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Original Article

Effects and mechanisms of miR-133a on invasion and migration of gastric cancer cells MKN28

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Abstract: Objective: To study the influence of miR-133a on the invasion and migration of gastric cancer cells and to explore the mechanisms contained within it. Methods: The expression of miR-133a in gastric cancer cell lines with high metastatic BGC-823 lines, gastric cancer cell lines with low metastatic MKN-28 lines and gastric cancer tissues were detected by qPCR; the miR-133a inhibitor was transfected into gastric cancer cells by liposome-mediated transfection method, and then a blank control group was set. The effect of miR-133a inhibitor on the migration and invasion of gastric cancer MKN-28 cell lines was determined by transwell migration assay and invasion assay lines; the expression amounts of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in gastric cancer MKN-28 cell lines influenced by the miR-133a inhibitor were detected by Western blot. Results: The result of qPCR showed that the expression of miR-133a in gastric cancer cell lines with high metastatic lines BGC-823 lines was significantly lower than that in gastric cancer cell lines with low metastatic MKN-28 lines ($P < 0.05$). The down-regulation of miR-133a expression significantly promoted MKN-28 cell lines Invasion and migration ability ($P < 0.05$), and at the same time the down-regulation of miR-133a expression up-regulated the expression of MMP-2 and MMP-9. Conclusion: The low expression of miR-133a can significantly promote the invasion and migration ability of gastric cancer cells, which may be related to up-regulation of matrix metalloproteinase.

Keywords: MiR-133a, invasion and migration, gastric cancer cells

Introduction

Gastric cancer is a kind of common cancer and with high incidence and its morbidity and mortality are in the forefront of the tumor [1, 2]. In recent years, with the development of science and technology, the work of gastric cancer diagnosis and treatment has made great progress, but studies have reported that the 5-year survival rate of gastric cancer patients has been still lower than 30% [3, 4]. The scholars reported that the main reason was the high recurrence rate and high metastasis rate [5, 6]. Degradation of extracellular matrix is necessary for the invasion and metastasis of gastric cancer cells, MMP-2 and MMP-9 which can degrade extracellular matrix play an important role in the invasion and migration ability of tumor cells. Therefore, it is of great significance to study the mechanisms of invasion and metastasis of gastric cancer by exploring the high sensitivity and high specificity of biological indicators. Previous studies found that a series of APC, P53 and other gastric cancer related to tumor suppressor genes and oncogenes, but the mechanisms of gastric cancer were still not so clear [7, 8]. Recently, microRNAs have become one kind of the hot biomolecules in tumor research. Numerous studies have shown that microRNAs have been intimately linked with the tumor in the process of its proliferation, metastasis, invasion, differentiation and apoptosis, especially playing a very critical role in the occurrence and development of many tumors, like the gastric carcinoma [9-11]. The latest study suggested that down-regulation of miR-133a expression in the breast cancer tissues was in negative relation with tumors' metastasis [12, 13]. Besides, miR-133a also has great inhibitory effects on invasion of bladder cancer cells and esophageal squamous cell carcinoma cells [14, 15]. Thus, it is clear that miR-133a can play a significant role in tumor suppression by regulating the expression of oncogenes.

In order to determine the effects of miR-133a on invasion and migration of gastric cancer cells, in this research, we compared the expression of miR-133a in gastric cancer cell lines with high metastatic BGC-823 lines, with its gastric cancer cell lines with low metastatic MKN-28 lines, and gastric carcinoma tissues. Respectively, and then, transfected the miR-133a inhibitor into gastric cancer cells through the transfection method of liposome-mediated to investigate the possible mechanisms and functions of miR-133a on the invasion and metastasis of gastric cancer cells.

Materials and methods

Cell lines

Human gastric cancer cell lines with high metastatic BGC-823 lines and low metastatic MKN-28 lines were kept and cultured in DMEM with 10% fetal bovine serum, being prepared for the experiments.

Tissues specimens

Patients have given their informed consent to the clinical samples before the experiment, which were also approved by the ethic committee. Among those specimens, the fresh gastric cancer tissues were from patients who underwent subtotal distal gastric to my operation, without any radiotherapy or chemotherapy. All cases were diagnosed as gastric carcinoma by pathological diagnosis. A total of 10 specimens were collected in this study, include the specimens of the cancer tissues and para-carcinoma tissues.

