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Original Article

Effects of miR-545-3p expression in esophageal cancer tissues on proliferation and apoptosis of esophageal cancer cells

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Abstract: Objective: The aim of this study was to discuss the effects of expression of miR-545-3p in esophageal cancer tissues on proliferation and apoptosis of esophageal cancer cells. Methods: This study collected samples of cancer tissues and para-carcinoma tissues from 121 esophageal cancer (EC) patients, admitted to the Central Hospital of Wuhan, between April 2013 and July 2014. Expression vectors were conducted for miR-545-3p, transfecting them into human esophagus cancer CaEs-17 cells. Cells with recombinant expression vectors were selected as the positive group, while those with empty expression vectors were selected as the control group. Real-time quantitative reverse transcription polymerase chain reaction was used to detect expression levels of miR-545-3p in EC tissues and CaEs-17 cells. Proliferation, apoptosis, and invasion of CaEs-17 cells were tested by MTT assay, TUNEL assay, and Transwell migration assay, respectively. Results: Relative expression levels of miR-545-3p in EC tissues were lower than in normal para-carcinoma tissues ($P<0.05$). Relative expression levels of miR-545-3p in the positive group were higher than in the control group ($t=3.026$, $P=0.039$). According to MTT assay, absorbance of CaEs-17 cells in the positive group was lower than that in the control group after 12 hours, 24 hours, 48 hours, and 72 hours of culturing, respectively (all $P<0.05$). Results indicated that the proliferative capacity of CaES-17 cells in the positive group was lower than in the control group. Comparison of absorbance, at different time points, between the two groups showed that the absorbance of both groups increased in the first 48 hours of culturing ($P<0.05$). Absorbance of the control group continued to increase after 72 hours of culturing ($P<0.05$). No significant differences were found in the absorbance of the positive group at 72 hours of culturing, compared with that at 48 hours of culturing ($P>0.05$). According to TUNEL assay the apoptosis rate of CaEs-17 cells in the positive group was significantly higher than that in the control group ($t=14.987$, $P<0.001$). According to Transwell migration assay, the number of migrated CaEs-17 cells in the positive group was significantly lower than that in the control group ($t=9.074$, $P=0.001$). Conclusion: Expression levels of miR-545-3p declined in esophageal cancer tissues, indicating that miR-545-3p could be a potential therapeutic target in the treatment of EC by inhibiting proliferation and invasion of EC cells and promoting apoptosis.

Keywords: Esophageal cancer, miR-545-3p, esophageal cancer cells, proliferation, apoptosis

Introduction

Esophageal cancer (EC) is one of the most common malignant tumors in the gastrointestinal tract, accounting for about 2% of all tumors [1]. In general, the prognosis of intermediate and advanced stage EC patients is poor. Prognosis of EC patients worsens as the cancer progresses, with overall five-year survival rates at about 30%. It is the second most lethal malignant cancer following gastric cancer, causing about 220,000 deaths annually [2, 3]. Treatment of EC usually involves surgery, supplemented by radiotherapy and chemotherapy. The resection rate of early-stage EC is quite high, but the effects of chemotherapy alone are not satisfactory. Despite the development of medical technology in recent years, the survival rate of EC patients remains relatively low [4, 5]. Therefore, it is very important to identify other therapeutic targets.

MicroRNAs (miRNA) are ubiquitously expressed in eukaryotic cells, regulating cell proliferation,

differentiation, and apoptosis. Abnormal changes in the biosynthesis of miRNAs are involved in a variety of pathophysiological processes [6, 7]. Many studies reported that miRNAs are closely related to biological behaviors of tumor cells, including proliferation and invasion [8, 9]. As a tumor-associated, small, and non-coding RNA newly discovered in recent years, miR-545-3p might be a new marker for the treatment and prognosis of colon cancer, according to Goblirsch [10]. Moreover, there have been reports on the relationship between miR-545-3p and lung adenocarcinoma [11]. There have been very few reports, however, on the relationship between miR-545-3p and EC.

The present study investigated the effects of miR-545-3p on the biological behavior of tumor cells, analyzing expression levels of miR-545-3p in cancer tissues of EC patients and regulating expression levels of miR-545-3p in human esophagus cancer CaEs-17 cells. Results of the present study may contribute to the clinical treatment of EC, prolonging survival times of EC patients and improving their quality of life.

