

Review Article

Effect of miR-182 targeting MTSS1 on the proliferation and metastasis of esophageal cancer

Qingfeng Jiang, Yingkun Ren, Jiwei Cheng, Jinhua Cheng, Jianjun Qin, Yin Li

Department of Thoracic Surgery, Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou, Henan, China

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Abstract: MTSS1 is a tumor metastasis suppressor which participates in the regulation of metastasis of a variety of malignancies. Studies have found that miR-182 plays a role in metastasis through regulation of MTSS1. Therefore, this article intends to study the role of miR-182 in the proliferation and metastasis of esophageal cancer by targeting MTSS1. A total of 89 patients with esophageal cancer were included. The levels of serum miR-182 were measured by qRT-PCR. The relationship between serum miR-182 level and tumor metastasis was analyzed. Targets of miR-182 were determined by bioinformatics and luciferase reporter gene assay. miR-182 was over-expressed in esophageal cancer KYSE410 cells. Protein levels of MTSS1 were measured. Flow cytometry was used to monitor the changes in cell cycle and transwell assay was used to measure the capability of cell invasion migration. The levels of serum miR-182 in esophageal cancer patients with metastasis were significantly higher than that of esophageal cancer patients without metastasis. Bioinformatics and luciferase reporter gene assay results showed that miR-182 binds to 3'UTR of MTSS1 mRNA. In vitro results showed that overexpression of miR-182 significantly decreased the expression level of MTSS1 in KYSE410 cells ($P < 0.05$), but significantly increased the proliferation and invasion capability of KYSE410 cells ($P < 0.05$). Patients with metastatic esophageal cancer had significant higher plasma levels of miR-182 than that of patients with non-metastatic esophageal cancer. Over-expression of miR-182 inhibited the expression of MTSS1 in KYSE410 cells to promote cell proliferation and enhance the capability of cell invasion and metastasis.

Keywords: miR-182, MTSS1, esophageal cancer, cell proliferation, cell invasion

Introduction

Esophageal cancer is one of the human malignancies originated from esophageal squamous cells or glandular epithelial cells. The incidence and mortality of esophageal cancer are ranked in the forefront of human malignancies [1]. In China, about 15 million people die from esophageal cancer each year, accounting for 21.8% of the mortality of cancer [2]. The cause of esophageal is not clear, but it is generally agreed that it is related to diet, genetic factors, and environmental carcinogens [3]. With the advances in medical science, esophageal cancer can be treated more effectively than in the past, but the high metastasis rate of esophageal cancer makes it difficult to improve the efficacy of treatment. But if it is treated in the early stages of metastasis, the survival rate of esophageal cancer patients will be greatly improved [4]. Therefore, screening biomarkers

for effective diagnosis and treatment of esophageal cancer in early stages is of great significance.

Belonging to the microRNA family, miR-182 is a small non-coding RNA with a length of 21-25 bp [5]. Studies have shown that, microRNA can affect the behavior of cells by regulating levels of target genes [6]. Recently, scholars have found that compared to normal cells, in esophageal cancer cells have abnormal miR-182 expression, suggesting that miR-182 plays an important role in the occurrence and development of esophageal cancer [7]. It has been shown that miR-182 can regulate the expression of various genes including MTSS1 [8]. MTSS1 is a tumor metastasis suppressor which involves in the invasion and metastasis of colorectal cancer, breast cancer, and other human malignancies [9, 10]. This study is designed to investigate the role of miR-182 and

MTSS1 in the proliferation and metastasis of esophageal cancer.

Material and methods

Subjects

A total of 89 patients with esophageal cancer from June 2010-June 2014 of The Affiliated Tumor Hospital of Zhengzhou University (Henan, China) were included in this study. There were 48 males and 41 females with an average age of 53.6 ± 6.2 years (range: 42 to 73 years). All subjects enrolled in this study were diagnosed by barium swallow exam, B-scan ultrasonography, and histopathological analysis. According to WHO classification criteria for grading esophageal cancer, 24 patients had I+II grade esophageal cancer, and 65 patients had III+IV grade of esophageal cancer. No patient received any chemotherapy, radiotherapy, or surgical treatment before admission. A total of 30 healthy adults with the similar age were included as controls. This study was approved by the ethics committee of The Affiliated Tumor Hospital of Zhengzhou University and all patients signed consent forms.

