

Original Article

miR-145 inhibits tumor proliferation and invasion and induces apoptosis in gastric cancer by targeting c-Myc

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Abstract: Gastric cancer is one of the most common digestive tract cancers with high incidence of mortality and poor prognosis. Recent evidence has demonstrated that miR-145 plays a vital role in the proliferation and prognosis of gastric cancer. This study aimed to investigate the effect of miR-145 on proliferation, invasion, and apoptosis of gastric cancer, and its relationship with the target gene c-Myc. In this study, human gastric cancer cell line SGC-7901 was selected as study object. miR-145 mimic was synthesized and transfected into SGC-7901 cells. The effects of miR-145 on proliferation, invasion, cell cycle and apoptosis of SGC-7901 cells were examined by colony formation assay, transwell chamber invasion assay and flow cytometry assay. The expression of c-Myc was detected by Western blot. The results showed that miR-145 mimic was successfully transfected into SGC-7901 cells. The number of colony-forming units and invasive cells in the miR-145 mimic group were obviously lower than the miR-145 NC and normal group ($P<0.05$). And the G0/G1 phase cell rate and apoptosis cell rate in the miR-145 mimic group was significantly higher than the miR-145 NC and normal group ($P<0.05$). The expression of c-Myc protein in miR145 mimic group were significantly lower than miR-145 NC and normal group ($P<0.05$). Therefore, we concluded that miRNA-145 may play an anti-oncogene role by inhibiting cell proliferation and invasion, and promoting cell apoptosis, arresting cell cycle at G0/G1 phase in gastric cancer partially through suppressing the expression of c-Myc. In brief, miRNA-145 may become a new early diagnosis mark and therapy target in gastric cancer.

Keywords: Gastric cancer, miR-145, c-Myc, proliferation, invasion, apoptosis

Introduction

In recent years, the incidence of gastric cancer is upward gradually in our country, especially in the young aged group. The younger patients have characteristics of non-specific symptoms, high rate of misdiagnosis and high malignancy [1]. Surgical resection is the first choice of the treatment for gastric cancer. In recent years, as radiotherapy, chemotherapy and surgical techniques continue to improve, the quality of life and prognosis of patients has been greatly improved. However, tumor metastasis is not only the malignant marker and characteristics of gastric cancer, but also the main cause of failure to cure and lose their life of the patients with gastric cancer [2].

MicroRNAs (miRNAs) were a group of small and single strand noncoding RNA gene products about 21-23 nt long that were very conserva-

tive in the evolution. They are usually processed by Dicer from precursors RNA with a characteristic hairpin secondary structure [3]. miRNAs regulate the expression of target genes at the post-transcriptional level and serve as one of the most important signaling pathways related to genetic network and epigenetic modification. A growing body of evidences have suggested that miRNAs play significant roles in tumor occurrence and development [4]. miR-145 acts as a anti-oncogene role located in chromosome 9q31. Recent study has shown that miR-145 is abnormally expressed in a variety of solid tumors, such as gastrointestinal cancer, colorectal cancer, head and neck cancer and pancreatic cancer [5]. miR-145 regulates tumor proliferation via multiple target genes, such as KRAS, c-Myc and FSCN1 [6].

c-Myc is a key oncogene in the myc family and localized to chromosome 8q24. c-Myc is asso-

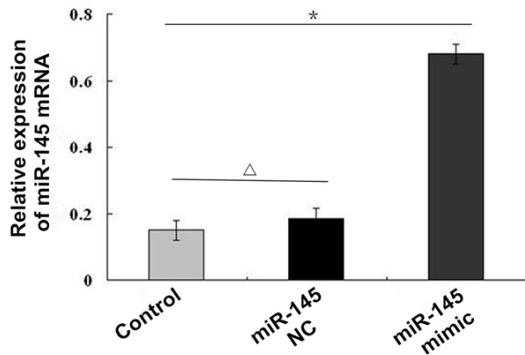


Figure 1. The level of miR-145 was significantly increased in SGC-7901 cells after miR-145 transfection (n=3). * $P < 0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $\Delta P > 0.05$ miR-145 NC group versus the Control group.

