Silencing of methyltransferase-like 3 inhibits oesophageal squamous cell carcinoma

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Abstract. Methyltransferase-like 3 (METTL3) is a methyltransferase responsible for N6-methyladenosine mRNA modifications, which has been demonstrated to serve oncogenic roles in various types of cancer; however, the exact function of METTL3 in oesophageal squamous cell carcinoma (ESCC) has not been determined. The present study aimed to explore the regulatory role of METTL3 in ESCC. In the present study, reverse transcription-quantitative PCR and western blotting were used to examine mRNA and protein expression, CCK-8 assays and flow cytometry were used to determine cellular viability and apoptosis, and wound healing and Transwell assays were conducted to study cellular migration and invasion. The expression levels of METTL3 were significantly higher in ESCC tissues and cell lines compared with adjacent non-tumour tissues and the normal oesophageal epithelial cell line HET-1A, respectively. Increased METTL3 expression was associated with an advanced clinical stage of ESCC and poorer prognosis. Furthermore, the genetic knockdown of METTL3 using small interfering RNA significantly suppressed ESCC growth, invasion and migration *in vitro*, and induced cellular apoptosis, in addition to reducing the phosphorylation levels of PI3K and AKT. In conclusion, the present study demonstrated that the upregulation of METTL3 promoted ESCC progression, and that inhibition of METTL3 significantly suppressed the malignant phenotypes of ESCC cells, at least in part, by downregulating PI3K/AKT signalling activity. Thus, it is suggested that METTL3 may be a promising therapeutic target for ESCC.

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Introduction

Oesophageal squamous cell carcinoma (ESCC) is a major type of oesophageal cancer and is one of the most common types of cancer, which causes a large number of cancer-related deaths worldwide (1-3). In recent decades, considerable attention has been paid to treatment innovation through surgical resection in combination with chemo- or radiotherapy; however, the overall survival time of patients with ESCC remains poor due to the high recurrence and metastasis rates (4,5). Thus, it is of great importance to explore the regulatory mechanisms underlying ESCC progression to guide the development of novel diagnostic and therapeutic strategies for ESCC (6-9).

N6-methyladenosine (m6A) is the most abundant type of methylation, and it serves a crucial role in RNA stability, localization, splicing and translation (10,11). The target genes of m6A are associated with cellular proliferation, organization and transport, as well as cancer-related signalling pathways (10). Methyltransferase-like 3 (METTL3) is the 70-kDa subunit of MT-A, which is part of N6-adenosine-methyltransferase; N6-adenosine-methyltransferase has been implicated in the post-transcriptional methylation of internal adenosine residues in eukaryotic mRNAs, and thus forms m6A (12-14). It has been widely reported that METTL3 is frequently increased and has an oncogenic role in common types of human cancer, including gastric (15), lung (16), bladder (17), ovarian (18), pancreatic cancer (19), melanoma (20) and breast cancer (21). For example, METTL3 is overexpressed in breast cancer and promotes breast cancer progression through inhibiting the tumour suppressor, let-7g (21). Taketo *et al* reported that METTL3 promoted chemoresistance and radioresistance in pancreatic cancer cells (19). In contrast, a recent study demonstrated that METTL3 functioned as a tumour suppressor in renal cell carcinoma; METTL3 inhibited renal cell carcinoma proliferation, cell cycle progression, migration and invasion (22). However, to the best of our knowledge, no previous study has focused on the expression pattern and function of METTL3 in ESCC. In the present study, the mRNA and protein expression levels of METTL3 in ESCC were investigated and the clinical significance of METTL3 expression in ESCC was explored. Subsequently, the function of METTL3 in regulating the malignant phenotypes of ESCC cell lines *in vitro*, and the molecular mechanism behind METTL3

in ESCC were investigated. The findings of this study may provide novel therapeutic strategies for the treatment of ESCC.

Materials and methods

Patient studies. A total of 53 ESCC and paired adjacent tissues were collected from 53 patients with ESCC who underwent resection at The Second Xiangya Hospital of Central South University, China between March 2012 and May 2013. These patients included 30 men and 23 women between 42 and 78 years old. The inclusion criterion was that all patients exhibited primary ESCC, and the exclusion criterion was patients that had received chemotherapy or radiotherapy before surgery. The tissues were stored at -80˚C until required for further experimentation. The follow-up time was 5 years following surgery.

Cell culture and reagents. Normal oesophageal epithelial cell line (HET-1A) and four ESCC cell lines (TE-9, Eca-109, KYSE150 and EC9706) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and incubated in a humidified atmosphere at 37˚C and 5% CO_2.

Cell transfection. Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection, according to the manufacturer's protocol. TE-9 and Eca-109 cells ($5x10^5$ cells/ml) in the logarithmic growth phase were transfected with 100 nM negative control (NC) small interfering RNA (siRNA) or 100 nM METTL3-specific siRNAs (Shanghai GenePharma Co., Ltd.) at 37˚C. Following transfection for 48 h, METTL3 expression levels were evaluated using reverse transcription-quantitative PCR (RT-qPCR).

