

# Downregulated miR-486-5p acts as a tumor suppressor in esophageal squamous cell carcinoma

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**Abstract.** microRNAs (miRNAs/miRs) are crucial regulators of gene expression at the post-translational level through promoting mRNA degradation or the repression of translation of target genes. miRs have been confirmed to serve a dominant role in tumor biology. miR-486-5p has been ascertained to be involved in non-small-cell lung cancer, breast cancer and hepatocellular carcinoma; however, the expression and function of miR-486-5p in esophageal squamous cell carcinoma (ESCC) has yet to be elucidated. The present study aimed to analyze the expression levels of miR-486-5p in ESCC tissues and paired normal adjacent tissues, and determine the effects of miR-486-5p on esophageal cancer cells using MTT, wound scratch and apoptosis assays. The current results showed that miR-486-5p was significantly downregulated in ESCC specimens. Ectopic expression of miR-486-5p by synthetic mimics reduced cell proliferation and migration and induced increased cell apoptosis. The results indicated miR-486-5p may function as a tumor suppressor in ESCC. The present study demonstrated that miR-486-5p was downregulated in ESCC and served an anti-oncogene role in ESCC via affecting cellular migration.

## Introduction

Esophageal cancer is the eighth most common type of cancer and the sixth leading cause of cancer-associated mortality worldwide (1-3). Esophageal cancer incidence in men is higher compared with that in women, and its occurrence increases with age, and mortality rate is ~90% for all cases (1,2,4). Esophageal squamous cell carcinoma (ESCC) is the predominant histological type amongst Chinese populations, resulting

in 150,000 cases of mortality annually (5). Despite advances in medical and surgical techniques, the prognosis for ESCC remains poor, and long-term survival is in the range of 26.2-49.4% due to local or distant recurrences (6). Therefore, it is critical to identify novel molecular mechanisms to elucidate oncogenesis and metastasis in ESCC.

MicroRNAs (miRNAs/miRs) are small (18-22 nucleotide) endogenous non-coding RNAs that serve crucial roles in various biological processes (7,8). Mature miRNAs usually bind to the 3'-untranslated regions of target genes to downregulate the expression of target genes at post-transcriptional levels through promotion of mRNA degradation or repression of the translation of target genes (7,9). Since miRNA-lin-4 was initially identified in 1993, numerous miRNAs have been ascertained to be involved in various physiological and pathological processes, including carcinogenesis (10). Several miRNAs such as miR-21, miR-34a and miR-155 have been found to be associated with carcinogenesis by targeting oncogenes or anti-oncogenes (11-13).

Recently, miR-486-5p has been reported to function as a tumor suppressor in non-small-cell lung cancer (NSCLC) (14), breast cancer (15) and hepatocellular carcinoma (16). However, the function and clinical significance of miR-486-5p in ESCC has yet to be elucidated (15). In the present study, the expression levels of miR-486-5p in ESCC tissues were determined, and the function of miR-486-5p in ESCC cells were investigated by cell migration, proliferation and apoptosis assays. The results indicated that miR-486-5p was downregulated in ESCC tissues and functioned as an anti-oncogene in ESCC by affecting cellular migration, proliferation and apoptosis.

## Materials and methods

**ESCC tissue sample collection.** All ESCC and adjacent normal tissues used in the present study were collected in Dongnan Affiliated Hospital of Xiamen University (Zhangzhou, China). Written informed consent was obtained from all patients. Ethical approval for the collection and use of all samples was approved by the Ethics Committee of Dongnan Affiliated Hospital of Xiamen University. Fresh tissues were immersed in RNAlater (Qiagen GmbH, Hilden, Germany) in 30 min after resection and subsequently stored at -80°C for future use.