ciated with the occurrence, development, drug resistance, invasion and metastasis of many cancers, including gastric cancer, colorectal cancer, cervical cancer, pancreatic cancer and breast cancer [7]. Realignment, over-expression or amplification of the c-Myc gene will influence tumor cell proliferation, cell cycle progression and apoptosis [8]. c-Myc affect tumor cells in many aspects, including tumor angiogenesis, extracellular matrix degradation, invasion and metastasis. It has been found that miRNA-145 is downregulated in gastric cancer by acting as a tumor suppressor gene. However, the specific mechanism and its correlation with c-Myc oncogene remain unknown [9].

In our study, human gastric cancer cell line SGC-7901 was taken as study object. The cells were transfected with miR-145 mimic to observe the effect of miR-145 on the proliferation, invasion and apoptosis of gastric cancer. To explore the significance and effects of miR-145 on the proliferation, invasion, and apoptosis of gastric cancer, and the relationship between the target gene c-Myc were also discussed.

Materials and methods

Materials

The human gastric cancer cell line SGC-7901 was gifted by Chinese Academy of Sciences. Fetal bovine serum, penicillin-streptomycin, RPMI1640, the thiazolyl blue kit (MTT), trizol and trypsin all were purchased from RiboBio Bio-chemistry Co. Ltd (China). LipofectamineTM 2000 were purchased from TAKARA Co. Ltd (USA). SYBR PrimeScriptTM miRNA RT-PCR Kit

was bought from Promega Co. Ltd (USA). The cell apoptosis kit was bought from Sigma Co. Ltd (USA). Rabbit anti-human c-Myc and mouse anti-human β -actin polyclonal antibody were bought from BD Co. Ltd (USA). The primers sequence of miR-145 was designed by PCR primer design software Primer Premier 5, and miRNA analogs (miR-145 mimics), and negative control (miR-145 NC) synthesized by Invitrogen company. miR-145 mimics: 5'-ATCGTCCAGTTTCCCAGG-3'/5'-CGCCTCCACACACTCACC-3', miR-let-7i NC: 5'-ATTGGAACGATACAG AGAAGATT-3'/5'-GGAACGCTTCACG AATTG-3'.

Methods

Transfection and RT-PCR: A total of 1 mL (1×10^3 cells/mL) of cells of each group was seeded in 6-well plate for the following experiment. Cells were cultured with transfection reagent medium. miR-145 mimics and miR-145 NC were transfected using Lipo-2000 transfection kit. The samples were divided into normal group (Normal), negative control group (miR-145 NC), and miR-145 mimic group (miR-145 mimics). After transfection for 24 hours, the total RNA of each group was extracted by Trizol reagent. RNA template was reverse-transcribed using a PrimeScript RT Master Mix kit for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and maintained at 4°C. The mRNA expression level of miR-145 was determined using RT-qPCR. Applied Biosystems 7900HT Fast Real-Time PCR system was employed using 2 μ l total RNA with SYBR Green PCR master mix. The following primers were used for RT-qPCR. The PCR parameters were as follows: 95°C for 45 sec, followed by 40 cycles of 95°C for 30 sec and 60°C for 45 sec. Analysis of relative mRNA level data was examined by $2^{-\Delta\Delta Ct}$. ΔCt computational method: $\Delta Ct = Ct_{miR-145} - Ct_{U6}$. Experiments were performed in triplicate and repeated three times.