Materials and methods

Subjects

Samples of cancer tissues and para-carcinoma tissues were collected from 121 EC patients admitted to The Central Hospital of Wuhan, between April 2013 and July 2014. All patients included in this study met the following inclusion criteria: Patients pathologically diagnosed with EC in The Central Hospital of Wuhan; Patients aged 45-65 years old; Patients had no abnormalities in leukocyte and lymphocyte counts; Patients whose cancer showed no signs of distant metastasis, according to medical imaging; Patients with a predicted survival time of more than 3 months; Patients had not accepted any anti-tumor therapy or had no large tumors before surgery; Patients with no history of other tumors, no family genetic diseases, and no immune defects; Patients with no cardiac, liver, kidney, and other organ dysfunction before surgery; Patients suffering no abnormal bleeding or coagulation disorders before surgery and patients with no history of alcohol abuse or nitroglycerin use. This study was approved by the Ethics Committee of the Central Hospital of Wuhan. All participants and relatives provided informed consent before entry into the study.

Human esophagus cancer cell line CaEs-17 (Shanghai Institute of Biochemistry and Cell Biology) was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Thermo Fisher Scientific) in an atmosphere with a temperature of 37°C, 5% CO_2 concentration, and humidity of 70-80%. The construction of miR-545-3p expression vector was carried out by Guangzhou LabGene Biotechnology Co., Ltd. using *E. coli* plasmid vector pGEX-4T-1 (Wuhan Miaoling Biotechnology Co., Ltd.). Recombinant expression vectors were selected as the positive group, while empty expression vectors were selected as the control group. Respectively, they were transfected into CaEs-17 cells and dissociated by trypsin in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C and a CO_2 concentration of 5% for 48 hours. After transfection, the cellswere cultured for further detection. miR-545-3p: Chromosome X, NC_000023.11 (74287104.74287209, complement).

Total RNA isolation

TRIzol Reagent (Thermo Fisher Scientific) was used to isolate total RNA from cancer tissue cells, para-carcinoma tissue cells, and CaEs-17 cells. Manufacturer instructions were followed for the reagent kit. GeneQuant 1300 ultraviolet spectrophotometer (GE Healthcare) was used to measure concentrations and purity of the total RNA.

Real-time quantitative reverse transcription polymerase chain reaction of miR-545-3p

Total RNA was then used in the synthesis of cDNA through reverse transcription (RT). Procedures were guided by manufacturer instructions of the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). The RT reaction was based on a 10 μL mixture, which included 1.0 μL of oligo (dt) primer, 1.0 μL of dNTPs, 2 μg of total RNA, and RNase-free water. The mixture was heated to 65°C for 5 minutes. Next, 2 μL of cDNA templates, 32.5 μL of SYBR Green Mix, 0.5 μL of forward primers, and 0.5 μL of reverse primers were added into the mixture. It was diluted to 50 μL with double-distilled water before PCR amplification. Samples were amplified for 30 cycles, with each cycle consisting of an initial denaturation of 3 minutes at 95°C, a denaturation of 30 seconds at 95°C, an annealing of 30 seconds at 55°C, and an extension of 60 seconds at 72°C. They

Table 1. Sequences of primers

	Forward primer	Reverse primer
miR-545-3p	5'-CGACAAGGGTCAGCAAACATT-3'	5'-GCAGGGTCCGAGGTATTC-3'
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Table 2. Baseline characteristics of EC patients

	Baseline characteristics
Age Range (Y)	58.42±12.14
Gender	
Male	85 (70.2)
Female	36 (29.8)
Tumor site	
Upper part of esophagus	22 (18.2)
Middle part of esophagus	79 (65.3)
Lower part of esophagus	20 (16.5)
TNM staging	
0	12 (9.9)
I	14 (11.6)
II	49 (40.5)
III	46 (38.0)
Types of EC	
Squamous cell carcinoma	104 (86.0)
Adenocarcinoma	3 (2.5)
Small cell carcinoma	6 (4.9)
Mucoepidermoid carcinoma	1 (0.8)
Other types	7 (5.8)
Grading	
Undifferentiated/Poorly differentiated	39 (32.2)
Moderately differentiated	41 (33.9)
Well differentiated	41 (33.9)

Note: EC: Esophageal cancer.

were processed with a final extension of 5 minutes at 72°C. GAPDH was used as internal control in real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). All samples were performed in triplicate. Results were analyzed using the $2^{-\Delta\Delta CT}$ method. Primers were designed and synthesized by Shanghai Daixuan Biotech Co., Ltd. Sequences are shown in **Table 1**.