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**Cell culture and transfection.** Human ESCC cell lines Eca109 and TE-1 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Eca109 and TE-1 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37°C for 24 h in a humidified incubator containing 5% CO<sub>2</sub>. For the restoration of miR-486-5p in ESCC tissues with endogenously downregulated miR-486-5p, synthesized miR-486-5p mimics (GenePharma Co., Ltd., Shanghai, China) was transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were trypsinized, and total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) 24 h after transfection.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays.** Total RNA was extracted from 36 ESCC tissue samples and adjacent normal esophageal tissues, or from the trypsinized ESCC cell lines Eca109 and TE-1, using TRIzol reagent (Invitrogen), and purified using an RNeasy Maxi Kit (Qiagen GmbH) according to the manufacturer's protocol. To obtain the cDNA templates, 1 µg total RNA of each sample was used for reverse transcription using an miScript Reverse Transcription kit (Qiagen GmbH). This reaction was performed at 37°C for 60 min, then 95°C for 5 min. The qPCR reaction of miR-486-5p was performed on an ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using miScript SYBR Green PCR Kit (Qiagen GmbH). The 20 µl reaction mixture contained 1 µl cDNA template, 2 µl 10X miScript Universal Primer, 0.4 µl of each of the specific miRNA primers, 10 µl 2X QuantiTect SYBR Green PCR Master mix and 6.6 µl RNase-free water. Primer sequences were as follows: Forward, 5'-TCCTGTACTGAGCTGCCCCGAG-3' [the reverse primer was provided by the miScript SYBR Green PCR Kit (Qiagen GmbH)] for miR-486-5p; and forward, 5'-CTCGCTTCG GCAGCAC-3' and reverse, 5'-ACGCTTCACGAATTT GCGT-3', for U6 (U6 was used as an endogenous control in the present study). Amplification conditions were set as follows: 95°C for 2 min, followed by 95°C for 15 sec, 58°C 30 sec and 72°C for 30 sec, for 40 cycles. This experiment was repeated 3 times, with accompanying no cDNA and no reverse transcriptase controls. The expression of miR-486-5p was analyzed using the  $\Delta\Delta C_q$  method (17), normalizing to U6 expression.

**Migration assay.** A wound scratch assay was used to assess the migratory ability of Eca109 and TE-1 cells *in vitro*. Cells (~150,000) were seeded into a 12-well dish and transfected with miR-486-5p mimics (60 pmol) or the negative, scrambled control (60 pmol) (GenePharma Co., Ltd.) 24 h later. A sterile 200 µl pipette tip was used to scrape a clear line through the cell monolayer 5 h post-transfection. The cells were then rinsed three times with phosphate-buffered saline (PBS) and cultured in an incubator at 37°C. Images of the wound scratches were acquired with an inverted light microscope (DM IRB; Leica Microsystems GmbH, Wetzlar, Germany) at 0 and 24 h after the wounds were made. The migration

distance (µm) was measured with a standard caliper and the experiments were performed in triplicate and analyzed by at least two observers.

**MTT assay.** The cell proliferation of Eca109 and TE-1 cells was determined with MTT assay kit (Sigma-Aldrich, St. Louis, MO, USA). To determine cell growth, ~5,000 cells were seeded into the wells of 96-well plates and transfected with miR-486-5p mimics (5 pmol) or the negative, scrambled control (5 pmol). MTT (20 µl; 5 mg/ml; Sigma-Aldrich) was added to each well at 0, 24, 48 and 72 h after transfection. Subsequent to incubation for 4 h, the MTT medium was removed and 150 µl dimethyl sulfoxide was added. After shaking for 15 min at room temperature, the optical density (OD) of each sample was determined with an Enzyme Immunoassay Instrument (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490/630 nm.

**Flow cytometry.** For apoptosis assays, Eca109 and TE-1 cells were cultured in 6-well plates at 37°C to a confluence of ~65% and transfected with miR-486-5p mimics or a negative control. After 48 h of transfection, Eca109 and TE-1 cells were harvested and washed twice with cold PBS, then resuspended in 10 µl 1X binding buffer (Invitrogen). Annexin V-FITC (5 µl; Invitrogen) and 10 µl propidium iodide was added to each sample. According to the manufacturer's protocol, the fluorescence of stained cells was then assessed by flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) using 488 nm excitation within 30 min.

**Statistical analysis.** Statistical analysis was performed using the SPSS statistical software package (version 17.0; SPSS, Inc., Chicago, IL, USA). Statistical significance was determined by paired and Student's t-tests.  $P < 0.05$  and  $P < 0.01$  were considered to indicate a statistically significant difference.