Colony formation assay: A total of 1 mL (1×10^3 cells/mL) of cells of each group were seeded in 6-well plate and were then cultured in DMEM at 37°C for 14 days to allow colonies to grow. The cells were then rinsed 3 times with PBS, fixed in 100% methanol at room temperature for 20 min, and stained with Giemsa stain (0.04%) for 30 min at room temperature. The number of the colonies containing at least 50 cells was counted using a light microscope at magnification, $\times 40$. The surviving fraction was calculated as the number of colonies counted/

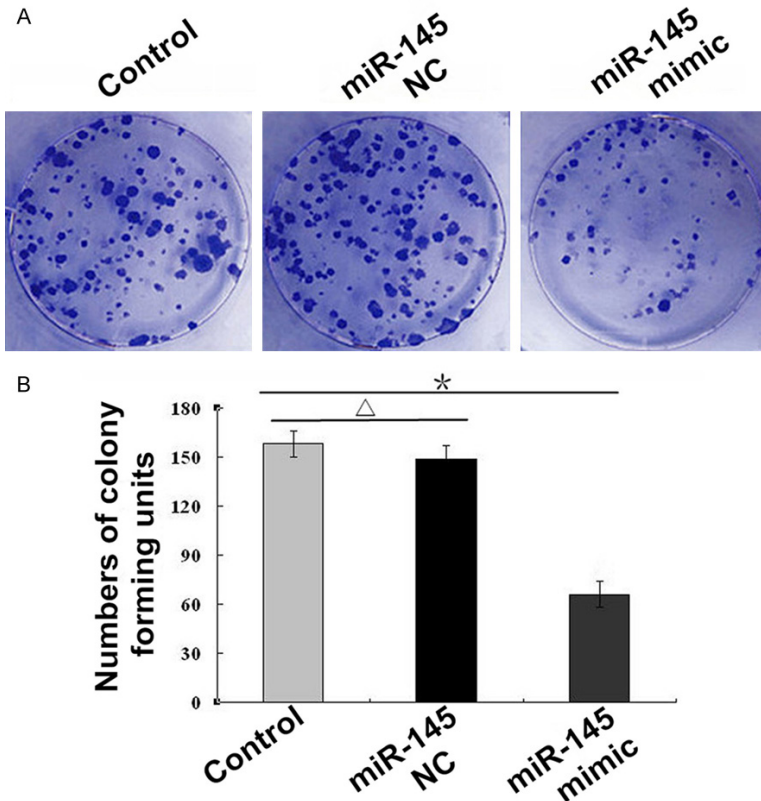


Figure 2. The effects of miRNA-145 on cell proliferation were detected by colony formation assay (n=3). A. Colony formation image ($\times 100$). B. The number of colony-forming units. * $P<0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $^{\Delta}P>0.05$ miR-145 NC group versus the Control group.

(the number of cells seeded \times plating efficiency). Experiments were performed in triplicate and repeated three times.

Transwell chamber invasion assay: Following 48 h transfection, 0.25% trypsin (containing EDTA) was used to digest cells. Cells were stained with 0.16% trypan blue dye solution for 3 min at room temperature. The cells at a concentration of $3-5 \times 10^5/\text{mL}$ of each group were suspended in serum-free RPMI 1640 medium and the lower layer of the Transwell chamber was plated with RPMI 1640 medium, supplemented with 10% fetal bovine serum. Cells were plated in the upper chamber and incubated at 37°C in an atmosphere containing 5% CO_2 for 24 h. The membrane was coated with Matrigel. Subsequently, the number of invaded cells were counted in 16 fields of view. Images were captured at a wavelength of 490 nm on a Leica DC 300F camera. Experiments were per-

formed in triplicate and repeated three times.

Cell apoptosis was analyzed by flow cytometry: After 48 h of the transfection, the cells in the logarithmic growth phase of each group were digested and counted. Then, the cells were collected by centrifugal tubes of 5 mL. After centrifugation, the cell precipitation were washed with PBS. Next, the cell suspension were supplemented with Annexin V-APC for staining and properly hid away from light at room temperature for 10 to 15 minutes. Experiments were performed in triplicate and repeated three times.

