

MiR-601 inhibits the proliferation and metastasis of esophageal squamous cell carcinoma (ESCC) by targeting HDAC6

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-601 (miR-601) in the proliferation and invasion of esophageal squamous cell carcinoma (ESCC) cells, thereby providing new thoughts for prognosis evaluation and targeted therapy of ESCC.

PATIENTS AND METHODS: 23 pairs of ESCC tissue samples and adjacent normal tissues were collected, and the expression level of miR-601 was detected. Biological information analysis and Luciferase report gene assay were used to verify the potential target genes of miR-601. Then, three groups were established in ESCC cell line (TE-1) to perform similar experiments, including the miR-NC group, the miR-601 mimics group and the mimics + HDAC6 group. Cell counting kit-8 (CCK-8) assay was used to detect cell proliferation ability. Meanwhile, transwell assay and scratch-wound assay were applied to observe the effect of miR-601 on cell invasion and migration. Quantitative reverse transcription Polymerase Chain Reaction (qPCR) and Western blot assay were applied to determine the mRNA and protein expression changes after transfection.

RESULTS: Compared with normal adjacent tissues and normal esophageal epithelial cells, the expression of miR-601 was significantly decreased in ESCC tissues and cells. HDAC6 was identified as a target gene of miR-601. The expression of HDAC6 in esophageal carcinoma cells transfected with miR-601 mimics was significantly down-regulated. The negative correlation between miR-601 and HDAC6 expression was assessed by qPCR and Western blot (WB) assay. Furthermore, miR-601 remarkably suppressed the proliferation of ESCC cells. Meanwhile, cell invasion and migration were also found markedly restricted after transfection of miR-601 mimics. However, the overexpression of HDAC6 significantly counteracted the effects of miR-601.

CONCLUSIONS: MiR-601 suppressed the proliferation, invasion and migration of esophagus

carcinoma cells by down-regulating HDAC6 expression.

Key Words:

MicroRNA-601 (MiR-601), Esophageal squamous cell carcinoma (ESCC), Histone deacetylase 6 (HDAC6).

Introduction

Esophageal cancer is a common and extremely invasive malignant tumor of the digestive tract¹. Esophageal squamous cell carcinoma (ESCC) accounts for about 80% of esophageal cancer. According to global tumor statistics, the incidence rate of esophageal cancer around the world is declining. However, the number of patients with esophageal cancer has not significantly decreased. There are approximately 48.9% of new cases of esophageal cancer and 49.3% of death cases in China². Patients often ignore the symptoms of esophageal cancer in the early stage. Therefore, most of them are diagnosed in the middle and advanced stage, missing the best time of treatment. In recent years, the levels of diagnosis and treatment for esophageal cancer have been greatly improved. However, the crueler reality is that the five-year survival rate of patients is still less than 20%^{1,3}. Therefore, it is of great significance to study and clarify the causes and molecular mechanism of esophageal cancer metastasis, eventually improving the survival rate of patients with esophageal cancer.

Micro-ribonucleic acids (miRNAs), discovered in recent years, are a type of non-coding small RNAs with 18-25 nucleotides in length. Previous studies have shown that miRNAs possess many biological functions⁴. For example, miRNAs can negatively regulate the translation and inhibit the

function of target genes through specific binding to the 3' untranslated region (3'-UTR) of target gene messenger RNAs (mRNAs) at the transcriptional level. Subsequently, they are involved in various biological processes such as cell metastasis, proliferation, apoptosis, differentiation and metabolism⁵. With the discovery of new species and functions of miRNAs, more and more attention has been paid to the role of miRNAs in a variety of diseases, especially in malignant tumors. Recent studies have demonstrated that miRNAs exert a crucial role in malignant biological behaviors^{3,6}.

