

Original Article

MiR-29a-3p suppresses the proliferation, migration and invasion of esophageal squamous cell carcinoma by targeting IGF-1

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Abstract: Esophageal squamous cell carcinoma (ESCC) is one of the most common and lethal cancers in the world. The function of miR-29a-3p has been reported in multiple human cancers as a key regulator of oncogenic processes. However, the function of miR-29a-3p in ESCC remains unclear. In this study, we found that enhanced expression of miR-29a-3p in ESCC cell lines not only suppressed cell proliferation, but also decreased the cellular motility including migration and invasion. Furthermore, luciferase reporter assay revealed insulin-like growth factor 1 (IGF-1) is the direct target gene of miR-29a-3p in ESCC cell lines. Experimental validation demonstrated that expression of IGF-1 was suppressed by miR-29a-3p on protein and mRNA levels. In addition, we detected the expression of miR-29a-3p and IGF-1 mRNA in 26 pairs early ESCC specimens which were at the stage of T₁₋₂N₀ and found that the expression of miR-29a-3p was significantly down-regulated in early ESCC specimens relative to adjacent normal specimens. Furthermore, we found a negative correlation between the expression of miR-29a-3p and IGF-1. This is the first time proving miR-29a-3p participates in suppressing oncogenic process in ESCC by targeting IGF-1. Taken together, these results suggested that miR-29a-3p was a tumor suppressor and might be a potential therapeutic candidate for the diagnosis and therapy of ESCC.

Keywords: miR-29a-3p, ESCC, proliferation, migration, invasion, IGF-1

Introduction

Esophageal cancer is nowadays the leading cause of cancer-related death worldwide. Esophageal squamous cell carcinoma (ESCC) contributes to majority in China [1]. Despite the continuous advancement in diagnosis and therapies for ESCC, five-year survival rate of ESCC was approximately 15-25% [2], owing to the most were at advanced stages when diagnosed [3, 4]. Although previous studies had elaborated on the basic mechanisms of ESCC cell proliferation, migration and invasion, the mechanism of ESCC tumorigenesis remained unclear and the five-year survival rate was not obvious improvement [5]. Therefore, it is of great significance to make a better understanding of early diagnosis of ESCC, discovering a novel molecular marker for ESCC is the critical for the development of therapeutic strategies.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules of 18-22 nucleotides which are known to play important regulatory role in gene expression [6]. MiRNAs exert effect mainly by binding to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs), leading to block translation of mRNA [7, 8]. Accumulating evidences showed miRNAs were involved in regulation of lots of cellular processes, including proliferation, migration, invasion and differentiation and function as tumor suppressors or oncogenes [9, 10]. Among them, miR-29a-3p is one of the most frequently down-regulated miRNAs in miRNA profiles of different types of cancer, including acute myeloid leukemia [11], chronic lymphocytic leukemia [12], nasopharyngeal carcinoma [13], breast cancer [14]. However, the molecular mechanisms of miR-29a-3p in ESCC are still poorly understood.

In this study, we investigated the role of miR-29a-3p in ESCC proliferation, migration and invasion. We found that miR-29a-3p was significantly down-regulated in ESCC cell lines and clinical tissue specimens, compared with esophageal epithelial cell and adjacent normal specimens, suggesting that miR-29a-3p might act as a tumor suppressor miRNA in ESCC. Furthermore, we identified that IGF-1 was the direct target gene of miR-29a-3p. Our research provided novel insight into the role of miR-29a-3p in ESCC which could be crucial for the development of new strategies for ESCC diagnosis and therapy.

Materials and methods

Clinical samples and cell culture

26 pairs of histopathologically confirmed early ESCC specimens which were at the stage of T₁₋₂N₀ and corresponding adjacent normal specimens were collected from patients between 2010 and 2013 who had undergone resection at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. None of patients received radiotherapy or chemotherapy in front of operation. All of the specimens were frozen immediately after surgery and stored at -80°C until RNA extraction. This study was approved by the Tongji Hospital, Tongji Medical College Ethics Committee, and written informed consent was obtained from all patients.

Human ESCC cell lines EC109, EC9706, KYSE510 and esophageal epithelial cell HET-1a were purchased from the First Affiliated Hospital of Zhengzhou University, China. Cells were cultured with containing 5% CO₂ at 37°C in RPMI 1640, supplemented with 10% fetal bovine serum (FBS, Invitrogen).

RNA extraction and quantitative real-time PCR

Total RNA of cells and tissue specimens, include miRNA, were extracted using Trizol (Invitrogen, Carlsbad, USA). And the concentration of total RNA was measured by a spectrophotometer (ND 2000, Nanodrop Inc, Wilmington, Del). RNA was reverse-transcribed into cDNA by using SuperScript First Strand cDNA System (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was con-

ducted to detect with sybr green mRNA assay by using ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The parameters of PCR were as follows: 95°C for 20 s, 40 cycles of 95°C for 3 s, 60°C for 30 s. The calculating method of relative expression level was the $\Delta\Delta C_t$ method. Reverse-transcribed and real-time primers for miR-29a-3p and IGF-1 mRNA were synthesized by Ribobio Co (Guangzhou, China). The real-time primer sequences of IGF-1 were as follows: forward, 5'-CTCAGACAGGCATCGTGG AT-3' and reverse, 5'-ACTTCC-TTCTGGGTCTTGGG-3'. β -actin and U6 were used as internal controls.

