## ORIGINAL ARTICLE

## MicroRNA-182 promoted esophageal squamous cell carcinoma cell growth and metastasis via targeting YWHAG

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## Summary

**Purpose:** To study the functioning mode of miR-182 on esophageal squamous cell carcinoma (ESCC) cell growth and metastasis and provide therapeutic targets for ESCC.

**Methods:** miR-182 expression level in ESCC cell lines was examined by quantitative real-time polymerase chain reaction (qRT-PCR). Using miR-182 inhibitor, we analyzed the effects of miR-182 down-expression on cell proliferation, invasion, cell apoptosis and cell cycle. Dual-luciferase activity assay was used to examine the potential target gene YWHAG which was predicted by several databases. Protein level was studied using western blotting.

**Results:** Decreased expression of miR-182 in ESCC cells

was accompanied by decreased cell invasion and proliferation, promotion of cell apoptosis and cell cycle arrest at GO/G1 phase. Dual-luciferase and western blot confirmed YWHAG as a target gene of miR-182. Furthermore, silence of YWHAG counteracted the suppressive effect of miR-182 down-expression on cell growth and metastasis.

**Conclusions:** miR-182 could suppress ESCC cell proliferation and metastasis via regulating YWHAG, which might provide a new target for ESCC diagnosis and therapy.

*Key words:* esophageal squamous cell carcinoma, metastasis, miR-182, proliferation, YWHAG

## Introduction

Esophageal cancer is currently one of the malignant tumors of digestive tract with higher morbidity (ranks 9<sup>th</sup>) and mortality (ranks 6<sup>th</sup>) rates among cancers worldwide [1]. The morbidity rate of esophageal cancer ranks 20<sup>th</sup> in developed countries and 8<sup>th</sup> in developing countries, while its mortality rate ranks 11<sup>th</sup> in developed countries and 5<sup>th</sup> in developing countries [1]. Esophageal cancer has become from the 7<sup>th</sup> major cancer in 1990 to the 6<sup>th</sup> major cancer in 2013, and both aging and the growing population contribute to the increasing cases of this disease [2]. ESCC and adenocarcinoma are the main pathological types of esophageal cancer, the former accounting for more than 90% of the cases in China [3]. World-

wide, about 400,000 people die of esophageal cancer each year and China is one of the high-prevalence areas of this disease in the world, with more than 150,000 people dying each year [4]. Due to the silent early symptoms of esophageal cancer and the lack of early diagnosis, most patients are in the late stage when first diagnosed. The 5-year overall survival rate after esophagectomy is 9.5-45%, and both tumor invasion and metastasis are the leading causes of death in patients with esophageal cancer [5]. Invasion and metastasis mainly depend on the activation of proto-oncogenes and inactivation of tumor suppressor genes, as well as the synergistic effect of various factors, such as cell proliferation and apoptosis. If the occurrence and

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development of esophageal cancer can be studied from the perspective of genetics and epigenetics, it would provide new targets and new strategies for the diagnosis and disease treatment.

Micro ribonucleic acid (miRNAs) are small non-coding RNA molecules discovered in recent years, which contain about 22 nucleotides and are existing widely in eukaryotes [6]. Although most miRNAs exist in cells, some have also been found in the extracellular environment, known as circulating miRNAs or extracellular miRNAs [7]. At present, the most common regulation of target genes is that miRNAs bind to some proteins to form miRNA-induced silencing complexes, thus leading to complete or incomplete pairing with the 3'-untranslated region (3'UTR) of messenger RNA (mRNA), after which specific mRNA is identified and degraded or its post-transcriptional translation is inhibited, in other words, the expression of target gene is inhibited at the post-transcriptional level. MiRNAs play an important role in cell proliferation, apoptosis and differentiation through regulating the expression of target genes, and also play a major role in maintaining the normal function of cells. Researchers have found that miRNAs dysregulation is associated with many diseases, including cancer [8,9].

Recent studies have found that miR-182 is abnormally expressed in a variety of tumors, and its abnormal expression is associated with the prognosis of patients. Zhao et al. [10] found that miR-182 is up-regulated in esophageal cancer cell line KYSE410, and the miR-182 level in the serum of esophageal cancer patients is also higher than that in normal controls. At the same time, the high expression of miR-182 is closely correlated with the grade of differentiation, depth of invasion, lymph node metastasis and clinical staging of esophageal cancer, suggesting that miR-182 is related to the tumor invasion and metastasis [10,11]. However, the specific mechanism of miR-182 in the occurrence and development of esophageal cancer has not been explored yet.