Reagent and instrument

High glucose medium DMEM and fetal bovine serum were obtained from the American company Gibico; crystal violet was from the American company Sigma; GAPDH antibody, rabbitanti-human matrix metalloproteinase-2 (MMP-2) and MMP-9 antibody were purchased from Santa cruz; Transwell chambers were from Corning; miR-133a was obtained from Jima company in Shanghai; Trizol Regent and Lipofectamine TM 2000 transfection reagents were obtained from the American company Invitrogen; the inverted microscope was from Japanese Nikon; Real-time PCR was obtained from ABI; Gel imaging system was purchased from Bio-Rad.

qPCR analysis of miR-133a expression in gastric cancer cell lines and gastric cancer tissues

Total miRNA of gastric cancer tissues and gastric cancer cell lines were extracted through the one-step method miRNA. Using TaqMan MicroRNA reverse transcription test kit, reverse transcriptase the above extraction of miRNA to cDNA and add miR-133a or internal reference U6 primers for amplification. The primers were designed as follows, miR-133a upstream primer: CTGCGATATGACCTCTCAC-3', downstream primer: 5'-AGATCTTGAGTGCTTTTCC-3'. The reaction system: upstream and downstream primers were 0.4 μL, SYBR Green Realtime PCR Master Mix 10 μL, cDNA 2 μL, ddH_2O 7.2 μL. The reaction requirement: pre denaturation 10 min in 95°C, denaturation 15 s in 95°C and annealing/extension 1 min at 60°C, a total of 40 cycles. After the completion of PCR, analyze the gene amplification conditions in ABI 7300 System software to obtain the corresponding Ct value, and correct the copy number of the PCR template by U6 as an internal reference, then calculate the relative expression amount of gene by 2-ΔΔCt method.

Cell migration experiment

We inoculated MKN-28 lines into 96-well culture plate. When the rate of cell fusion reached 60%, the miR-133a inhibitor transfected cells were mediated by Lipo2000 transfection reagent, then miR-133a transfected cells were set as a control group. About 48 h after transfection, trypsin were used to digest the cells, then they were inoculated into transwell chambers, with 3 complex wells in each group. The upper chamber was set 100 μL serum-free DMEM culture medium, and the lower chamber was set 500 μL DMEM culture medium which contained 10% fetal bovine serum. After having continued to culture for 24 h in 37°C incubator, the transwell chambers were taken out and set in 0.1% crystal violet staining liquid for 10 min, washed 3 times by PBS, then the upper unmigrated cells were wiped off with cotton swap. Under the light microscope, observation field were randomly selected and pictures were taken. Then with 33% acetic acid dehydrating, the crystal violet were eluted completely and the OD value of the elution liquid was detected in Microplate Reader at 570 nm, which indicated the migration ability of the cells.

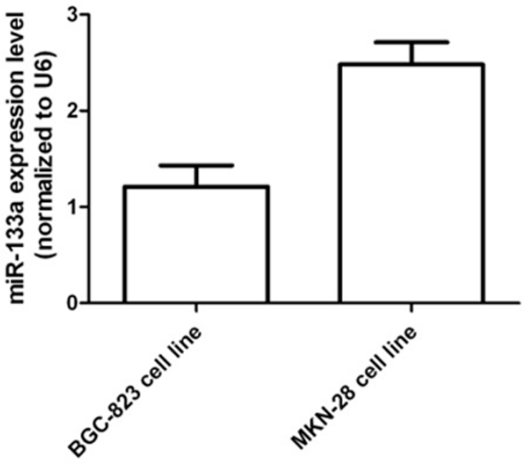

Figure 1. The expression of miR-133a in gastric Cancer Cells ($P < 0.05$).

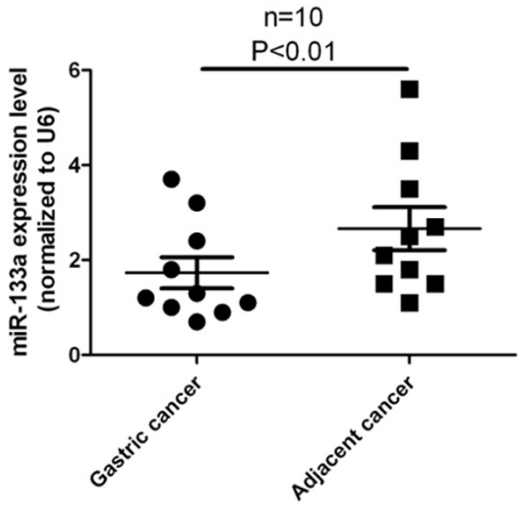

Figure 2. The expression in gastric cancer tissues.