Transfection of miR-29a-5p mimic and inhibitor, IGF-1 siRNA

MiR-29a-3p mimic and inhibitor with respective negative controls were transfected into cells with Lipofectamine 2000 transfection reagent (Invitrogen) in accordance with the manufacturer's instructions. MiR-29a-3p mimic was transfected at 50 nM, while miR-29a-3p inhibitor was transfected at 100 nM. IGF-1 siRNA and negative control were transfected at 100 nM.

Cell proliferation assay

The cell proliferation effects of miR-29a-3p on cells were determined by Cell counting kit-8 assay. Logarithmic growing EC109 cells were seeded into each well of 96-well plates at a density of 5.0×10^3 cells/well and transfected with miR-29a-3p mimic, miR-29a-3p inhibitor, IGF-1 siRNA and respective negative controls at an appropriate concentration. The optical density (OD) on cell proliferation was measured at different time points (0, 24, 48, 72, 96 h) by a microplate reader (Beckman Coulter, Fullerton, CA, USA) at 492 nm. Each well was carried out in three-replicate wells and triplicate independent experiments were performed.

Wound healing assay

After transfection for 24 hours, the EC109 cells seeded in six-well plates had reached approximately confluence of 100%. The wound was vertically scratched by using a 10- μ l pipette tip, and then used phosphate buffer solution (PBS) repeatedly washed each of well. The EC109 cells were covered with serum-free RPMI 1640 and incubated for 48 h. At 1, 24, 48 hours after scratching the images were captured and tripli-