This study aimed to investigate the role of miR-182 in invasion, metastasis and proliferation at the cellular level, so as to confirm that miR-182 may be used as a new target for the treatment of esophageal cancer.

## Methods

#### Cell lines

Human esophageal cancer cell lines Eca109 and EC9706 were purchased from the Shanghai Institute for Cells, Chinese Academy of Sciences. Those cells were subcultured with Roswell Park Memorial Insti-

tute (RPMI)-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 pg/mL streptomycin (Sigma, St. Louis, MO, USA) in a cell incubator with 5% CO<sub>2</sub> and saturated humidity at 37°C. Then, the medium was replaced by fresh medium every 2-3 days, and cells in logarithmic growth phase were selected for the experiments. Cells needing to be transfected were firstly cultured with RPMI-1640 medium containing 10% FBS, then transfected and cultured with RPMI-1640 medium containing 5% FBS.

#### Cells transfection

Cells were digested with 0.25% trypsin (Gibco, Rockville, MD, USA), counted and seeded into a 96-well plate at  $2 \times 10^3$  cells per well overnight. On the day of transfection, cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS in the 96 wells was replaced by fresh medium and 150 µL RPMI-1640 medium containing 5% FBS was added into each well. Then, 50 µL serum-free RPMI-1640 medium was used to dilute 0.5 µL corresponding miRNA-inhibitor or normal control (NC)-inhibitor, and 0.75 µL Hiperfect transfection reagent was added. After that, the 96-well plate was taken out and the above mixture was added dropwise with cell suspension and gently mixed to evenly distribute the transfection complex in the cell suspension.

#### RNA extraction and quantitative real-time PCR

Total RNA was extracted under aseptic RNase-free conditions in strict accordance with the instructions of RNA extraction kits (Invitrogen, Carlsbad, CA, USA). Then, the extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of Prime-Script<sup>™</sup> reverse transcription kits (TaKaRa, Tokyo, Japan). Real-time fluorescent quantitative polymerase chain reaction (PCR) reaction system was prepared according to the instructions of SYBR Green. After the reaction was completed, the cycle threshold (Ct) of each reaction tube was calculated, and the relative expression quantity of miR-182 was calculated by 2<sup>-ΔΔC</sup>t method.

#### CCK8 assay

Human esophageal cancer cell lines ECA109 and EC9706 were inoculated into a 96-well plate. 200  $\mu$ L Dulbecco's modified Eagle Medium (DMEM) containing 10% FBS was added into each well. The cell proliferation was detected after culture for 12, 24, 48 and 72 hrs. During detection, 20  $\mu$ L CCK-8 reagent was added into each well of the 96-well plate for incubation at 37°C for 2 hrs. The optical density (OD) value of each well was detected at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

#### Cell apoptosis analysis

Cells were seeded into a 6-well plate, transfected with miR-182 inhibitor or NC-inhibitor for 72 hrs and subjected to apoptosis assay. Cells were digested with ethylene diamine tetraacetic acid (EDTA)-free trypsin and the digestion was stopped using DMEM containing 10% FBS. Then, cells were collected, 250  $\mu$ L 1×binding buffer was added into each Eppendorf tube to resuspend cells, and cell concentration was adjusted to 1×10<sup>6</sup>/mL. Following that, 100  $\mu$ L cell suspension was taken, added with 5  $\mu$ L Annexin V/fluorescein isothiocyanate (FITC) and 10  $\mu$ L propidium iodide (PI) solution, and incubated at room temperature in the dark for 15 min. Lastly, 300  $\mu$ L binding buffer was added into the reaction Eppendorf tube, and the suspension was loaded onto a flow cytometer for detection of cell apoptosis.

#### *Cell cycle analysis*

Cells were inoculated into the 6-well plate and transfected with miR-182 inhibitor or NC-inhibitor for 72 hrs, followed by cell cycle detection. Cells were collected after conventional trypsinization and fixed in 70% ethanol at 4°C for 48 hrs. Then, they were harvested after centrifugation and stained with 500  $\mu$ L PI staining solution containing RNase for 1 hr. Lastly, flow cytometer was used for detection of cell cycle.

