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**MOLECULAR BIOLOGY** 

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Received: Accepted: Published:	2015.07.31 2015.08.21 2015.11.18		Effect of miR-503 Down and Invasion of Esopha Related Immune Function	n-Regulation on Growth gus Carcinoma and on	
Authors' St Data Statistic Data Int Manuscript Litera Funds	Contribution: udy Design A a Collection B cal Analysis C erpretation D Preparation E ture Search F 5 Collection G	BC AD FG CE BF AG	Ke Zhao Bao-Jun Chen Zhi-Guo Chen Yong-Jian Zhang Di Xu Qi Liu	Department of Thoracic Surgery, The Central Hospital of Wuhan, Wuhan, Hubei, P.R. China	
Corresponding Author: Source of support:		g Author: f support:	Bao-Jun Chen, e-mail: baoJun20158@163.com Departmental sources		
Background: Material/Methods:		rground: Nethods:	MicroRNA (miR) has been proved to be an important biomarker for tumors because it can regulate occurrence, progression, invasion, and metastasis of cancer. A previous study has shown the involvement of miR-503 in multiple gastrointestinal tumors. Its detailed role and immune regulatory function in esophagus carcinoma, however, remains unknown. This study thus investigated the effect of miR-503 in regulating growth, proliferation, and invasion of esophagus cancer and its influence on cytokine secretion. Esophagus carcinoma cell line EC9706 and normal esophageal epithelial cell line HEEC were transfected with miR-503 inhibitor. MTT assay was used to quantify the cell proliferation, and a Transwell chamber was used to evaluate cell invasion. Release of cytokines, including interleukin-2 (IL-2), IL-4, IL-10, and interferon- $\gamma$ (IFN- $\gamma$ ), was measured by enzyme-linked immunosorbent assay (ELISA).		
Results:		Results:	MiR-503 expression was significantly elevated in esophagus carcinoma cells ( $p<0.05$ ). The specific inhibition of miR-503 expression remarkably suppressed proliferation and invasion of tumor cells. It can also down-regulated IL-2 and IFN- $\gamma$ expression and facilitate secretion of IL-4 and IL-10 when compared to the control group ( $p<0.05$ in all ceases).		
Conclusions:		lusions:	The inhibition of miR-503 can effectively inhibit tumor progression and improve immune function, suggesting its potency as a novel drug target for esophagus cancer treatment.		
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# Background

As one of the most common malignant tumors derived from the gastrointestinal tract, esophagus carcinoma has a high incidence and mortality, and more than 300 000 people died from this cancer, making it the sixth leading cause of cancer death worldwide [1,2]. Due to various factors, including environment, diet, and modern life-styles, China has one of the world's highest incidence rates of esophagus cancer. Most patients had primary diagnosis above 40 years old, with significant male bias. Esophagus carcinoma has been reported as one of top 10 cancers in Chinese [3–5]. The etiology of esophagus carcinoma has revealed the involvement of multiple factors, including genetics, diet, and environmental influences [6,7]. In its early stage, esophagus cancer normally manifests as only minor discomfort when chewing solid food and lacks typical features, causing a high rate of misdiagnosis. It is thus usually diagnosed in late stage, when opportunity for optimal treatment has passed, leading to difficulty of treatment and unfavorable prognosis, and severely compromising patient life quality and survival rate [8]. The etiological mechanism of esophagus carcinoma has been receiving much research interest.

Current studies agree on the genetic component underlying risk of malignant tumors, which are the results of accumulation of minor genetic mutations across different genes in a cascade manner [9]. Micro RNA, which is a type of small molecule, also named as miRNA or small molecule RNA, is widely expressed in animal and plant cells for gene expression regulation [10]. Via its base paring with a target gene or to inhibit its downstream protein expression, miRNA mediates mRNA degradation and protein expression [11]. A close relationship between miRNAs and tumor pathogenesis has been suggested, as certain miRNAs facilitate tumor proliferation and metastasis, while the silencing of certain miRNA may cause it to lose its tumor-suppressor activity [12,13]. Studies have discovered the dysregulation of miRNAs in dozens of tumors. Recent finding indicated the role of miR-503 in various tumor cells, including tumor cell growth, differentiation, and invasion, as well as prognosis [14,15]. The detailed function of miR-503 and its effects on the immune system, however, remain unclear. This study thus investigated the role and effects of miR-503 in cell growth, proliferation, and invasion of esophagus cells and measured its influences on immune cytokine secretion, in an attempt to study the related mechanism and to develop novel drug targets against esophagus cancer.