Function of miR-29a-3p in ESCC

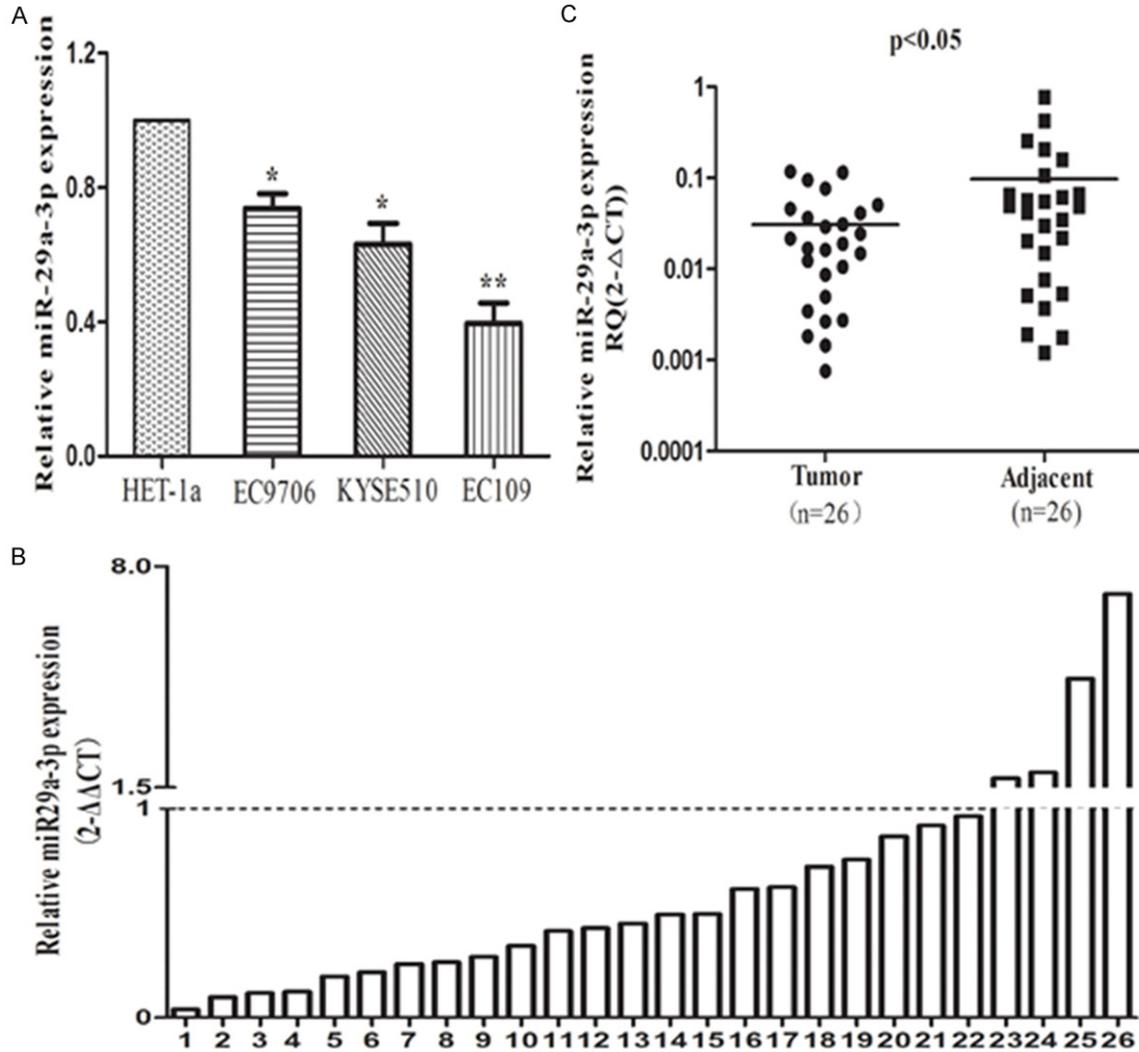


Figure 1. Expression of miR-29a-3p in ESCC cell lines and 26 pairs of early ESCC specimens. A. Compared with esophageal epithelial cell, expression of miR-29a-3p was significantly in EC9706, KYSE510 and EC109. B. The expression of miR-29a-3p in 26 pairs of early ESCC specimens compared with adjacent normal specimens. C. The expression of miR-29a-3p was significantly lower in early ESCC than in adjacent normal specimens, * $P < 0.05$, ** $P < 0.01$.

cate independent experiments were performed.

Transwell assay

Transwell assay was used to measure cell migration and invasion. Briefly, the transfected EC109 cells were at a density of 5.0×10^4 cells/well seeded into Matrigel-coated chambers in serum-free RPMI 1640 medium. The lower chambers in 24-well plate were contained with 10% FBS RPMI-1640 medium. After 24 hours of incubation, the cells number of EC109

cells attaching to the lower chambers was counted to quantify cell invasion ability. The migration ability of cells was measured in the equal condition without Matrigel.

Western blot

After transfection three days, equal amounts of protein were separated on 10% SDS-PAGE gels and transferred to PVDF membrane. Anti-Akt, p-Akt, Erk1/2, p-Erk1/2, GAPDH antibodies were totally purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IGF1

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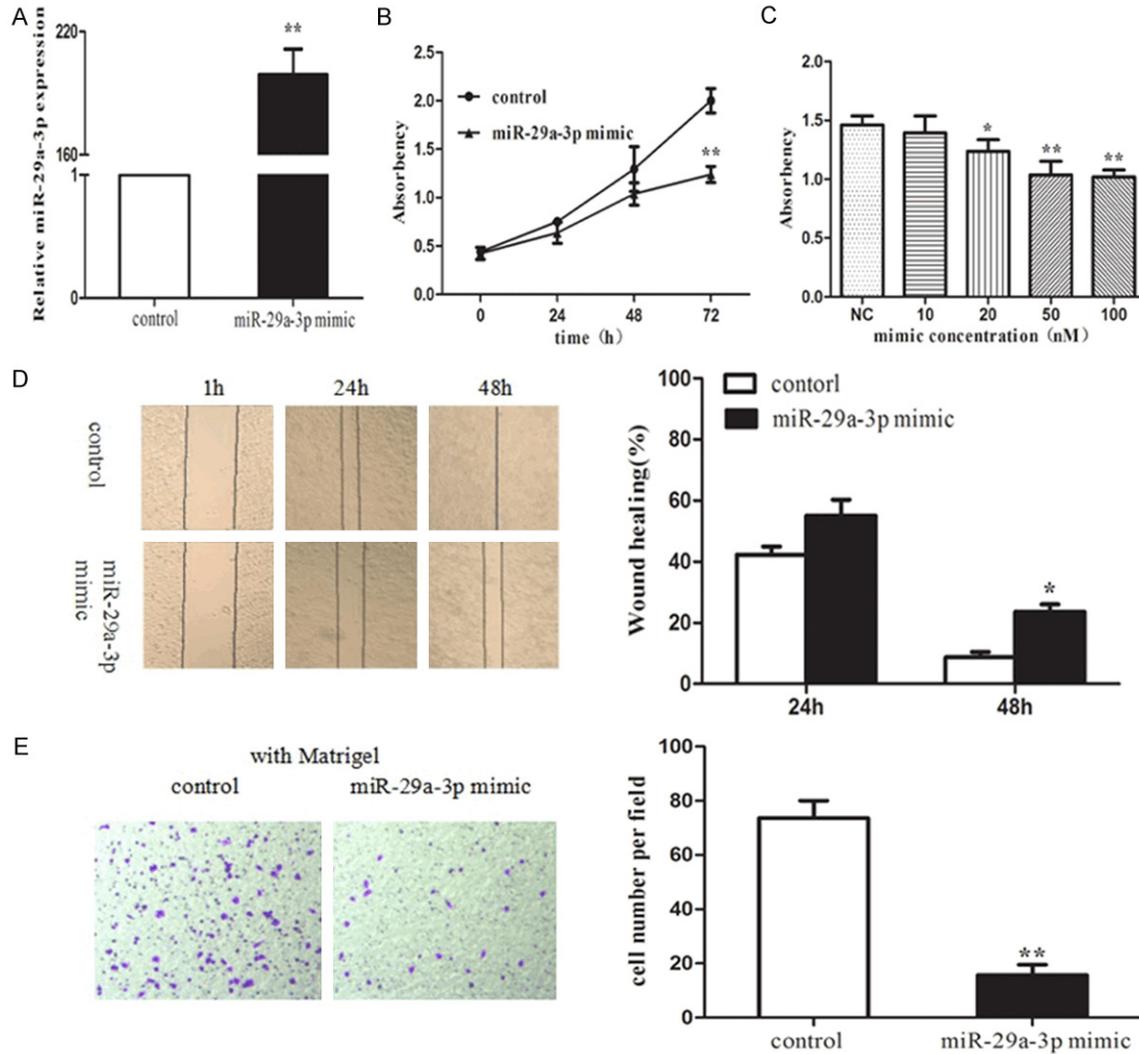