#### Transwell assay

The upper chamber surface of the bottom membrane of the Transwell chamber was dried with 50 mg/L Matrigel with 1:8 dilution at 4°C. A diluted Matrigel (3.9  $\mu$ g/ $\mu$ L) 60-80  $\mu$ L was added to the polycarbonate film on the upper chamber, and the Matrigel was polymerized into a gel at 37°C for 30 min. After 48 hrs of transfection, the cells were digested by trypsin, washed by PBS twice and suspended with serum containing bovine serum albumin (BSA) medium. The cell density was adjusted to  $5 \times 10^4$  /mL. Six mL FBS containing medium were added into the lower chamber. After 24 hrs of normal culture, the basement membrane of the lower chamber was removed and images were captured using an inverted microscope (Nikon, Tokyo, Japan).

#### Dual-luciferase assay

Dual luciferase reporter plasmids inserted with wild-type and mutant YWHAG -3'UTRs were established. Human embryonic kidney 293T (HEK 293T) cells were seeded into the 96-well plate and co-transfected with the reporter plasmids and miR-182 inhibitor or NC-inhibitor according to experimental methods described above. After 48 hrs of transfection, luciferase activity was measured by Promega (Madison, WI, USA) dual luciferase reporter system.

#### Western blotting

Cells were collected after 72 hrs of transfection, added in the cell lysis solution, and centrifuged at 15000 r/min for 30 min to extract total protein. Then, the concentration of the extracted protein was determined by bicinchoninic acid (BCA) method and adjusted, followed by conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, the target protein was transferred onto a polyvinylidene fluoride (PVDF) membrane by semi-dry method and blocked with 5% nonfat milk. Lastly, the protein was incubated with primary antibodies [anti- YWHAG (1:500) and  $\beta$ -actin (1:2000)] overnight, and then with secondary antibodies [goat anti-rabbit-horseradish peroxidase (HRP) and goat anti-mouse-HRP (1:5000)] at room temperature for 1 hr, followed by X-ray film color development.

#### Statistics

The target gene prediction software (TargetScan) was used to screen the miRNAs that could be directly targeted to YWHAG. The experimental data were expressed as mean ±standard deviation, and the SPSS17.0 (Chicago, IL, USA) statistical software was used to analyze the data. Comparison between groups was done using One-Way anova test followed by *post hoc* test (least significant difference). p<0.05 was considered as statistically significant.

## Results

Downregulation of miR-182 inhibited ESCC cell growth and metastasis in vitro

To identify the mode of action of miR-182 in ESCC tumorigenesis *in vitro*, Eca109 and EC9706 cell lines were transfected with miR-182 inhibitor and NC-inhibitor for downregulation of miR-182 (Figure 1A). Compared with NC-inhibitor, the viability of ESCC cells was significantly suppressed after been transfected with miR-182 inhibitor (p<0.05) (Figure1B).

The invasion capacity of the cells in each group was detected by transwell assay. The experimental results showed that the invasive ability of the experimental group was significantly lower than that of the NC group (Figure 1C). These results showed that miR-182 could suppress ESCC proliferation and invasion.

## Downregulation of miR-182 suppressed cell apoptosis and attenuated cell cycle arrest at G0/G1 phase

As shown in Figure 2A, compared with NC-inhibitor, miR-182 inhibitor significantly increased the apoptosis rate of ESCC cells (p<0.01).As shown in Figure 2B, compared with NC-inhibitor, miR-182 inhibitor induced increase of cell proportion at G0/G1 phase in ESCC cells, but induced also decrease of cell proportion at S phase. The difference was statistically significant (p<0.01) indicating that miR-182 strengthened the G0/G1 phase arrest of ESCC cells. These results indicated that miR-182 inhibitor inhibits the growth of ESCC cells by inducing cell apoptosis and G0 / G1 phase arrest of the cell cycle.

#### YWHAG was directly targeted by miR-182

Conserved miRNAs that might act on 3'UTR of YWHAG was predicted using TargetScan. To verify whether miR-182 reduces the expression



**Figure 1.** Downregulation of miR-182 inhibited ESCC cell growth and metastasis *in vitro*. **(A)** Analysis of transfection efficiency in ESCC cells transfected with miR-182 inhibitor and NC-inhibitor. The relative expression of miR-182 in ESCC cells transfected with mir-182 inhibitor was significantly lower than that with NC-inhibitor. **(B)** CCK8 assay was performed to determine the viability of transfected ESCC cells. Compared with NC-inhibitor, the viability of ESCC cells was significantly suppressed after been transfected with miR-182 inhibitor. **(C)** Transwell invasion assay was used to detect the invasion ability of transfected ESCC cells. Total RNA was detected by qRT-PCR and GAPDH was used as internal control. The invasive ability of the Experimental group was significantly lower than that of the NC group. Data are presented as the mean ± SD of three independent experiments. \*\*p<0.01.