RNA extraction and qRT-PCR

10 mL of fasted blood was collected from each patient and plasma was collected by centrifugation. Total RNA was extracted by Rneasy MiNi Kit (QIAGEN). mirVanat qRT-PCR miRNA Detection Kit (Ambion) was used to perform qRT-PCR with the following profile: an initial 3 min incubation at 95°C, 40 cycles of (95°C/15 s and 60°C/30 s). Results were analyzed using software V2.02. 2- $\Delta\Delta C_t$ method was used to calculate relative quantities [11].

Function prediction of miR-182

TargetScan 5.1 (www.targetscan.org) software was used to predict the function of miR-182. Luciferase reporter gene assay was used to verify possible miR-182 targets.

Luciferase reporter gene assay

Primers were synthesized according to 3'UTR region of MTSS1 mRNA sequence (Genbank accession number NM_001282971). MTSS1-F: 5'CTAGCGGCCGCTA-GTCTTTTGATTTTCTGAAGGTGCCAAAT3', MTSS1-R: 5'AGATTTGGCACCTT-

CAGAAAAATCAAAGACTAGCGGCCGCTAGTTT3'. Amplified MTSS1-3'UTR was inserted into the downstream of coding region of pmirGLO firefly luciferase vector to construct pmirGLO-MTSS1. pmirGLO-MTSS1 and pmirGLO were transfected into HEK293 cells, followed by transfection of miR-182 mimics. 48 h after transfection, dual luciferase reporter assay system (Promega) and MicroLumatPlus LB96V luminometer (Berthold) were used to analyze fluorescence intensity [12].

Overexpression of miR-182

miR-182 mimics were synthesized according to the sequence of miR-182 and transfected into cells at a final concentration of 50 nM using INTERFER in TM liposome transfection kit (Polyplus transfection). KYSE410 cells and HEK293 cells were purchased from cell bank of Chinese Academy. Transfection was performed 24 h after inoculation of cells according to manufacturer's instruction.

Western-blot

Cells were collected, lysed, and centrifuged to get supernatant. Cell lysates were resolved by SDS-PAGE. Proteins were then transferred to PVDF membrane, blocked, washed, and incubated with mouse anti-human MTSS1 antibodies or mouse anti-human β -actin antibodies. Horse radish peroxidase-labeled goat anti-mouse secondary antibody was used for development. Gray values were analyzed with β -actin as an internal reference.

Flow cytometry

Flow cytometry was used to measure the effect of miR-182 overexpression on the proliferation of KYSE410 cells. Cells were cultured to logarithmic phase, washed with PBS, and fixed by 90% ethanol overnight at 4°C. The ethanol was removed and RNase was added to cells and incubated at 37°C for 30 min. Propidium iodide (PI) was used to stain cells. Cells were then analyzed by a flow cytometer (Becton Dickinson) with excitation wavelength of 488 nm and emission wavelength of 630 nm. Modifit software was used to analyze data. All experiments were repeated three times.

Transwell assay of cell invasion and migration

60 μ L of 5 mg/mL Matrigel (BD Company) was added to each of the upper chamber of Trans-

miR-182 and MTSS1 in esophageal cancer

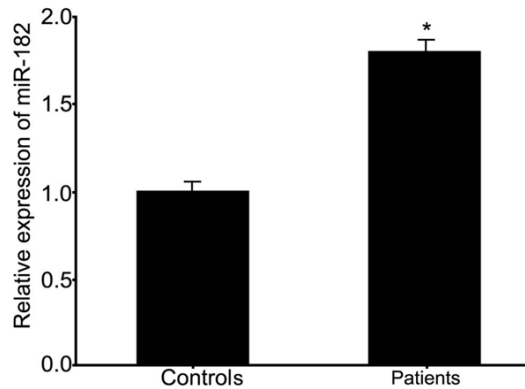


Figure 1. Expression levels of miR-182. *P<0.05, compared with control.