Figure 2. Up-regulation of miR-29a-3p inhibits cell proliferation, migration and invasion. A. The transfection efficiency of miR-29a-3p mimic in EC109 was confirmed by qRT-PCR analysis. B. Up-regulation of miR-29a-3p inhibited the proliferation of EC109. C. the inhibition tendency was depended on the miR-29a-3p mimic concentration when transfected. D. Up-regulation of miR-29a-3p inhibited the migration of EC109 and confirmed by wound healing assay. E. Up-regulation of miR-29a-3p inhibited the invasion of EC109 and confirmed by transwell assay, * $P < 0.05$, ** $P < 0.01$.

antibody was from Proteintech (Chicago, CA, USA). Triplicate independent experiments were performed.

Enzyme-linked immunosorbent assay

To determine the IGF-1 concentration, the culture supernatants of EC109 cells incubated after three days infection were collected and quantified by the IGF-1 Elisa kit (Abcam; USA) according to the manufacture's instruction. Optical density (OD) value of each well was measured at 450 nm and then established the

standard curve the value of standard samples. Concentration of tested samples was determined according to the standard curve.

Dual-luciferase reporter assay

The IGF-1 3'-UTR (wide type) luciferase reporter plasmids or mutant type were co-transfected with miR-29a-3p mimic or control respectively by using lipofectamine 2000 reagent. After 48 hours of transfection, the Dual-Luciferase Reporter Assay System (Promega) was used to measure luciferase activities according to the

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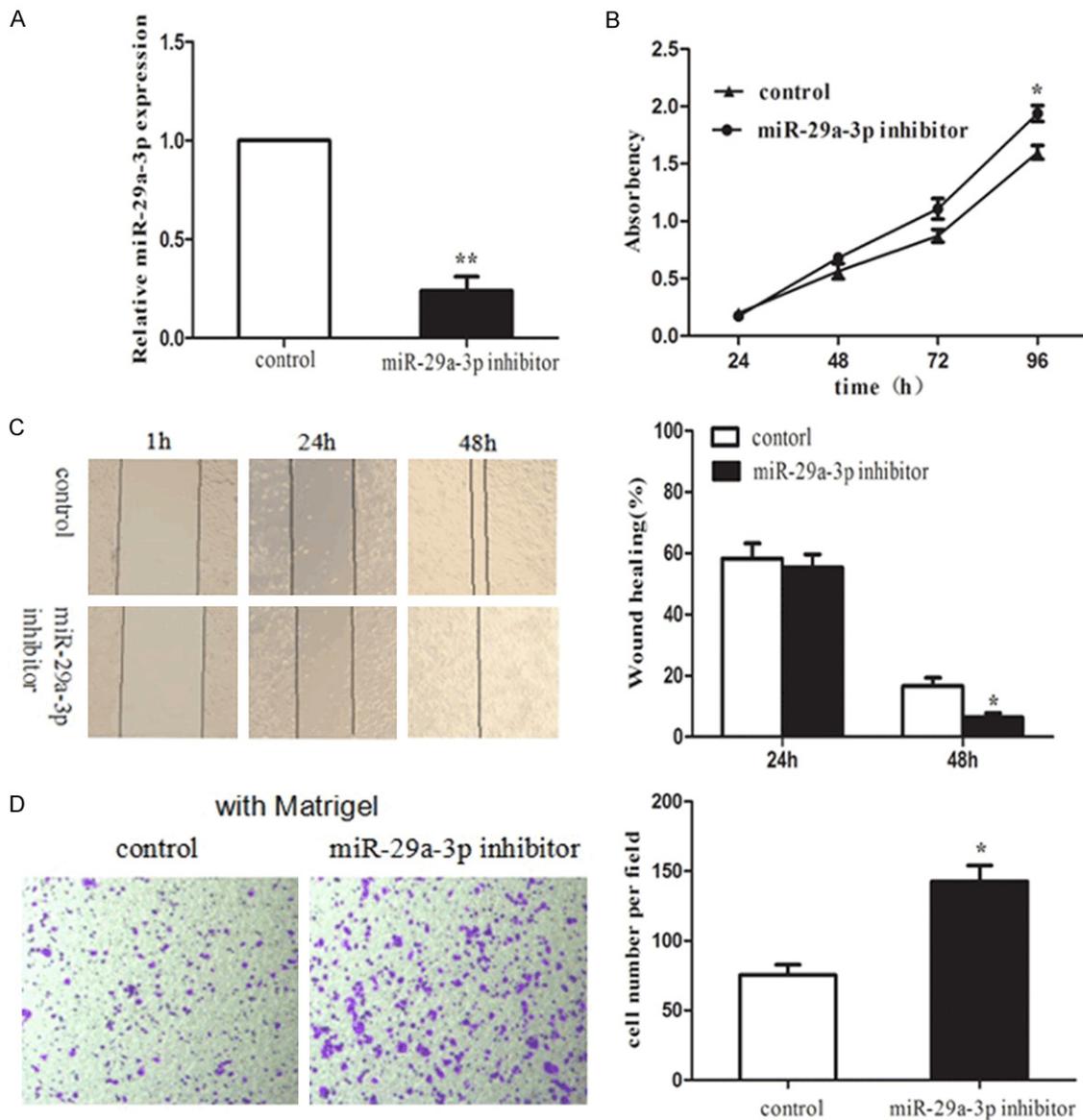