**Figure 2.** Downregulation of miR-182 suppressed cell apoptosis and attenuated cell cycle arrest at G0/G1 phase. **(A)** Flow cytometric analysis was performed to detect the apoptotic rates of transfected ESCC cells. Compared with NC-inhibitor, miR-182 inhibitor significantly increased the apoptosis rate of ESCC cells. **(B)** Flow cytometric analysis was performed to detect cell cycle progression of transfected ESCC cells. Compared with nc-inhibitor, mir-182 inhibitor induced the increase of cell proportion at g0/g1 phase in ESCC cells, but the decrease of cell proportion at S phase. \*\*p<0.01.

of YWHAG by directly targeting it, dual luciferase reporter plasmids were established in this study to express wild-type YWHAG mRNA 3'UTR and mutant YWHAG 3'UTRm at the miR-182 binding site, respectively (Figure 3A). In HEK293T cells, co-transfection of YWHAG3'UTR/miR-182 inhibitor significantly lowered the luciferase activity in comparison with co-transfection of YWHAG3'UTR/ NC inhibitor, while there was no obvious difference in luciferase activity between co-transfection of YWHAG3'UTRm/NC-inhibitor and co-transfection of YWHAG3'UTRm/miR-182 inhibitor (Figure 3B), indicating that YWHAG mRNA 3'UTR is a direct target of miR182, and miR-182 downregulates the expression of YWHAG by directly targeting YWHAG mRNA 3'UTR.

Meanwhile, we further detected the expression level of YWHAG in transfected ESCC cells. The results indicated that YWHAG was upregulated in ESCC cells transfected with miR-182 inhibitor on mRNA level and protein level when compared with NC-inhibitor (Figures 3C and 3D). All these results indicated that YWHAG was directly targeted by miR-182.

# Silencing of YWHAG recovered the carcinogenesis role of miR-182

To further identify the relationship of miR-182 and YWHAG, we explored whether YWHAG is responsible for the functional effects of miR-182 in ESCC tumorigenesis. We silenced the YWHAG expression by transfecting with siRNA- YWHAG in miR-182-decreased ESCC cells (Figure 4A). YWHAG silencing not only increased the proliferation and invasion capacities (Figures 4B and 4C), but also attenuated cell apoptosis and weakened cell cycle distribution at G0/G1 phase (Figures 4D and 4E). These results implied that miR-182 promoted ESCC tumorigenesis by partially inhibiting YWHAG expression.



**Figure 3.** YWHAG is directly targeted by miR-182. **(A)** YWHAG was selected as the potential downstream of miR-182 via using bioinformatics analysis. Dual luciferase reporter plasmids were established in this study to express wild-type YWHAG mRNA 3'UTR and mutant YWHAG 3'UTRM at the miR-182 binding site, respectively. **(B)** Luciferase activities of ESCC cells transfected with the wild-type or the mutated YWHAG 3'UTR together with miR-182 inhibitor or NC-inhibitor. Co-transfection of YWHAG 3'UTR/miR-182 inhibitor significantly lowered the luciferase activity in comparison with co-transfection of YWHAG 3'UTR/NC-inhibitor. **(C)** Analysis of YWHAG mRNA expression level of ESCC cells transfected with miR-182 inhibitor or NC-inhibitor. YWHAG was upregulated in ESCC cells transfected with miR-182 inhibitor or NC-inhibitor. TWHAG protein expression level of ESCC cells transfected with miR-182 inhibitor or NC-inhibitor. **(D)** Analysis of YWHAG protein expression level of ESCC cells transfected with miR-182 inhibitor. Data are presented as mean ± SD of three independent experiments. \*\*p<0.01.