Cell cycle was detected by flow cytometry: For cell-cycle analysis, the method was same as above. After 48 h of the transfection, the cells in the logarithmic growth phase of each group were cultured in complete DMEM at 37°C in triplicate in 6-well plates. The cell-cycle distribution was analyzed by 0.05

mg/mL propidium iodide staining (37°C for 30 min) and flow cytometry.

The expression of c-Myc protein was detected by western blot: The cells of each group transfected with miR-145 in the logarithmic growth phase were prepared using a lysis buffer containing 10 mg/L aprotinin and 10 mg/L leupeptin. Protein content was quantified using the Bradford dye-binding procedure. Proteins were separated by SDS-PAGE (10% gels) followed by electrotransfer onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h and incubated with Rabbit anti-human c-Myc and mouse anti-human β -actin polyclonal antibody overnight at 4°C . All primary antibodies were used at a dilution of 1:1,000. Membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit IgG for 1 h at room temperature following washing with

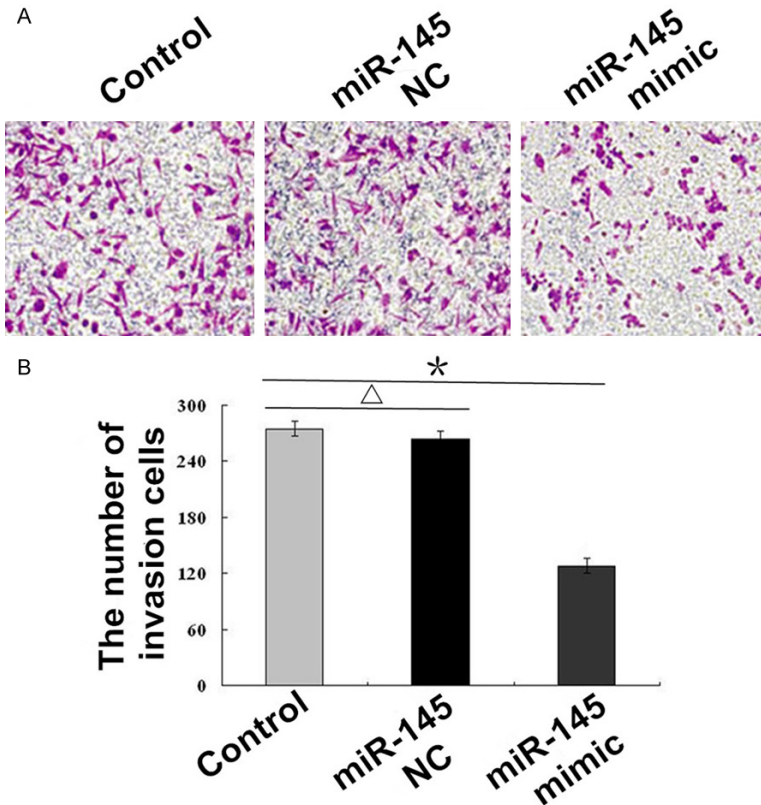


Figure 3. The effects of miRNA-145 on cellular invasion detected by Transwell invasion chambers (n=3). A. Transwell invasion chambers image ($\times 400$). B. The number of invasion cells. * $P < 0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $\Delta P > 0.05$ miR-145 NC group versus the Control group.

TBST for 15 min. Immunoblots were visualized by Odyssey V3.0 image scanning. Experiments were performed in triplicate and repeated three times.

Statistical analysis

SPSS17.0 statistics software was employed to undergo statistical analysis. The measurement data are represented by mean \pm standard deviation. The differences in expression between three groups (normal group, miR-145 NC group, miR-145 mimic group) were examined by one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant difference. * $P < 0.05$, $\Delta P > 0.05$.

