

MicroRNA-216a-5p suppresses esophageal squamous cell carcinoma progression by targeting KIAA0101

TUANHE SUN¹, QIAN^{2*}, RONG YAN¹, KANG LI¹, KUN ZHU¹, CHENGXUE DANG¹ and DAWEI YUAN¹

Departments of ¹Surgical Oncology and ²Rheumatism and Immunology,
First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shannxi 710061, P.R. China

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Abstract. The KIAA0101 protein (also referred to as NS5ATP9 or Paf15) is overexpressed in esophageal squamous cell carcinoma (ESCC) and is associated with disease progression and poor patient survival, but how KIAA0101 expression is regulated remains unknown. The relationship between tumor miR-216a-5p expression and prognosis in patients with ESCC was revealed by survival analyses. Quantitative reverse-transcriptase PCR and western blot analysis were used to evaluate miR-216a-5p and KIAA0101 expression in human ESCC tissues and cell lines. The targeting of KIAA0101 by miR-216a-5p was verified by dual-luciferase reporter assays. The EC9706 and TE1 cell lines were transfected with miR-216a-5p mimics and inhibitor, or KIAA0101-specific shRNA and KIAA0101-expressing plasmids, in order to evaluate the effect of manipulating miR-216a-5p and KIAA0101 expression on ESCC cell proliferation, cell cycle progression, migration, and invasion. miR-216a-5p was lowly expressed and inversely correlated with KIAA0101 protein expression in ESCC tissues and cell lines. Lower miR-216a-5p expression was associated with worse prognosis in patients with ESCC. miR-216a-5p negatively regulated KIAA0101 expression by directly targeting the 3'-untranslated region of the KIAA0101

mRNA. Overexpression of miR-216a-5p suppressed the proliferation, migration, and invasion of the ESCC cell lines, whereas inhibition of miR-216a-5p had the opposite effects. Meanwhile, KIAA0101 promoted ESCC migration and invasion, and its overexpression abolished the antitumor effects of miR-216a-5p mimics. As a tumor suppressor, miR-216a-5p targets KIAA0101 to inhibit the proliferation, migration, and invasion of ESCC. Therefore, the miR-216a-5p/KIAA0101 axis may be a potential target for ESCC treatment.

Introduction

Esophageal cancer is one of the most commonly diagnosed malignant tumors; 572,034 cases of newly diagnosed esophageal cancer were estimated in 2018 worldwide (1,2). Esophageal squamous cell carcinoma (ESCC) accounts for approximately 90% of all esophageal cancers in Asian countries (3). Despite advances in therapeutic methods, the prognosis for patients with ESCC remains poor due to the late stage at diagnosis in most cases. Studies during the past three decades have identified a number of genetic alterations involved in regulating the initiation and progression of ESCC (4). Nevertheless, the molecular mechanisms involved in the progression of ESCC are not fully understood.

KIAA0101 (also referred to as NS5ATP9 or Paf15) is involved in multiple biological activities, including DNA repair, cell proliferation, cell cycle progression, and cell migration (5). Previous studies have shown that the KIAA0101 protein is dysregulated in a variety of malignant tumors and that its overexpression is associated with poor prognosis in patients with cancer (6-8). A recent study by our group showed that KIAA0101 protein expression was upregulated in ESCC tissues, and is associated with the pathological Tumor-Node-Metastasis (pTNM) stage, resistance to chemotherapy, tumor recurrence, and poor survival of patients with ESCC (7). The overexpression of KIAA0101 was also found to enhance ESCC cell proliferation and to upregulate the expression of cyclins A and B, thus leading to a reduced percentage of cells in the G1 phase (7). Nevertheless, the molecules that could regulate the expression of KIAA0101 in ESCC cells have not been elucidated to date.

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNA molecules that control specific gene expression by translational repression or degradation of the

Correspondence to: Dr Dawei Yuan, Department of Surgical Oncology, First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shannxi 710061, P.R. China
E-mail: dwyuan@mail.xjtu.edu.cn

*Contributed equally

Abbreviations: ESCC, esophageal squamous cell carcinoma; pTNM, pathological Tumor-Node-Metastasis; miRNA, microRNAs; NSCLC, non-small cell lung cancer; AJCC, American Joint Committee on Cancer; OS, overall survival; DFS, disease-free survival; OD, optical density; PBS, phosphate-buffered solution; SEM, standard error of the mean; CDDP, cis-diamminedichloroplatinum/cisplatin; TCTN1, tectonic family member 1

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complementary messenger RNAs (mRNAs) (6). miRNAs play important regulatory roles in cell differentiation, proliferation, invasion, metastasis, and apoptosis (9). Currently, data are available on the miRNA signature of ESCC, and there are studies attempting to point out the potential value of miRNAs in ESCC screening, diagnosis, treatment, and prognosis (10).

miR-216a-5p is a member of the miR-216 family (miR-216a/miR-216b), which has been shown to be dysregulated in several types of human cancers (11-16). For example, miR-216a is significantly upregulated in hepatocellular carcinoma and was found to induce epithelial-mesenchymal transition (EMT) by suppressing the expression of PTEN, SMAD7, and TSLC1, thus contributing to carcinogenesis and recurrence (17). On the other hand, miR-216a displays a low expression in non-small cell lung cancer (NSCLC) and could inhibit the cell activity of NSCLC by directly targeting eIF4B and ZEB1 (18). The role of miR-216a and the underlying molecular mechanisms in ESCC have rarely been explored.

A previous study by our group indicated that the overexpression of KIAA0101 induced ESCC cell viability and was a marker for poor prognosis of ESCC, including early recurrence and short survival. In addition, KIAA0101 enhanced resistance to cisplatin by upregulating cell mitosis (7). Therefore, we conducted a preliminary exploration of its upstream regulation. By using TargetScan and miRanda, we chose to study miR-216a-5p. In the present study, we evaluated the expression level of miR-216a-5p in clinical specimens of ESCC and human ESCC cell lines and observed that lower expression of miR-216a-5p was associated with malignancy in comparison with the control tissues or cells. We examined the association between miR-216a-5p expression and prognosis of patients with ESCC. KIAA0101 was confirmed to be a downstream target of miR-216a-5p, and the *in vitro* experimental results demonstrated that miR-216a-5p inhibited tumor proliferation, migration, and invasion by directly decreasing the expression of KIAA0101 in ESCC. Our study demonstrated a critical role of the miR216a-5p/KIAA0101 axis in the progression of ESCC.

Materials and methods

Study population, tissue samples, and collection of survival data. Between July 2014 and June 2017, 83 patients with unifocal primary ESCC (histologically diagnosed) were recruited at the Department of Oncologic Surgery of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, Shaanxi, China). During the operation, tumor and corresponding normal tissues (>5 cm from the edge of the tumor) were collected and immediately stored at -80°C until use. All patients underwent margin-negative complete tumor resection (R0 resection) and extensive lymphadenectomy. Patients with advanced-stage cancer received 5-FU/leucovorin-based post-operative chemotherapy and radiotherapy. Tumor stage and histological grade were recorded according to the 7th edition of the classification guidelines of the American Joint Committee on Cancer (AJCC) (19). After surgery, the patients were followed routinely for more than 36 months or until death. The follow-up was censored in February 2019. The overall survival (OS) and disease-free survival (DFS) rates were calculated with the Kaplan-Meier method. The study was approved by

the Ethics Committee at the First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from all patients.

Cell culture. Six human ESCC cell lines (EC9706, EC109, KYSE150, KYSE450, TE1, and TE10) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The human esophageal epithelial cell line (HET1A) was obtained from the American Type Culture Collection (ATCC). EC9706, EC109, KYSE150, KYSE450, TE1, and TE10 cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare) containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA). The HET1A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown in a humidified atmosphere with 5% CO₂ at 37°C.