Figure 3. Down-regulation of miR-29a-3p promotes cell proliferation, migration and invasion. A. The transfection efficiency of miR-29a-3p inhibitor was confirmed by qRT-PCR analysis. B. Down-regulation of miR-29a-3p promoted the proliferation of EC109. C. Down-regulation of miR-29a-3p promoted the migration of EC109 and confirmed by wound healing assay. D. Down-regulation of miR-29a-3p promoted the invasion of EC109 and confirmed by transwell assay, * $P < 0.05$, ** $P < 0.01$.

manufacturer's instruction. Triplicate independent experiments were performed.

Statistical analysis

Student's t test was used to estimate the differences between two groups. The relationship between miR-29a-3p expression and IGF1 mRNA was analyzed by using the Pearson's test. P values < 0.05 was considered statistically significant. All of the statistical analyses were performed using SPSS version 18.0.

Results

MiR-29a-3p expression is down-expressed in ESCC cell lines and specimens

qRT-PCR analysis was performed and confirmed that expression of miRNA-29a-3p was down-regulated in ESCC cells, compared with esophageal epithelial cell (Figure 1A). We further measured the miR-29a-3p expression in 26 pairs early ESCC specimens and found that the expression of miR-29a-3p in ESCC specimens

Function of miR-29a-3p in ESCC