MiR-601 has been found to have a variety of biological functions. A number of studies have suggested that miR-601 is abnormally expressed in a variety of tumors. Meanwhile, it may play a different regulatory role in the pathological processes of different tumors⁷⁻¹⁰. In general, it tends to play a role in tumor suppression. However, whether miR-601 is involved in the proliferation and metastasis of ESCC is unclear. Furthermore, its role in the development of ESCC and the underlying mechanism remain to be clarified.

The aim of this study was to clarify the role of miR-601 in ESCC, especially in the metastasis of ESCC, and to explore the possible underlying molecular mechanism. Our study might provide a new effective therapeutic target for the clinical treatment of ESCC.

Patients and Methods

ESCC Cases and Cells

From February 2015 to February 2017, ESCC tissues and cancer-adjacent normal tissues were collected from 23 patients in the Cancer Hospital Affiliated to Zhengzhou University. Cancer-adjacent normal tissues were >5 cm away from the tumor, and all the tissues were confirmed by pathology. The patients enrolled received no treatment before specimen collection. This research was approved by the Ethics Committee of Cancer Hospital Affiliated to Zhengzhou University.

Human ESCC cell line (TE-1) together with normal esophageal epithelial cell line (Het-1A) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Grand Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in a 37°C, 5% CO₂ incubator. The medium was changed

every 2 days. The cells were subjected to passage for 2-3 days, and those in the logarithmic growth phase were selected for subsequent experiments.

Luciferase Reporter Gene Assay

TargetScan, miRDB and mi-Randa websites predicted that histone deacetylase 6 (HDAC6) was a target gene of miR-601. The binding sequence of miR-601 at the 3'-UTR of HDAC6 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Mutated HDAC6 (Mut-type) and non-mutant HDAC6 (WT-type) were connected to the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant HDAC6 was transfected into TE-1 cells after lentivirus intervention on 24-well plates. The same treatment was performed on the pGL3-Basic vector connected with non-mutant HDAC6 according to the instructions of the Luciferase Reporter Gene Assay Kit. Finally, Luciferase activity was detected by a multi-function microplate reader.

Cell Transfection

The cells were first seeded into 6-well plates at a density of 4×10^5 cells/well. After the cells adhered to the wall, cell transfection was conducted according to the instructions of LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, cells in each well were transfected with 5 µL LipofectamineTM 2000 and 5 µL NC mimics, miR-601 mimics or HDAC6 mimics. 6 h after transfection, the medium containing 10% FBS was replaced for further culture. Transfection efficiency was detected by a fluorescence microscope. After 24 h, the expression changes of miR-601 before and after transfection were detected via quantitative reverse transcription Polymerase Chain Reaction (qPCR).

qPCR Analysis

Total RNA was extracted in accordance with the manufacturer's protocol of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SYBR green qPCR assay (TaKaRa, Otsu, Shiga, Japan) was used to measure the expression level of HDAC6. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to measure the expression level of miR-601 normalized to miRNA U6. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$

method. Primers used in this study were: HDAC6, F: 5'-AGGTAAAGGGGAAGAAACAAA-3', R: 5'-TGCGGATGAGTTGTTTCTGGC-3'; miR-601, F: 5'-AAACATCCTGGGGTGTCTGACTG-3', R: 5'-GTGTCGTGGATTGCAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCTCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

TE-1 cells in different groups were collected, and total protein was extracted by radioimmuno-precipitation assay lysate. The concentration of extracted proteins was measured *via* the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Briefly, 20 µg proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After sealing for 1 h, the membranes were incubated with primary antibodies of HDAC6 and GAPDH (diluted at 1:1000, Cell Signaling Technology (CST) Inc., Danvers, MA, USA) at 4°C overnight. The next day, the membranes were incubated with secondary antibodies (diluted at 1:1000) at room temperature for 2 h. Subsequently, Beyo-ECL chemiluminescence detection reagents were applied for color development. The chemiluminescence apparatus was used for color development and photographing. The relative expression level of the target protein was calculated, and GAPDH was used as an internal reference.