Cell transfection. In order to overexpress KIAA0101, the full-length KIAA0101 cDNA was cloned into the mammalian expression vector p-EGFP (RiboBio, China) to generate the KIAA0101-overexpressing plasmid. The empty p-EGFP plasmid was used as a control. The plasmids were transfected into the cell lines using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. At 48 h after transfection, G418-sulfate (600 ng/ml for EC9706, and 800 ng/ml for TE1; Sigma-Aldrich; Merck KGaA) was added to the cell culture medium throughout the experimental periods, as previously described (7). In order to knock down KIAA0101, the ESCC cells were transfected with the pSIREN-Shuttle vector (RiboBio) encoding the KIAA0101-targeted shRNA (5'-GCA ACCTGATCACACAAATGA-3') using Xtreme HP (Roche), according to the manufacturer's instructions. For the transfection of small RNA molecules, ESCC cells were cultured in 6-well plates (2x10⁵ cells per well), and the miR-216a-5p mimic (30 nM), or the miR-216a-5p inhibitor (300 nM), or the negative controls (all were from RiboBio) were transfected into cells using Lipofectamine RNAiMAX (Life Technologies; Thermo Fisher Scientific, Inc.). The cells were used at 48 h after transfection.

Real-time qPCR. Total RNA was extracted from the cultured cells and tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RNA concentrations were measured using a NanoDrop1000 spectrophotometer (NanoDrop Technologies). RNA integrity was assessed by gel electrophoresis. To detect the expression levels of miR-216a-5p, the miR-216a-5p-specific primers (RiboBio) were used for the reverse transcription of the total RNA. To detect the expression levels of KIAA0101, total RNA (1 µg each sample) was used to synthesize cDNA using the PrimeScript® RT Master Mix Perfect Real-Time Reagent Kit (Takara Bio Inc.). Quantitative reverse transcription PCR (RT-qPCR) for miR-216a-5p and KIAA0101 was performed using a standard protocol based on the SYBR Green PCR kit (Toyobo) on an iQ5 system (Bio-Rad Laboratories, Inc.). The relative expression of miR-216a-5p was normalized against U6, while that of KIAA0101 was normalized to β-actin, both using

the $2^{-\Delta\Delta Cq}$ method (20). When comparing the ΔCq between tumor and normal tissue, $2^{-\Delta Cq}$ was used. When normal cells or tissue were used as references, $2^{-\Delta\Delta Cq}$ was compared. The $2^{-\Delta\Delta Cq}$ of normal tissue was 1. Each sample was run in triplicates, and the experiment was repeated at least twice.

Western blotting. Total protein was extracted from cultured cells or tissues using the radioimmunoprecipitation assay lysis buffer (RIPA; Beyotime Institute of Biotechnology). Protein concentration was measured using the BCA kit (Beyotime Institute of Biotechnology). Western blotting was performed as previously described (7). The primary antibodies included KIAA0101 (dilution 1:500, 15 kDa; cat. no. H0009768-M01; Abnova) and β -actin (dilution 1:1,500, 42 kDa; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). The secondary antibody was goat anti-mouse antibodies conjugated to horseradish peroxidase (HRP; dilution 1:1,000; cat. no. 31430; Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Proteins of interest were visualized using an enhanced chemiluminescence kit (EMD Millipore). The band intensities were quantified by densitometry using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA). Each experiment was repeated at least three times.

Immunohistochemistry. Immunohistochemistry was carried out as previously described (21), using the primary antibody for KIAA0101 (dilution 1:300; Abnova) and a secondary antibody (goat anti-mouse IgG; Pierce Biotechnology; Thermo Fisher Scientific, Inc.). By calculating the percentage of KIAA0101-positive cancer cells as previously described (7), all samples were classified into two groups: Negative or weak KIAA0101 expression (<15% or 16-30%) and positive or strong KIAA0101 expression (31-60% or >60%). All slides were independently assessed by at least two pathologists who were unaware of the clinicopathological features of the tumors.

Dual-luciferase reporter assay. The KIAA0101 3'-UTR containing the binding sites of miR-216a-5p was amplified by PCR and inserted into the firefly luciferase reporter vector pmiR-RB-REPORT (RiboBio). Following the manual of Gene Tailor Site-Directed Mutagenesis System (Invitrogen; Thermo Fisher Scientific, Inc.), the construct with mutations in the miR-216a-5p seed sequence (the sequence of TGAGATT at 357-363 bp was mutated to TCACAAT) was produced by the mutagenic oligonucleotide primers (forward: 5'-GTTGTATTTCACAAATTGCTTAGATTGTTGTAC-3' and reverse: 5'-CTAAGCAATTGTGAAATACAACATACTTGTA-3'). EC9706 and TE1 cells were co-transfected with 30 ng of pmiR-RB-KIAA0101 3'-UTR or their mutant constructs, 50 ng luciferase reporter, and 10 ng *Renilla* luciferase reporter using Lipofectamine 2000. At 24 h after transfection, the firefly and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega Corp.). For each analysis, the *Renilla* luciferase signal was standardized to the firefly luciferase signal.

Cell proliferation. At 24 h before transfection, the EC9706 and TE1 cells were seeded into 96-well plates at 5,000 cells/well. At 48 h after transfection with the KIAA0101-expressing or KIAA0101-specific shRNA expressing plasmids, the cells were assessed using the Cell Counting Kit 8 reagent (CCK-8,

Sigma-Aldrich; Merck KGaA) for proliferation, following the manufacturer's instructions. Cell viability was quantified by measuring absorbance at 450 nm on a microplate reader. The optical density (OD) values of the samples in the experimental groups were normalized to that of the groups with control transfection at each time point. Each experiment was repeated at least three times.

Colony formation. EC9706 and TE1 cells were trypsinized and incubated in 6-well plates (1,000 cells/well) in a humidified 5% CO₂ atmosphere for 10 days. The colonies were fixed with 70% ethanol for 15 min at room temperature and stained with 2% crystal violet. The positive colonies (more than 50 cells or 3 mm² per colony) were counted. Images of stained sections were obtained using an Olympus BX51 optical microscope (Olympus Corp.) equipped with a digital camera (PD71; Olympus). The experiments were repeated at least three times.

Flow cytometry. The cultured EC9706 and TE1 cells were digested with trypsin, washed with phosphate-buffered solution (PBS), and fixed with 70% ethanol overnight at 4°C. The cells were incubated with DNase-free RNase (100 μ g/ml; Thermo Fisher Scientific, Inc.) for 20 min at 37°C, and stained with propidium iodide (PI) (50 μ g/ml; Sigma-Aldrich; Merck KGaA). The DNA contents were determined using the FACSCalibur™ platform (BD Biosciences), following the manufacturer's instructions. The data were generated from 20,000 events (cells) using the ModFit LT software (Verity Software House, USA).

Cell migration and invasion assays. The migration and invasion assays were carried out using Transwells with 6.5-mm polycarbonate membranes and 8.0- μ m pores (Corning, Inc.) with and without Matrigel (BD Biosciences). A total of 1×10^5 EC9706 or TE1 cells were seeded in the upper chambers. In the migration and invasion experiments, we used a serum-free RPMI-1640 medium in the upper chamber, and a medium containing 10% FBS in the lower chamber to avoid the influence of cell proliferation. We determined the time of observation and counting according to the proliferation cycle and growth of the two types of cells and avoided the effects of cell proliferation as much as possible. After incubation at 37°C for 24 h, the cells in the chambers were stained with 0.1% crystal violet, observed under a light microscope (IX71; Olympus), and photographed at x200 magnification.

Statistical analysis. Data are presented as mean \pm standard error of the mean (SEM). The differences between ESCC and corresponding normal tissues were compared using the Wilcoxon t-test. The correlations between miR-216a-5p expression and the clinicopathological features of the patients with ESCC were evaluated using the Chi-square test. The independent risk factors associated with the survival of the patients with ESCC were identified by Cox proportional hazards regression analysis. The survival curves of the patients with ESCC were obtained using the Kaplan-Meier method, and the survival rates were compared using the log-rank test. The significance level was set to $P < 0.05$. Data analysis was performed using SPSS 20.0 (IBM, Corp.) and Graph Pad Prism 5 (GraphPad Software, Inc.).