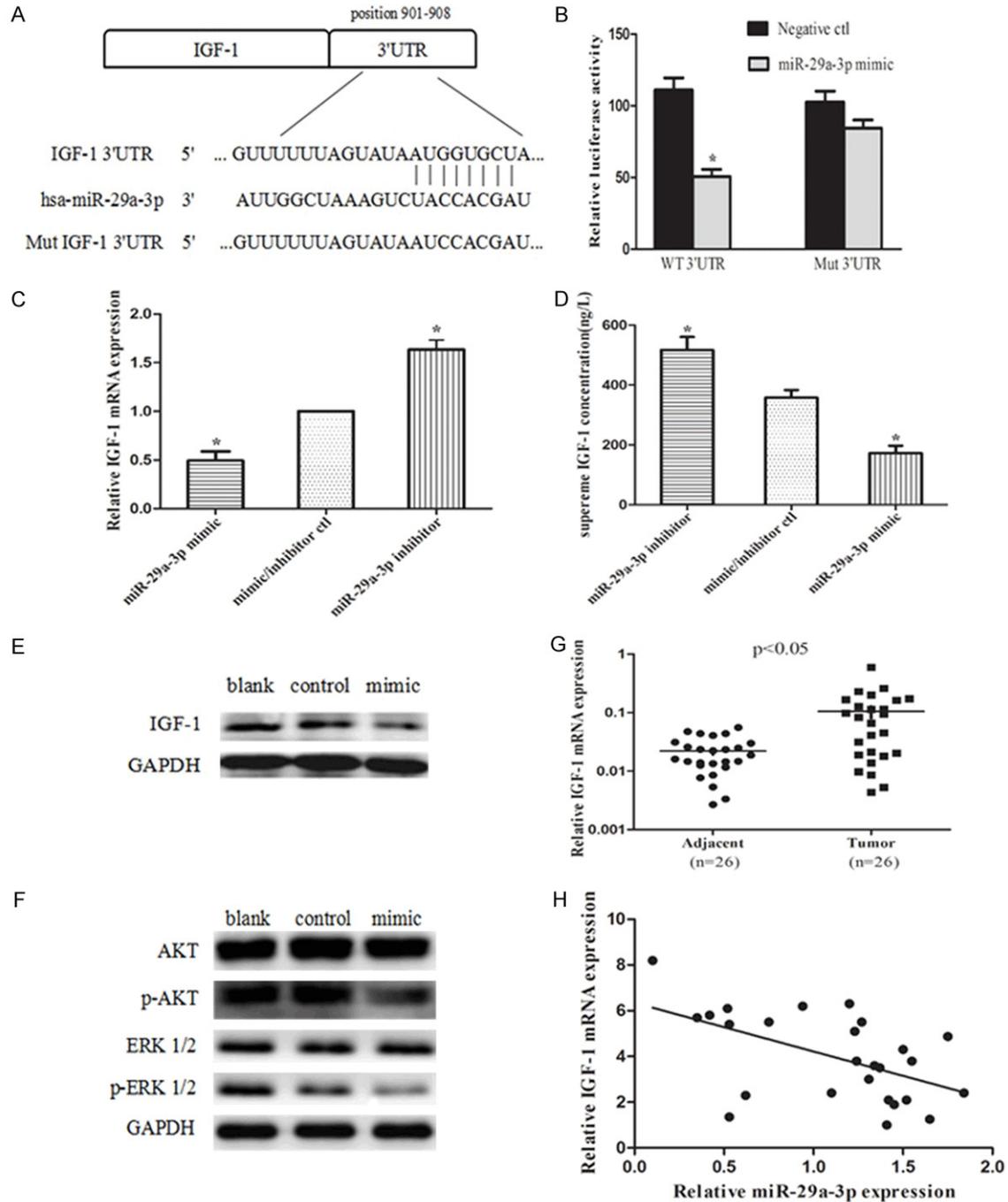


Figure 4. IGF1 is a direct target regulated by miR-29a-3p via Akt and Erk1/2. A. The putative miR-29a-3p binding sequence in the 3'-UTR of IGF-1 mRNA. Mutation was generated on the 3'-UTR sequence of IGF-1 in the complementary site for the seed region of miR-29a-3p. B. Luciferase activity of wild-type 3'-UTR of IGF-1 was suppressed by miR-29a-3p mimic in EC109. C. The expression of IGF-1 mRNA was influenced by miR-29a-3p mimic/inhibitor in EC109 and confirmed by qRT-PCR. D. The expression of IGF-1 was influenced by miR-29a-3p mimic/inhibitor in EC109 and confirmed by Elisa assay. E. The expression of IGF-1 regulated by miR-29a-3p mimic. F. PI3K/Akt and MAPK signaling pathways were involved by miR-29a-3p mimic in EC109. G. The expression of IGF-1 mRNA was significantly higher in early ESCC than in adjacent normal specimens. H. An inverse correlation between IGF-1 mRNA and miR-29a-3p expression in ESCC specimens (Pearson's correlation $r=-0.534$, $P=0.049$).

was significantly lower than which in corresponding adjacent normal specimens (**Figure 1B**,

1C). These data suggested that miR-29a-3p might play a role in esophageal carcinogenesis.

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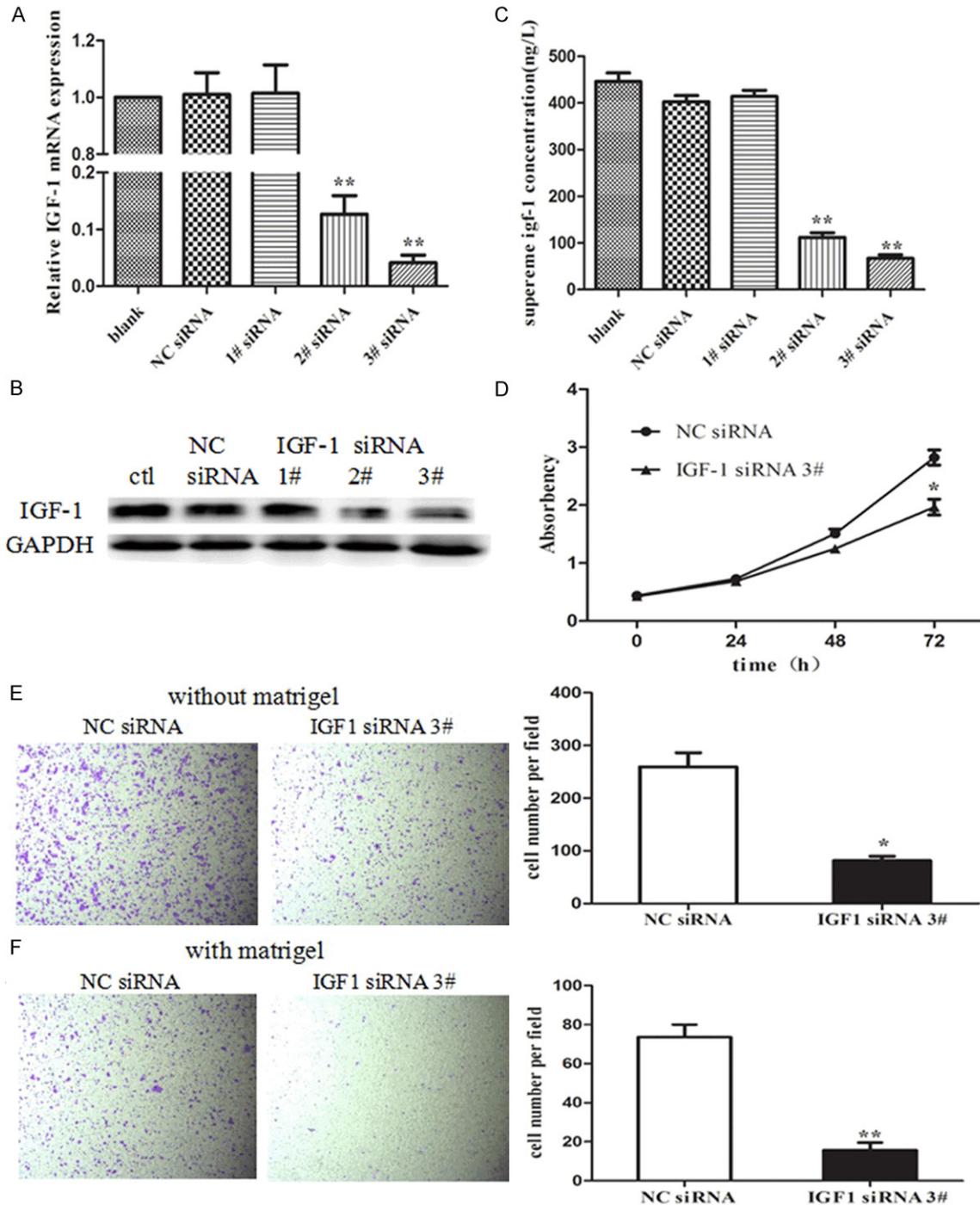


