

## ORIGINAL ARTICLE

# Effects of MiR-210 on proliferation, apoptosis and invasion abilities of esophageal cancer cells

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## Summary

**Purpose:** To investigate the effects of microRNA-210 (miRNA-210) on the biological behaviors (proliferation and invasion) of EC109 cells of highly metastatic human esophageal cancer (EC).

**Methods:** The EC109 genomic DNA of human EC was used as a template to amplify the precursor sequence of miRNA-210 by polymerase chain reaction (PCR). The precursor sequence of miRNA-210 was sub-cloned into the eukaryotic expression vector pcDNA3.1(-) via double digestion by BamH I and Hind III restriction enzymes. Then the pcDNA3.1 (-)-pri-miRNA-210 vector (named as p-miRNA-210) that was constructed successfully was transiently transfected into EC109 cells of human EC in vitro. Quantitative real-time PCR (qRT-PCR) was used to detect the expression level of mature miR-210. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay and scratch method were adopted to detect the proliferation and in vitro migration of EC109 cells, and flow cytometry was performed to detect the degree of cell apoptosis.

**Results:** The eukaryotic expression vector carrying miRNA-210 was constructed successfully. Compared with that in the blank group (Mock) and the control group (P-Blank), miRNA-210 was overexpressed in the transfected EC109 cells. The cell apoptosis was significantly increased compared with that in the control group ( $p < 0.05$ ); the inhibition of proliferation of EC109 cells in the p-miRNA-210 vector transfected group was remarkably elevated ( $p < 0.05$ ), and wound healing ability was also significantly increased ( $p < 0.05$ ).

**Conclusion:** The overexpression of miRNA-210 can significantly inhibit the proliferation of EC109 cells of human EC and accelerate the migration ability and the rate of apoptosis, providing a potential strategy for the treatment of EC.

**Key words:** esophageal cancer, MiR-210, MTT, qRT-PCR, scratch method

## Introduction

Esophageal cancer (EC) is a malignant tumor ranking 8<sup>th</sup> in incidence and 6<sup>th</sup> in mortality worldwide. It can be mainly divided into two types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC accounts for up to 90% among all the EC patients in China [1-

3]. In recent years, breakthroughs have been made in the treatment of EC. However, the prognosis is poor, the overall survival rate is low, and the post-operative 5-year survival rate does not exceed 20% [4]. China is a country with high incidence of EC. The incidence of EC in China takes up more than

50% of the global incidence. Early disease symptoms are usually overlooked contributing thus to moderate or late disease stages when the patients come to the hospital for treatment, missing the best chance for surgical treatment. Moreover, some patients have poor prognosis after surgery. Generally, this disease seriously affects people's health and life [5,6]. The occurrence of EC is the result of interaction of multiple factors. However, the pathogenesis of EC as well as its migration and invasion ability are not clear yet.

In recent years, micro-ribonucleic acids (miRNAs), as a class of non-coding endogenous RNAs with a length of 17-23 nucleotides, can directly regulate the target genes through translation triggered by their combination with the 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs). MiRNAs can be divided into two types in tumors. One type displays a carcinogenic role, and its expression level is significantly increased, reducing the role of tumor suppressor genes. The other type exerts an anti-cancer effect, and is lowly expressed in the tumor tissues, thereby inhibiting the activity of proto-oncogenes [7-9]. Some studies have shown that miRNA-210 is expressed in a variety of tumors such as breast cancer [10], lung cancer [11], laryngeal cancer [12] and renal clear cell carcinoma [13]. However, few studies have been conducted over its expression in EC as well as its proliferation, apoptosis and migration abilities. In this study, miRNA-210 was transfected in the EC109 cells of human EC by eukaryotic expression transfection method to investigate the expression level of miRNA-210 in *in vitro* cell lines and its impact.

## Methods

### Main reagents

The following main reagents were used in this study: reverse transcription kit (miScript II RT Kit) was purchased from Qiagen Corporation, CA, USA; fetal bovine serum (FBS) was purchased from Gibco Corporation, USA; methyl thiazolyl tetrazolium and dimethyl sulfoxide were purchased from Sigma Corporation, St. Louis, MO, USA; AnnexinV-FITC was purchased from Bestbio Corporation, Shanghai, China; Lipofectamine™2000 transfection reagent was purchased from Invitrogen, Waltham, MA, USA; miRNA primers and mimics Hiperfect transfection reagent were purchased from Qiagen Corporation, CA, USA.

### Cell culture

EC109 cells of human EC were purchased from Shanghai Aolu Biotech Co., Ltd. Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS was used for cell culture in an incubator at 37°C with 5% CO<sub>2</sub>.