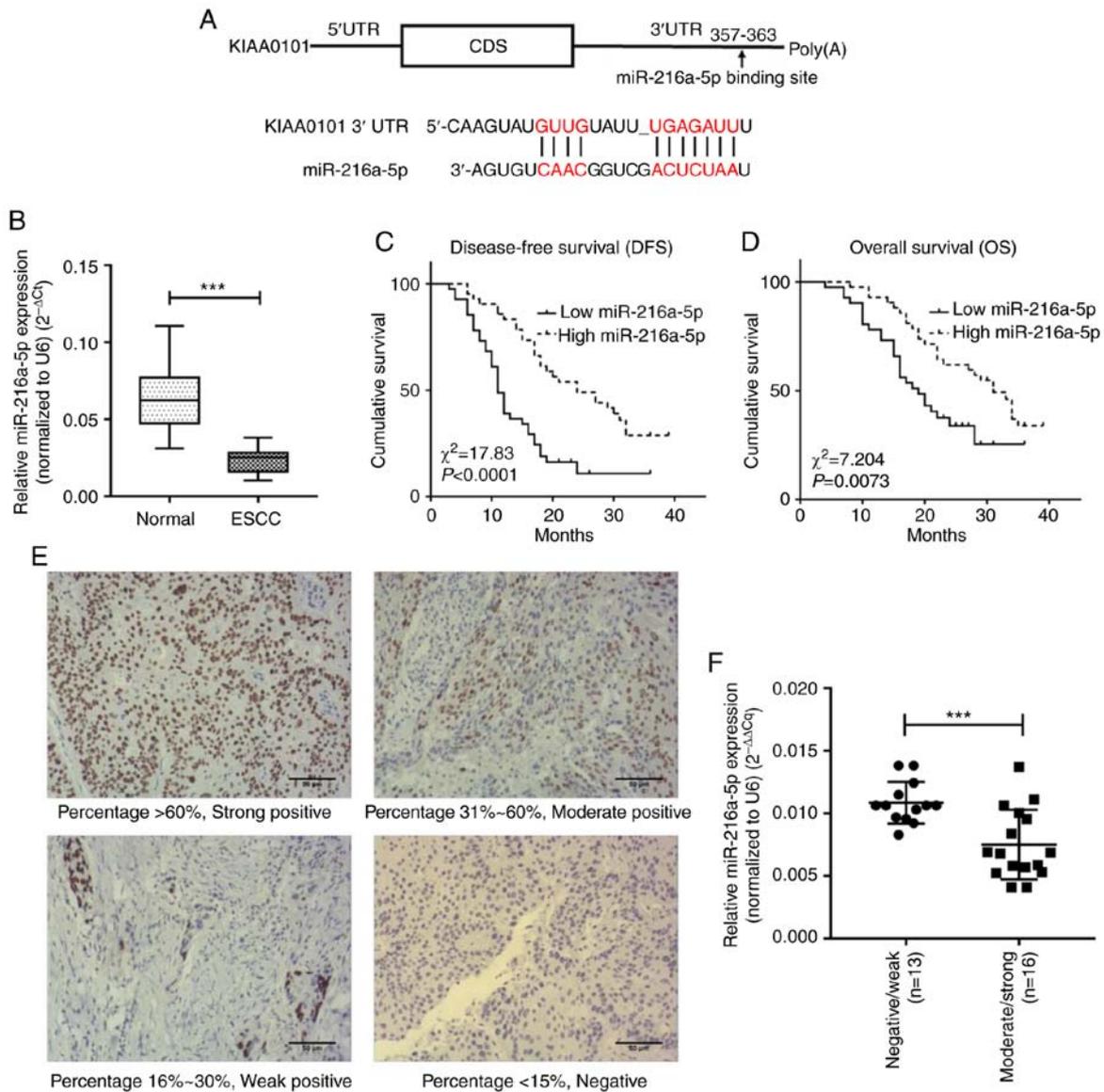


Figure 1. miR-216a-5p is expressed at a lower level in ESCC specimens, and its low expression predicts a worse prognosis of ESCC. (A) The potential binding site of miR-216a-5p in the KIAA0101 3'UTR. (B) The relative expression levels of miR-216a-5p in ESCC specimens and adjacent normal tissues were quantified by qPCR; n=29 for the control group; n=29 for the ESCC group. (C and D) The Kaplan-Meier cumulative survival curves of 83 patients with ESCC who were grouped by their median expression levels of miR-216a-5p. The ESCC patients with lower miR-216a-5p expression displayed shorter disease-free survival (DFS; C) and overall survival (OS; D) (both $P < 0.0001$ for DFS, $P = 0.0073$ for OS); n=41 for the miR-216-5p low group; n=42 for the miR-216-5p high group. (E) Representative IHC images showing the different staining patterns of KIAA0101 expression with negative/weak/moderate/strong intensities. Scale bar, 50 μm . (F) miR-216a-5p expression in the ESCC specimens with moderate/strong KIAA0101 staining (n=16) and negative/weak KIAA0101 staining (n=13) was quantified by qPCR. *** $P < 0.001$. ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry.

Results

Lower expression of miR-216a-5p predicts worse prognosis of the ESCC patients. To explore the potential roles of miR-216a-5p in the pathogenesis and chemoresistance of ESCC, we first evaluated the binding site of miR-216a-5p to the 3'UTR of KIAA0101. We found that miR-216a-5p could bind the 3'UTR of KIAA0101 (Fig. 1A), which suggests that miR-216a-5p directly targets the KIAA0101 mRNA. Then, the expression levels of miR-216a-5p were examined in 29 ESCC specimens. RT-qPCR analysis demonstrated that compared with the adjacent normal tissues, the tumor tissues had significantly lower expression of miR-216a-5p (Fig. 1B). We next examined the miR-216a-5p expression in 83 human ESCC specimens to investigate the relationship between miR-216a-5p

expression and the prognosis of patients with ESCC. The patients with ESCC were categorized into high- or low-expression groups using a cut-off value according to the median miR-216a-5p expression level. The miR-216a-5p expression levels in ESCC were not correlated with sex, age, pTNM stage, or preoperative CEA levels, but were correlated with histology ($P = 0.022$), tumor depth ($P = 0.006$), and lymph node metastasis ($P = 0.018$; Table I). In addition, the Cox regression analysis (Table II) revealed that the lymph node metastasis ($P = 0.001$), pTNM stage ($P < 0.001$), and miR-216a-5p levels ($P < 0.001$) were independent risk factors for shorter overall survival in patients with ESCC. Furthermore, patients with ESCC and lower miR-216a-5p expression displayed reduced DFS ($P < 0.0001$; Fig. 1C) and OS ($P = 0.0073$; Fig. 1D). Immunohistochemistry was carried out to examine the expression

Table I. Association between miR-216a-5p expression and the clinicopathological characteristics of the ESCC patients.

| Characteristics | All cases n (%) | miR-216a-5p expression | | P-value |
|-----------------------|-----------------|------------------------|-----------|--------------|
| | | High n (%) | Low n (%) | |
| Total | 83 (100) | 42 (50.6) | 41 (49.4) | |
| Sex | | | | 0.679 |
| Male | 59 (71.1) | 29 (49.2) | 30 (50.8) | |
| Female | 24 (28.9) | 13 (54.2) | 11 (45.8) | |
| Age (years) | | | | 0.573 |
| <60 | 37 (44.6) | 20 (54.1) | 17 (45.9) | |
| ≥60 | 46 (55.4) | 22 (47.8) | 24 (52.2) | |
| Histology | | | | 0.022 |
| Differentiated | 64 (77.1) | 28 (43.8) | 36 (56.3) | |
| Undifferentiated | 19 (22.9) | 14 (73.7) | 5 (26.3) | |
| Tumor depth | | | | 0.006 |
| T1-T2 | 40 (48.2) | 14 (35.0) | 26 (65.0) | |
| T3-T4 | 43 (51.8) | 28 (65.1) | 15 (34.9) | |
| Lymph node metastasis | | | | 0.018 |
| Negative | 30 (36.1) | 10 (33.3) | 20 (66.7) | |
| Positive | 53 (63.9) | 32 (60.4) | 21 (39.6) | |
| pTNM stage | | | | 0.097 |
| I-II | 33 (39.8) | 13 (39.4) | 20 (60.6) | |
| III | 50 (60.2) | 29 (58.0) | 21 (42.0) | |
| Preoperative CEA | | | | 0.588 |
| <3.5 | 45 (54.2) | 24 (53.3) | 21 (46.7) | |
| ≥3.5 | 38 (45.8) | 18 (47.4) | 20 (52.6) | |

ESCC, esophageal squamous cell carcinoma; pTNM, pathological tumor/node/metastasis; CEA, carcinoembryonic antigen. P-values in bold print indicate significance.