RT-qPCR. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reversed transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). The RT conditions were 16˚C for 30 min, followed by 42˚C for 30 min and 85˚C for 5 min. qPCR was subsequently performed using the All-in-One qPCR mix (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The following primer pairs were used for the qPCR: METTL3, forward 5'-TTGTCTCCAACCTTCCGTAGT-3', reverse 5'-CCAGATCAGAGAGGTGGTGTAG-3'; and GAPDH, forward 5'-CTGGGCTACACTGAGCACC-3' and reverse 5'-AAGTGGTCGTTGAGGGCAATG-3'. Relative METTL3 mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (23) and normalized to the internal reference gene GAPDH.

Western blotting. Total protein was extracted using RIPA buffer (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Proteins (50 μg/lane) were separated via SDS-PAGE on a 12% gel and the separated proteins were subsequently transferred onto a PVDF membrane (Roche Diagnostics) and blocked overnight at 4˚C with 5% milk. The membranes were incubated with primary antibodies against METTL3 (1:500; cat. no. ab66660; Abcam), Bax (1:250; cat. no. ab182733; Abcam), caspase-3 (1:300; cat. no. ab13847; Abcam), Bcl-2 (1:300; cat. no. ab32124; Abcam), PI3K (1:500; cat. no. ab40755; Abcam), phosphorylated (p)-PI3K (1:200; cat. no. ab182651; Abcam), AKT (1:500; cat. no. ab8805; Abcam), p-AKT (1:500; cat. no. ab8933; Abcam) and GAPDH (1:500; cat. no. ab9485; Abcam) for 4 h at room temperature. Following the primary antibody incubation, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Protein bands were visualized using Pierce ECL Western Blotting substrate (Thermo Fisher Scientific, Inc.) and protein expression was semi-quantified using ImageJ software (version 1.46; National Institutes of Health) with GAPDH as the loading control.

Cell viability assay. A total of $5x10^3$ cells/well of transfected TE-9 and Eca-109 cells were seeded into 96-well plates and cultured in a humidified incubator containing 5% CO_2 for 0, 24, 48 or 72 h. CCK-8 solution (10 μl; Thermo Fisher Scientific, Inc.) was added to each well, and after incubation at 37˚C for 2 h, the absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. A total of $1x10^3$ transfected TE-9 and Eca-109 cells/well were seeded into 6-well plates and cultured in an incubator containing 5% CO_2 at 37˚C for 14 days. Subsequently, cells were fixed with 75% ethanol at room temperature for 1 h and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. The colonies were visualised and counted using a light microscope (magnification, x10).

Flow cytometric analysis of apoptosis. Transfected TE-9 and Eca-109 cells ($1x10^6$ cells/ml) were fixed in 75% ethanol at 4˚C for 3 h and subsequently washed with PBS three times. The cells were stained with Annexin V-FITC and propidium iodide using the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences), according to the manufacturer's protocol. Apoptotic cells were subsequently analysed using a FACScan flow cytometer (BD Biosciences) and BD Accuri™ C6 software (version 1.0; BD Biosciences).

Would healing assay. Transfected TE-9 and Eca-109 cells were plated at a density of $5x10^5$ cells/well were seeded in 12-well plates and cultured to ≥95% confluence. A 200-μl sterile pipette tip was used to generate the wounds. The cells were washed with Dulbecco's PBS and DMEM (Gibco; Thermo Fisher Scientific, Inc.) was subsequently added. The cells were visualized using a light microscope (magnification, x40) at 0 h and after 24 h incubation at 37˚C. The width of the wounds at 0 and 24 h were determined using ImageJ software (version 1.5; National Institutes of Health).

Matrigel invasion assay. Cell invasion was determined using Matrigel-coated Transwell chambers with an 8-μm pore size membrane (BD Biosciences). In brief, transfected TE-9 and