### Vector construction

The miR-210 primer was designed and constructed by Shanghai GenePharma Co. Ltd. (Table 1). MiRNA-155 primers were amplified using high-fidelity polymerase chain reaction (PCR), followed by the construction of template primer and empty vector pcDNA3.1(-). The vector that was successfully constructed using double digestion by BamH I and Hind III restriction enzymes was pcDNA3.1(-)-pri-miRNA-210 (also named as P-miRNA-210). The control group (P-Blank) and the blank group (Mock) were set.

### Cell transfection

EC109 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 1% penicillin and streptomycin, transferred to a constant-temperature incubator containing 5% CO<sub>2</sub> at 37°C for culture, digested and passaged. Cells were counted using trypsin. The cell density was adjusted and sequentially inoculated into a 6-well plate with 2×10<sup>5</sup> cells in each well, transferred to the DMEM (containing 10% FBS without 1% penicillin and streptomycin) and cultured for 24 hrs. When the total cell confluence reached about 80%, transfection was performed using the Lipofectamine™ 2000 kits.

### Detection of miRNA-210 expression via quantitative real-time PCR (qRT-PCR)

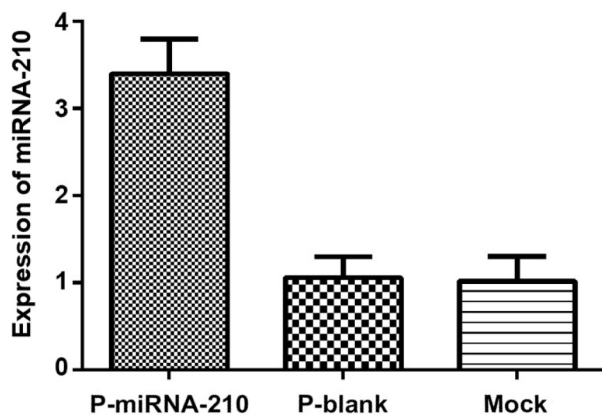
Total RNA was extracted from each group of cells using TRIzol reagent. The purity and concentration of the extracted RNA were detected by an ultraviolet spectrophotometer and protein electrophoresis. The reverse transcription of miRNA-155 was performed according to the instructions of the manufacturer, and the extracted total RNA was transcribed. Then, detection via qRT-PCR was performed. The reaction system was configured according to the kits. The reaction system was as follows: 10 μL 2×SYBR green; 1.5 μL 10 miscript; 1.5 μL 10× miscript primer assay; 1.5 μL template complementary DNA (cDNA). Finally, diethylpyrocarbonate (DEPC) was supplemented to 20 μL. Primer sequences were designed and synthesized by Shanghai GenePharma Co. Ltd. (Table 1). PCR reaction conditions: pre-degeneration at 95°C for 10 min, 95°C for 15 s, 65°C for 30 s and 72°C for 30 s, which were repeated 45 times. U6 was used as a control gene. The obtained expression amount was calculated using the 2<sup>-ΔΔCt</sup> method.

**Table 1.** Primer sequences

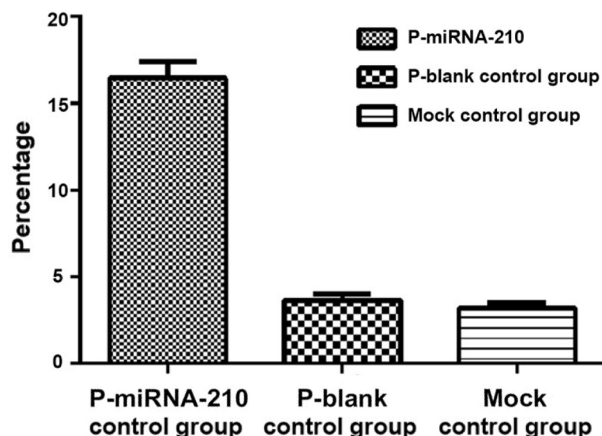
| Primer sequence   | miRNA-210                              | U6                         |
|-------------------|--|----------------------------|
| Upstream primer   | 5'-ACACTCCAGCTGGGCTGTGCGTGTGACAGCGG-3' | 5'-CTCATTTCCGGCCAGTCA-3'   |
| Downstream primer | 5'-CTCAACTGGTGTGCTGGA-3'               | 5'-AACGCATCACGAAGCTGCGT-3' |

#### Detection of cell apoptosis via flow cytometry

Each group of cells in the culture plate was transferred to a conical tube and placed on ice after they had been transfected for 48 hrs. Cells in the plate were rinsed with 2 mL phosphate buffer saline (PBS) solution. PBS was removed, and 0.5 mL 0.25% trypsin [without ethylene diamine tetraacetic acid (EDTA)] was added for incubation. The cells started to detach under the microscope. The culture plate was slightly shaken so as to make the cells completely fall off from it. The obtained cells were re-suspended in 1×buffer solution at a density of  $1 \times 10^6$  cells/mL and transferred to a clean centrifuge tube. The solution for cell apoptosis detection (Annexin-V-FITC) was added and protected from light at room temperature for 15 min. The cells were centrifuged at 1500 r/min for 5 min and the supernatant was discarded. Re-suspending with 1×buffer solution was performed. Finally, 10  $\mu$ L propidium iodide (PI) was added. Flow cytometry was used for analysis and the experiment was repeated 3 times.



