationship between miR-613 expression and patients' age and sex (**Table 1**).

There after, we investigated the relationship between miR-613 expression and the survival time. We found that patients with lower expression of miR-613 tended to have a shorter life expectancy and a shorter disease-free survival time (**Figure 1B**).

Decreased miR-613 in colon cancer contributes to cell proliferation and metastasis

We also measured miR-613 expression in the two colon cancer cell lines HCT8 and DLD1 by qRT-PCR. We found that miR-613 expression was relatively lower in HCT8. Therefore, we performed a gain-of-function study using miR-613 mimics in HCT8 cells, and performed a loss-of-function study using miR-613 inhibitors in DLD1 cells (**Figure 2A**). CCK-8 assays revealed that miR-613 overexpression significantly inhibited HCT8 cell proliferation, while depletion of miR-206 in DLD1 cells showed the opposite effects (**Figure 2B**). We next assessed the effect of miR-613 on the invasion and metastasis of colon cancer cells. The transwell assays showed that miR-613 overexpression in HCT8 cells markedly decreased the invasive abilities, while suppression of miR-613 in DLD1 cells showed the opposite effect (**Figure 2C**). These results suggested that miR-613 could inhibit cell proliferation, and profoundly decrease invasion and metastasis of colon cancer cells.

MiR-613 directly targets neurokinin-1

To explore the potential mechanisms underlying miR-613-mediated colon cancer cell behaviors, we used a common bioinformatic algorithm (TargetScan 7.0) to predict the mRNA targets of miR-613. Among those predicted mRNAs, neurokinin-1 (5'...UAUGGGUUAGGGAA-AACAUUCCA...3', miR-613, 3'-CCGUUUCUUCC-UUGUAAGGA-5'; the underlining sequence is the binding sites)was previously investigated in our institution and reported as a potential target for colon cancer therapy [10-12]. Therefore, it was selected as a candidate target for further validation. We cloned the wild-type (wt) 3'-UTR of neurokinin-1 into luciferase constructs. Reporter assays revealed that miR-613 repressed the