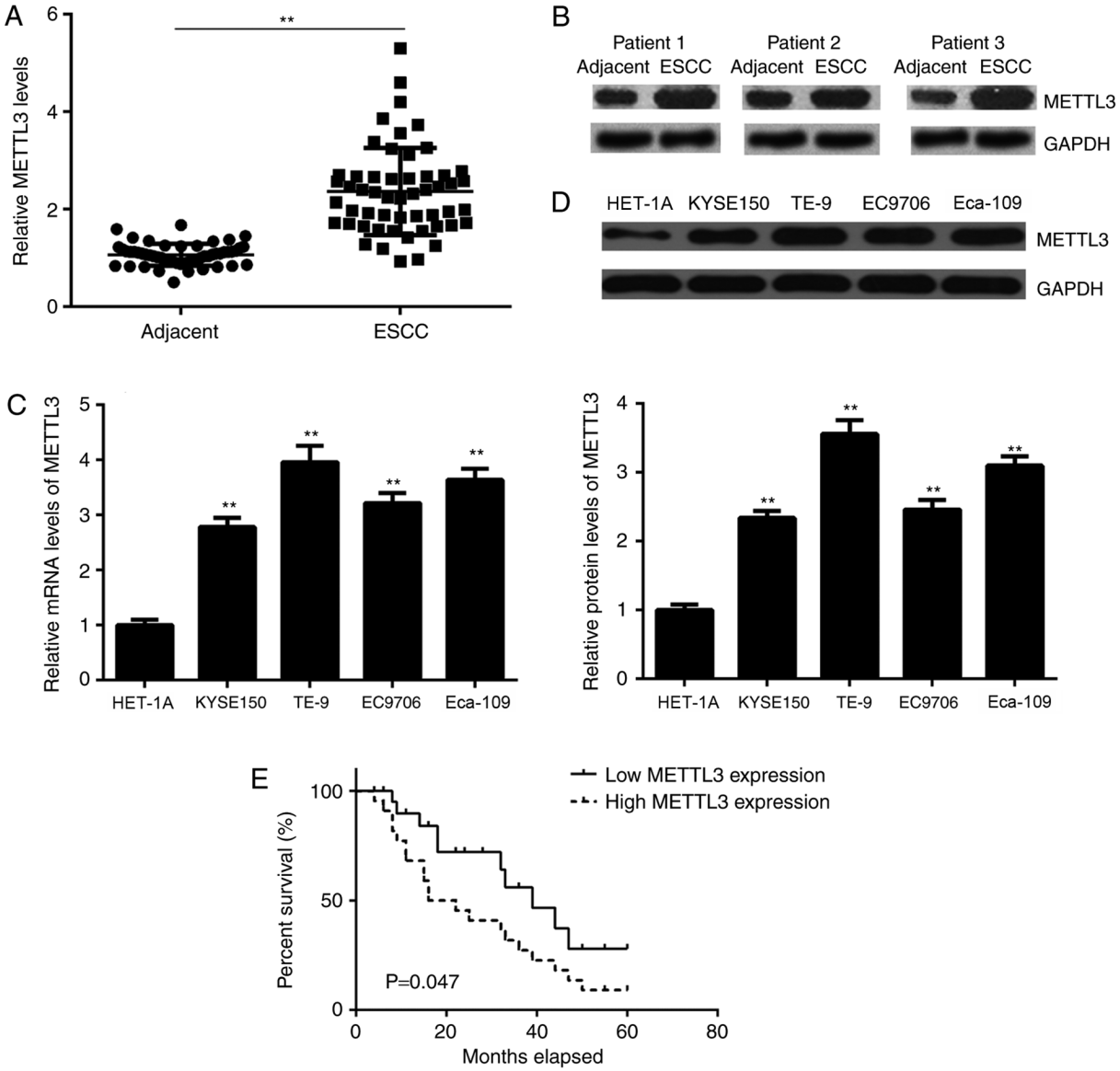

Figure 1. Upregulation of METTL3 promotes ESCC progression. (A and B) mRNA and protein expression levels of METTL3 in ESCC tissues and adjacent tissues, as assessed by reverse transcription-quantitative PCR and western blotting, respectively. **P<0.01 vs. adjacent tissues. (C and D) mRNA and protein expression levels of METTL3 in ESCC cell lines and the normal cell line HET-1A, as assessed by reverse transcription-quantitative PCR and western blotting, respectively. **P<0.01 vs. HET-1A cells. (E) Kaplan-Meier analysis of overall survival time of patients with ESCC with high METTL3 expression compared with that of patients with ESCC with low METTL3 expression. METTL3, methyltransferase-like 3; ESCC, oesophageal squamous cell carcinoma.

Eca-109 cells (1x10^5 cells/well) and 300 μl serum-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) were plated in the upper chamber. DMEM (500 ml) supplemented with 10% FBS was plated in the lower chamber. After 24 h at 37˚C, the invading cells on the lower surface of the chamber were stained with 0.1% crystal violet at room temperature (Thermo Fisher Scientific, Inc.) for 5 min and subsequently washed with PBS (Thermo Fisher Scientific, Inc.). Stained cells were counted using a light microscope (magnification, x200).

Statistical analysis. All experiments were repeated at least three times. All data are expressed as the mean ± SD. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.). Differences between groups were determined using Student's t-test or one-way ANOVA followed by Tukey's post hoc test. A χ^2 test was used to analyse the clinical significance of METTL3 expression in ESCC, and Kaplan-Meier analysis and log-rank test were applied for survival analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of METTL3 promotes ESCC progression. The expression levels of METTL3 were determined using RT-qPCR and western blotting in ESCC and adjacent non-tumour tissues. The mRNA expression levels of METTL3 were significantly increased in ESCC tissues compared with adjacent non-tumour tissues (Fig. 1A). Similar results were obtained by western blotting; with METTL3 protein levels increased in ESCC tissues (Fig. 1B). Consistent with the clinical data, the METTL3 mRNA and protein expression levels

Table I. Association between METTL3 expression and clinicopathological features in patients with oesophageal squamous cell carcinoma.