Results

miR-145 mimic was successfully transfected

After 48 h transfection, the RT-PCR results showed that the relative level of miR-145 in miR-

145 mimic group was obviously higher than that in the miR-145 NC and normal group ($P < 0.05$, **Figure 1**). No statistical difference was observed between miR-145 NC and normal group ($P > 0.05$). It was suggested that miR-145 was successfully transfected into SGC-7901 cell.

miR-145 inhibits cell proliferation

The number of colony-forming units in the miR-145 mimic group were obviously lower than that in the miR-145 NC and normal group ($P < 0.05$, **Figure 2**). It was indicated that the miR-145 can inhibit colony formation of SGC-7901 cells.

miR-145 inhibits cell invasion and migration ability

The transwell chamber invasion results demonstrated that the number of invasive cells that passed through transwell chamber in miR-145 mimic group were significant-

ly lower than that in the miR-145 NC and Normal group ($P < 0.05$, **Figure 3**). It was demonstrated that miR-145 could inhibit cell invasion ability.

miR-145 promotes cell apoptosis

The flow cytometry results showed that the apoptosis cells rate in the miR-145 mimic group were significantly higher than that in the miR-145 NC and normal group ($P < 0.05$, **Figure 4**). It was suggested that the miR-145 could promote cell apoptosis.

miR-145 arrests tumor cells in G0/G1 phase

The flow cytometry results showed that the rate of G0/G1 phase in the miR-145 mimic group were $(85.08 \pm 2.5)\%$, whereas miR-145 NC group and normal group were $(62.81 \pm 3.2)\%$ and $(70.03 \pm 1.5)\%$, respectively. The G0/G1 phase cells in miR-145 mimic group were apparently increased as compared with

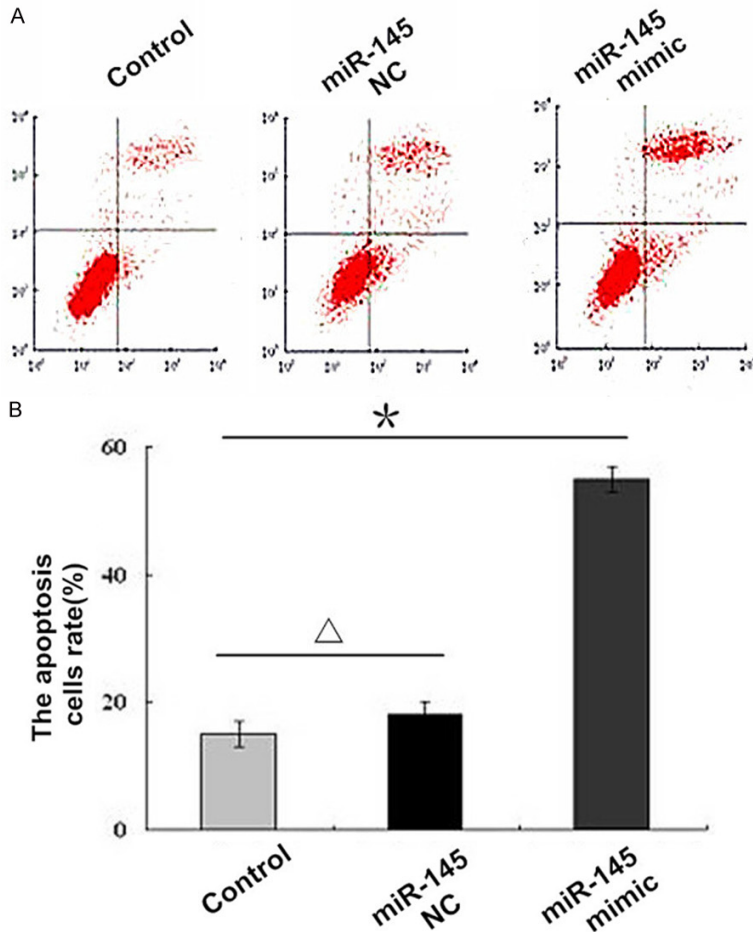


Figure 4. The effects of miRNA-145 on cell apoptosis detected by detected by Flow Cytometry (n=3). A. Flow Cytometry image ($\times 400$). B. The apoptosis cells rate. * $P < 0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $\Delta P > 0.05$ miR-145 NC group versus the Control group.