Cell Proliferation

24 h after transfection, the cells were prepared into the cell suspension, and a total of 4×10^3 cells were inoculated into 96-well plates. 5 replicates were set in each group. The cells were irradiated after adhering to the wall. After further culture for 48 h, cell counting kit-8 (CCK-8) reagents (10 µL CCK-8 solution per 100 µL medium, Dojindo, Kumamoto, Japan) were added in each well, followed by incubation for 4 h in the dark. After that, optical density (OD) value was measured using a microplate reader.

Cell Invasion and Migration Assays

Cell invasion assay was performed by transwell plates (Corning, Corning, NY, USA) with 8-µm-pore size membranes with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 2×10^4 cells were seeded into the upper chambers with serum-free medium. Meanwhile, the lower chamber was added

with complete medium containing 10% FBS as a chemoattractant. After incubation for 2 days, cells on the top of the membrane were wiped by a brush. Subsequently, the membrane was stained with 0.2% crystal violet and drenched with 95% ethanol. Invasive cells were observed by an inverted microscope.

Cell migration assay was performed by scratch-wound assay. After transfection, cells were placed in an incubator for further culture. After cell passage, scratches were made with a 100 µL pipette head along a ruler. The scratched cells were then washed with phosphate-buffered saline. Subsequently, the cells were cultured in an incubator, followed by sampling and photographing 24 h after culture. 5 sets of scratch spacing were randomly selected for statistical analysis.

Statistical Analysis

Prism 6.02 software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. Student's *t*-test or *F*-test was used to compare the differences among different groups. All *p*-values were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Decreased Expression of MiR-601 in ESCC Tissues and Cells

In this study, we first detected the expression levels of miR-601 in ESCC tissues and corresponding cancer-adjacent tissues by qPCR. The results manifested that the expression level of miR-601 in ESCC tissues was significantly lower than that of cancer-adjacent normal tissues (Figure 1A). Meanwhile, the same results were obtained at the cellular level (Figure 1B). These data demonstrated that miR-601 might have some regulatory effects in ESCC.

Transfection Efficiency of MiR-601

MiR-601 mimics and NC mimics were transfected into TE-1 cells. Transfection efficiency, as well as the expression changes of miR-601 in transfected cells, was detected. Results illustrated that the expression level of miR-601 in the miR-601 mimics group was remarkably higher than that of the NC group (Figure 1C).

HDAC6 Was a Direct Target of MiR-601 in ESCC Cells

Target Scan Human, mi-Randa and miRDB speculated that HDAC6 was one of the possi-

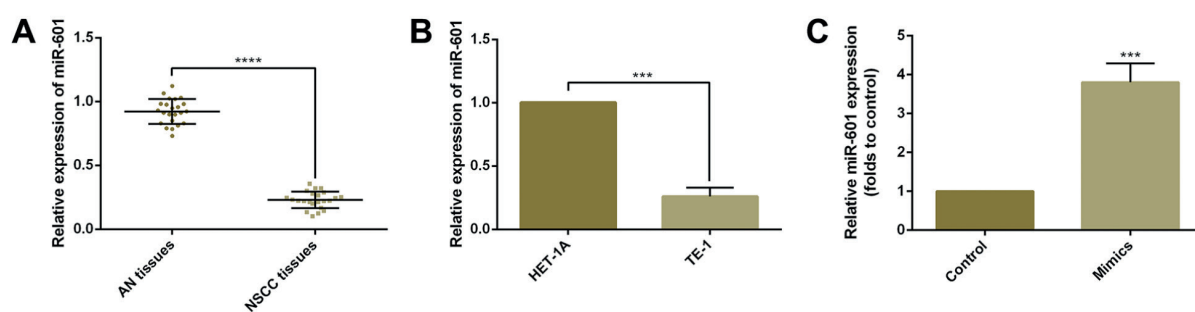


Figure 1. The expressions of miR-601 and HDAC6 in ESCC tissues and cells comparing with corresponding adjacent normal tissues and normal esophageal epithelial cells. **A**, Difference in the expression of miR-601 between ESCC tissues and corresponding adjacent normal tissues ($***p < 0.0001$ compared with adjacent normal tissues). **B**, The expression of miR-601 in ESCC cells (TE-1) and normal esophageal epithelial cells (Het-1A) ($***p < 0.01$ compared with Het-1A). **C**, Transfection efficiency was detected by qPCR ($***p < 0.001$).