Table II. Cox proportional hazards regression model of the prognostic variables for overall survival.

| Prognostic variable | P-value | Risk ratio (95% CI) |
|------------------------|------------------|---------------------|
| miR-216a-5p expression | <0.001 | 10.46 (4.58-23.88) |
| Sex | 0.172 | 1.55 (0.83-2.93) |
| Age | 0.656 | 0.87 (0.47-1.62) |
| Histology | 0.159 | 1.73 (0.81-3.70) |
| Tumor depth | 0.315 | 0.72 (0.38-1.37) |
| Lymph node metastasis | 0.001 | 0.18 (0.06-0.49) |
| pTNM stage | <0.001 | 0.18 (0.08-0.42) |
| Preoperative CEA | 0.809 | 0.93 (0.50-1.71) |

CI, confidence interval; pTNM, pathological tumor/node/metastasis; CEA, carcinoembryonic antigen. P-values in bold print indicate significance.

of KIAA0101 in ESCC specimens (Fig. 1E). Lower miR-216a-5p expression in ESCC specimens was accompanied by higher KIAA0101 protein expression (P<0.001, Fig. 1E and F). Taken together, miR-216a-5p expression was low in ESCC specimens, and its expression was inversely correlated with KIAA0101 protein expression.

miR-216a-5p regulates the protein expression of the downstream target KIAA0101 by binding its 3'UTR in ESCC. We next examined the miR-216a-5p expression levels in ESCC cell lines. The six common ESCC cell lines, including EC109, TE1, TE10, KYSE150, KYSE450, and EC9706, demonstrated a marked reduction in miR-216a-5p expression (Fig. 2A) and

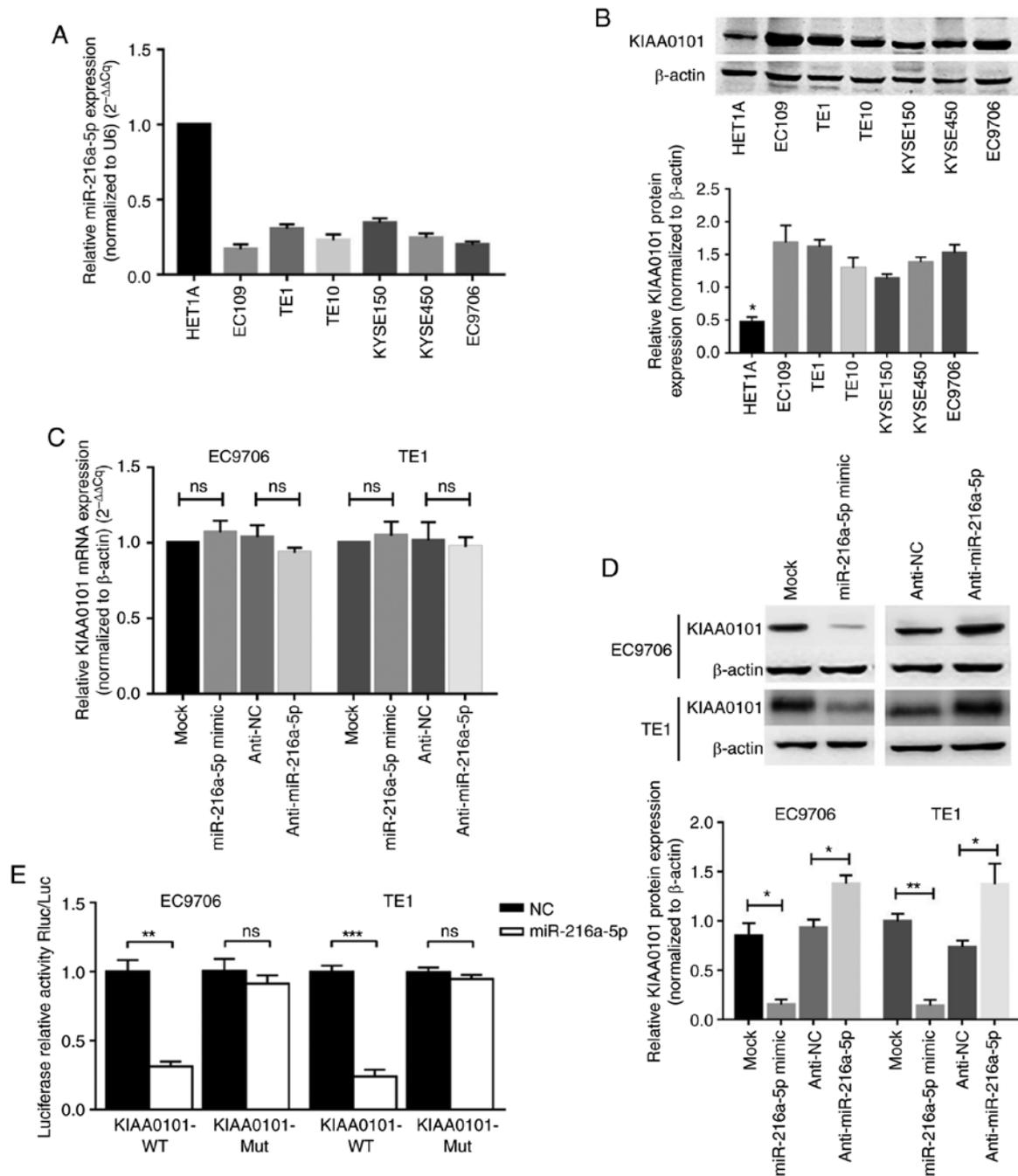


Figure 2. KIAA0101 is a downstream target of miR-216a-5p, and its expression is inversely correlated with miR-216a-5p expression level in ESCC. (A) miR-216a-5p expression level in the human normal esophageal epithelial HET1A cells and six human ESCC cell lines (EC109, TE1, TE10, KYSE150, KYSE450, and EC9706) were quantified by qPCR. Values were calculated using the $2^{-\Delta\Delta Cq}$ method, normalized to U6. (B) Expression of KIAA0101 protein (15 kDa) and β -actin (42 kDa) in the indicated cell lines was quantified by western blot analysis. Representative western blot images are shown (upper panel), and the band intensities were summarized (lower panel; $n=3$ for each group). * $P<0.05$, HET1A cells compared with the ESCC cell lines. (C) The mRNA level of KIAA0101 in EC9706 and TE1 cells transfected with miR-216a-5p mimic, or inhibitor (Anti-miR-216a-5p), or the corresponding controls (Mock and Anti-NC) was determined by qPCR. $n=3$ for each group. Values were calculated using the $2^{-\Delta\Delta Cq}$ method, normalized to β -actin. ns, not significant. (D) The protein levels of KIAA0101 in EC9706 and TE1 cells with the indicated transfection were determined by western blot analysis. Representative western blot images are shown (upper panel), and the band intensities were summarized (lower panel; $n=3$ for each group). * $P<0.05$ and ** $P<0.01$. (E) Compared with the controls, miR-216a-5p inhibited the reporter activities of the vector with the wild-type (WT), but not the mutant-type (MUT) of 3' UTR of KIAA0101 in EC9706 and TE1 cells in the dual-luciferase assays. ** $P<0.01$ and *** $P<0.001$; ns, not significant. ESCC, esophageal squamous cell carcinoma.

significant increase in KIAA0101 protein expression (Fig. 2B) compared with the normal esophageal epithelial HET1A cells.