that in the miR-145 NC and normal group ($P < 0.05$, **Figure 5**). It was demonstrated that the miR-145 could arrest cell at G0/G1 phase.

miR-145 suppresses the expression of c-Myc

The western blot outcome demonstrated that the expression of c-Myc protein in miR-145 mimic group were significantly lower than that in the miR-145 NC group and normal group ($P < 0.05$, **Figure 6**). It was indicated that miR-145 can suppress the expression of c-Myc.

Discussion

MicroRNAs are a class of small non-coding RNA molecules, about 21-23 nucleotides in length. miRNAs can inhibit protein translation by binding to the 3'UTR region of the target gene, or by directly degrading mRNA of the target gene. miRNAs are involved in the occurrence and

development of many diseases [1], and much is known about the roles of miRNAs in tumors. Increasing studies suggested that abnormal expressions of miRNA are observed in many tumors, such as non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, etc. miRNA plays important roles by regulating its target genes. One miRNA may regulate several target genes, and one target gene receives regulation from multiple miRNAs. miRNAs only account for 2% of total gene number in humans, but regulate the expression of over 30% of total genes. miRNAs along with its target genes form an extensive genetic network that regulates the growth, development, differentiation and apoptosis of the organisms [10]. Therefore, miRNAs and its target genes are among the hot topics in cancer research.

miR-145 is localized at chromosome 9q31 and displays some common features of miRNAs, including chronology, conservativeness, and tissue specificity. It participates in the growth, proliferation, development and apoptosis of tumor cells [2]. Under physiological conditions, miR-145 promotes protein secretion in the liver [11], and upregulates the expression of smooth muscle cell markers and facilitates the differentiation of vascular smooth muscle cells [12]. miRNA even plays a regulatory role in embryonic stem cells and nuclear transcription factors [13]. But under pathological conditions, miR-145 is involved in tumor occurrence and development. Studies have shown that miR-145 is downregulated in many solid tumors, especially in lung cancer, ovarian cancer, colorectal cancer, cervical cancer and gastric cancer. Thus miR-145 may act as a tumor suppressor gene that negatively regulates tumor occurrence and progression [14]. Further research have indicated that miR-145 can regulate multiple target ge-

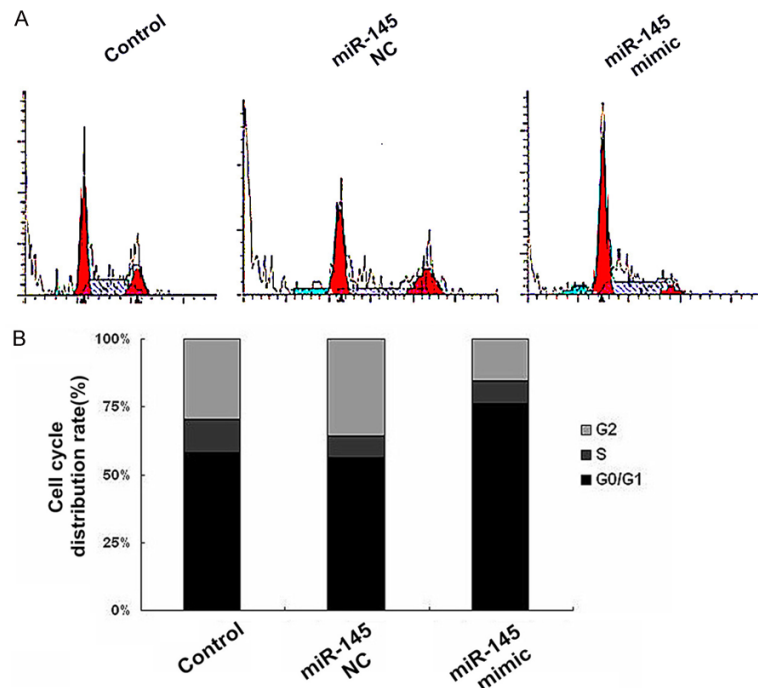


Figure 5. The effects of miRNA-145 on cell cycle were detected by Flow Cytometry (n=3). A. The distributions figure of cell cycle. B. Cell cycle distribution rate. * $P < 0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $\Delta P > 0.05$ miR-145 NC group versus the Control group.