ble acting target genes of miR-601 (Figure 2A). Luciferase reporter gene assay revealed that miR-601 inhibited the fluorescent expression of pGL3-HDAC6 (HDAC6-3'UTR-wild) but not the fluorescent expression of pGL3-HDAC6-mut (HDAC6-3'UTR-mut) (Figure 2B). The above results indicated that miR-601 inhibited the expression of HDAC6 by binding to the 3'-UTR of HDAC6.

MiR-601 Decreased the Expression Level of HDAC6

Three groups were established in TE-1 cells to perform similar experiments, including the miR-NC group, the miR-601 mimics group and the mimics + HDAC6 group. To further verify that HDAC6 was a target gene for miR-601, the protein and mRNA levels of HDAC6 in TE-1 cells after overexpression of miR-601 were detected. The results demonstrated that the protein and mRNA expression levels of HDAC6 in TE-1 cells were remarkably down-regulated in the miR-601 mimics group. Meanwhile, the effect of miR-601 on HDAC6 could be reversed by HDAC6 mimics transfection (Figure 3A, 3B). These data further illustrated the regulatory effect of miR-601 HDAC6 expression.

MiR-601 Suppressed the Proliferation of ESCC Cells

After the transfection, CCK-8 assay was performed to detect the proliferation rates of cells. CCK-8 results suggested that the proliferation rates of TE-1 cells were significantly inhibited by miR-601 mimics transfection. In contrast, the growth of ESCC cells was significantly increased in the mimics + HDAC6 group (Figure 3C).

MiR-601 Inhibited the Invasion and Migration of ESCC Cells

Migration and invasion are the two most key factors in cancer cell proliferation¹¹. In this study, the scratch-wound assay was conducted to study the effect of miR-601 overexpression on the migration ability of ESCC cells. The results showed that after 24 h of scratching, the scratch spacing of cells in the miR-601 mimics group was significantly larger than that of the NC group. This indicated that overexpression of miR-601 remarkably suppressed the migration ability of ESCC cells. Meanwhile, transwell assay showed that the invasion ability was also affected by the intervention of miR-601. The invasive cells were markedly reduced. However, HDAC6 could reverse the effects of miR-601 on the migration and invasion of ESCC cells (Figure 4).

Discussion

Tumor forms gradually, and epigenetics exerts a crucial role in its occurrence and development. The regulation of non-coding RNAs is a new field of epigenetic research, which has also become a research hotspot in life science^{12,13}. As a class of non-coding RNAs, miRNAs inhibit the expression of genes by modifying histones¹⁴⁻¹⁷. In the present study, we first detected the expression levels of miR-601 in ESCC tissues and cancer-adjacent normal tissues. The results revealed that the expression level of miR-601 in ESCC tissues was significantly declined, demonstrating a low expression of miR-601 in ESCC. Subsequently, ESCC cell line (TE-1) was selected as a research object. As expected, compared with the con-

trol cells, miR-601 was also found remarkably down-regulated in TE-1 cells. These results implied the regulatory role of MIR-601 in ESCC.