## Results

**miR-486-5p expression levels were downregulated in ESCC tissues.** RT-qPCR was used to determine expression levels of miR-486-5p in 35 paired ESCC tissues and adjacent normal tissues. The relative expression of miR-486-5p in the 35 paired ESCC tissues and adjacent normal tissues are shown in Fig. 1A. miR-486-5p expression in ESCC tissues was significantly downregulated compared with those of the paired adjacent normal tissues ( $P < 0.05$ ) (Fig. 1B).

**Transfection efficiency.** To analyze the function of miR-486-5p in ESCC, miR-486-5p mimics and negative controls were transfected into the ESCC cell lines, Eca109 and TE-1. Images of cells transfected with Fam-labeled negative control were obtained 6 h after transfection. As shown in Fig. 2A, the transfection efficiency was ~80 and ~85% in Eca109 and TE-1 cells, respectively. Compared with the negative control, the relative expression levels of miR-486-5p in Eca109 and TE-1 cells transfected with miR-486-5p mimics were 94- and 103-fold, respectively ( $P < 0.05$ ; Fig. 2B). The results demonstrated that miR-486-5p mimics were effective in upregulating the expression of miR-486-5p.

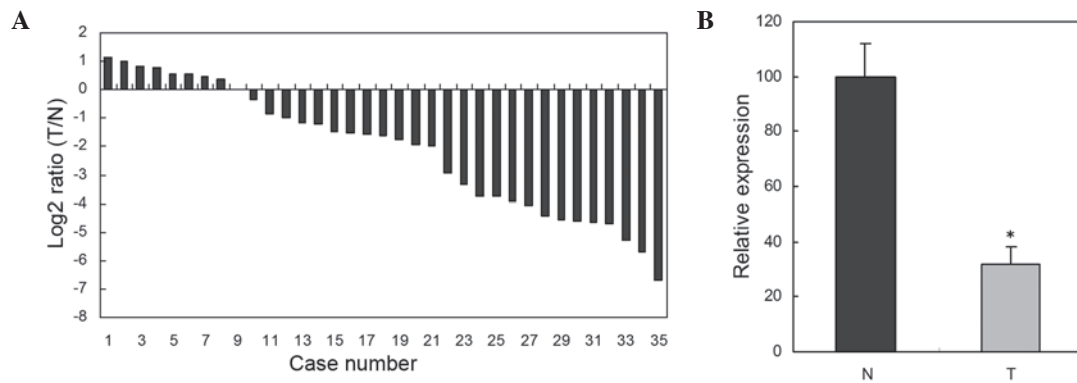


Figure 1. Expression levels of miR-486-5p in 35 paired esophageal squamous cell carcinoma (ESCC) tissues and adjacent normal esophageal tissues. (A) Log<sub>2</sub> ratios of miR-486-5p expression levels in 35 paired ESCC tissues (T) to adjacent normal esophageal tissues (N). (B) The relative expression of miR-486-5p in ESCC (T) and adjacent normal esophageal tissues (N). Values presented are the mean  $\pm$  standard deviation of three independent experiments ( $P < 0.05$ ).

