

Original Article

miR-21 is negatively correlated with miR-203 in esophageal cancer tissues and functions oppositely in tumor progression

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Abstract: Objectives: It is well-known that microRNAs (miRNAs) function as vital regulators in the development of human malignancies as tumor suppressors or oncogenes. miR-21 and miR-203 have been both characterized as functional miRNAs in esophageal cancer. This study aimed to evaluate the internal relationship between the two miRNAs. Methods: A total of 56 patients of esophageal cancer were enrolled and expression of miR-21 and miR-203 were detected by qRT-PCR. Associations between the two miRNAs expression in different categories of these esophageal cancer samples were analyzed. Inhibitor of miR-21 and/or mimics of miR-203 was introduced into EC9706 cells and cell proliferation was measured by crystal violet staining assay. Results: miR-21 expression was significantly elevated while miR-203 was decreased in tissue samples of esophageal cancers compared to those in the paired normal adjacent tissues. Negative correlation was found in these two miRNAs expression. Crystal violet staining assays validated that inhibition of miR-21 or forced expression of miR-203 promoted cell proliferation. In addition, inhibition of miR-21 expression led to obvious upregulation of miR-203, and forced miR-203 expression caused miR-21 repression. Conclusion: Negative correlation and opposite functions of miR-21 and miR-203 were established in esophageal cancer. The future combined assay of miR-21 and miR-203 may help to treat this disease.

Keywords: Esophageal cancer, miR-21, miR-203, cell proliferation, negative correlation

Introduction

Esophageal cancer is a common malignant tumor of the digestive tract, ranking the eighth most common type of all malignancies and the sixth leading cause of cancer-associated mortality worldwide [1-3]. According to the different etiological and pathological characteristics, esophageal cancers can be divided into two main forms: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. As is known, China is one of high-incidence areas of esophageal cancer, where ESCC is the dominant pathological subtype leading to more than 150,000 cases of mortality annually [4, 5]. Despite the great progress in surgery, chemotherapy and radiotherapy options during the last decades, esophageal cancer still presents with poor prognosis, mainly due to local or distant recurrences [6-8]. Currently, the pathogenesis and carcinogenesis of esophageal cancer has not been fully figured out. And we believe that an in-depth understanding of the oncogen-

esis of esophageal cancer is of profound significance for esophageal cancer therapy.

MicroRNAs (miRNAs), a group of endogenous, small (18-22 nucleotide) non-coding RNAs, have attracted increasing attention in cancer research [9, 10]. By mediating post-transcriptional regulation of target genes through translation repression or promoting RNA degradation, miRNAs are involved in various biological and pathological processes, and considered as a novel species of oncogenes or tumor suppressor genes in human cancers [11, 12].

As two frequently studied miRNAs related with esophageal cancer, miR-21 and miR-203 promotes and suppresses tumor development, respectively [13-15]. Although their expression levels and functions have been identified, the internal correlation between the two miRNAs remained unclear. This study thus investigated the correlation between miR-21 and miR-203 both *in vivo* and *in vitro* in an attempt to provide