Table 1. Relationship between serum miR-182 level and clinicopathological parameters

| | High miR-182 | Low miR-182 | P |
|------------|--------------|-------------|-------|
| Age (year) | | | |
| ≥60 | 16 | 36 | >0.05 |
| <60 | 11 | 27 | |
| Gender | | | |
| Male | 14 | 34 | >0.05 |
| Female | 13 | 28 | |
| Grading | | | |
| I+II | 3 | 21 | <0.05 |
| III+IV | 24 | 41 | |
| Metastasis | | | |
| Yes | 15 | 2 | <0.05 |
| No | 12 | 60 | |



Figure 2. Sequence homology analysis of MTSS1 3'UTR and miR-182.

well and dried at 4°C. KYSE410 cells in logarithmic phase were trypsinized and re-suspended with fresh serum-free medium. 200 µL cell suspension was added to each upper chamber. 600 µL of culture medium containing 10% fetal calf serum was added to each of the lower Transwell chamber. Cells were then incubated at 37°C incubator for 24 h. Cotton wipe was used to remove residual cells and Matrigel. Cells migrated the membrane were stained by crystal violet and washed with 10% acetic acid. OD570 was measured using a microplate reader. All experiments were repeated three times.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. All data are expressed as mean ± standard error. Differences between groups were analyzed by t-test. P<0.05 was considered statistically significant.

Results

Serum levels of miR-182

Levels of serum miR-182 were measured and the results were shown in **Figure 1**. Compared with the healthy controls, patients with esophageal cancer had significantly higher levels of serum miR-182 (P<0.05).

Correlation between miR-182 and metastasis of esophageal cancer

A 21-month telephone follow-up with esophageal cancer patients was performed and the results were shown in **Table 1**. Results showed that 7 out of 89 patients had liver metastasis (7.87%), 3 patients had liver metastases and lung metastasis, 3 patients had peritoneal metastasis, and 4 patients had other metastasis. Of the 17 patients with metastasis, 15 patients (72.2%) showed higher serum levels of miR-182. In patients without metastasis, 12 out of 72 patients (21.4%) showed higher serum levels of miR-182 (P<0.05).

Prediction of miR-182 targets

TargetScan Release 5.1 (www.targetscan.org) was used to predict the potential targets of miR-182. Results showed that 3'UTR region of MTSS1 was a potential target for miR-182. As shown in **Figure 2**, there was a certain homology between the sequence of 3'UTR region of MTSS1 mRNA and the sequence of miR-182.

Cell transfection

Total RNA was extracted from transfected cells to perform qRT-PCR to detect the relative expression of miR-182 (**Figure 3**). Results showed that the level of miR-182 was significantly increased 24 h after transfection in KYSE410 cells (P<0.05), suggesting that miR-182 was successfully overexpressed in KYSE-410 cells.

miR-182 and MTSS1 in esophageal cancer

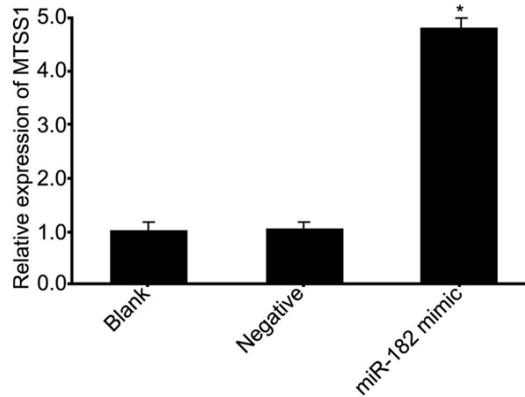


Figure 3. Overexpression of miR-182 in KYSE410 cells. * $P < 0.05$, compared with control.

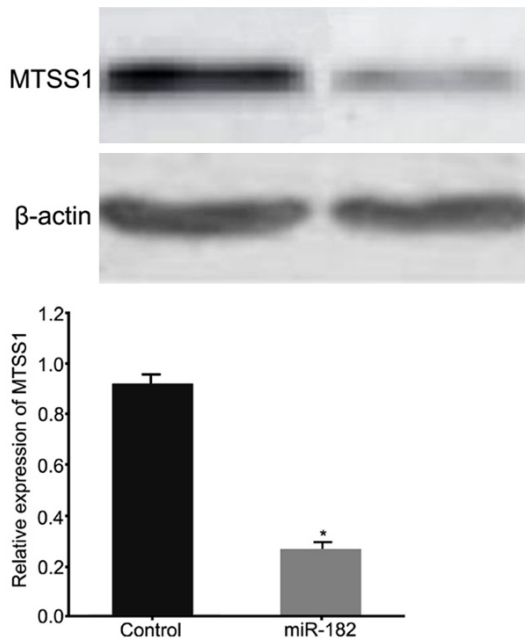


Figure 4. Over expression of miR-182 suppressed the expression of MTSS1. * $P < 0.05$, compared with control.