**Figure 1.** Expression of transfected miRNA-210 in the cells. It can be seen from the figure that the number of miRNA-210 in the EC109 cells after transfection is increased significantly, which is 3.20 times as high as that in the control group and 3.33 times as high as that in the blank group. The comparison between the two groups has statistical difference ( $p < 0.05$ ).



**Figure 2.** Cell apoptosis of highly expressed miRNA-210 cells. The apoptosis of P-miRNA-210 cells was significantly different after transfection compared with that in the other two groups ( $p < 0.05$ ).

#### Detection of cell proliferation via MTT assay

The density of the cultured cells was adjusted to  $1 \times 10^4$ . EC109 cells were inoculated in a 96-well plate and cultured in an incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) for 24 hrs. Three duplicate wells were set for P-miRNA-210 plasmid and P-Blank control group after transfection with Lipo2000. Seventy-two hrs later, 20  $\mu$ L reaction solution (MTT reagent) (5 mg/mL) was added to each well to culture continuously for 2 hrs. A pipette was used to suck the supernatant and 100  $\mu$ L DMSO were added. Then the plates were shaken for 15 min. A microplate reader was used to detect and calculate the results, and the cell growth curve was drawn.

#### Cell scratch test

The cells transfected for 24 hrs in each group were selected and inoculated to a 6-well plate and a total of 3 groups of duplicate wells were set. When the cell confluence reached about 90%, the tip of a 20  $\mu$ L spearhead was placed perpendicularly to the 6-well culture plate, and scratching was performed as per the prepared horizontal line. The plate was washed for 3 times using PBS and the cells were cultured continuously in DMEM containing 1% FBS. The experiment was repeated 3 times.

#### Statistics

The statistical analyses of the collected data were performed using the SPSS 20.0 (Beijing Sichuang Weida Co., Ltd.). The data were expressed as mean  $\pm$  standard deviation (SD). The least significant difference (LSD) method was used for the pairwise comparison of each group of data, and *t*-test was adopted for analyses. A *p* value  $< 0.05$  suggested statistical difference.

## Results

#### Expression of miRNA-210 in EC109 cells after transfection

After EC109 cells in each group had been transfected for 48 hrs, the qRT-PCR was performed. The results are shown in Figure 1. The expression level in P-miRNA-210-EC109 cells was significantly higher than that in the control group (P-Blank) and the blank group (Mock) ( $p < 0.01$ ). There was no difference in the expression level between the control group (P-Blank) and the blank group (Mock) ( $p > 0.05$ ).

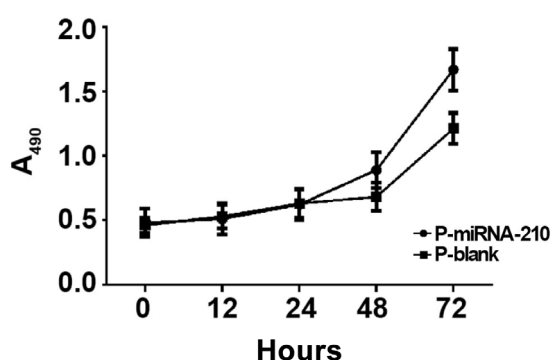
#### Cell apoptosis of highly expressed miRNA-210 cells

The flow cytometry was used to detect cell apoptosis in each group after transfection. The apoptosis rate of P-miRNA-210 cells was  $16.45\% \pm 1.67$  after transfection, and those in the control group (P-Blank) and the blank group (Mock) were  $3.67\% \pm 0.64\%$  and  $3.21\% \pm 0.57\%$ , respectively. The apoptosis of P-miRNA-210 cells was significantly increased compared with that in the control group (P-Blank) and the blank group (Mock) ( $p < 0.05$ ). The

expression level in the control group (P-Blank) and the blank group (Mock) had no difference ( $p > 0.05$ ) (Figure 2).

#### *Proliferation ability of highly expressed miRNA-210 in EC109*

After the miRNA-210 cells were transfected into EC109 cells for 48 hrs, MTT test results showed that there was no difference in proliferation between the two groups at 12-24 hrs ( $p > 0.05$ ). Forty-eight hrs later the difference between P-miRNA-210 and P-Blank control group was statistically significant ( $p < 0.05$ ) (Figure 3).