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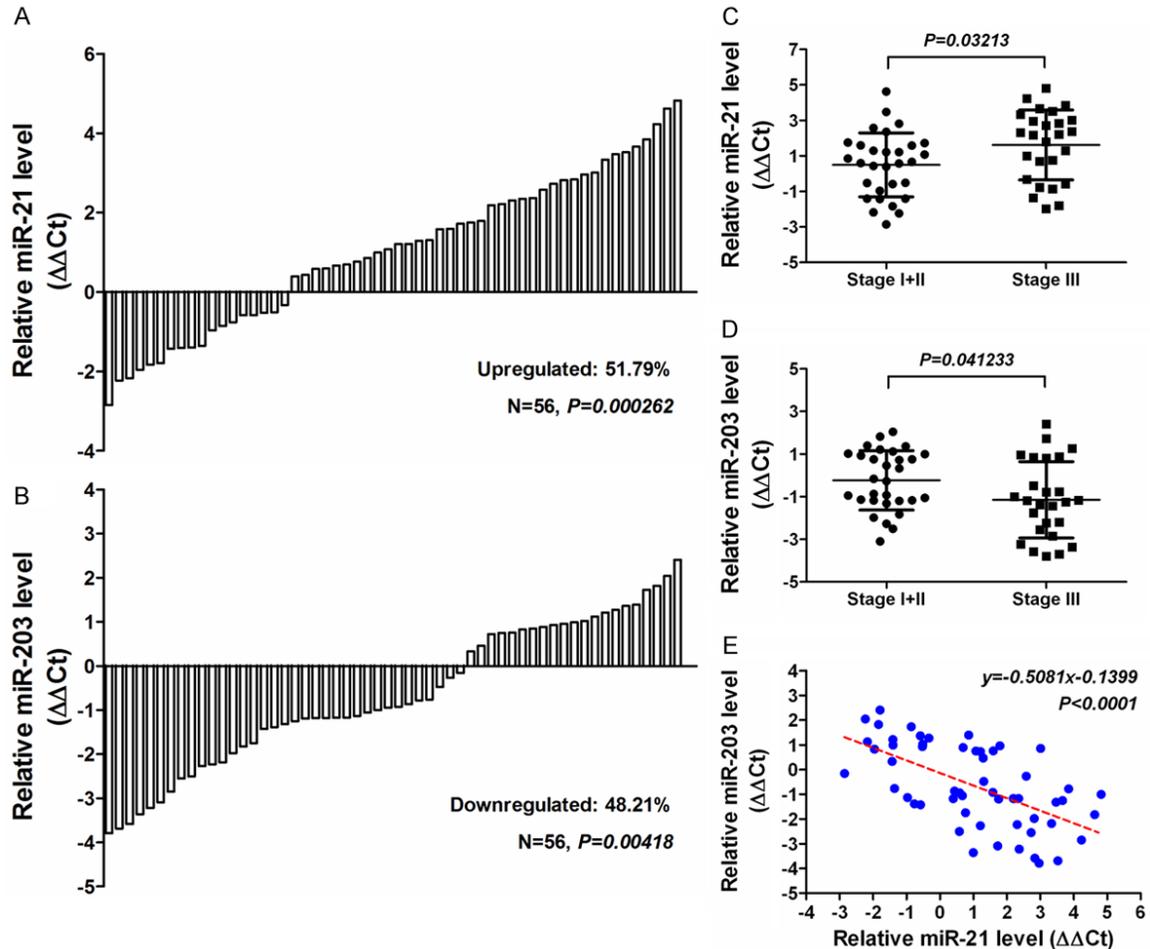


Figure 1. Expression and correlation of miR-21 and miR-203 in 56 paired esophageal squamous cell carcinoma tissues and adjacent normal esophageal tissues. A: $\Delta\Delta\text{Ct}$ of miR-21 expression levels in 56 paired ESCC tissues to adjacent normal esophageal tissues. B: $\Delta\Delta\text{Ct}$ of miR-203 expression levels in 56 paired ESCC tissues to adjacent normal esophageal tissues. C: MiR-21 expression level in stage I+II and stage III ESCC tissues. D: MiR-203 expression level in stage I+II and stage III ESCC tissues. E: Negative correlation between miR-21 and miR-203 expression in 56 paired ESCC tissues to adjacent normal esophageal tissues.

new insights for the internal regulation network among miRNAs.

Materials and methods

Patients and tissues

All esophageal squamous cell carcinoma tissues and adjacent normal esophageal tissues used in this study were collected in Ningbo First Hospital. Written informed consent was obtained from all patients. The Ethics Committee of Ningbo First Hospital offered the ethical approval for the collection and use of patients' samples. Fresh tissues were immediately frozen in liquid nitrogen for 10 min, followed by stored at -80°C for future use.

Cell culture

Normal human esophageal epithelial HEEpic cells and ESCC cell lines EC9706, Ec109, EC-1 and TE-1 were from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 or DMEM medium (Gibco) supplemented with 10% FBS (Hyclone), 100 U/ml penicillin and 100 g/ml streptomycin (Gibco), at 37°C in a humidified incubator containing 5% CO_2 .

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from the frozen tissues or cell pellets was extracted using the TRIzol reagent