Expression levels of MTSS1

Western Blot was used to detect the expression levels of MTSS1 in transfected KYSE410 cells. Results showed that overexpression of miR-183 significantly decreased the expression levels of MTSS1 in KYSE410 cells compared with that of control (Figure 4, $P < 0.05$), indicating that overexpression of miR-183 inhibited the expression of MTSS1 in esophageal cancer cells.

Cell cycle assay

Flow cytometry was used to analyze the effect of over-expression of miR-182 on the prolifera-

tion of KYSE410 cells. Modifit software was used to analyze the data. As shown in Figure 5, compared with control, over-expression of miR-182 significantly increased the number of cells in S phase ($P < 0.05$), but showed no significant effect on the number of cells in G0/G1 phase or G2/M phase ($P > 0.05$).

Cell invasion capability assay

The results of invasion capability of KYSE410 cells with or without overexpression of miR-182 were shown in Figure 6. Overexpression of miR-182 significantly enhanced the invasion capability of KYSE410 cells ($P < 0.05$). Absorbance at OD570 showed that overexpression of miR-182 increased the invasion capability of KYSE410 cells by 1.25 times.

Discussion

In order to explore the levels of serum miR-182 and its role in the proliferation and metastasis of esophageal cancer, we used RT-PCR and found that patients with metastatic esophageal cancer had significant higher levels of serum miR-182 than that of patients with non-metastatic esophageal cancer. It has been shown that microRNAs including miR-150, miR-145, miR-200 and miR-182 were abnormally expressed in esophageal cancer cells [13-15]. Studies found that miR-182 were significantly up-regulated in a variety of human malignancies such as prostate cancer and breast cancer. Therefore, some scholars believe that miR-182 is a proto-oncogene [11, 16, 17]. How is miR-182 down-regulated in esophageal cancer cells remains unclear. Some scholars believe that abnormal methylation in the CpG island of promoter region of miR-182 leads to the down regulation of miR-182 [18], but this conclusion needs more experimental data to support.

Luciferase gene reporter assay and bioinformatics analysis showed that miR-182 could interact with 3'UTR region of MTSS1 to regulate the expression of MTSS1. Then, miR-182 was over-expressed in KYSE410 cells. Western blot results showed that overexpression of miR-182 significantly decreased the expression of MTSS1, increased the proliferation capability, and shortened cell cycle in KYSE410 cells. In addition, overexpression of miR-182 enhanced the invasion capability of KYSE410 cells. These results suggested that overexpression of miR-182 increased the proliferation and invasion of KYSE410 cells via down-regulation of MTSS1.

miR-182 and MTSS1 in esophageal cancer

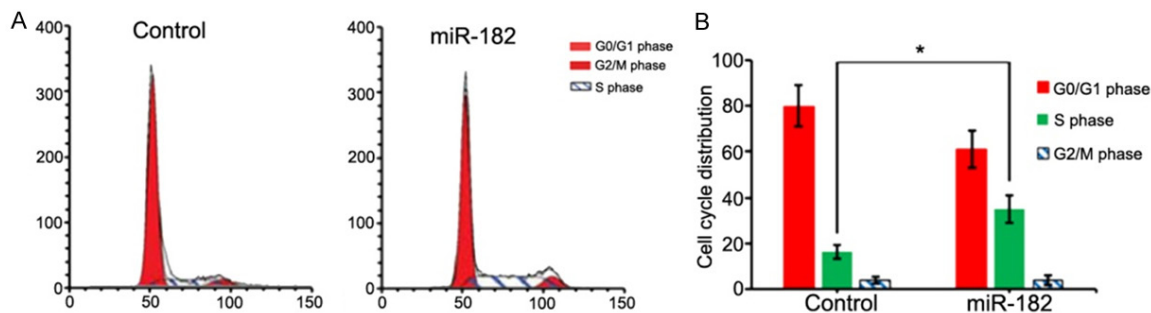


Figure 5. Cell cycle assay. A. FL2 area and DNA histogram; B. Cell cycle changes, * $P < 0.05$, compared with control.