Cell invasion experiment

Precooling DMEM culture solution were applied without fetal bovine serum to dilute Matrigel, and spreaded evenly on the membranella (8 μm) of Transwell cell. The Subsequent operations should be operated according to cell migration experiment method. Finally, the crystal violet were eluted completely with 33% acetic acid and OD value on Microplate Reader was detected at 570 nm, which indicated the invasion ability of the cells.

The protein expression of MMP-2 and MMP-9 detected by Western blot

When the rate of cell fusion reached 60%, miR-133a inhibitor was transfected into cells by Lipo2000 transfection reagent. 48 h after transfection the protein lysis buffer RIPA were added, the cell total protein was extracted and the protein concentration by BCA method was detected. After SDS-PAGE gel electrophoresis, the protein samples were transferred to the PVDF membrane. Then the membrane was placed in the TBST of 5% non-fat milk powder, and blocked at room temperature for 1 h. The first antibody was incubated overnight in the shaker at 4°C. After being rinsed for three times, PVDF membrane was placed into the second antibody to incubate at room temperature for an hour. Besides, ECL reagent was added to develop the color and the Gel imaging system was used to image in chemical exposure mode. Bio-Rad image software was applied to measure the integrated optical density (IOD value) of the target brand. GAPDH served as a loading control.

Statistical methods

SPSS 18.0 software was used to conduct statistical analysis of data. The mean ± standard deviation ($\bar{X} \pm S$) was applied to express measurement data and T test was used for the comparison between two groups. Enumeration data was expressed by rate and χ^2 test was used for the comparison between the two groups. A $P < 0.05$ indicates difference with statistical significance.

Results

The expression of miR-133a in gastric cancer cells

According to qPCR test, the expression level of miR-133a in high metastatic gastric cancer BGC-823 lines (1.21±0.22) was much lower than that of low metastatic MKN-28 (2.48±0.23) lines, the difference between the expression of the two cell lines had statistical significance (**Figure 1**, $P < 0.05$). The expression of MKN-28 cells was higher and subsequent experiments were conducted.

The expression in gastric cancer tissue

qPCR test was used to measure the expression level of the ten samples of gastric cancer tissues (para-carcinoma and cancer tissues). The expression of miR-133a in cancer tissues was much lower than that in para-carcinoma (**Figure 2**). As there was an obvious difference between

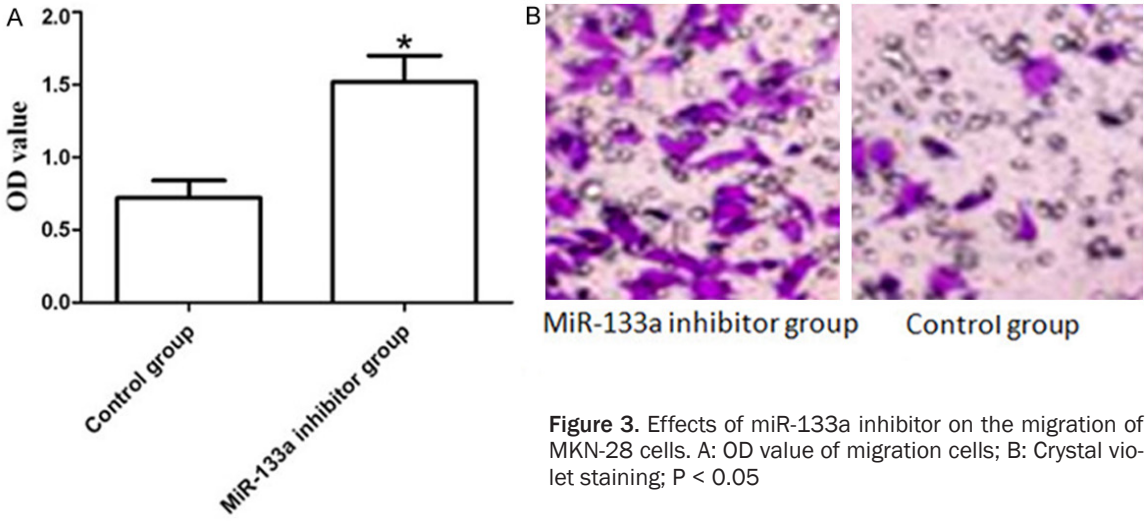

Figure 3. Effects of miR-133a inhibitor on the migration of MKN-28 cells. A: OD value of migration cells; B: Crystal violet staining; $P < 0.05$