Currently, researches on miRNAs have primarily verified that miRNAs can inhibit the function of miRNAs by binding to target mRNAs. The premise is to predict the target genes of miRNAs. In this study, online miRNA bioinformatics databases such as TargetScanHuman, mi-Randa and miRDB predicted that HDAC6 was a targeted functional gene of miR-601. Dual-Luciferase reporter gene assay, as the gold standard for miRNA target gene identification, confirmed our hypothesis. Furthermore, Western blot and qPCR results suggested that the overexpression of miR-601 could significantly inhibit the expression of HDAC6. This indicated that miR-601 could regulate the expression of HDAC6 in a targeted way.

HDAC6 exists in cells and is a functional protein located in the cross system of two signals, including protein ubiquitination in the cytopla-

sm and protein lysine acetylation. It can bind to ubiquitinated proteins, which also has histone or non-histone deacetylase activity. Meanwhile, HDAC6 interacts with substrates, α -tubulin, heat shock protein 90 (Hsp90) and dermal actin¹⁸⁻²⁰, and deacetylates them. Through these interactions, HDAC6 participates in the regulation of a variety of important biological processes, including intracellular transportation, cell migration, stability of microtubules, formation of immune synapses and degradation of abnormal proteins²¹⁻²³. Studies have showed that HDAC6 dysfunction is closely associated with the occurrence and development of malignant tumors. The expression level of HDAC6 has been verified to be significantly up-regulated in oral squamous cell carcinoma, acute myeloid leukemia, ovarian cancer and hepatocellular carcinoma. Moreover, its expression level is correlated with tumor stage²⁴⁻²⁷. Meanwhile, HDAC6 can activate oncogenic Ras signaling pathway and tumor cell survival signaling pa-