We then selected the EC9706 and TE1 cells since they maintained moderate expression levels of both miR-216a-5p and KIAA0101 molecules among the six cell lines. To ascertain whether miR-216a-5p functionally affects KIAA0101

expression, miR-216a-5p was overexpressed and knocked down by transfecting the ESCC cell lines EC9706 and TE1 with the miR-216a-5p mimics and miR-216a-5p inhibitor. The transfections of the miR-216a-5p mimic and anti-miR-216a-5p were confirmed by RT-PCR (Fig. S1A). When compared to those transfected with the control mimic (Mock) or

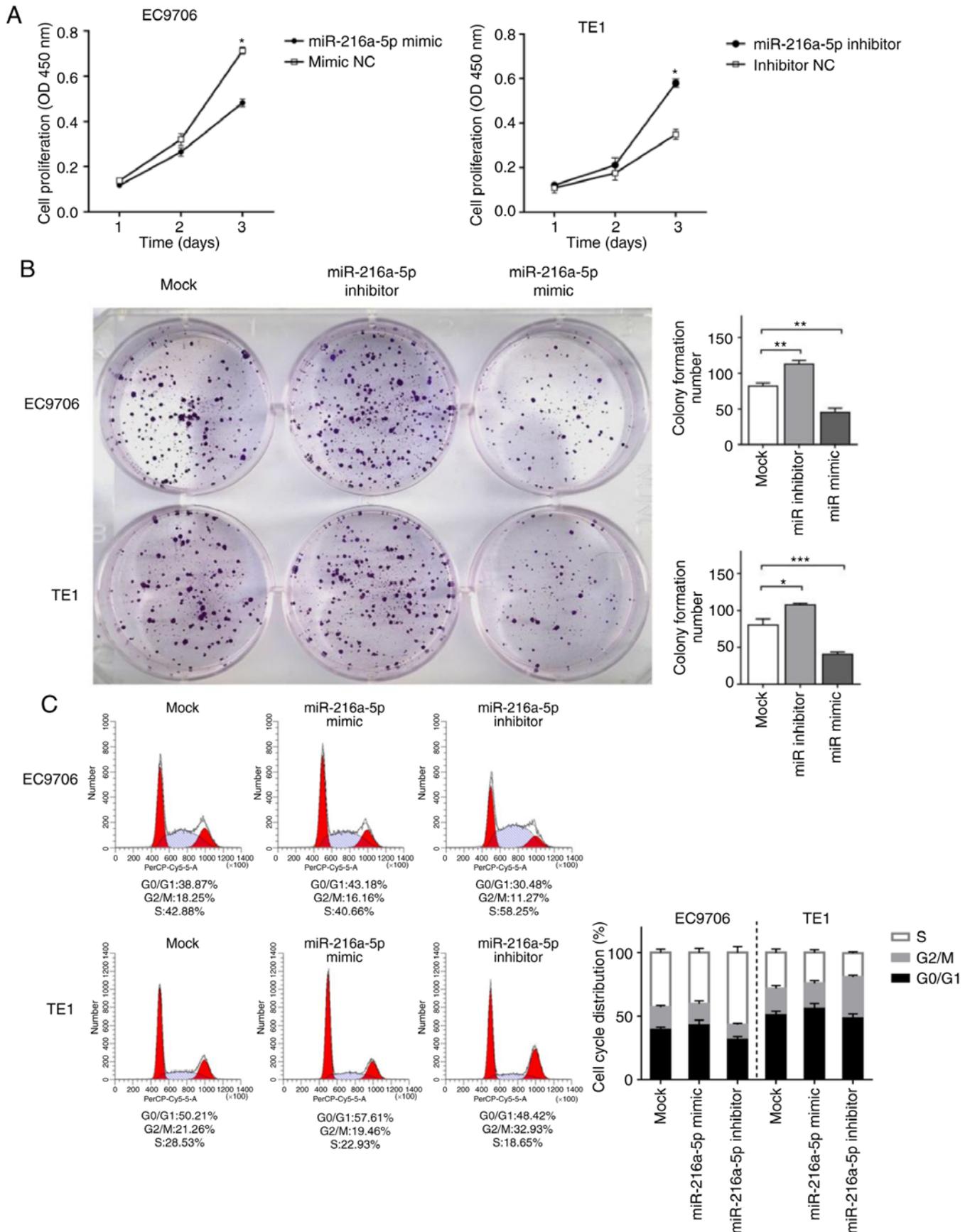


Figure 3. miR-216a-5p suppresses the proliferation of ESCC cells *in vitro*. (A) The proliferation ability of EC9706 and TE1 cells transfected with the miR-216a-5p mimic, or the miR-216a-5p inhibitor, or the corresponding controls (mimic NC or inhibitor NC) were determined by CCK-8 assays. * $P < 0.05$, compared with the mimic NC or inhibitor NC. (B) The proliferation abilities of EC9706 and TE1 cells after the indicated transfections were evaluated by colony formation assays. The representative images of cells in a 6-well plate after crystal violet staining are shown. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (C) The cell cycle progression of EC9706 and TE1 cells after the indicated transfections were determined by flow cytometry. The representative flow profiles of DNA content after PI staining are shown. ESCC, esophageal squamous cell carcinoma.

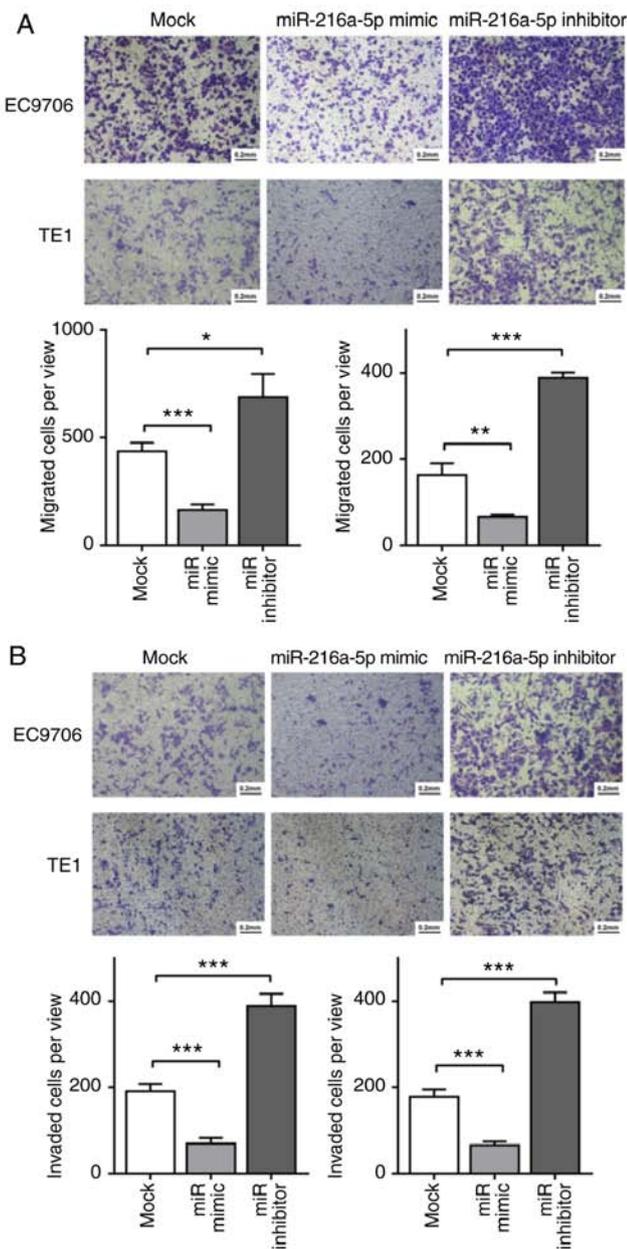


Figure 4. miR-216a-5p suppresses the migration and invasion of ESCC cells. (A and B) The migration (A) and invasion (B) of EC9706 and TE1 cells with mock transfection, or transfection of the miR-216a-5p mimic, or transfection of the miR-216a-5p inhibitor were evaluated by Transwell assays without Matrigel and Transwell assays with Matrigel, respectively. Representative images after crystal violet staining in each group are shown, and the numbers of migrated cells (A) or invaded cells (B) are summarized from three independent experiments with three replicates in each group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. ESCC, esophageal squamous cell carcinoma.