Figure 5. Knockdown of IGF1 inhibits cell proliferation, migration and invasion. A. The expression of IGF-1 mRNA was influenced by IGF-1 siRNA. B. The expression of IGF-1 on protein level was influenced by IGF-1 siRNA. C. The expression of IGF-1 on protein was influenced and confirm by Elisa assay. D. Down-regulation of IGF-1 inhibited the proliferation of EC109. E. Down-regulation of IGF-1 inhibited the migration of EC109 and confirmed by transwell assay. F. Down-regulation of IGF-1 inhibited the invasion of EC109 and confirmed by transwell assay, * $P < 0.05$, ** $P < 0.01$.

Up-regulation of miR-29a-3p inhibits cell proliferation, migration and invasion

All of the results above suggested that miR-29a-3p may act as a tumor suppressor in ESCC.

To test the biological role of miR-29a-3p, we transfected ESCC cell line EC109 with miR-29a-3p mimic. The transfection efficiency of miR-29a-3p mimic was confirmed by qRT-PCR analysis (**Figure 2A**). CCK8 assay showed that the

growth of EC109 infected with miR-29a-3p mimic was significantly inhibited, compared to the control group (**Figure 2B**). Furthermore, we found that the inhibition tendency was closely associated with the miR-29a-3p mimic concentration when transfected (**Figure 2C**). To explore whether miR-29a-3p could influence the migration and invasion capacity of EC109, wound healing assay was actualized and showed (**Figure 2D**), over-expression of miR-29a-3p resulted in retarded wound closing compared to the mimic control. Similarly, as the invasion assay showed (**Figure 2E**), the number of EC109 cells in transwell inserts migration through membrane was much less in the miR-29a-3p mimic group compared to the control group. All the data suggested that over-expression miR-29a-3p could effectively inhibit ESCC proliferation, migration and invasion.

Down-regulation of miR-29a-3p promotes cell proliferation, migration and invasion

To ascertain whether down-regulation miR-29a-3p could reverse the phenomena, we transfected EC109 cells with miR-29a-3p inhibitor. The transfection efficiency of miR-29a-3p inhibitor was confirmed by quantitative real-time PCR analysis (**Figure 3A**). CCK8 assay showed that the growth of EC109 infected with miR-29a-3p inhibitor was promoted, compared to the control group (**Figure 3B**). Wound healing assay showed that the migration of EC109 in miR-29a-3p inhibitor was faster than the control group (**Figure 3C**). Similarly, as the invasion assay showed (**Figure 3D**), the number of EC109 cells in transwell inserts migration through membrane was more in the miR-29a-3p inhibitor group compared to the control group. All the data suggested that down-regulation of miR-29a-3p could effectively promote ESCC proliferation, migration and invasion.

IGF1 is a direct target regulated by miR-29a-3p via Akt and Erk1/2

To explore the target genes of miR-29a-3p which may participate in regulating ESCC tumorigenesis, we searched for putative targets in miRNA target prediction software, including TargetScan, miRanda and Pictar. And finally IGF-1 was predicted to be a potential target gene of miR-29a-3p. To confirm the relationship between miR-29a-3p and IGF-1, we analyzed the 3'-UTR of IGF-1 and identified one