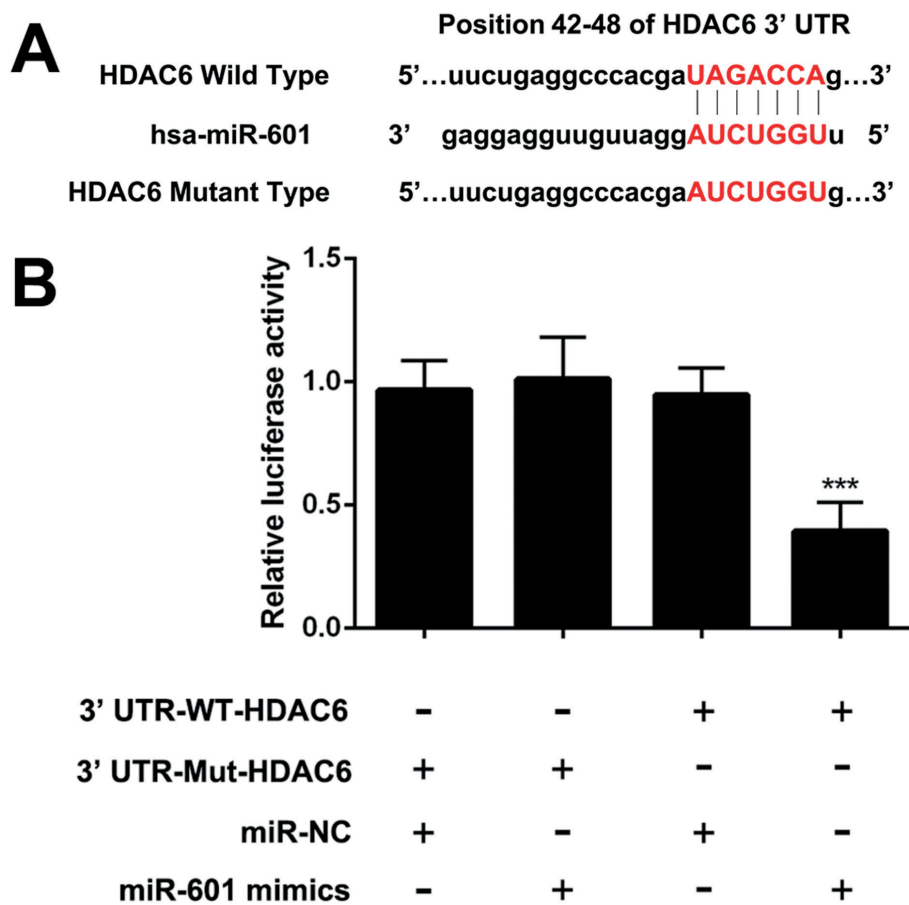


Figure 2. HDAC6 was a direct and functional target of miR-601. TE-1 cells were transfected with miR-601 mimics and inhibitor. **A**, Diagram of putative miR-601 binding sites of HDAC6. **B**, Relative activities of Luciferase reporters (** $p < 0.001$).

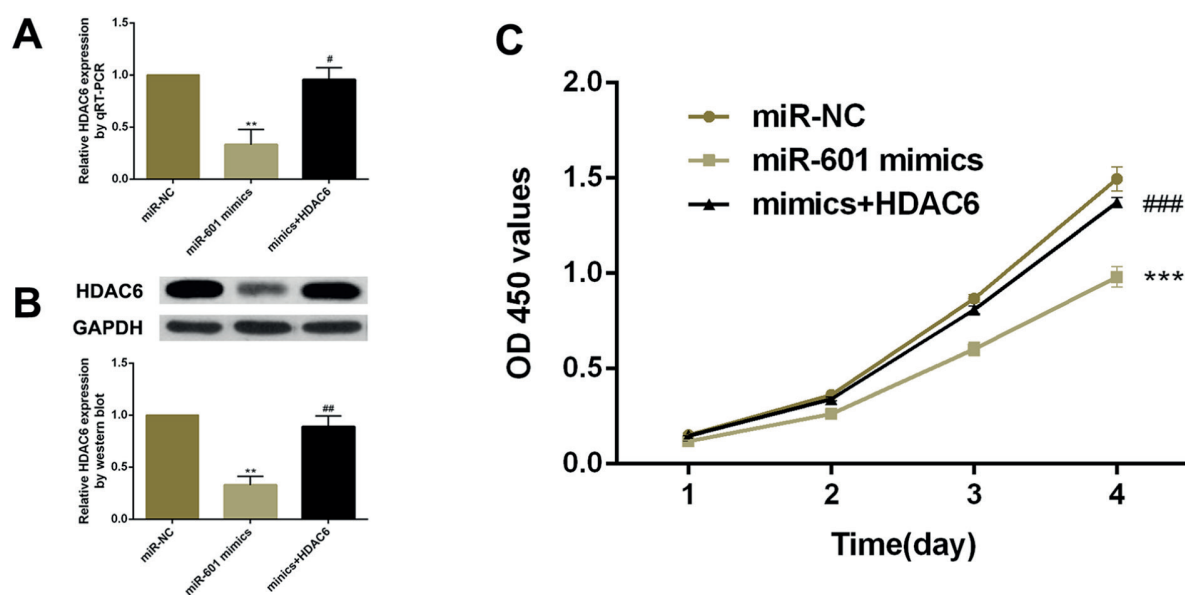


Figure 3. A-B, MiR-601 decreased the expression level of HDAC6. Data were expressed as means \pm standard deviations (** p <0.01 vs. NC group; # p <0.05, ## p <0.01 vs. Mimics group). C, MiR-601 inhibited the proliferation of ESCC cells (***) p <0.0001 vs. NC group; ### p <0.001 vs. Mimics group).

thway, which also enables transformed cells to undergo anchor-independent proliferation. This is beneficial to the evasion of cells from anoikis and survival, thus stimulating the occurrence and development of tumor²⁸. Additionally, the involve-

ment of HDAC6 substrate Hsp90 is essential for the structural maturation and activity of various oncogenic proteins. Under the inactivation state of HDAC6, Hsp90 undergoes super acetylation and tumor growth is inhibited²⁹.