inhibitor (Anti-NC), the miR-216a-5p-specific mimic or inhibitor (Anti-miR-216a-5p) did not significantly change the mRNA levels of KIAA0101 (Fig. 2C). Transfection of the miR-216a-5p mimics resulted in downregulation of the KIAA0101 protein levels in EC9706 and TE1 cells, and transfection of the miR-216a-5p inhibitor upregulated the KIAA0101 protein levels (Fig. 2D). These results indicated that miR-216a-5p regulated the expression of KIAA0101 in ESCC cells in a post-transcriptional manner. We then co-transfected the luciferase reporter constructs into EC9706 and TE1 cells

together with the miR-216a-5p mimics or NC controls. The relative luciferase activity was significantly decreased in the cells transfected with the KIAA0101-WT-3'-UTR vector together with miR-216a-5p mimics, compared with that in the cells transfected with the plasmid of corresponding mutant (Mut) sites and NC controls (Fig. 2E). Therefore, this finding revealed that miR-216a-5p could reduce the expression of KIAA0101 protein by directly binding to the 3'UTR of KIAA0101 and possible translation suppression.

miR-216a-5p regulates cell cycle progression and inhibits the proliferation, migration, and invasion of ESCC cells by targeting KIAA0101. The inverse correlation between miR-216a-5p and KIAA0101 expressions drove us to investigate the possible biological functions of miR-216a-5p in ESCC cells. The CCK-8 assays (Fig. 3A), colony formation assays (Fig. 3B), and flow cytometry assays (Fig. 3C) revealed that miR-216a-5p overexpression through transfection of the miR-216a-5p mimics significantly decreased ESCC cell proliferation, suppressed colony formation, and induced cell cycle arrest at G0/G1 phase in EC9706 and TE1 cells, whereas inhibition of miR-216a-5p through transfection of the miR-216a-5p inhibitor had the opposite effects. In addition, Transwell assays were carried out on the ESCC cells to examine the roles of miR-216a-5p in cell migration and invasion. Overexpression of miR-216a-5p significantly suppressed the ESCC invasion and reduced the ESCC migration. In contrast, the migration and invasion of ESCC cells increased when endogenous miR-216a-5p was inhibited (Fig. 4A and B).

Ectopic KIAA0101 expression attenuates the effects of miR-216a-5p in ESCC cells. As shown in Fig. S1B, transfection with KIAA0101 alone increased the protein expression of KIAA0101 in EC9706 and TE1 cells, while transfection with the KIAA0101-targeted shRNA decreased the expression of KIAA0101. As shown in Fig. 5A, transfection of KIAA0101 reversed the decreased expression of KIAA0101 that was mediated by the transfection of the miR-216a-5p mimic. The CCK-8 assays (Fig. 5B), colony formation assays (Fig. 5C), and flow cytometry assays (Fig. 5D) also demonstrated that KIAA0101 ectopic expression significantly attenuated the anti-proliferative effects of miR-216a-5p. Meanwhile, additionally forced KIAA0101 expression in ESCC cells restored the migration and invasion inhibition induced by overexpression of miR-216a-5p (Fig. 6A and B).

KIAA0101 promotes the migration and invasion of ESCC cells. These results also indicate that KIAA0101 functions as an oncogene in ESCC. Thus, we further evaluated the impact of KIAA0101 overexpression and knockdown on the migration and invasion of EC9706 and TE1 cells. Compared with the control cells without transfection, KIAA0101 overexpression significantly enhanced ESCC cell migration and invasion, while KIAA0101 knockdown remarkably inhibited ESCC cell migration and invasion (Fig. 7). Collectively, overexpression of miR-216a-5p can inhibit the *in vitro* proliferation, migration, and invasion of ESCC cells through targeting the oncogene KIAA0101.

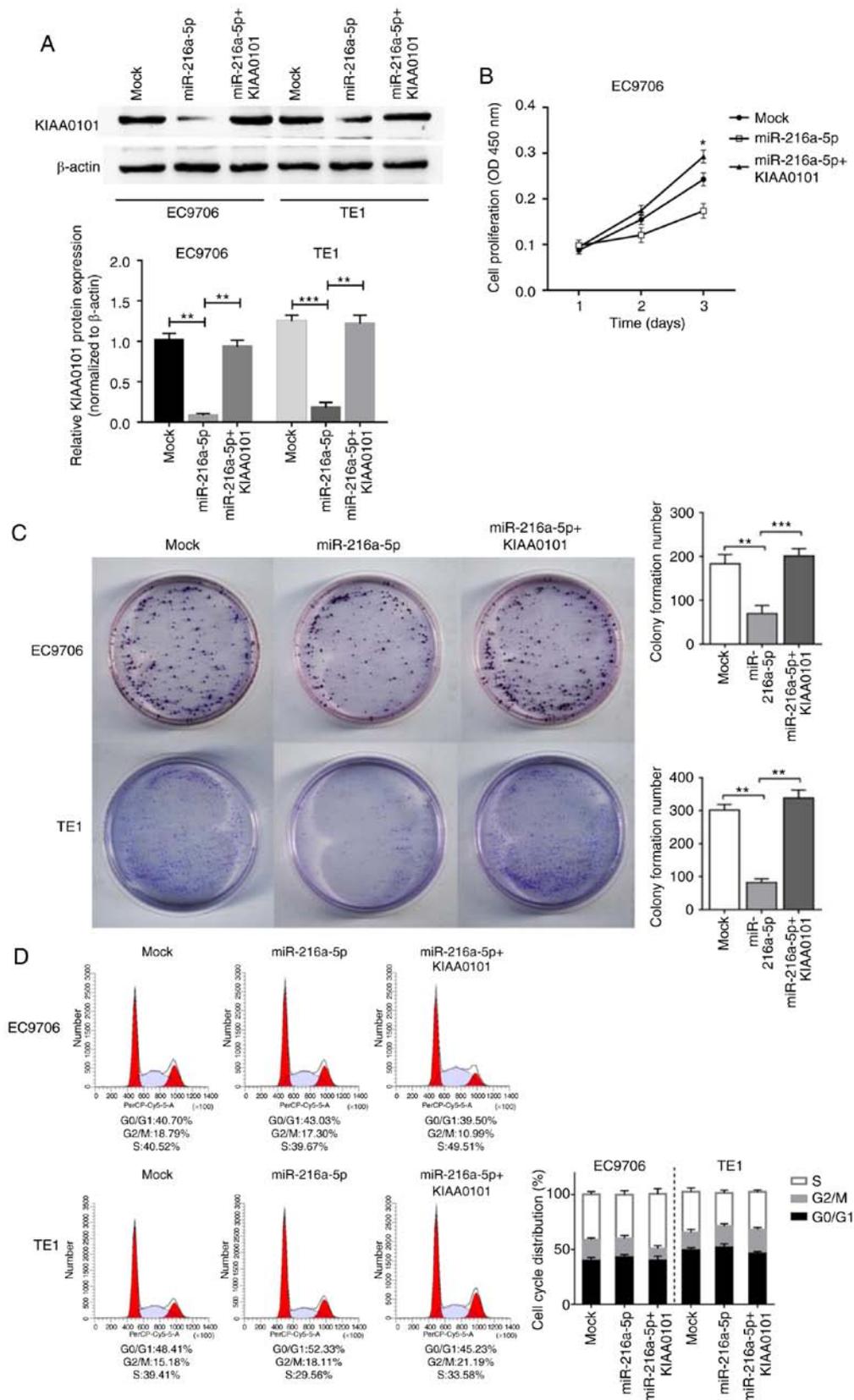


Figure 5. Ectopic KIAA0101 expression attenuates the anti-proliferative effects of miR-216a-5p in ESCC cells. (A) Western blot analysis was used to determine the expression level of KIAA0101 protein after mock transfection, or transfection of miR-216a-5p mimic alone (miR-216-5p), or combined transfection of miR-216a-5p mimic and KIAA0101-expressing plasmid (miR-216-5p+KIAA0101). Representative western blot images are shown, and the relative expression level of KIAA0101 from three independent experiments was summarized. ** $P < 0.01$ and *** $P < 0.001$. (B) The proliferation of EC9706 cells after the indicated transfections was determined by CCK-8 assay. $n = 3$ for each group at each time point. * $P < 0.05$, compared with the Mock group. (C) The proliferation of EC9706 cells after the indicated transfections was determined by colony formation assays. The representative images of crystal violet staining are shown, and the numbers of colonies per well were summarized. $n = 3$ for each group. ** $P < 0.01$ and *** $P < 0.001$. (D) The cell cycle progression of EC9706 and TE1 cells after the indicated transfections was determined by flow cytometry. The representative flow profiles of DNA content after PI staining are shown. ESCC, esophageal squamous cell carcinoma.