Variables	Cases	METTL3 expression		χ^2	P-value
		Low (n=28)	High (n=25)		
Age (years)				2.369	0.124
<65	25	16	9		
≥65	28	12	16		
Sex				0.408	0.523
Male	30	17	13		
Female	23	11	12		
Tumor location				0.821	0.365
Upper, middle	31	18	13		
Lower	22	10	12		
Lymph node metastasis				8.355	0.004[b]
Negative	34	23	11		
Positive	19	5	14		
TNM stage				6.718	0.010[a]
I+II	33	22	11		
III+IV	20	6	14		

METTL3, methyltransferase-like 3. [a]P<0.05; [b]P<0.01.

were significantly higher in ESCC cell lines compared with the normal oesophageal cell line HET-1A (Fig. 1C and D). To study the clinical significance of METTL3 expression in ESCC, the patients with ESCC were divided into high and low METTL3 expression groups based on the median mRNA expression value (2.58) of METTL3. A χ^2 test reported that high METTL3 expression was significantly associated with advanced clinical stage and metastasis of ESCC (Table I). Kaplan-Meier analysis indicated that patient survival was worse in those patients with high METTL3 levels compared with patients with low METTL3 levels (Fig. 1E). These findings suggested that the upregulation of METTL3 promoted tumour progression and predicted a poor prognosis in ESCC.

Silencing METTL3 gene expression inhibits ESCC cell viability and colony formation, while inducing cellular apoptosis. The role of METTL3 in regulating the malignant phenotypes of ESCC cells was studied. TE-9 and Eca-109 cells were transfected with two METTL3 siRNAs to knockdown the expression of METTL3. Transfection with METTL3 siRNAs significantly decreased METTL3 mRNA and protein expression levels compared with the cells transfected with NC siRNA in both cell lines (Fig. 2A and B). METTL3 siRNA1 demonstrated a more potent suppressive effect over mRNA and protein expression and was thus selected as the siRNA of choice for further experiments. CCK-8 assays indicated that gene silencing of METTL3 expression significantly suppressed the viability of TE-9 and Eca-109 cells at 72 h compared with NC siRNA-transfected cells (Fig. 2C and D). In addition, the number of colonies formed of TE-9 and Eca-109 cells was significantly reduced in the presence of METTL3 siRNA compared with NC siRNA (Fig. 2E and F).

It was hypothesized that apoptosis may be involved in METTL3-mediated ESCC cell viability. Therefore, the effects of METTL3 inhibition on ESCC cell apoptosis were examined. The apoptotic rate of ESCC cells was significantly enhanced following METTL3 knockdown with siRNA compared with the NC siRNA transfected cells in both cell lines (Fig. 3A and B), indicating that silencing METTL3 gene expression induced ESCC cell apoptosis. In addition, silencing METTL3 gene expression significantly increased the protein levels of pro-apoptotic Bax and caspase-3 and decreased those of anti-apoptotic Bcl-2 in TE-9 and Eca-109 cells compared with the controls (Fig. 3C and D).

METTL3 knockdown with siRNA suppresses ESCC cellular migration and invasion. To further study the function of METTL3 in ESCC metastasis, wound healing and Matrigel Transwell assays were used to assess cellular migration and invasion following METTL3 knockdown with siRNA. As shown in Fig. 4A and B, the migratory capacity of TE-9 and Eca-109 cells were significantly inhibited following siRNA METTL3 knockdown compared with the NC siRNA-transfected cells in both cell lines. Similarly, following knockdown of METTL3 expression by siRNA, the invasion of TE-9 and Eca-109 cells was also significantly repressed (Fig. 4C and D). These findings suggested that the genetic knockdown of METTL3 with siRNA may inhibit ESCC metastasis.

METTL3 inhibition decreases PI3K/AKT signalling pathway activity. The effects of METTL3 knockdown with siRNA on the activity of the PI3K/AKT signalling pathway in ESCC cells was investigated; the PI3K/AKT signalling pathway