**Figure 3.** MTT proliferation curve. It can be seen from the figure that the proliferation rate between the two groups has no obvious difference at 0-24 h. 24 hrs later, the curve starts to separate. At 48 hrs there is significant difference in the proliferation rate between the two groups ( $p < 0.05$ ), and at 72 hrs there is obvious difference in the proliferation rate between the two groups ( $p < 0.05$ ).

#### *Scratch test of highly expressed miRNA-210*

The width of the scratch wound was observed under an inverted optical microscope. In this test, the migration of cells in the P-miRNA-210 group was significantly accelerated, while the scratch in the uninhibited P-Blank control group was healed slowly; the migration distance of the cells in the P-miRNA-210 group was significantly shorter than that of the cells in the P-Blank control group. The differences were statistically significant ( $p < 0.05$ ).

## Discussion

ESCC is a malignant tumor derived from EC epithelial tissues. Its characteristics include relatively high mortality rate, mainly due to the relatively poor prognosis, low 5-year survival rate, and higher incidence in male than in female patients, which may be associated with their living habits to some extent [14]. In China, ESCC is a disease with high incidence. Although the level of medical care has been improving and the mortality of

the patients has been declining in recent years, there are still some difficulties in the diagnosis and treatment of ESCC. The patient's condition was not improved after surgery and other treatments [15]. These are mainly due to the difficulty in distinguishing ESCC in early stage, leading to delay in the treatment of disease. A study has shown that the proliferation, apoptosis and invasion abilities of cancer cells can also affect the prognosis and survival of the patients [16]. Proliferation, apoptosis and invasion are multi-step biological processes including several factors. Therefore, finding out the molecular markers of early stage EC will help provide more effective methods for the diagnosis and treatment of EC. At present, a study in China [17] and other countries have shown that the occurrence of ESCC is induced by a large number of tumor suppressor genes and oncogenes, but the exact mechanism of ESCC is still not clear yet.

MiRNA, as a single-stranded RNA processed by endogenous expression, mainly affects the stability of mRNA by suppressing the translation, thus inhibiting the expression of target genes [18]. The expression of miRNAs is found in almost all tissues of the human body. A current study [19] showed that miRNAs are involved in almost all the biological processes of the body to maintain normal function and regulate the genes. With the deepening of research, more and more miRNAs have been found to participate in the development and progression of tumors through their roles in the proliferation, migration, apoptosis and angiogenesis of tumor cells [20].

MiRNA-210, located on chromosome 11p15.5, is an oxygen-deficient miRNA whose expression is consistently increased under hypoxic environment [21]. In this study, miRNA-210 was transfected into EC109 cells through successful construction of eukaryotic vector to detect its expression in cells as well as its biological functions. It was found that the high expression of miRNA-210 in the successfully constructed miRNA-210-EC109 cells was significantly different from those in the control group and the blank group ( $p < 0.05$ ). However, Liu et al. reported in their studies that miRNA-210 is highly expressed in the serum of EC patients, demonstrating that the expression of miRNA-210 in cancer cells is consistent with that in the serum [22]. A study has shown that the high expression of miRNA-210 can inhibit the expression of interleukin-6 (IL-6) and other inflammatory factors caused by the toll-like receptors 4 (TIR4) [23]. The expression level of IL-6 is proportional to tumor staging and pathological type. In order to detect the proliferation, migration and apoptosis of EC109 cells transfected with miRNA-210, experiments

such as flow cytometry, MTT cell proliferation and cell scratch test were performed, respectively. The results showed that the cell apoptosis in P-miRNA-210 group and other two groups had obvious statistical significance ( $p < 0.05$ ). The existence of hypoxic environment in the tumor site can promote the increase in the expression of miRNA-210, making a variety of hypoxic pathways to promote cell apoptosis, thereby accelerating the occurrence and development of tumors [24]. The experiment of cell proliferation showed that the proliferation rate in the P-miRNA-210 group at 0-24 hrs after transfection had no difference with that in other two groups. With the prolongation of culture time, the proliferation rate of cells was significantly increased. It might be related to the participation of miRNA-210 in the regulation of cells, which inhibited the rate of cell proliferation. Finally, scratch test was performed for the cell migration ability. The results showed that high expression of miRNA-210 could promote the migration rate of cancer cells which was significantly faster than that in the control group.

Some limitations of this study should be acknowledged. We did not carry out in-depth as-

essment and discussion on the mechanism of miRNA-210 in the body. Meanwhile, this study was an *in vitro* experiment. The specific impacts in the tumor were not clear. It is hoped that the mechanism of miRNA-210 and its occurrence and development can be explored in a more detailed and in-depth way in future studies.

In conclusion, transfected cells were successfully constructed and expressed in this study. The biological effect of miRNA-210 on EC were investigated by qRT-PCR, flow cytometry, MTT and cell scratch test, which proved that miRNA-210 can inhibit cell proliferation and decelerate cell migration and apoptosis.

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### Conflict of interests

The authors declare no conflict of interests.

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