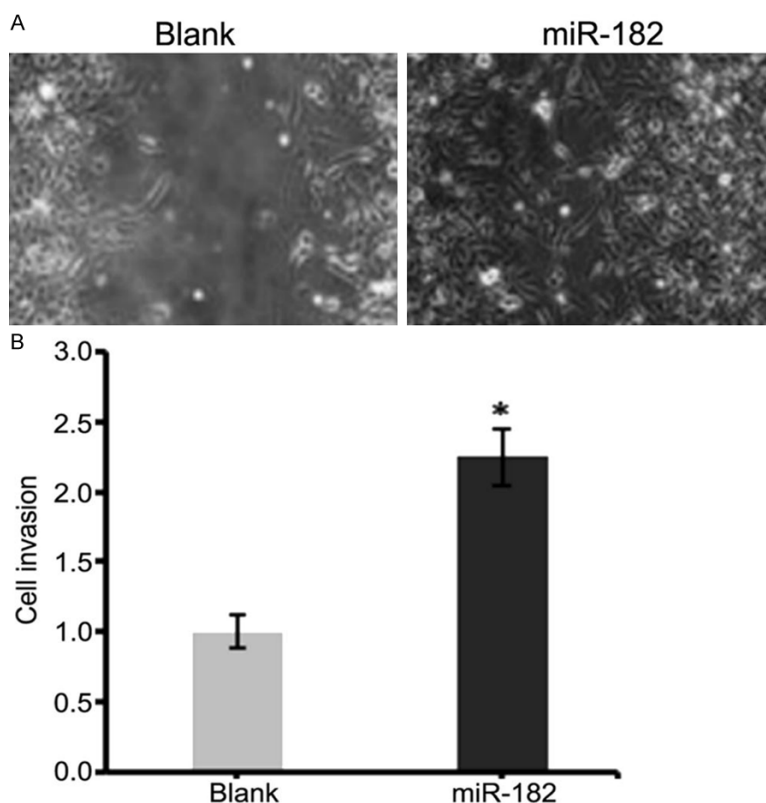


Figure 6. Effect on over-expression of miR-182 on invasion capability. A. Representative figure of invasion; B. Quantification of invasion. * $P < 0.05$, compared with control.

Studies have shown that microRNA can regulate cell behavior through RNA interference [6]. Hirata et al found that miR-182 regulated cell proliferation and invasion in prostate cancer through regulation of MTSS1 [12]. MTSS1 is a tumor metastasis suppressor which plays an important role in a variety of human malignancies including prostate cancer and breast cancer [19]. As a co-transcription factor, MTSS1 can bind Gli protein to up-regulate gene expression [20]. MTSS1 has been found down-regulated in a variety of human malignancies and low

expression of MTSS1 was closely associated with poor prognosis of cancer patients [21]. The study found that miR-182 promoted the proliferation and invasion of esophageal cancer cells via inhibition of MTSS1 expression, which is consistent with other findings [22].

Due to high degree of malignancy and easy metastasis, esophageal cancer seriously threatens people's lives. Therefore, the study of mechanisms of invasion and metastasis of esophageal carcinoma cells has become a hot point [2]. These results confirmed the relationship between miR-182 and MTSS1 in esophageal carcinoma cells, providing a theoretical basis for the future use of RNA interference treatment for esophageal cancer.

Conclusion

miR-182 levels were significantly increased in the plasma of patients with metastatic esophageal cancer compared to that of patients with non-metastatic esophageal cancer. miR-182 enhanced the proliferation and invasion of esophageal cancer cells via down-regulation of MTSS1.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yin Li, Department of Thoracic Surgery, Affiliated Tumor Hospital of Zhengzhou University, 127 Dongming Road, Zhengzhou 450003, Henan, China. Tel: +86-371-655-88414; Fax: +86-371-65588414; E-mail: YinLiqwe@163.com

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