## **Material and Methods**

### **Cell culture**

Resuscitated EC9706 and HEEC cells (ATCC cell bank, USA) were thawed at 37°C, followed by 1 000 rpm centrifugation for 3 min and re-suspension in 1 mL DMEM medium (Hyclone, US). Cells were incubated in a humidified 37°C chamber with 5%  $CO_2$  perfusion for 24~48 h. Both cell lines were then inoculated into a culture dish at 1×10<sup>7</sup>/cm<sup>2</sup> using high-glucose DMEM medium containing 10% fetal bovine serum (FBS, Evergreen, China), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged every 2~3 days. Log-phased HEEC cells were used as the control group, while EC9706 cells were randomly divided into a tumor cell control group and a miR503 inhibitor group.

#### Transfection of miR-503 inhibitor

MiR-503 inhibitor oligonucleotide (5'-GAGCAUUUCGGU CUGGAA-3', Gimma, Shanghai) was mixed with 0.2 mL serum-free DMEM medium and Lipo2000 transfection reagent (Invitrogen, USA), and was added into cultured EC9706 cells (confluence: 70~80%) for 30-min incubation. After the removal of serum and rinsing by PBS, 1.6 mL of serum-free medium was added for further 6-h incubation.

#### **Real-time PCR**

Trizol reagent (Invitrogen, USA) was used to extract mRNA from all groups. After measuring concentration and purity by ultraviolet spectrometer, total RNA was used as the template for *in vitro* reverse-transcription using a test kit (Invitrogen, US) to synthesize cDNA. Real-time PCR was then used to amplify the target gene using specific primers (Table 1, Sangon, China) under the following condition: 55°C for 1 min, followed by 35 cycles each with 92°C denaturing for 30 s, 58°C annealing for 45 s, and 72°C elongation for 35 s. Using GAPDH as the internal reference, fluorescent signals were quantified to determine CT values of all samples and standards. Semi-quantification of target gene amplification was performed by  $2^{-\Delta Ct}$  method.

#### MTT assay

Log-phased EC9706 cells were seeded into 96-well plates at 5 X  $10^3$  density in DMEM medium containing 10% FBS. After

Table 1. Primer sequence.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
MiR503	TACGACTATGTGACCTGCCTG	TACCGATGTCTGGTAGACGAT



Figure 1. MiR-503 expression level. \* p<0.05 compared to HEEC cells; # p<0.05 compared to EC9706 cells.

24-h incubation, 20  $\mu$ L of sterile MTT reagents was added into each well. Four hours later, supernatants were removed, followed by the addition of 150  $\mu$ L DMSO. The plate was shaken until complete resolving of the crystal violet. A microplate reader (Bio-Rad, USA) was used to measure the absorbance value at 570 nm.

### **Cell invasion assay**

A transwell chamber (Hyclone, USA) was coated with 50 mg/L Matrigel dilution. After 48-h transfection, cells were incubated using serum-free medium for 24 h. The transwell chamber was put into a 24-well plate which containing 0.5 mL DMEM medium (with 10% FBS). We added a 100- $\mu$ L suspension of tumor cells inside the chamber with serum-free medium. Controlled treatment was performed in a transwell chamber without coating. After 48-h incubation, the chamber was rinsed in PBS and fixed in cold ethanol. Crystal violet solution was added for 30-min staining. The number of cells translocated to the lower side of the membrane was counted using an inverted microscope. We counted 10 randomly selected fields to calculate the average cell number in triplicate.

#### Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels including IL-2, IFN- $\gamma$ , IL-4 and IL-10 were quantified by ELISA using test kits (eBioscience, USA) following the manual instructions. In brief, serially diluted standard samples were added into 96-well plates, along with tested samples in triplicates. After washing and rinsing 5 times, enzyme-linked reagent was applied to each well, followed by 37°C incubation for 30 min. Chromogenic substrate A and B were sequentially added into each well for development in the dark (10 min). The reaction was stopped by adding quenching buffer to each well. Optical density (OD) value at 450 nm was measured by the microplate reader within 15 min. A linear regression function was



Figure 2. Cell proliferation assay. \* p<0.05 compared to HEEC cells.

approximated by standard samples. Concentration of tested samples was then deduced from the standard curve.

### Statistical analysis

The SPSS16.0 software package was used to analyze all collected data, of which measurement data are presented as mean  $\pm$  standard deviation (SD). Multiple group comparison was performed by analysis of variance (ANOVA). Statistical significance was defined as p<0.05.

## **Results**

## **Expression of miR-503**

Using real-time PCR, we found significant elevation of miR-503 expression in EC9706 esophagus carcinoma cells compared to normal HEEC cells (p<0.05, Figure 1). The transfection of miR-503 inhibitor effectively inhibited miR-503 expression (p<0.05).