luciferase activities of wt-neurokinin-1 3'-UTR (**Figure 3A**). We also introduced mutant (mut) neurokinin-1 3'-UTR into luciferase constructs and found that miR-613 had no effect on the activity of mut-neurokinin-1 3'-UTR (**Figure 3A**). In addition, ectopic miR-613 in HCT8 cells reduced neurokinin-1 expression (**Figure 3B**), while knockdown of miR-613 in DLD1 cells led to increased expression of neurokinin-1 (**Figure 3B**). miR-613 also inhibited the mRNA levels of neurokinin-1 (**Figure 3C**). These results demonstrate that miR-613 could
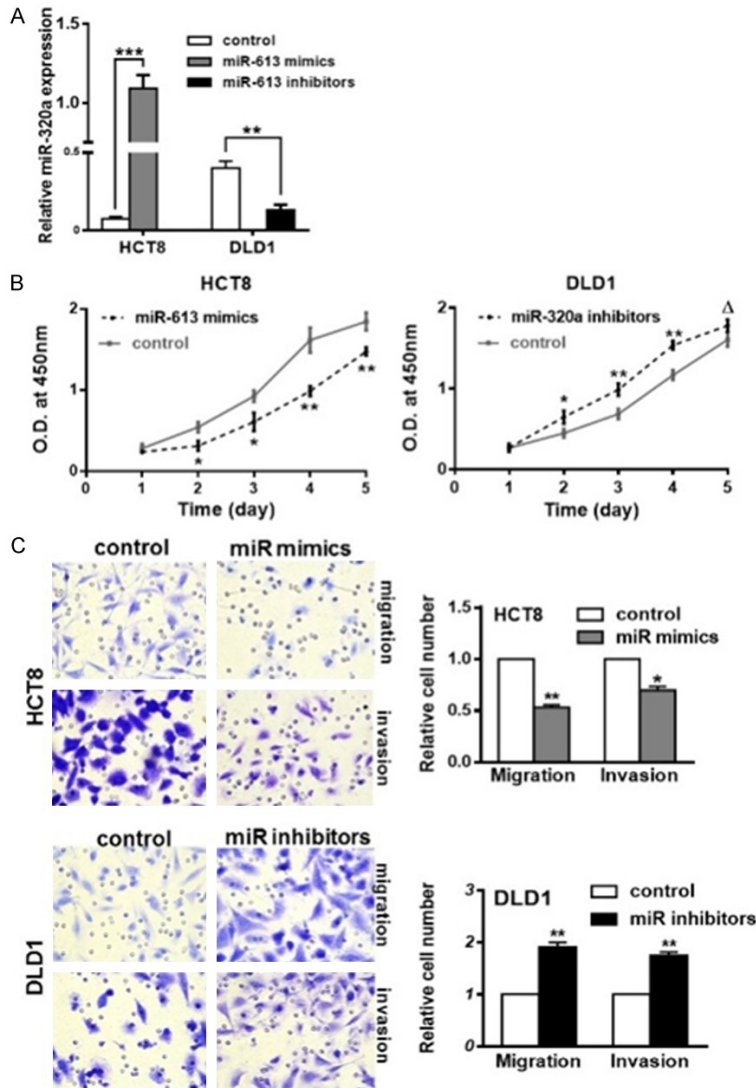
Figure 2. MiR-613 inhibits colon cancer growth and migration/invasion. A. The transfection efficiencies for miR-613 mimics andinhibitors were validated by qPCR. After transfection for 24 h, total RNAs were extracted and PCR for miR-613 was performed. miR-613 mimics transfection in HCT8 cells significantly upregulated miR-613 expression, and miR-320a inhibitors transfection in DLD1 cells significantly downregulated miR-613 expression. Data were analyzed by student's t test. B. MiR-613 mimics were introduced in HCT8 cells to upregulate miR-613 expression, and miR-613 inhibitors were introduced in DLD1 cells to downregulate miR-613 expression. CCK8 assay was performed in the following days to detect the effect of miR-613 on cell proliferation. The results showed that miR-613 could inhibit colon cancer growth. Data were analyzed with one-way analysis of variance. C. Migration/invasion inhibition in HCT8 cells after miR-613 mimics transfection. Cells were seeded in chambers after transfection for 24 h. After seeding for 28 h and 48 h, cells pass through the membrane were calculated respectively for migration assay and invasion assay. The results showed that the passed cells significantly decreased after miR-613 mimics transfection, while increased after inhibitors transfection. The value of "relative cell number" was calculated by experimental group/control group. Data were analyzed by student's t test. Vertical bars indicate SD. *, P<0.05; **, P<0.01; △, no significant difference.