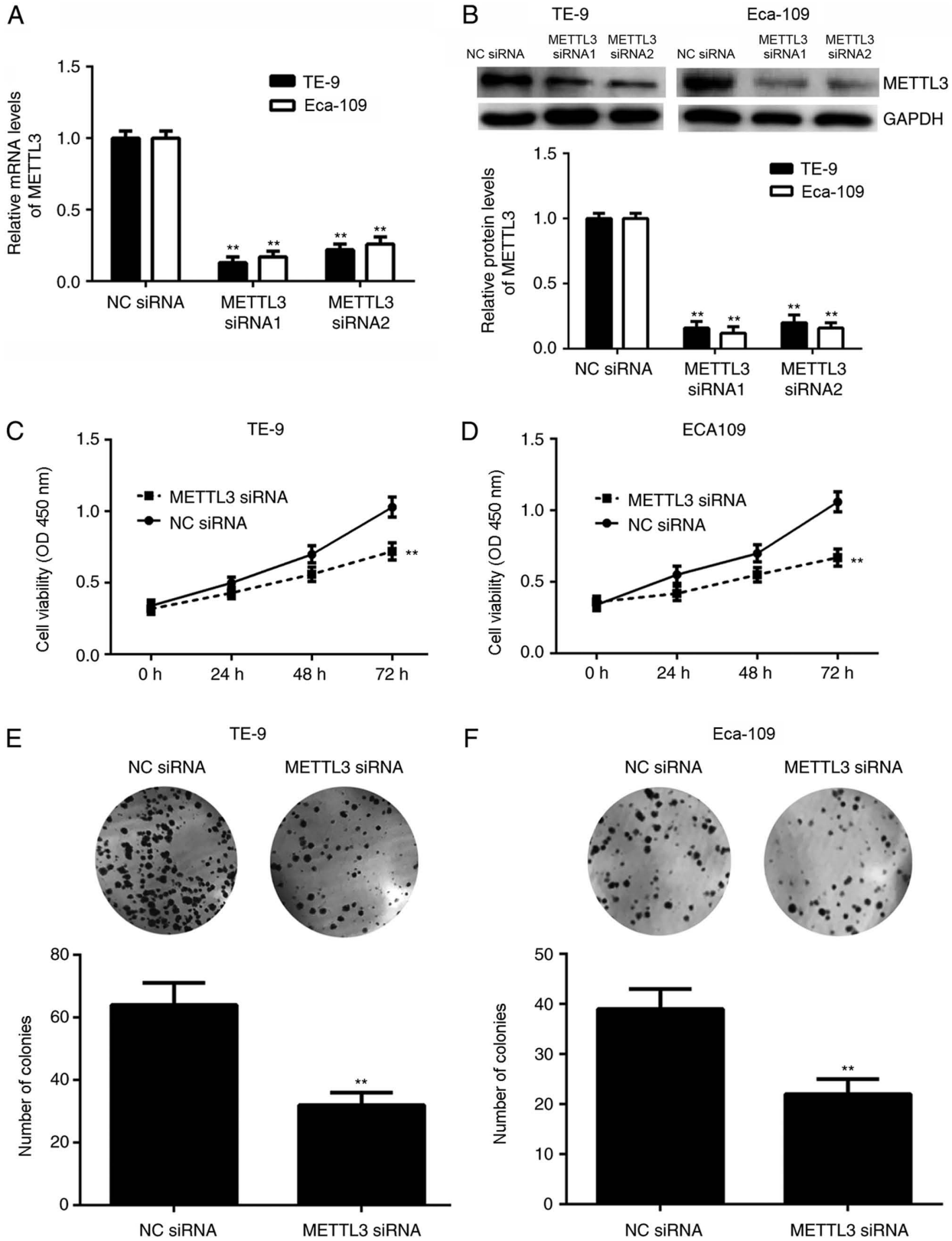

Figure 2. METTL3 gene knockdown with siRNA decreases ESCC cell viability and colony formation ability. TE-9 and Eca-109 cell lines were transfected with two METTL3 siRNAs or NC siRNA. (A and B) mRNA and protein expression levels of METTL3 were examined following cell transfection with siRNAs against METTL3 compared to NC siRNA in TE-9 and Eca-109 cell lines. (C and D) Cell viability and (E and F) colony formation ability of TE-9 and Eca-109 cell lines transfected with either METTL3 siRNA or NC siRNA were determined. Magnification, x40. **P<0.01 vs. NC siRNA. METTL3, methyltransferase-like 3; ESCC, oesophageal squamous cell carcinoma; siRNA, small interfering RNA; NC, negative control; OD, optical density.

serves a crucial role in cancer cell growth and metastasis (22). In Fig. 5, the quantitative analysis refers to the ratio between total and phosphorylated protein levels. Our data indicated that the phosphorylated protein levels of PI3K and AKT were