## Effect of miR-503 on EC9706 cell proliferation

MTT assay was used to describe the effect of miR-503 on the proliferation of esophagus carcinoma cells. Results showed significantly depressed proliferation rate after the transfection of miR-503 inhibitor compared to the control group (p<0.05, Figure 2), suggesting the participation of miR-503 in cell proliferation of EC9706.

## **Cell invasion ability**

We used a transwell chamber to evaluate the invasion ability of EC9706 cells. After the transfection of miR-503, the percentage of invasive cells was significantly decreased compared to controlled EC9706 cells (p<0.05, Figures 3, 4). This



Figure 3. Representative figures of transwell chamber assay. (A) EC9706 cells; (B) miR-503 scramble RNA transfected EC9706 cells; (C) miR-503 inhibitor transfected cells.



Figure 4. Cell invasion ability. \*, p<0.05 compared to HEEC cells.

result suggests the potency of miR-503 in regulating invasion of esophagus carcinoma cells.

# Cytokine secretion from EC9706 cells

The transfection of miR-503 inhibitor significantly decreased the secretion of Th1 cytokines IL-2 and INF- $\gamma$  as compared to control group (p<0.05, Figure 5A, 5B), suggesting the potentiation of miR-503 in Th1 cytokine secretion from esophagus carcinoma cells.

Opposite effects occurred when checking the level of Th2 cytokines including IL-4 and IL-10, both of which had elevated secretion after miR-503-specific silencing (p<0.05, Figure 5C, 5D). Therefore, miR-503 can inhibit the secretion of Th2 cytokines.

# Discussion

As one of the most common malignant tumors in China, esophagus carcinoma has relatively high invasion and recurrence rates, both of which cause unfavorable prognosis. Currently available treatment against esophagus cancer, including surgical resection and chemo- and radio-therapy, cannot significantly improve its 5-year recurrence rate. Such difficulty, plus the insidious onset of esophagus cancer, make it an important focus in cancer research [16,17]. Therefore, the illustration of the biological mechanism underlying occurrence and progression of esophagus cancer may help early diagnosis and treatment of cancer, thus improving the prognosis and patient life quality.

MiRNA is a kind of pluripotent small molecule involved in multiple cellular processes, including cell proliferation, apoptosis, signal transduction, differentiation, hormone secretion, lipid metabolism, and maintaining potency of embryonic stem cells, all of which can regulate body growth and environmental acclimation. Recent studies have revealed the participation of miRNA in tumor occurrence, progression, invasion, and metastasis, as well as other biological features [18]. As a newly discovered miRNA, miR-503 has been suggested to be abnormally expressed in various tumors and can be the target for regulating tumor biology. Its expressional profiles, however, vary across different tumors. In hepatocellular carcinoma, miR-503 can inhibit tumor growth and proliferation via inducing G1 phase arrest [19]. In other tumors, such as osteosarcoma and brain glioma, miR-503 was shown to have aberrant expression [20,21]. In most other malignant tumors, however, miR-503 has decreased expression for potentiating tumor growth. Therefore, it is generally believed that miR-503 acts like a tumor-suppressor gene. However, in a study of esophagus carcinoma tissue, miR-503 expression was found to facilitate tumor progression [22], although its detailed role and mechanism in esophagus cancer has not been defined.



Figure 5. Cytokine secretion from esophagus cancer cells. (A) IL-2; (B) IFN- $\gamma$ ; (C) IL-4; (D) IL-10. \* p<0.05 compared to HEEC cells.

This study demonstrated the elevation of miR-503 in esophagus carcinoma, in agreement with a previous observation [22]. Further *in vivo* gene silencing assay by transfecting miR-503 inhibitor into esophagus cancer cell revealed significantly inhibited cell proliferation and lowed invasion ability, suggesting the participation of miR-503 in facilitating tumor proliferation and metastasis. We also analyzed the effect of miR-503 on cytokine release from cancer cells and found decreased Th1 cytokines (IL-2 and IFN- $\gamma$ ) but elevated Th2 cytokines (IL-4 and IL-10) in miR-503 inhibitor transfected cells. It has been shown that Th1 sub-population of T cells mainly induce pathogenic immune response via secreting IL-2 and tumor necrotic factor- $\alpha$  (TNF- $\alpha$ ) to facilitate cell apoptosis, increased expression of adhesion molecules, and degrading normal tissues. Th2

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sub-type of lymphocytes, however, can inhibit Th1-induced immune damage and stimulate the differentiation of B cells into plasma cells for secreting antibody and production of memory B cells, thus potentiating humoral immunity [23].

# Conclusions

miR-503 is up-regulated in esophagus carcinoma cells. The inhibition of miR-503 can depress proliferation and invasion ability of tumors, in addition to improving body immune function. This study suggests the potency of miR-503 as a novel molecular target for diagnosis and treatment of esophagus cancer.

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