binding site for miR-29a-3p at the position 901-908nt (**Figure 4A**). We constructed the IGF-1 3'-UTR fragment into luciferase vector. Treatment of EC109 with miR-29a-3p mimic significantly reduced relative luciferase activity, while mutation binding site of miR-29a-3p reduced the response to miR-29a-3p (**Figure 4B**). qRT-PCR showed the transfection of the miR-29a-3p mimic to EC109 resulted in a dramatic decrease in IGF-1 expression. Treatment of EC109 cells with miR-29a-3p inhibitor resulted in an increase in IGF-1 expression (**Figure 4C**). Elisa assay also confirmed that transfection of the miR-29a-3p mimic to EC109 resulted in a dramatic decrease in supreme IGF-1 concentration, and reversed when treatment with miR-29a-3p inhibitor (**Figure 4D**). These changes in IGF-1 expression were also confirmed by western blot assay (**Figure 4E**). Furthermore, PI3K/Akt and MAPK signaling pathways were dramatically inhibited by up-regulated miR-29a-3p in ESCC (**Figure 4F**). All of the data showed an inverse correlation between miR-29a-3p and IGF-1 expression in ESCC cells. To further determine, we analyzed 26 pairs of early ESCC specimens and corresponding adjacent normal specimens. We found the relative expression of IGF-1 mRNA was significantly higher in ESCC specimens compared with corresponding adjacent normal specimens (**Figure 4G**). Moreover, the relative expression of miR-29a-3p was significantly lower in ESCC. Analysis of the expression of miR-29a-3p and IGF-1 mRNA showed a significant and inverse correlation between miR-29a-3p and IGF1 (**Figure 4H**; $r=-0.534$, $P=0.049$).

Knockdown of IGF1 inhibits cell proliferation, migration and invasion

Previous evidences had demonstrated that miR-29a-3p inhibited ESCC cell proliferation and invasion by targeting IGF-1. Then we silenced IGF-1 in EC109 cells using RNA interference (IGF-1 siRNA). The effect of IGF-1 siRNA was detected by qRT-PCR (**Figure 5A**), western blot (**Figure 5B**) and Elisa (**Figure 5C**). CCK8 assay showed IGF-1 siRNA significantly inhibited EC109 cell proliferation compared to control group (**Figure 5D**). Furthermore, as the migration and invasion assays showed (**Figure 5E**, **5F**), the number of EC109 cells in transwell inserts migration through membrane was much less in the IGF-1 siRNA group compared to the control group.

Discussion

Previous studies reported that miR-29a-3p as a member of the miR-29 family which included hsa-miR-29a, miR-29b and miR-29c acted as a tumor suppressor in various types of cancer [15, 16]. In this study, we explored the expression and biological function of miR-29a-3p in ESCC. Our data showed that miR-29a-3p was significantly down-regulated in ESCC cell lines involving in EC109, EC9706 and KYSE510 compared with esophageal epithelial cell HET-1a. Moreover, we found that miR-29a-3p might act as a tumor suppressor in ESCC. Up-regulated miR-29a-3p dramatically suppressed proliferation, migration and invasion of EC109 cells by targeting IGF-1. Furthermore, the data of 26 pairs early ESCC which were at the stage of T₁₋₂N₀ showed that the expression of miR-29a-3p in ESCC specimens was significantly lower than which in corresponding adjacent normal specimens. Our results indicated that miR-29a-3p might be critical for the regulation of proliferation and metastasis in ESCC.

Insulin-like growth factor 1 (IGF-1) is a growth factor which is involved in many pathological and physiological processes such as angiogenesis, differentiation, apoptosis [17]. And the metastatic activity of tumor cells was significantly promoted at the condition of abnormal expression of IGF-1 [18]. Despite miR-29a had been reported through down-regulating IGF-1 to promote myocardial cell apoptosis induced by high glucose [19], there were no previous reports that proved the function of miR-29a-3p in regulating the expression of IGF-1 in ESCC. In this study, we found a significantly inverse correlation between miR-29a-3p and its target IGF-1 in ESCC specimens. Furthermore, both western blot and Elisa proved the up-regulation of miR-29a-3p significantly decreased the protein expression of IGF-1 in ESCC cell line. The miR-29a-3p/IGF-1 interaction was confirmed, as miR-29a-3p interacted with the 3'-UTR of IGF-1 through a specific binding site. This is the first report showing that IGF-1 is regulated by miR-29a-3p in ESCC.

As reported, PI3K/Akt and MAPK signaling could be induced by IGF-1 as downstream signal transduction pathways and involved variety of tumorigenesis [20, 21]. Our results revealed that it might involve in the mechanisms of ESCC

cell proliferation and invasion. Here we found phosphorylation of Akt and Erk1/2 involving in PI3K/Akt and MAPK signaling were decreased by up-regulated miR-29a-3p, which might clarify the mechanisms of proliferation, migration and invasion inhibited by miR-29a-3p in ESCC.

In summary, we demonstrated that miR-29a-3p was significantly down-regulated in ESCC cell lines and early ESCC tissues. Up-regulated miR-29a-3p suppressed the proliferation, migration and invasion of EC109 by targeting IGF-1 via inhibition of PI3K/Akt and MAPK signaling. Therefore, the results described here showing miR-29a-3p may be the potential molecular target for ESCC treatment.

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Disclosure of conflict of interest

None.

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