MTT assay

CaEs-17 cells of both groups were harvested, respectively, and suspended at $4*10^6$ per mL. The cells were then added into 96-well cell culture plates and incubated for 6 hours before incubating for another 4 hours at 37°C with 20 μL of MTT reagent (5 mg/mL) added into each well. After incubation, the supernatant of each well was removed to discard foreign substances and dimethyl sulfoxide was added into each well before shaking the plate on a horizontal shaker for 10 minutes. Finally, the absorbance of CaEs-17 cells in both groups was measured on an ELISA plate reader at a wavelength of 570 nm after 12 hours, 24 hours, 48 hours, and 72 hours of culturing, respectively. The MTT Assay Kit was purchased from Beijing Biofine Co., Ltd.

TUNEL assay

CaEs-17 cells of both groups were fixed by 4% neutral-buffered formalin at room temperature for 10 minutes, then washed twice with phosphate buffer saline (PBS) for 5 minutes each. The cells were later treated with 2% H_2O_2 in PBS at room temperature for 5 minutes, then washed twice with PBS for 5 minutes each. Cell staining was carried out according to manufacturer instructions of the TUNEL Reagent Kit (Shanghai Yanqi Biotech Co., Ltd.). Image-pro Plus 5.0 software was used to calculate the number of TUNEL-positive cells in 5 distinct microscope fields of 400X magnification. The integrated optical density represented the total number of TUNEL-positive cells. This procedure was repeated three times.

Transwell migration assay

The cultured cell suspension ($5*10^5$/mL) of both groups was pipetted with 100 μL in Transwell chambers, respectively. The number of cells migrating through the insert was measured after 24 hours. The assay was conducted in triplicate. The Transwell chamber was purchased from Shanghai Shengbo Biopharmaceutical Technology Co., Ltd.

Statistical analysis

Data were analyzed with SPSS 19.0 statistical software (AsiaAnalytics (formerly SPSS China)).

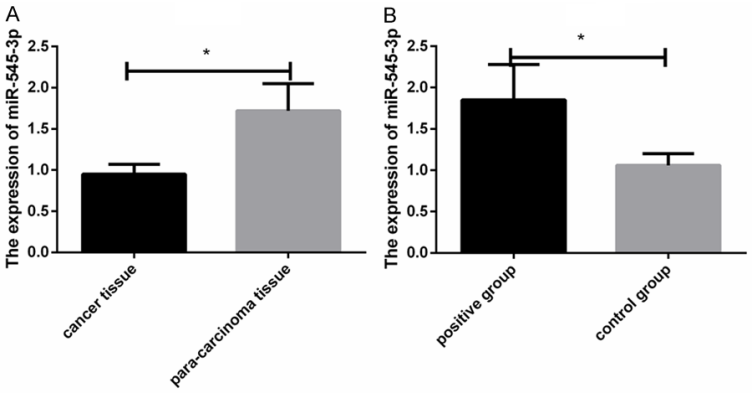

Figure 1. Relative expression levels of miR-545-3p. A. Relative expression levels of miR-545-3p in cancer tissues and para-carcinoma tissues were (1.06±0.14). Relative expression levels of miR-545-3p in cancer tissues were significantly lower than that in normal para-carcinoma tissues between the two groups (t=24.121, P<0.001). *: P<0.05. B. Relative expression levels of miR-545-3p in the positive group and the control group. Relative expression levels of miR-545-3p in CaEs-17 cells of the positive group were higher than that in CaEs-17 cells of the control group (t=3.026, P=0.039). *: P<0.05.

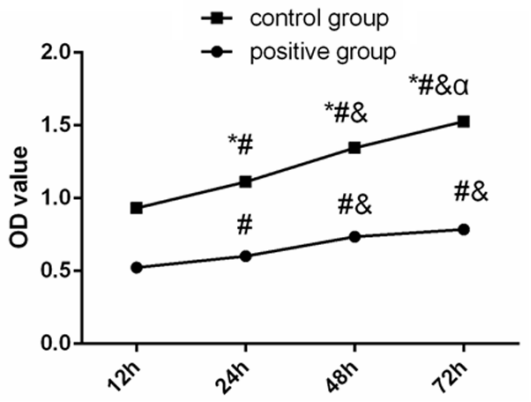

Figure 2. Results of MTT assay. The absorbance of CaEs-17 cells in the control group increased at every time point (all P<0.05). In the first 48 hours of culturing, the absorbance of CaEs-17 cells in the positive group continuously increased (P<0.05). No significant differences were found between the absorbance of the positive group at 48 hours and 72 hours of culture (P>0.05). The absorbance of the positive group was lower than that of the control group at every time point (all P<0.05). *: Compared with the control group, P<0.05; #: Compared with the absorbance at 12 hours of culturing, P<0.05; &: Compared with the absorbance at 24 hours of culturing, P<0.05; α: Compared with the absorbance at 48 hours of culturing, P<0.05.