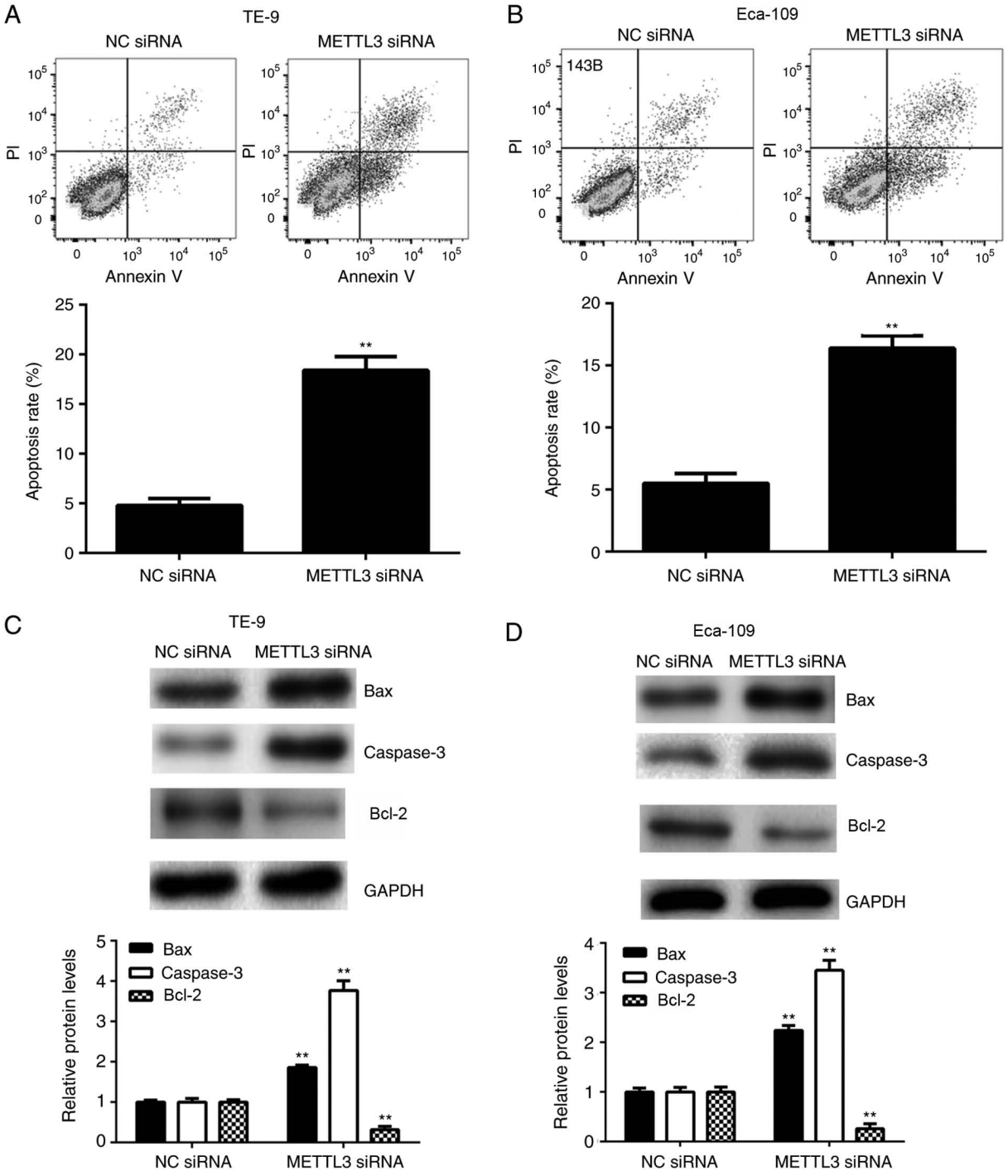

Figure 3. METTL3 gene knockdown with siRNA induces ESCC cellular apoptosis. TE-9 and Eca-109 cells were transfected with METTL3 or NC siRNA. (A and B) Flow cytometric analysis of the rate of apoptosis of TE-9 and Eca-109 cells transfected with METTL3 or NC siRNA. (C and D) Protein expression levels of apoptosis-related proteins were examined by western blotting in TE-9 or Eca-109 cells transfected with METTL3 or NC siRNA. **P<0.01 vs. NC siRNA. METTL3, methyltransferase-like 3; ESCC, oesophageal squamous cell carcinoma; siRNA, small interfering RNA; NC, negative control; PI, propidium iodide.

significantly reduced following transfection with METTL3 siRNA compared with NC siRNA in TE-9 and Eca-109 cells, indicating that METTL3 inhibition decreased the PI3K/AKT signalling pathway activity (Fig. 5A and B).

Discussion

The expression pattern and function of METTL3 in ESCC has previously not been reported. The present study demonstrated that METTL3 was significantly upregulated in ESCC, which was associated with ESCC progression and poorer prognoses, and that the genetic knockdown of METTL3 with siRNA inhibited ESCC cell viability, colony formation, cellular migration and invasion, induced apoptosis, and decreased PI3K/AKT signalling.

The upregulation of, and the oncogenic function of METTL3 has been reported in various types of human cancer (16-18). For example, METTL3 is upregulated in