**Figure 4.** Silencing of YWHAG recovered the carcinogenetic role of miR-182. **(A)** Analysis of transfection efficiency in ESCC cells transfected with miR-182 negative control (NC), inhibitor and/or si-YWHAG. The YWHAG expression was silenced by transfecting with siRNA- YWHAG in miR-182-decreased ESCC cells. **(B)** Reduced YWHAG rescued suppressed cell proliferation by miR-182. YWHAG silencing increased the proliferation capacities. **(C)** Reduced YWHAG rescued suppressed cell invasion by miR-182. YWHAG silencing increased the invasion capacities [describe the results of this subfigure]. **(D)** Reduced ywhag attenuated cell apoptosis. ywhag silencing attenuated cell apoptosis. **(E)** Reduced YWHAG silencing weakened cell cycle distribution at GO/G1 phase. Data are presented as mean ± SD of three independent experiments. \*p<0.05, \*\*p<0.01.

## Discussion

Esophageal cancer is one of the common malignant tumors in clinical practice with a high mortality rate and poor prognosis. At present, many clinicopathological features can be used as important prognostic indicators for patients with esophageal cancer, but they cannot predict the prognosis of patients accurately and individually [12-15]. Therefore, it is of great clinical significance to study key molecular biological events during the occurrence and development of esophageal cancer, and search for important molecular markers for early diagnosis, targeted therapy and prognosis prediction.

Now, more than 1,900 strands of miRNAs have been found in mammals, which can regulate more than 60% of gene functions [16]. In tumor research it has been confirmed that abnormally expressed miRNAs can play a cancer-promoting or anti-cancer role, and the abnormally expressed miRNAs can also be used as potential diagnostic and prognostic markers. In recent years, more and more abnormally expressed miRNAs have been found in the study on esophageal cancer, among which the upregulation of miR-21 [17], miR-192 [18] and miR-106b [19] can promote proliferation, invasion and metastasis of tumor cells and the downregulation of miR-375 [20] and miR-203 [21] can inhibit tumor cell cycle and promote its apoptosis. In addition, the roles of miR-21 and miR-375 in the estimation of prognosis of esophageal cancer have also been confirmed [22].

Current studies have found that miR-182 plays different roles in different tumors. MiR-182 can promote tumor progression in cervical cancer [23], breast cancer [24] and liver cancer [25]. On the contrary, miR-182 can inhibit tumor progression in renal cancer [26] and uveal melanoma [27]. The mechanism of action of miRNAs is binding to target mRNA to inhibit its functions, so its overall biological effect depends on the sum of different genes regulated by it. Therefore, the same miRNA can exert different biological effects in different tumors. In a variety of tumors, the cancer-promoting mechanism of miR-182 has been reported. MiR-182 can promote the proliferation of tumor cells in medulloblastoma through activating the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway [26]. In breast cancer, miR-182 can inhibit

the expressions of a variety of tumor suppressor genes, such as breast cancer susceptibility gene 1 (BRCA1), forkhead box protein O1 (FOXO1) and FOXO3 [24]. In uveal melanoma cells, miR-182 acts as tumor suppressor gene mainly through the targeted regulation of Microphthalmia-Associated Transcription Factor (MITF), B-cell lymphoma 2 (BCL2) or cyclin D2 [27]. This study focused on the potential mechanism of miR-182 in the development of ESCC and found that downregulated miR-182 inhibited ESCC cell proliferation and invasion, promoted cell apoptosis and induced cell cycle arrest at the G0/G1 phase. All these findings suggested that downregulated miR-182 exerted its suppressive effect on cell growth and metastasis of ESCC.

To further identify the underlying mechanism of how downregulated miR-182 suppressed ESCC cell tumorigenesis, YWHAG was predicted and selected as the novel target gene of miR-182 by bioinformatics analysis. YWHAG, as a member of 14-3-3 protein family, has been reported as a tumor suppressor and reduces cell proliferation and metastasis in different cancers including glioblastoma [28] and breast cancer [29]. However, the underlying upstream mechanism of YWHAG in ESCC has not been well identified yet. In our present study, we initially revealed that YWHAG was directly targeted by miR-182, and silencing of YWHAG could rescue tumor suppression role by downregulated miR-182 on ESCC cell proliferation. The evidence indicated that miR-182 might be the upstream of YWHAG involved in ESCC tumorigenesis.

## Conclusions

In summary, the present study demonstrated that downregulated miR-182 had tumor-suppressive effect on ESCC proliferation via targeting YWHAG *in vitro*, which might function as a useful biological target for ESCC therapy.

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## **Conflict of interests**

The authors declare no conflict of interests.

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