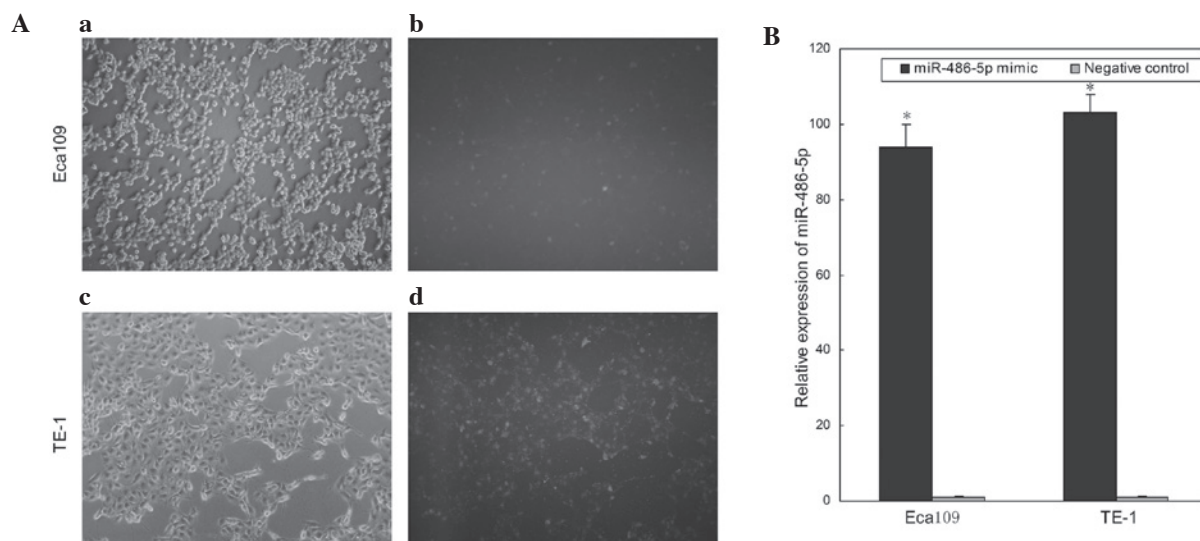


Figure 2. Analysis of transfection efficiency and miR-486-5p expression levels by fluorescence microscopy and reverse transcription-quantitative polymerase chain reaction. (Aa-Ad) The transfection efficiency was  $\sim 80$  and  $\sim 85\%$  in Eca109 and TE-1 cells, respectively. Images of (Aa) Eca109 cells transfected with the Fam-labeled negative control. (Ab) Eca109 cells exhibiting green fluorescence 6 h post-transfection. (Ac) Images of TE-1 cells transfected with the Fam-labeled negative control. (Ad) TE-1 cells exhibiting green fluorescence 6 h after transfection. (B) The fold changes of miR-486-5p expression in Eca109 and TE-1 cells treated with miR-486-5p mimics or the negative control were determined 24 h post-transfection. Values presented are the mean  $\pm$  standard deviation of three independent experiments ( $P < 0.05$ ).

**Overexpression of miR-486-5p suppressed ESCC cell migration *in vitro*.** The effects of overexpression of miR-486-5p on cell migration of ESCC cells *in vitro* was determined by the use of a wound scratch assay. As shown in Fig. 3, the wound widths of Eca109 and TE-1 cells transfected with miR-486-5p mimics were wider ( $P < 0.05$ ) compared with those of the negative control group at 24 h. Thus, it was indicated that upregulation of miR-486-5p inhibited the cell migration of ESCC cells.

**miR-486-5p mimics inhibited cell proliferation.** The impact of miR-486-5p on cell proliferation in ESCC cells was analyzed using an MTT assay. The OD values of the miR-486-5p mimic and negative control groups were measured at 0, 24, 48 and 72 h after transfection. The results showed that the proliferation of Eca109 cells decreased by 9.09, 15.71 and 19.84% (all  $P < 0.05$ ) at the respective aforementioned time-points, while the proliferation of TE-1 cells decreased by 8.89, 14.47 and 19.17% (all  $P < 0.05$ ; Fig. 4). These results suggest that the

upregulation of miR-486-5p by mimics suppressed proliferation of ESCC cells *in vitro*.

**Restoration of miR-486-5p induced ESCC cell apoptosis.** To determine the effects of miR-486-5p on ESCC cell apoptosis, flow cytometry was used to determine the apoptosis rates after transfection. As shown in Fig. 5, apoptosis rates of Eca109 cells transfected with miR-486-5p mimics and those of the negative control were 9.7 and 3.4%, respectively ( $P < 0.01$ ) 48 h after transfection. The apoptosis rates of TE-1 cells were 10.4 and 4.4%, respectively ( $P < 0.01$ ) subsequent to transfection (Fig. 5). Thus suggesting that the restoration of miR-486-5p expression levels induced ESCC cell apoptosis.

## Discussion

Carcinogenesis involves the activation of numerous oncogenes and anti-oncogenes. In the complex network involving the