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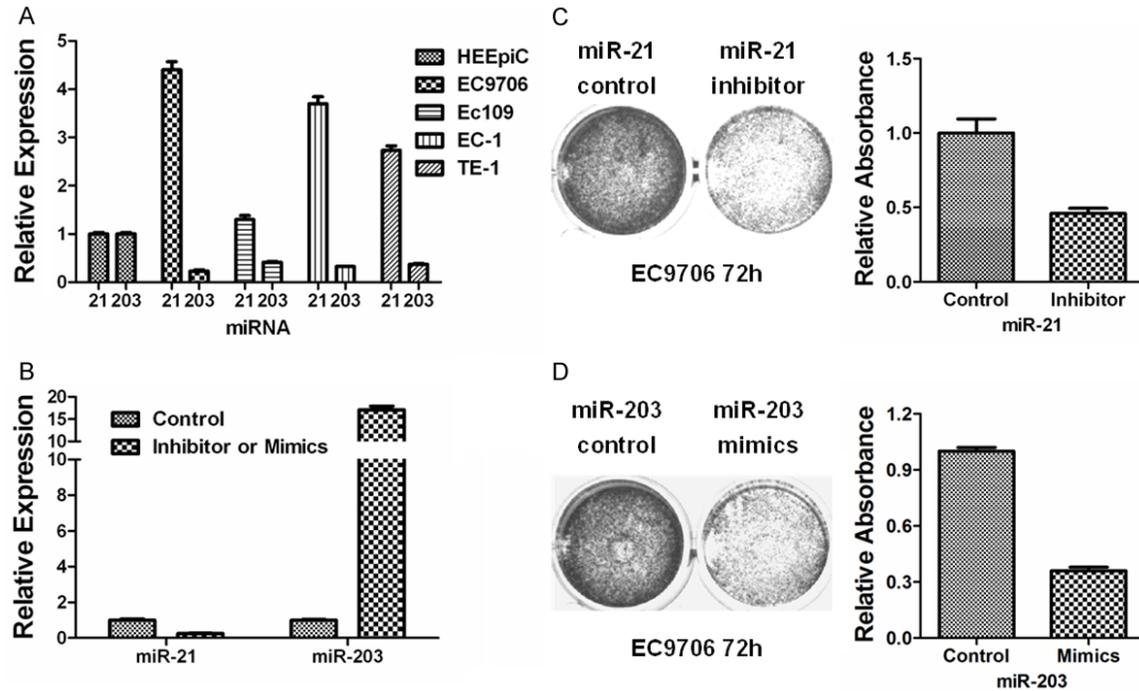


Figure 2. Opposite expression and effects of miR-21 and miR-203 in esophageal cancer cells. A: Different expression of miR-21 and miR-203 in normal human esophageal epithelial HEEpIC cells and four ESCC cells. B: Knock-down or overexpression effects of miR-21 or miR-203 were examined by qRT-PCR. C: Crystal violet staining assay was performed to examine the promoting effect of miR-21 in cell proliferation. The right panel showed the quantitative results. D: Crystal violet staining assay was performed to examine the inhibitory effect of miR-203 in cell proliferation. The right panel showed the quantitative results.

(Life Technologies) according to the manufacturer's instructions. RNA was quantified using a NanoDrop2000 instrument and stored at -80°C . For miR-21 and miR-203 expression, a stem-loop reverse transcription-polymerase chain reaction was carried out by using specific primer kits from Ribobio (Guangzhou, China) according to the manufacturer's protocols. QRT-PCR was carried out with SYBR Green Mix (TOYOBO, Japan). U6 small nuclear RNA was used as an internal control.

MicroRNA mimics, inhibitors and transfections

MiR-21 inhibitor, miR-203 mimics and controls were purchased from Ribobio (Guangzhou, China). Transfections were performed by Lipofectamine2000 as per the manufacturer's protocols.

Crystal violet staining assay

Crystal violet staining was performed to examine the cell proliferation and cell viability. Briefly, cells were transfected with microRNA inhibitor

or mimics. After 72 h, cells were fixed with a 4% paraformaldehyde solution, followed by staining with 0.5% crystal violet and photographed by using a camera.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism v6.0 software. Differences between pairs or groups were determined by paired or grouped Student's t-tests. Linear regression was performed to test the correlation between different groups. A P value <0.05 indicated a statistically significant difference.

Results

Expression profiles of miR-21 and miR-203 in esophageal cancer patients

qRT-PCR experiments were carried out to confirm the expression of miR-21 and miR-203 in a total of 56 pairs of esophageal cancer samples. **Figure 1A** revealed significantly increased expression of miR-21, while **Figure 1B** revealed

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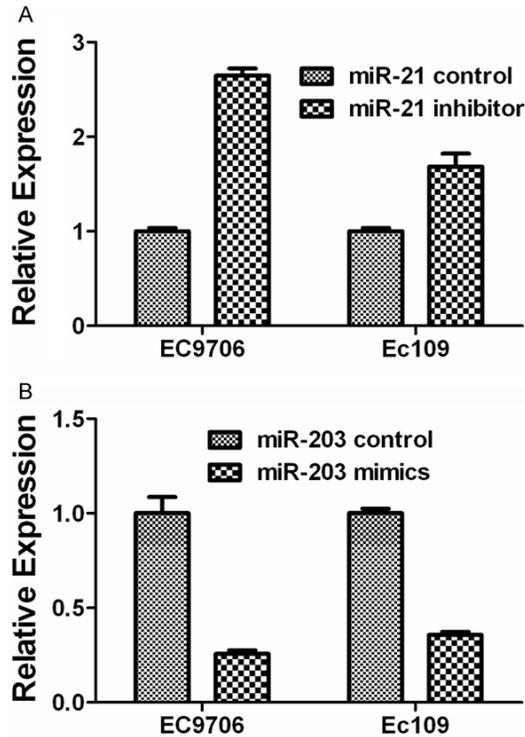