directly target neurokinin-1 in colon cancer.

Neurokinin-1 is necessary for miR-613 induced cell proliferation and invasion

Neurokinin-1 is regarded as a positive regulator of cell proliferation in colon cancer [11]. Thus, we speculated that neurokinin-1 might act as an effector of miR-613 in the progression of colon cancer. To determine whether cancer cell phenotypes associated with miR-613 expression could be reversed via restoration of neurokinin-1, we stably transduced miR-613-expressing HCT8 cells with neurokinin-1 construct lacking the 3'-UTR regions and confirmed its over-expression. Results of CCK-8 assays showed that neurokinin-1 reversed at least partially miR-613-mediated repression of proliferation in vitro at the first three days (**Figure 4A**). In addition, the constitutive expression of neurokinin-1 rescued miR-613 induced invasion of HCT8 cells (**Figure 4B**). These data make it obvious that miR-613 inhibits proliferation and invasion by targeting neurokinin-1.

Discussion

Colon cancer is one of the most common malignancies with a high incidence and mortality worldwide. Patients with early cancer may have no symptoms, and most are diagnosed at late stage, even at autopsy [13]. The present study demonstrated that down-regulation of miR-613 contributed to colon cancer proliferative, invasive and metastatic capacities. To the be-
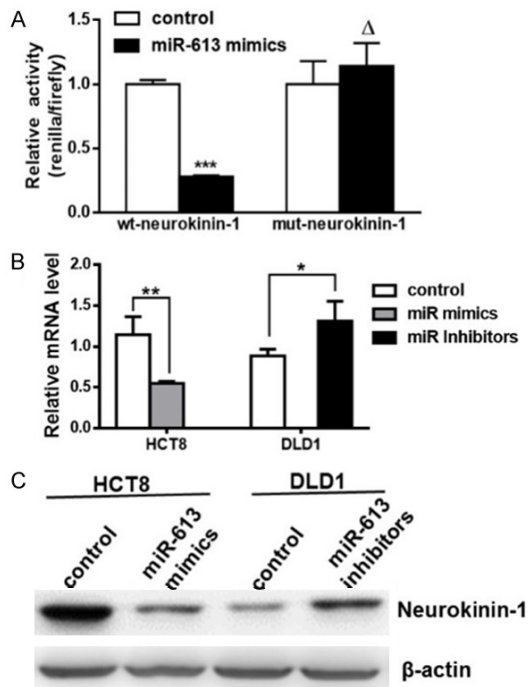
Figure 3. Neurokinin-1 is one target of miR-613. (A) Dual luciferase activity in 293T cells upon co-transfection of wild-type (wt) or mutant (mt) 3'-UTR-driven reporter construct and miR-613 mimics. We found that luciferase activity was decreased by miR-613 in wt-neurokinin1-3'UTR vector, whereas mutation of the binding sites in the 3'UTR-containing vector abolished responsiveness to miR-613. Data were analyzed by student's t test. (B and C) Neurokinin-1 mRNA (B) and protein expressions (C) in colon cancer cells after miR-613 overexpression and downregulation. MiR-613 alteration could significantly affect neurokinin-1 expression. Data were analyzed by student's t test. Vertical bars indicate SD *, P<0.05; **, P<0.01; **, P<0.001; △, no significant difference.

st of our knowledge, this is the first report about the biological function of miR-613 in colon cancer. In addition, we found that neurokinin-1 is one of the functional targets of miR-613. Given that the regulatory effect of neurokinin-1 in colon cancer [10], our study provides a novel perspective from the interaction between miR-613 and neurokinin-1 to explain underlying mechanisms of colon cancer carcinogenesis.

In this study we found that miR-613 expression in colon cancer tissues is significantly lower than that in normal colon mucosa tissues, and its expression was correlated strongly with tumor size and metastasis. Previously, miR-613 has been found to be deregulated in several tumors such as ovarian cancer [6, 14], non-small lung cancer [8], prostate cancer [9],

and esophageal squamous cell carcinoma [15]. Nonetheless, miR-613 can be severed as a prognostic factor for several cancers [14, 15]. In consistent with these results, the present study showed miR-613 can also be served as a predictor of colon cancer.

Previous data demonstrated that miR-613 functions as a tumor suppressor in the carcinogenesis and aggressiveness of several cancers. For example, miR-613 suppressed ovarian cancer cell proliferation, colony formation, and invasion, and KRAS was identified as a target of miR-613 [6]. In prostate cancer, miR-613 overexpression significantly suppressed prostate cancer cell proliferation and invasion through downregulating Wnt signaling pathway, and Frizzled7 could abrogate the inhibitory effect of miR-613 on cell proliferation and invasion as well as Wnt signaling pathway [9]. In non-small lung cancer, miR-613 reduced *in vitro* cell viability and colony formation by inducing cell cycle arrest by targeting CDK4 [8]. Consistent with these reports, our study showed that miR-613 negatively regulated the proliferation and invasion of colon cancer cells. It is necessary to further investigate the function of miR-613 in other malignancies to confirm if miR-613 is a cancer specific oncogenic makers.