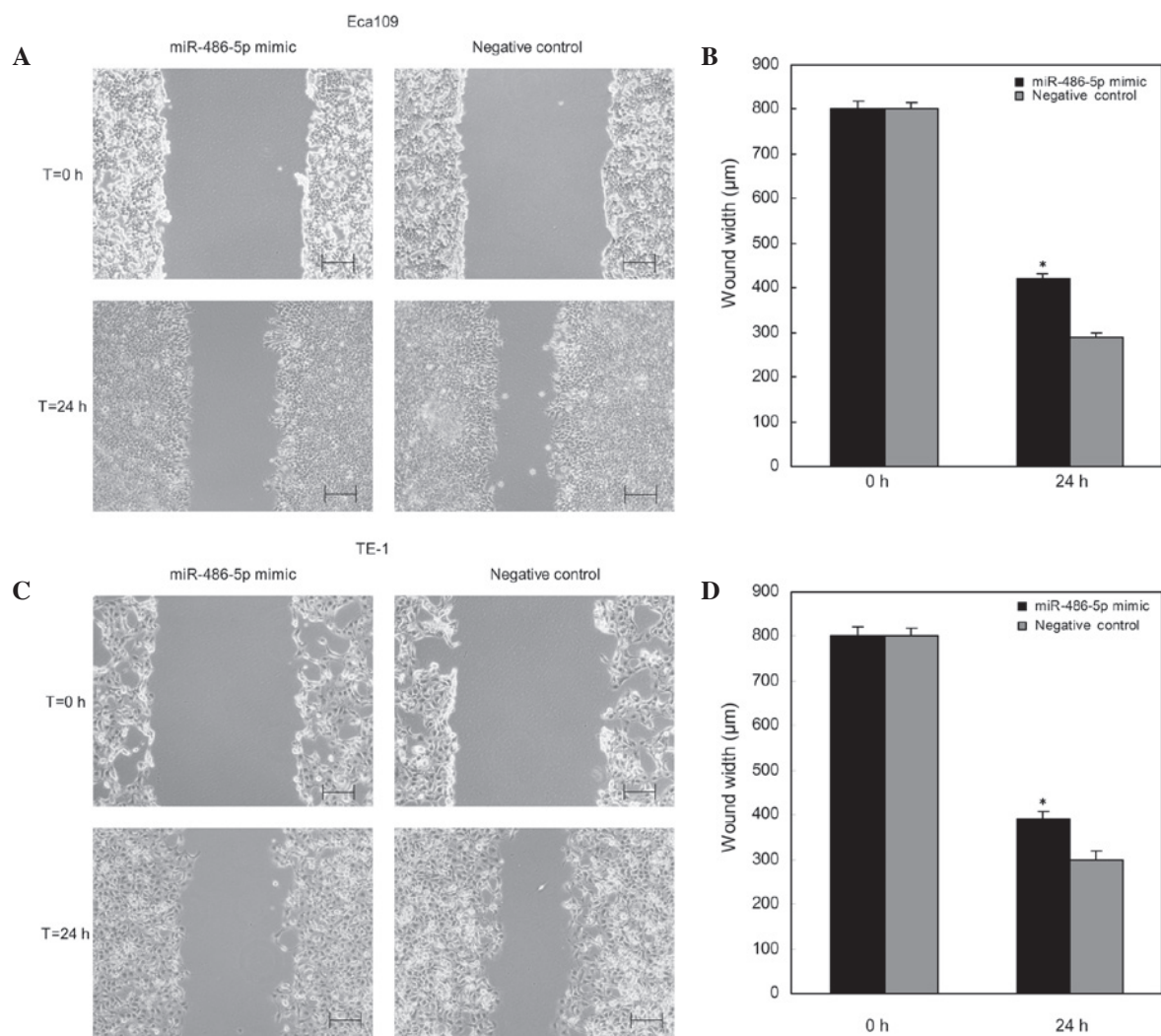


Figure 3. Wound scratch assay for Eca109 and TE-1 cells 24 h post-transfection. (A) Images of the Eca109 cells transfected with miR-486-5p mimics or negative control 0 and 24 h after the scratches were made at the same point. (B) Quantification of the migration distances ( $\mu\text{m}$ ) in Eca109 cells using a standard caliper ( $P<0.05$ ). (C) Images of the TE-1 cells transfected with miR-486-5p mimics or with the negative control 0 and 24 h after the scratches were made at the same point. (D) Quantification of the migration distances ( $\mu\text{m}$ ) in TE-1 cells using a standard caliper. Values presented are the mean  $\pm$  standard deviation of three independent experiments ( $P<0.05$ ).