Enumeration data are expressed as percentages (%) and were compared using Chi-squared tests. Measurement data are expressed as mean ± standard deviation ($\bar{x}$ ± sd). Comparisons of normally-distributed data between the two groups were based on t-test. Comparisons within the groups, at different time points, were based on repeated measures analysis of variance. *P* values <0.05 indicate statistical significance.

Results

General information

A total of 121 EC patients (mean age 58.4±12.14 years) were recruited, consisting of 85 male patients and 36 female patients. Tumors mostly occurred in the middle section of the esophagus. Stages of EC in these patients mainly ranged from stage II-III, according to the TNM system. Squamous cell carcinoma was the major type of EC in the patients enrolled. Of these, 39 patients were graded as undifferentiated/poorly differentiated, 41 patients moderately differentiated, and 41 patients well differentiated. See **Table 2**.

Comparison of relative expression levels of miR-545-3p

Relative expression levels of miR-545-3p in EC patients were 0.95±0.12. Relative expression levels of miR-545-3p in normal para-carcinoma tissues were 1.72±0.33. Differences between expression levels were statistically significant (t=24.121, P<0.001). Relative expression levels of miR-545-3p in EC tissues were lower than in normal para-carcinoma tissues (P<0.05). Relative expression levels of miR-545-3p in CaEs-17 cells of the positive group were 1.85±0.43, while relative expression levels of miR-545-3p in CaES-17 cells of the control group were 1.06±0.14. Differences in relative expression levels of miR-545-3p were statistically significant between the two groups. Relative expression levels of miR-545-3p in the positive group were higher than in the control group (t=3.026, P=0.039). See **Figure 1**.

Comparison of MTT assay results

Results of MTT assay showed that the absorbance of CaEs-17 cells in the positive group was lower than in the control group after 12

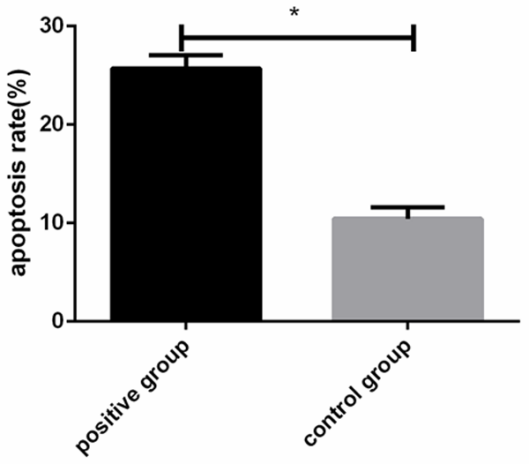

Figure 3. Results of TUNEL assay. The apoptosis rate of CaEs-17 cells in the positive group was significantly higher than that in the control group. *: P<0.05.

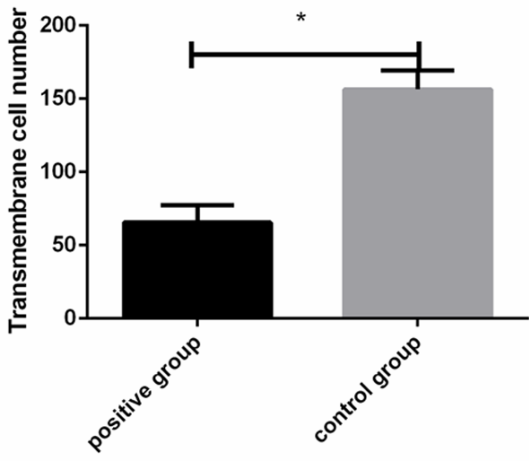

Figure 4. Results of Transwell assay. The number of migrated cells in the positive group was significantly lower than that in the control group (t=9.074, P=0.001). *: P<0.05.

hours, 24 hours, 48 hours, and 72 hours of culturing, respectively (all P<0.05). Results indicate that the proliferative capacity of CaES-17 cells in the positive group was lower than in the control group. Comparison of absorbance, at different time points, within the two groups showed that the absorbance of both groups increased in the first 48 hours of culturing (P<0.05). Absorbance of the control group continued to increase until 72 hours of culturing (P<0.05). No significant differences were found in absorbance of the positive group at 72 hours of culturing, compared with that at 48 hours of culturing (P>0.05). See **Figure 2**.