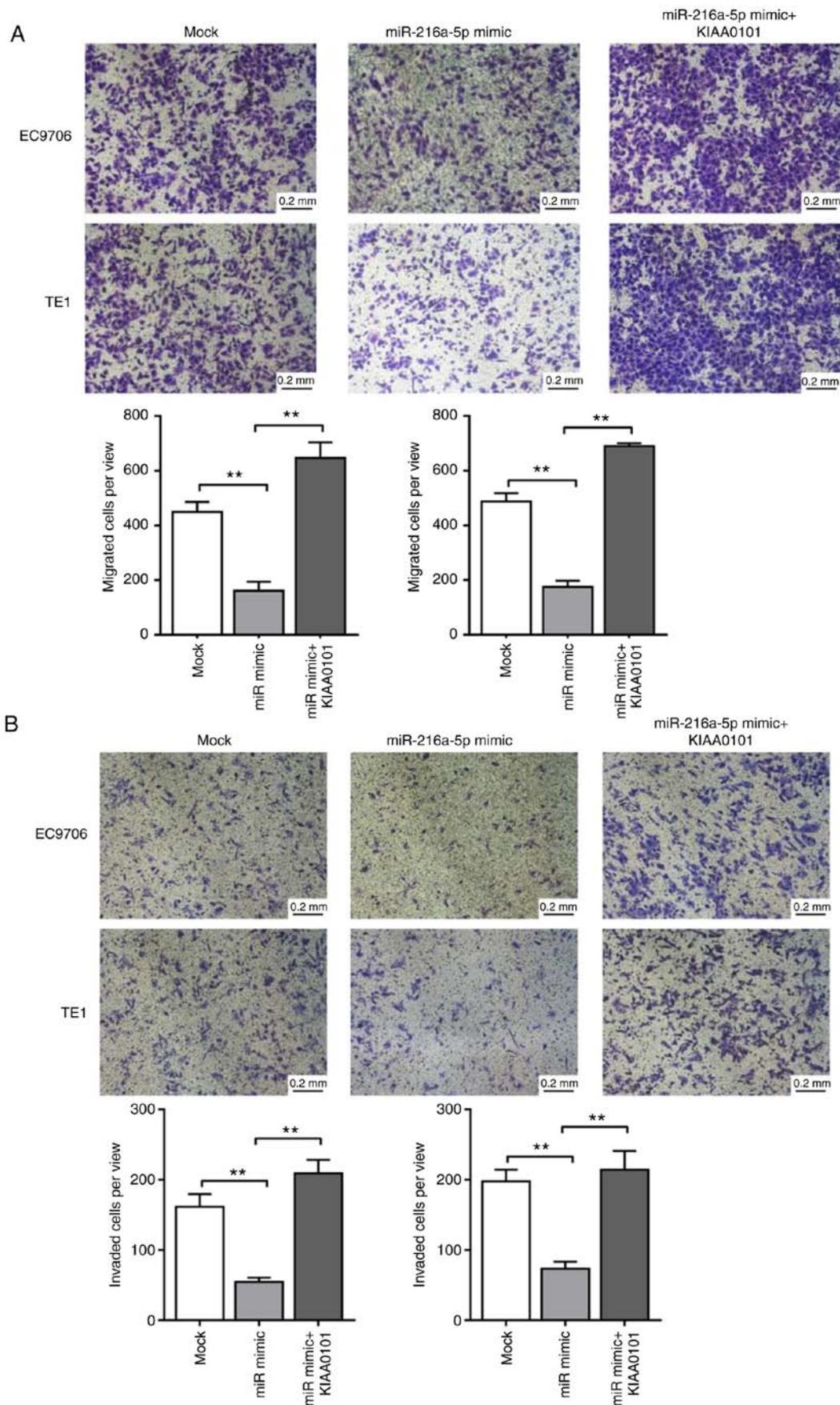


Figure 6. Ectopic KIAA0101 expression attenuates the effects of miR-216a-5p on the migration and invasion of ESCC cells. (A and B) The migration (A) and invasion (B) of EC9706 and TE1 cells with mock transfection, or transfection of the miR-216a-5p mimic alone, or combined transfection of the miR-216a-5p mimic and KIAA0101-expressing plasmid were evaluated by Transwell assays without Matrigel and Transwell assays with Matrigel, respectively. Representative images after crystal violet staining in each group are shown, and the numbers of migrated cells (A) or invaded cells (B) are summarized from three independent experiments with three replicates in each group. **P<0.01. ESCC, esophageal squamous cell carcinoma.