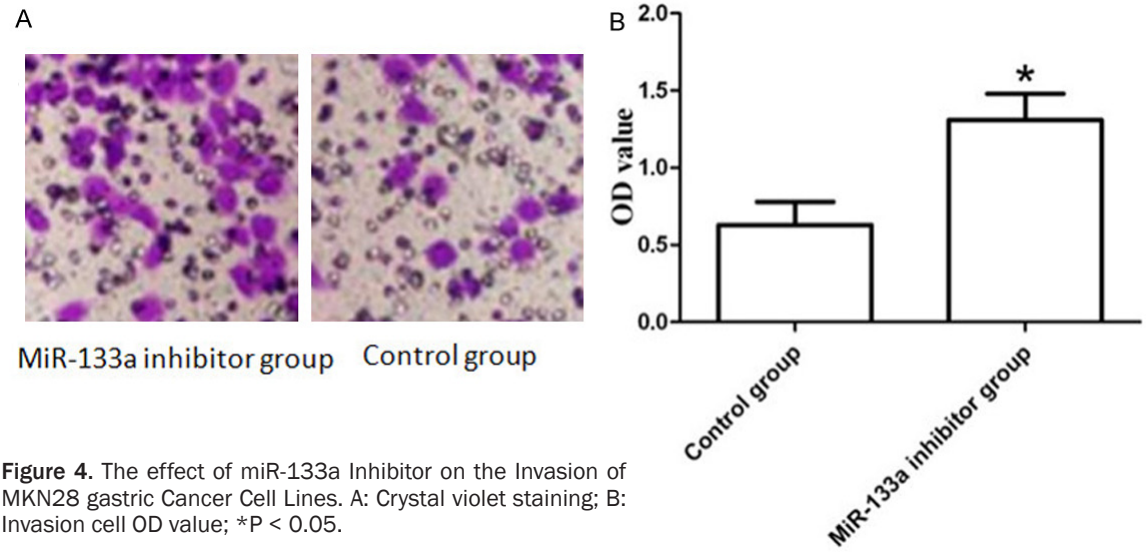

Figure 4. The effect of miR-133a Inhibitor on the Invasion of MKN28 gastric Cancer Cell Lines. A: Crystal violet staining; B: Invasion cell OD value; *$P < 0.05$.

the two groups ($P < 0.05$), miR-133a might express in disorder in the gastric cancer tissue.

Effects of miR-133a inhibitor on the migration of MKN-28 cells

48 hours after miR-133a inhibitor transfection, compared with control group (OD value: 0.72±0.12), miR-133a inhibitors significantly promoted the migration ability of MKN-28 cells (OD value: 1.52±0.18) (**Figure 3**, $P < 0.05$).

Effects of miR-133a inhibitor on the invasion of MKN-28 cells

The application of invasion assay showed that the invasion ability of MKN-28 cells was markedly improved (1.31±0.17) when miR-133a was down-regulated (**Figure 4**). Compared with the control group, there was an obvious difference with $P < 0.05$. So miR-133a inhibitor was able to accelerate the invasion of gastric cancer cells.

The effects of miR-133a inhibitor on the expression of matrix metalloproteinases in MKN28 gastric cancer cell lines

As shown in **Table 1** and **Figure 5**, miR-133a inhibitor significantly increased the expression of MMP-2 and MMP9 in gastric cancer MKN-28 cell lines compared with the control group ($P < 0.05$).

Table 1. The effect of miR-133a inhibitors on MMPs expression in MKN28 gastric cancer cell liness

Group	MMP-2 Relative expression amount	MMP-9 Relative expression amount
Control group	1.10±0.11	0.98±0.07
miR-133a Inhibitor group	1.72±0.12*	1.53±0.10*

Compared with the control group, *P < 0.05.

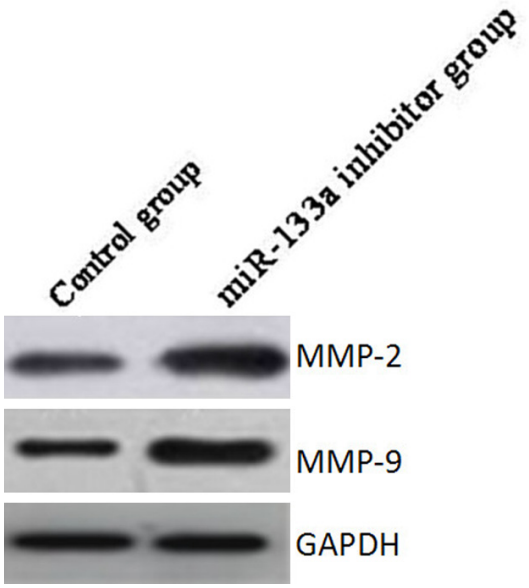

Figure 5. The effect of miR-133a inhibitor on the expression of MMP-2 and MMP-9 in MKN28 gastric cancer cell lines.