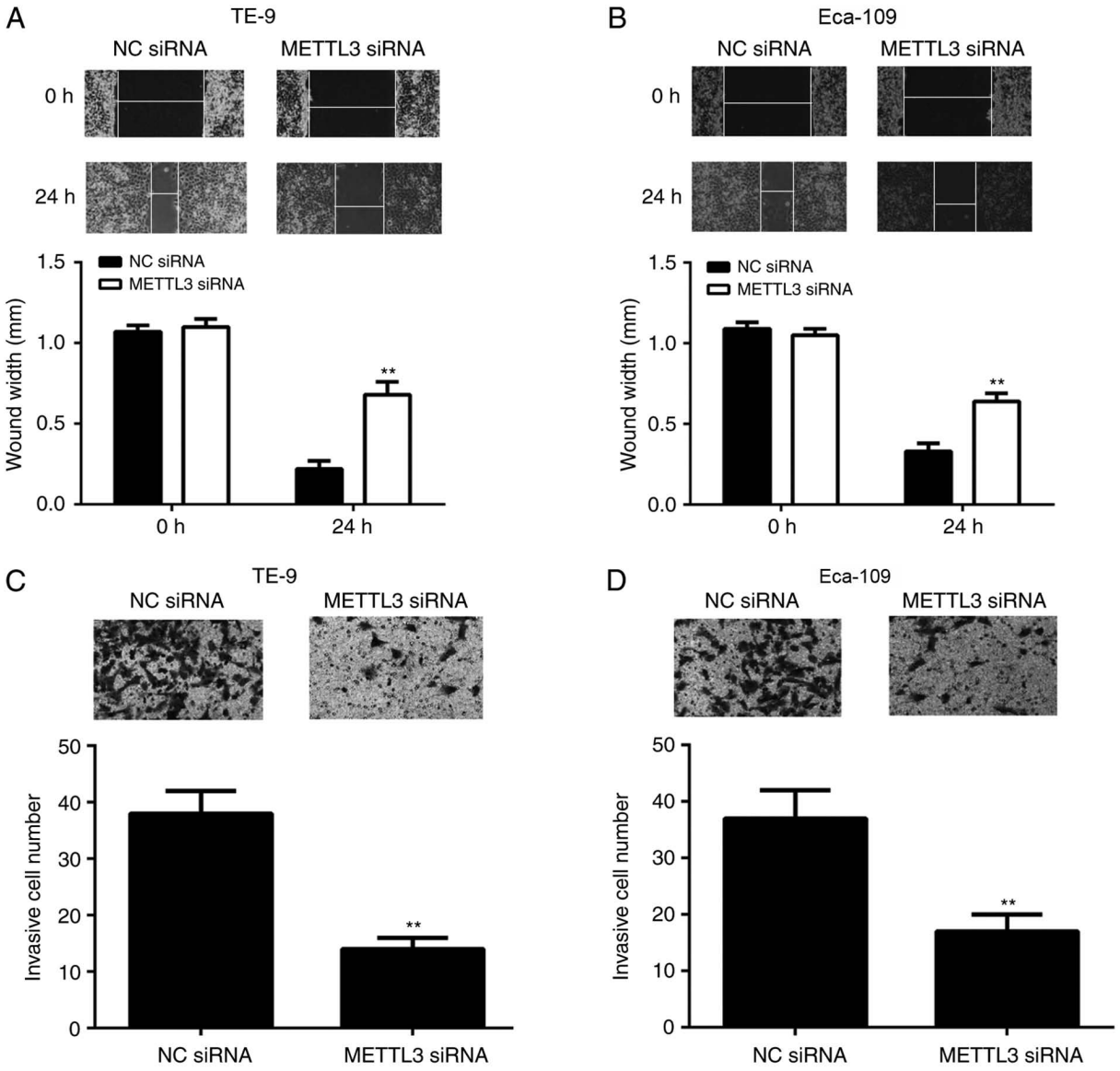

Figure 4. METTL3 gene knockdown with siRNA suppresses ESCC cellular migration and invasion. TE-9 and Eca-109 cells were transfected with METTL3 or NC siRNA. (A and B) Cell migration (magnification, x40) and (C and D) invasion (magnification, x200) capacities were determined in TE-9 and Eca-109 cells transfected with METTL3 or NC siRNA. **P<0.01 vs. NC siRNA. METTL3, methyltransferase-like 3; ESCC, oesophageal squamous cell carcinoma; siRNA, small interfering RNA; NC, negative control.

bladder cancer tissues, and promotes bladder cancer progression via regulation of AF4/FR2 family member 4/NF-κB/MYC signaling (17). METTL3 is upregulated in ovarian carcinoma; it promotes the malignant phenotype of ovarian cancer cells and increases tumour formation in nude mice (18). Regarding the underlying molecular mechanism, a previous study reported that METTL3 promoted mRNA translation of multiple oncogenes, such as the epidermal growth factor receptor and the Hippo pathway effector TAZ, through recruiting translation initiation factors in human cancer cells (11). In addition, Choe *et al* (24) identified a direct interaction between METTL3 and eukaryotic translation initiation factor 3 subunit H and revealed that this interaction served a crucial role in translation, densely packed polyribosome formation and oncogenic transformation. However, the exact role of METTL3 in ESCC remains to be elucidated. In the present study, it was demonstrated that the mRNA and protein expression levels of METTL3 were significantly increased in tumour tissues and cell lines compared with adjacent normal tissues and the normal oesophageal epithelial cell line, HET-1A. It was further observed that METTL3 upregulation was associated with advanced TNM stage, metastasis and poorer prognoses in ESCC. These findings suggested that upregulation of METTL3 may contribute to the malignant progression of ESCC, and that METTL3 expression may be used as a predictor for worse outcomes of patients with ESCC.