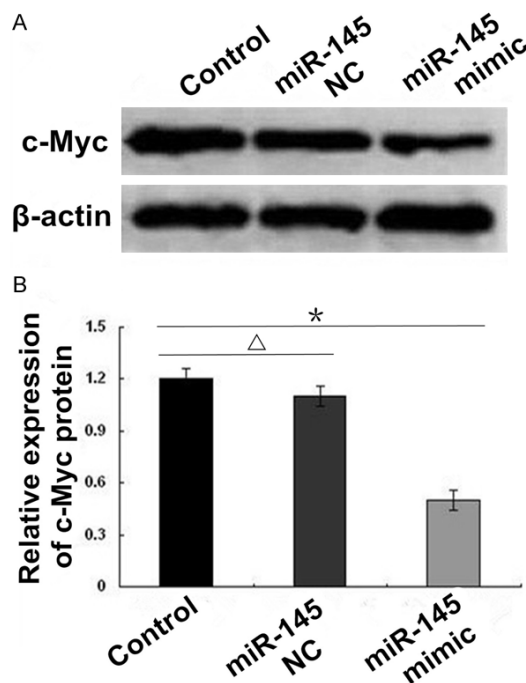


Figure 6. The expression of c-Myc were detected by Western blot (n=3). A. Protein blotting stripe. B. The relative protein content. * $P < 0.05$ miR-145 mimic group versus the miR-145 NC group and Control group, $\Delta P > 0.05$ miR-145 NC group versus the Control group.

nes, such as KRAS, c-Myc, FSCN1 and OCT4, thereby serving as a potential therapeutic target [15].

c-Myc oncogene is an important target gene regulated by miR-145 and localized at chromosome 8q24. c-Myc can encode for transcription factor and display DNA-binding activity, thus activating transcription and promoting cell proliferation [16]. c-Myc is thought to relate to the occurrence, development and drug resistance of many tumors. Realignment, overexpression or amplification of c-Myc have been detected in many cancers, including gastric cancer, colorectal cancer, cervical cancer, pancreatic cancer and breast cancer, which leads to overexpression of the c-Myc protein. An overexpression of c-Myc under physiological conditions can promote

transcriptional activation and uncontrolled cell proliferation, which finally results in cancer occurrence. Its overexpression in cancers can promote cell cycle progression, accelerate tumor cell growth and increase the invasiveness. This will probably lead to tumor recurrence and metastasis. In a word, c-Myc is an important therapeutic target in tumor therapy [17].

A growing body of evidence has proved that miR-145 is significantly downregulated in gastric cancer, while the c-Myc protein is upregulated. miR-145 and c-Myc are probably the key factors in invasion and metastasis of gastric cancer [18]. In our study, we found that miR-145 was downregulated in SGC-7901 cells, while its overexpression can greatly inhibited the cell proliferation and invasion ability and promote cell apoptosis. Furthermore, the expression of the c-Myc protein was also down-regulated considerably.

To conclude, miR-145 was down-regulated in gastric cancer and play a anti-oncogene role by inhibiting the cell proliferation, invasion and migration, and promoting cell apoptosis, arresting cell cycle at G0/G1 phase in gastric cancer partially through suppressing the expression of

c-Myc. In brief, miRNA-145 may become a new early effective diagnosis mark and potential therapy target in gastric cancer, its deserves further studying.

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Disclosure of conflict of interest

None.

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