miRNAs can bind to 3'-UTR of target mRNA transcripts of protein-coding genes and negatively control their translation or cause mRNA degradation. There are biological tools specifically designed for predicting these interactions. TargetScan is widely used to predict biological targets of miRNAs by searching for the presence of sites that match the seed region of each miRNA. In this study, we used this tool to screen potential target of miR-613. Indeed, a miRNA may produce targeting effects on multiple mRNAs, and vice versa. Our results demonstrated that neurokinin-1 was a functional target of miR-613 in colon cancer. The neurokinin-1 receptor is functionally coupled with G-protein and acts as a mediator of the biological activities encoded by the C-terminal sequence of tachykinins, for which substance P is the most effective agonist. Nonetheless, neurokinin-1 is overexpressed in tumor cells, including colon cancer [11, 16, 17]. A recent study demonstrated that neurokinin-1 can regulate proliferation of colon cancer cells [10]. A broad array of effector mechanisms is respon-
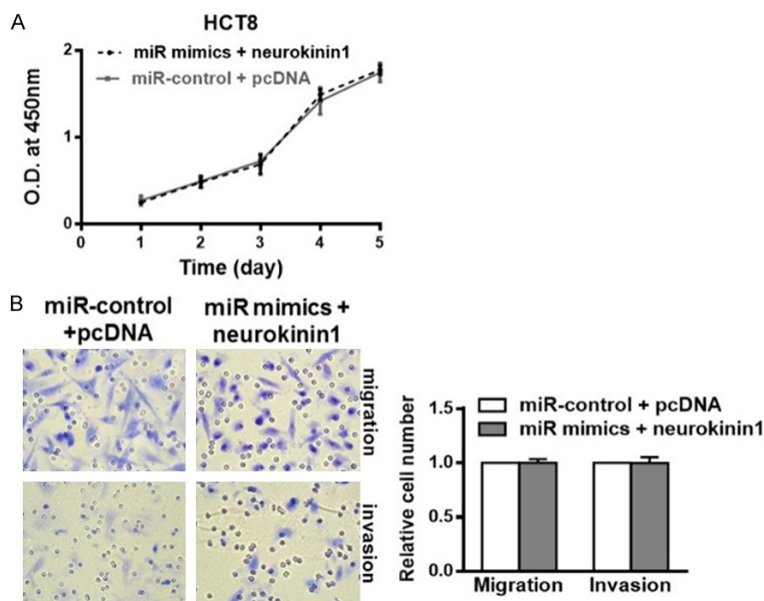
Figure 4. The interaction between miR-613 andneurokinin-1. Neurokinin-1 transfection could counteract miR-613 induced growth inhibition (A) and migration/invasion inhibition (B). HCT8 cells were co-transfected with miR-613 mimics and pcDNA3.1-neurokinin1. CCK8 assay and transwell assay for cell migration and invasion were performed after transfection. There was no significant difference between experimental group (co-transfection) and control group (pcDNA + miR-negative control), Data were analyzed by student's t test. Vertical bars indicate SD.

sible for the effects of neurokinin-1 in cancer cells, among which the activation of the mitogen-activated protein kinase cascade (MAPK) pathway is very important [18]. However, the other potential targets of miR-613 still need to be further investigated. According to a primary Wnt-reporter screen, miR-613 may function at upstream of β-catenin and then repress Wnt pathway [19]. So, the biological function of miR-613 may be executed by multiple effectors.

We must acknowledge that the present data have some limitations. (1) whether miR-613 is down-regulated in precancerous lesions (Intraepithelial neoplasia tissues, such as villous adenoma) is still not investigated; (2) other targets of miR-613 were not taken into this study; (3) the underlying mechanisms of miR-613 downregulation were still unclear.

In summary, our findings suggest that miR-613 is down-regulated in colon cancer and may affect proliferation, invasion and metastasis of colon cancer cells by inhibiting neurokinin-1. Restoration of miR-613 is a promising therapeutic approach for colon cancer treatment. Thus, our data demonstrate a potential mecha-

nistic connection between miR-613 dysregulation and colon cancer progression and may shed light on therapeutic strategies for colon cancer prevention and treatment.

Authors' contribution

Ji-Lin Hu designed the research; all authors wrote the paper; and Yun Lu critically revised the paper; all authors read, contributed to, and approved the final manuscript.

Address correspondence to: Dr. Yun Lu, Department of General Surgery, The Affiliated Hospital of Qingdao University, No. 1677 Wutaishan Road Huangdao District, Qingdao 266555, Shandong, PR China. Tel: +86-18661802231; Fax: +86-532-82911999; E-mail: cloudylucn@126.com

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