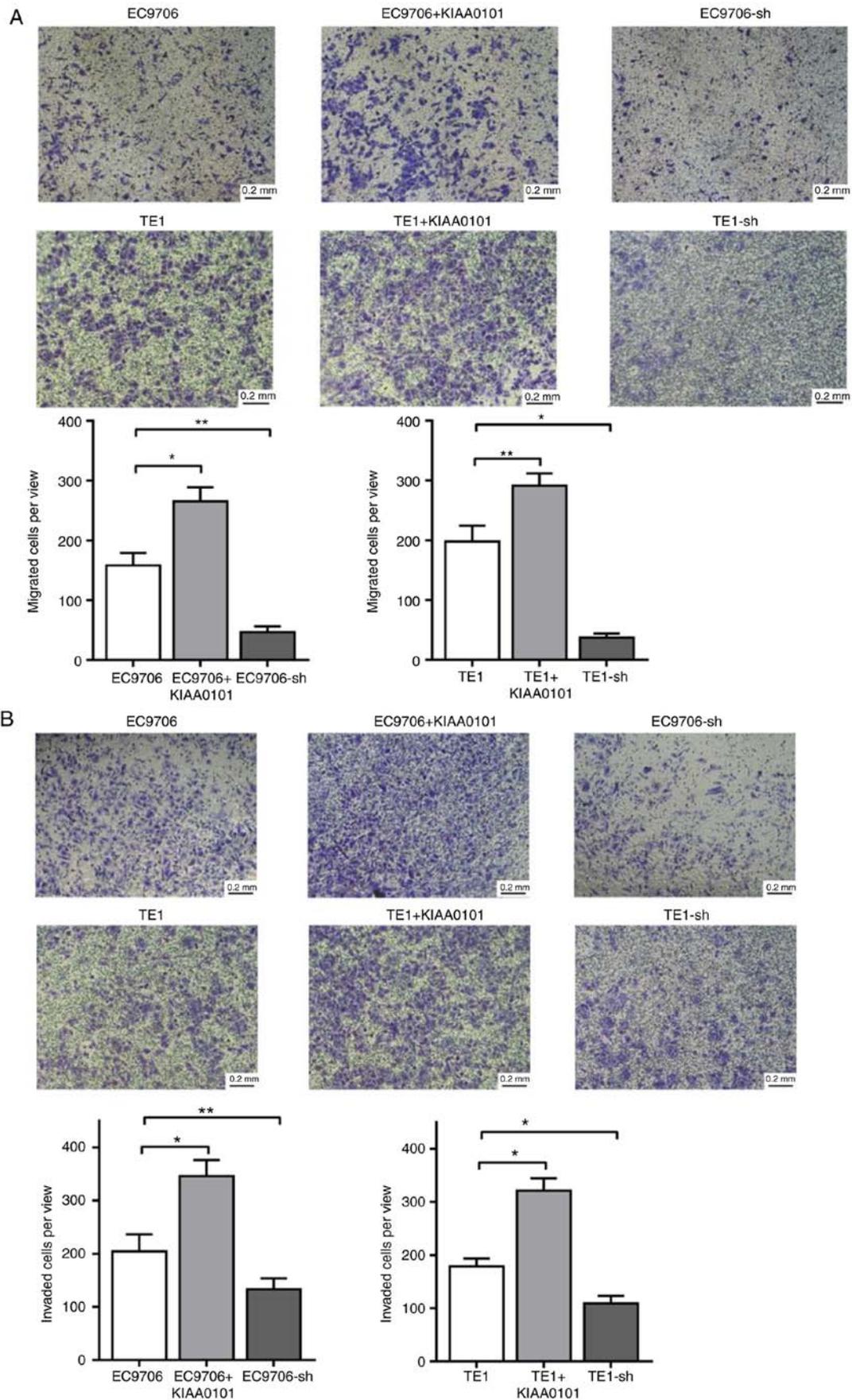


Figure 7. KIAA0101 promotes the migration and invasion of ESCC cells. (A and B) The migration (A) and invasion (B) of EC9706 and TE1 cells without transfection, or with transfection of the KIAA0101-expressing plasmid (+KIAA0101), or with transfection of the KIAA0101-specific shRNA-expressing plasmid (-sh), were evaluated by Transwell assays without Matrigel and Transwell assays with Matrigel, respectively. Representative images after crystal violet staining in each group are shown, and the numbers of migrated cells (A) or invaded cells (B) per well are summarized from three independent experiments with three replicates in each group. *P<0.05, **P<0.01. ESCC, esophageal squamous cell carcinoma.

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the most commonly diagnosed malignant tumors of the digestive tract in Asian countries, especially in China (22). It is urgently needed to understand the precise molecular mechanisms underlying ESCC tumorigenesis in order to develop more accurate diagnosis and therapy for patients. In the present study, we identified that miR-216a-5p expression was significantly downregulated in primary human ESCC specimens and cell lines, and demonstrated an inverse correlation with KIAA0101 expression. The patients with lower miR-216a-5p expression in tumors had worse disease-free survival (DFS) and overall survival (OS) than the patients with higher miR-216a-5p expression. KIAA0101 was validated to be a direct downstream target of miR-216a-5p, and its expression in ESCC cells was regulated by miR-216a-5p at the translational level. miR-216a-5p overexpression through the transfection of miR-216-5p mimics markedly inhibited cell proliferation, cell cycle progression, migration, and invasion of EC9706 and TE1 cells, and additional overexpression of the oncogene KIAA0101 remarkably reversed the antitumor effects of miR-216a-5p mimics. Our data demonstrated that the miR-216a-5p/KIAA0101 axis contributes to the progression of ESCC, and represents a valuable prognosis marker and therapeutic target for patients with ESCC.

As a PCNA-associated protein, KIAA0101 plays an important role in DNA replication, DNA repair, cell cycle progression, and cell proliferation (5,23-25). KIAA0101 overexpression is associated with the progression and poor prognosis of several cancers, including hepatocellular carcinoma (26), pancreatic cancer (27), breast cancer (28), lung cancer (29), gastric cancer (30), and esophageal cancer (9). A previous study by our group indicated that the overexpression of KIAA0101 induced ESCC cell viability and was a marker for poor prognosis of ESCC, including early recurrence and short survival. In addition, KIAA0101 was found to enhance resistance to cisplatin by upregulating cell mitosis (7). Herein we identified miR-216a-5p as an upstream regulator of KIAA0101 that was inversely correlated with KIAA0101. Consistent with the previous results, lower levels of miR-216a-5p in ESCC specimens corresponded to a higher level of KIAA0101 and less favorable prognosis for patients with ESCC.

Our findings suggest that by repressing KIAA0101 protein, miR-216a-5p plays an essential role in suppressing the proliferation and metastasis of ESCC. Evidence reveals that the abnormal expression of miR-216a is involved in tumorigenesis and cancer progression (11-16), but the specific role of miR-216a seems to be cell type-dependent. miR-216a has a low expression in NSCLC specimens and could inhibit cell activity of NSCLC (18), which was in accordance with observations in oral squamous cell carcinoma (31). In addition, Wang *et al* identified that miR-216a negatively regulated the progression of pancreatic cancer by direct interaction with JAK2 (32). These reports highlight the tumor-suppressive role of miR-216a. Nevertheless, miR-216a was also found to demonstrate a tumor-promoting role, and its expression affected the therapeutic effects of anticancer treatments. In hepatocellular carcinoma (HCC), upregulated expression of miR-216a induced resistance to sorafenib (17). In NSCLC,

it enhanced the sensitivity of cells to cis-diamminedichloroplatinum/cisplatin (CDDP) (33). In addition, the diagnostic value of circulating miR-216a has also been discovered. In acute pancreatitis, the expression of miR-216a/b in plasma was upregulated and had higher diagnostic specificity than amylase and lipase (34). Link *et al* found that decreased expression of fecal miR-216a could be used as a non-invasive miRNA biomarker for pancreatic cancer (35). Similarly, compared to healthy subjects, HCC patients had significantly downregulated miR-216b in plasma (36).

In the present study, miR-216a-5p had lower expression in ESCC specimens and cell lines than in the normal control tissues and cells. In support of our findings on the tumor-suppressive role of miR-216a-5p, a recent report also identified the significantly downregulated miR-216a-5p expression in ESCC specimens and cell lines (13). In addition, tectonic family member 1 (TCTN1) was proven to be another downstream target of miR-216a-5p in ESCC, and the restoration of TCTN expression reversed the effects of miR-216a-5p on cell proliferation and apoptosis (13). Therefore, further investigation of potential targets of miR-216a-5p is needed to fully elucidate the functions of miR-216a-5p in ESCC pathogenesis.

The current investigation has several strengths and limitations. Its major strength is its novelty; to the best of our knowledge, this is the first report to unveil the critical role of the miR-216a-5p/KIAA0101 axis in ESCC pathogenesis. The present research also provides a feasible framework for validating ESCC progression in relation to candidate miRNA genes to facilitate the diagnosis and implementation of more effective therapy of patients with ESCC. One limitation of the present study is its sample size of 83 patients and a single data source when evaluating the impacts of tumor miR-216a-5p expression on patient survival. In addition, due to the limited amount of material, extensive staining and immunohistochemistry assays could not be carried out on the ESCC specimens. This study only used cell lines, and future studies should include animal models. Further work on validating the therapeutic effects of administration of miR-216a-5p mimics and/or knockdown of KIAA0101 in animals is warranted to substantiate the potential of the miR-216a-5p/KIAA0101 axis-targeted therapy in patients with ESCC. In addition, the lack of a CCK-8 assay prevents the confirmation of the viability of the cells. Finally, miR-216a-5p has targets other than KIAA0101 (36), and it is possible to modulate the expression of miR-216a-5p affecting ESCC proliferation through proteins other than KIAA0101. Nevertheless, modulating the expression of KIAA0101 at least confirmed the role of KIAA0101 in ESCC, but without excluding the potential involvement of other proteins.

In summary, this is the first report that reveals the targeting effect of miR-216a-5p on KIAA0101 expression in ESCC. Our results demonstrated the antitumor effects of miR-216a-5p and oncogenic effects of KIAA0101 in ESCC cells in terms of regulating cell proliferation, cell cycle progression, migration, and invasion. In addition, lower miR-216a-5p expression in tumors predicts worse prognosis in patients with ESCC. Our study improves the understanding of miRNA functions in ESCC pathogenesis and suggests that the miR-216a-5p/KIAA0101 axis is an attractive target for ESCC therapeutic intervention.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DY and KL conceived and designed the study. CD was responsible for the administrative support. TS and RY provided the study materials and patients. KL and RY collected and assembled the data. KL and DY analyzed and interpreted the data. All authors participated in the manuscript writing. All authors gave their final approval of the manuscript.

Ethics approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present experiment was approved by the Ethics Committee at the First Affiliated Hospital of Xi'an Jiaotong University. All subjects were completely informed about the study goals and procedure and provided their signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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