Discussion

In recent years, the morbidity of gastric cancer has been kept increasing in China. The main causes of death in patients with gastric cancer are invasive growth and metastasis. Studies have shown that metastasis and invasion of tumor is a complex biological process in which multi-factors interact with each other and influence on each other and this process may involve extracellular matrix and basement membrane decomposition, migration of tumor cells, the decreasing of adhesion ability between cells and other factors [16, 17].

miRNAs are a class of highly conserved endogenous non coding small molecule RNA [18, 19]. They can participate in tumor angiogenesis, epithelial-mesenchymal transition, extracellular matrix degradation to influence tumor invasion and metastasis through post-transcriptional inhibition of target gene expression. In recent years, more and more miRNAs have been proved to be involved in tumorigenesis and development [20, 21]. For example, miR-143 was down-regulated in carcinoma of colon, miR-26a was down-regulated in lung cancer, miR-21 was up-regulated in breast cancer through the regulation of TIMP-3 expression to promote the migration and invasion ability of breast cancer cells. It could be clearly seen that the effect of down-regulation or up-regulation of the expression of different miRNAs suggested that the gene encoding the miRNA might act as a tumor suppressor gene or an oncogene in tumor.

In the past few years, previous studies showed that miR-133a expression in a variety of tumors was significantly reduced [22], but the role of miR-133a in gastric cancer still remained unclear. To determine the effects of miR-133a on the invasion and migration of gastric carcinoma cells, the expression of miR-133a was detected in gastric cancer cell lines with high metastatic BGC-823 lines and gastric cancer cell lines with low metastatic MKN-28 lines and gastric carcinoma tissues by qPCR in this study; the effects of miR-133a inhibitor on migration and invasion of gastric carcinoma cell lines were observed by Transwell migration assay and invasion assay. The results showed that the expression of miR-133a in gastric cancer cell lines with high metastatic BGC-823 lines was significantly lower than that in gastric cancer cell lines with low metastatic MKN-28 lines ($P < 0.05$), and the down-regulation of miR-133a expression significantly promoted the invasion and migration ability of MKN-28 cells ($P < 0.05$), indicating that miR-133a negatively regulated the migration and invasion of gastric carcinoma cells. This is consistent with the role of miR-133a in other types of tumors [23].

Matrix metalloproteases (MMPs) are a class of proteolytic enzymes that degrade extracellular matrix and play an essential role in tumor cell migration and invasion. Among them, MMP-9 can not only degrade extracellular matrix, but also break down the basement membrane, thereby increasing cell migration ability to pro-

mote tumor proliferation and metastasis. MMP-2 is an invasive marker of malignancy, which can not only degrade Collagen IV which will promote the infiltration of tumor cells to surrounding environment, but also advance the spread of tumor through nascent capillaries. Studies have shown that MMP-2 and MMP-9 in gastric carcinoma tissue expression is significantly higher than those in normal tissue [24]. MMP-9 overexpression can increase the invasion of the tumor, affecting the prognosis of patients [25]. Previous studies showed that miRNAs could regulate the expression of MMP-2 and MMP-9 in the migration and infiltration process of tumor cells [26, 27]. In this study, we found that miR-133a inhibitor could significantly upregulate the expression of MMP-2 and MMP-9 in gastric carcinoma cell lines by Western blot. This suggested that miR-133a might affect the invasion and migration ability of tumor cells through the expression of MMP-2 and MMP-9 in gastric carcinoma, thus affecting the prognosis of patients.

In summary, on the one hand, our research found that miR-133a low expression could significantly promote the invasion and migration ability of gastric carcinoma cells, and the upregulation of matrix metalloproteases. On the other hand, we also speculated and confirmed the role that miR-133a played in the gastric carcinoma. This study will make further exploration of miR-133a regulated target genes and related signaling pathways, so as to provide new ideas for the treatment of gastric carcinoma.

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Disclosure of conflict of interest

None.

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