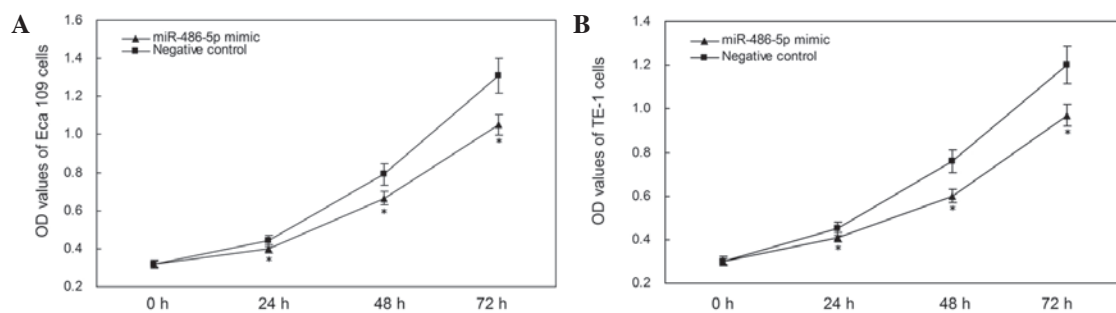


Figure 4. MTT assay for cell proliferation of Eca109 and TE-1 cells transfected with miR-486-5p mimics or the negative control. (A) Cell proliferation of Eca109 cells. (B) Cell proliferation of TE-1 cells. Values presented are the mean  $\pm$  standard deviation of three independent experiments ( $P<0.05$ ).

regulation of oncogenes and anti-oncogenes, miRNAs are associated with gene regulation at the transcriptional and translational level through base-pairing to complementary mRNA sequences in their target genes (18,19). A miRNA may fulfil the role of an anti-oncogene or an oncogene by regulating the levels of oncogenes or anti-oncogenes (9). miRNAs serve important

roles in diverse cellular processes, including cell proliferation, cellular differentiation, apoptosis, motility, invasion and morphogenesis (7,20-25). Numerous miRNAs have been found to be upregulated in ESCC, including miR-10b (26), miR-21 (27-29), miR-192, miR-93 and miR-194 (29), miR-23a, miR-26a, miR-27b, miR-96, miR-128b and miR-129 (30), and