Comparison of TUNEL assay results

Results of TUNEL assay showed that the apoptosis rate of CaEs-17 cells in the positive group was 25.72±1.33%. This was significantly higher than the control group, 10.45±1.16% (t=14.987, P<0.05). See **Figure 3**.

Comparison of Transwell migration assay results

Results of Transwell migration assay showed that the number of migrated CaEs-17 cells in the positive group was 65.58±11.64, while the number of migrated CaEs-17 cells in the control group was 156.45±12.86. The number of migrated cells in the positive group was significantly lower than that in the control group (t=9.074, P=0.001). See **Figure 4**.

Discussion

Occurrence and progression of EC is a multistage process involving a variety of molecules. Its most important biological characteristics, the malignant tumor cells of EC are capable of proliferating limitlessly and are highly invasive [12, 13]. However, early-stage EC patients generally show no typical clinical signs. EC is slow in progression and shares some symptoms with esophagitis and esophageal varices [14, 15]. Accordingly, EC is usually diagnosed at an intermediate or advanced stage. Chemotherapy is currently not a satisfactory treatment for EC patients of intermediate and advanced stages [16, 17]. Therefore, it is of great significance to identify new therapeutic targets for the treatment of EC. A tumor-associated gene newly discovered in recent years, miR-545-3p has been reported to be a potential biological marker for the treatment and prognosis of colorectal cancer and lung adenocarcinoma [10, 11]. The purpose of this study was to investigate expression levels of miR-545-3p in EC patients and the effects of miR-545-3p on EC cells, providing guidance for the clinical treatment of EC.

In this study, 121 EC patients were included, in strict accordance with inclusion criteria. Cancer tissues and para-carcinoma tissues were collected. Expression levels of miR-545-3p in the two tissues were detected by qRT-PCR. Results showed that expression levels of miR-545-3p in EC tissues were significantly lower than that in normal para-carcinoma tissues. Therefore, it

was assumed that miR-545-3p might function as tumor suppressor gene in EC. Culturing of CaEs-17 cells further verified this hypothesis. The EC cell line CaES-17 has been well recognized as an easily cultured cell line. It has a high survival rate and can be continuously passaged in culture with no morphological changes, being capable of proliferating rapidly [18, 19]. Expression vectors of miR-545-3p were conducted, successfully transfecting them into CaEs-17 cells. Results of qRT-PCR showed that expression levels of miR-545-3p in CaEs-17 cells were higher in the positive group than the control group.

There have been very few reports on miR-545-3p. Over the past five years, only Cosín-Tomás et al. reported that miR-545-3p might be an early biomarker of Alzheimer's disease [20]. Goblirsch et al. performed genetic screening over colorectal cancer. They found that expression levels of miR-545-3p were declined in colorectal cancer and that increased miR-545-3p expression was associated with increased disease-free survival in colorectal cancer patients [10]. The present study investigated the effects of miR-545-3p on the biological behavior of CaEs-17 cells. Results showed that miR-545-3p may inhibit the proliferation and invasion of CaEs-17 cells and contribute to apoptosis. Zhu et al. found that inhibited expression of miR-545-3p could promote the proliferation of lung adenocarcinoma cells [11]. Jeong et al. reported that PM2.5 could promote expression of miR-545-3p in normal alveolar epithelial cells. They assumed that the gene might help resist cell damage [21]. These results are consistent with present results. However, the present study still had some limitations. First, there were relatively few patients included in this study. Second, CaEs-17 cells might not fully reflect the characteristics of EC cells. Due to experimental limitations, the 121 EC patients included in this study were not followed-up. Therefore, more experimental data are necessary to verify whether miR-545-3p is associated with survival rates of EC patients. More clinical data and experiments are required to confirm present results and conclusions.

In conclusion, expression levels of miR-545-3p were declined in esophageal cancer tissues. Results indicate that miR-545-3p could be a potential therapeutic target for the treatment of EC by inhibiting the proliferation and invasion of EC cells and promoting apoptosis.

Disclosure of conflict of interest

None.

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