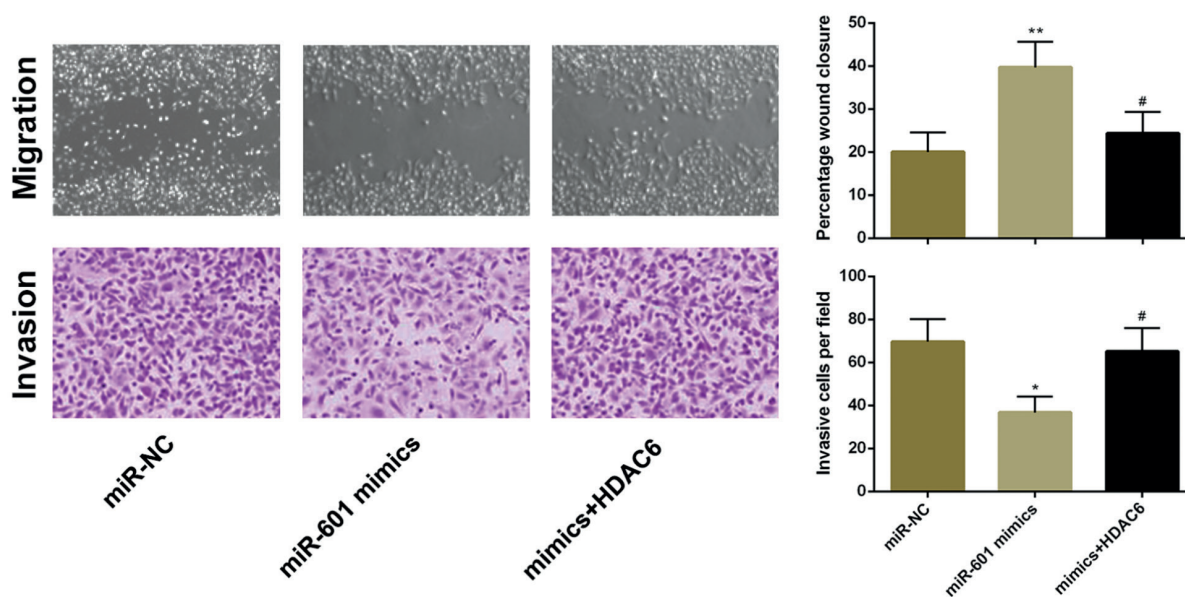


Figure 4. MiR-601/HDAC6 axis inhibited the invasion and migration of ESCC cells. HDAC6 overexpression attenuated the suppressive effect of miR-601 on TE-1 cells. A, The migration of cells was detected by scratch-wound assay. B, The invasion of cells was measured by transwell assay (* p <0.05, ** p <0.01 vs. NC group; # p <0.05 vs. Mimics group).

In vitro study demonstrated that the expression levels of miR-601 and HDAC6 in TE-1 cells were both significantly up-regulated after transfection with miR-601 mimics and sHDA6 mimics. Functional tests were further conducted to detect the effects of miR-601 and HDAC6 mimics on the proliferation, migration and invasion of ESCC cells. The results proved that the expression level of miR-601 in TE-1 cells was significantly increased after transfection with miR-601 mimics. However, the proliferation, migration and invasion abilities of TE-1 cells overexpressing miR-601 were remarkably declined. The above results proved that highly expressed miR-601 suppressed the occurrence and development of ESCC cells. However, the malignancy of ESCC was restored to some extent after the overexpression of HDAC6 in TE-1 cells.

In our study, we demonstrated the correlation between miR-601 and HDAC6 in ESCC. Meanwhile, the expression changes of miR-601 and HDAC6 were detected to elucidate the role of the miR-601/HDAC6 axis in ESCC cells. This work might provide new targets for the treatment of ESCC.

Conclusions

We found that the miR-601 suppressed the proliferation, invasion and migration of ESCC cells through down-regulation of HDAC6 expression, which could serve as a target molecular in the treatment of ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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