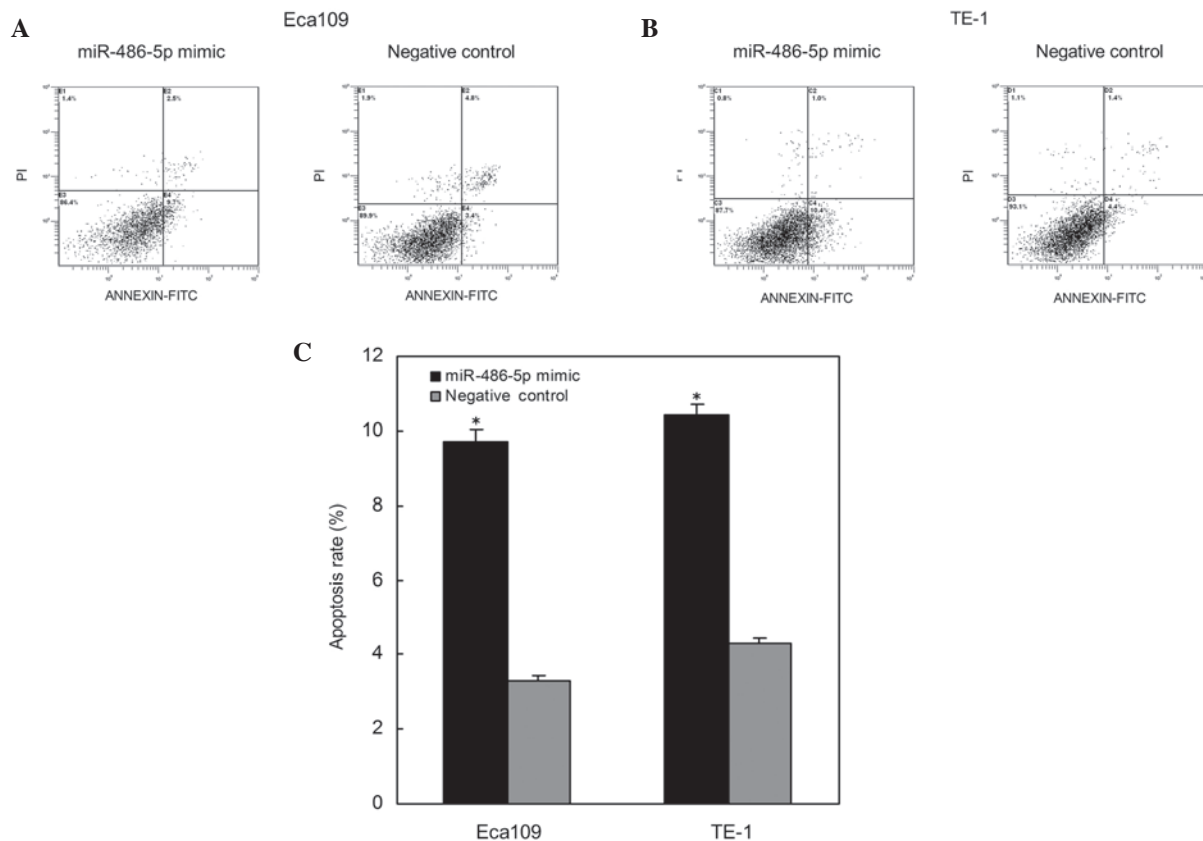


Figure 5. Flow cytometry analysis for cell apoptosis of Eca109 and TE-1 cells transfected with miR-486-5p mimics or the negative control. (A) Eca109 cells transfected with miR-486-5p mimics and negative control. (B) TE-1 cells transfected with miR-486-5p mimics and negative control. (C) Comparison of the apoptosis rates of Eca109 and TE-1 cells transfected with miR-486-5p mimics and the negative control. Data are presented as the mean  $\pm$  standard deviation of three measurements (\* $P < 0.01$ ).

miR-205 (31). By contrast, downregulation of miR-375 (27), miR-205, miR-203, miR-125b, miR-100 and miR-27b (29) have been detected in ESCC. Furthermore, numerous miRNAs were found to serve oncogenic or anti-oncogenic roles, including the facilitation of ESCC growth by miR-21 through targeting PTEN and PDCD4 (28,32), in addition to the ability of miR-145, miR-133a and miR-133b to converge to target Fascin 1, reducing cell growth and invasion (33). Furthermore, miR-210 targets FGFR1, exerting a negative effect on the cell cycle and proliferation (34), while miR-296 contributes to ESCC growth by targeting cell CCND1 and p27 (35). Finally, miR-593 may contribute to carcinogenesis through serine/threonine-protein kinase (36).

The downregulation of miR-486-5p is a frequent molecular event in certain human malignancies (14-16,37-40). Furthermore, miR-486-5p may function as tumor suppressor through contributing to the progression and metastasis of NSCLC by targeting ARHGAP5 (14), in addition to the fact that miR-486-5p exerts its antiproliferative function by directly downregulating PIM-1 expression in breast cancer cells (15), miR-486-5p suppresses tumor growth by targeting PIK3R1 in hepatocellular carcinoma (16). However, the expression and role of miR-486-5p in ESCC has yet to be elucidated.

To determine the expression and role of miR-486-5p in ESCC, RT-qPCR was used to quantify miR-486-5p expression levels in 35 cases of ESCC tissues and paired normal tissues. The present study showed that miR-486-5p expression levels were significantly downregulated in ESCC tissues, compared

with the expression levels in paired normal esophageal tissues. The effects of miR-486-5p on ESCC cell migration, proliferation and apoptosis were then analyzed by transfection of ESCC cell lines with synthetic miR-486-5p mimics. Transfection of miR-486-5p mimics into the ESCC cell lines Eca109 and TE-1, inhibited cellular proliferation, migration and induced apoptosis, compared with the negative control group. The data indicates that miR-486-5p may be characterized as an anti-oncogene in ESCC by inhibiting cellular proliferation and migration, and promoting cellular apoptosis. Further identification of miR-486-5p target genes is warranted to clarify the mechanism of action of miR-486-5p in ESCC.

In conclusion, the present study revealed that miR-486-5p was downregulated in ESCC and served a vital anti-oncogenic role in ESCC by affecting cellular migration, proliferation and apoptosis. Further studies are required to determine the mechanism of action of miR-486-5p in ESCC.

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