Figure 3. MiR-21 and miR-203 regulated each other in ESCC cells. A: MiR-203 expression was upregulated after transfected with miR-21 inhibitor in two ESCC cell lines. B: MiR-21 expression was down-regulated after transfected with miR-203 mimics in two ESCC cell lines.

significantly reduced expression of miR-203 in tumor tissues compared to adjacent tissues. Moreover, we found that expression of miR-21 was higher in advanced TNM stage (III) than in early TNM stage (stage I or stage II) (Figure 1C). Also, miR-203 expression pattern exhibited the opposite trend (Figure 1D). This finding led us to suppose that the two miRNAs might have negative correlation with each other. As shown in Figure 1E, Pearson's test revealed negative correlation between miR-21 and miR-203 expression in tumor tissues.

Opposite effects of miR-21 and miR-203 in esophageal cancer cell proliferation

Although it is clear that miR-21 exerts tumor promoting functions while miR-203 inhibits esophageal cancer progression, we still verified these in our systems. For choice of cell lines, we firstly determined the expression of both miR-21 and miR-203 in four esophageal cancer cell lines (EC9706, Ec109, EC-1 and TE-1), and one non-cancerous HEEpiC cell line. We found

the highest miR-21 meanwhile the lowest miR-203 expression in EC9706 cells (Figure 2A). Then we inhibited miR-21 by introducing its inhibitor or overexpressed miR-203 by introducing its mimics in EC9706 cells (Figure 2B). As shown in Figure 2C and 2D, the proliferation of EC9706 cells in the miR-21-inhibitor group was significantly decreased, while miR-203-mimics expressing cells had significantly lower growth rate, compared with that in the control groups ($P < 0.01$). These results indicated that miR-21 and miR-203 had important and opposite effects on EC9706 cell growth.

Correlation between miR-21 and miR-203 expression in vitro

In order to investigate the association between miR-21 and miR-203 in esophageal cancer cells, qRT-PCR was performed. As shown in Figure 3A, in the miR-21-inhibitor transfected EC9706 and Ec109 cells, we observed that the endogenous miR-203 expression was elevated slightly, by 1.5-3 fold upregulation. In addition, in the miR-203-mimics transfected EC9706 and Ec109 cells, we found that miR-21 expression was significantly decreased, by more than 3-4 fold downregulation ($P < 0.01$, Figure 3B). These results provided direct evidence that miR-21 and miR-203 had negative correlation and targeted each other in esophageal cancer cells.

Discussion

A large body of literatures have indicated that altered expression of miRNAs contribute to the initiation and progression of cancers [16-18]. And miRNA has significant value in tumor diagnosis, treatment efficacy and prognosis evaluation.

In esophageal cancer, miR-21 was shown to promote proliferation, migration, invasion, and cell cycle, and inhibit apoptosis via targeting key proteins in PTEN/PI3K/AKT signaling pathway [13]. In addition, dysregulation of miR-203 has been observed in esophageal cancer. Specifically, studies showed that miR-203 is a novel transcriptional target of E2F1 and that it regulates cell cycle arrest [19], suppresses tumor growth and invasion, through down-regulating the expression of Ran and miR-21 [15] in esophageal cancer. In the latter report, it was the first time we noticed miR-21 might be a tar-

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get of miR-203 [15]. Since few studies have reported the correlation between the two miRNAs, we intended to investigate the correlation between miR-21 and miR-203 expression in esophageal cancer.

Our results showed remarkably higher expression of miR-21 in esophageal cancer tissues, as consistent with former reports [13, 14]. In contrast with miR-21, miR-203 expression was significantly lower in tumor samples compared to adjacent tissues. We also observed negative correlation between miR-21 and miR-203 expression, and their opposite biological functions in esophageal cancer cell proliferation. More importantly, we found that inhibition of miR-21 expression led to obviously upregulation of miR-203, and forced miR-203 expression caused miR-21 repression. Hence, we have clearly showed that these two miRNAs targeted to each other, and further suggested that their oncogenic or tumor suppressing functions might be mediated by each other, forming a circulating feedback in esophageal cancer cells.

In conclusion, the present study has shown that miR-21 was upregulated while miR-203 was downregulated in esophageal cancer, with an internal negative correlation. And they oppositely regulated the growth of esophageal cancer cells. Further study using the mouse model would provide novel insights into their relationship and functions in esophageal cancer.

Disclosure of conflict of interest

None.

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