To further clarify the exact role of METTL3 in ESCC, TE-9 and Eca-109 cell lines were selected for further *in vitro* experiments owing to their high METTL3 expression levels. As METTL3 is upregulated in ESCC cells, TE-9 and Eca-109 cells were transfected with METTL3-specific siRNAs to knockdown its expression. Downregulating METTL3 significantly inhibited ESCC cell viability and colony formation. Similarly, Lin *et al* (15) reported that METTL3 promoted the

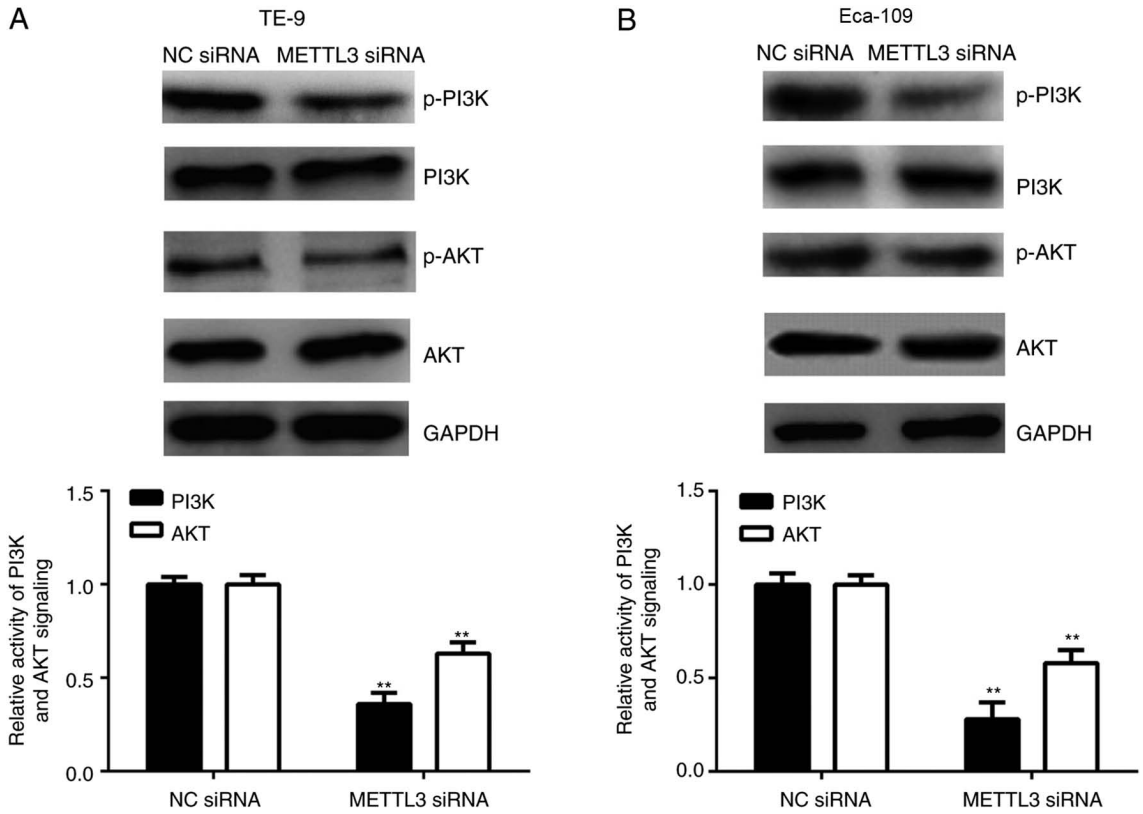

Figure 5. METTL3 knockdown with siRNA decreases PI3K/AKT signalling pathway activity. TE-9 and Eca-109 cells were transfected with METTL3 or NC siRNA. (A and B) Protein expression levels of PI3K, p-PI3K, AKT and p-AKT were evaluated by western blot analysis following transfection of TE-9 and Eca-109 cells with METTL3 or NC siRNA. The quantitative analysis refers to the ratio between total and phosphorylated protein levels. **P<0.01 vs. NC siRNA. METTL3, methyltransferase-like 3; siRNA, small interfering RNA; NC, negative control; p, phosphorylated.

proliferation of gastric cancer cells. In addition, knockdown of METTL3 drastically reduced bladder cancer cell proliferation and survival *in vitro* and tumorigenicity *in vivo* (17). Overexpression of METTL3 significantly promoted ovarian cancer cell proliferation *in vitro* as well as tumor formation in nude mice (18). Therefore, the present findings suggested that METTL3 plays a promoting role in ESCC growth. As reduced cell viability may be due to increased cellular apoptosis, the effects of METTL3 downregulation on ESCC apoptosis were studied. The data demonstrated that apoptosis of TE-9 and Eca-109 cells was significantly higher following METTL3 gene knockdown. These findings suggested that METTL3 may inhibit cell apoptosis. Similarly, knockdown of METTL3 also induced breast cancer cell apoptosis (21). To further confirm this, the expression levels of several key factors associated with cell apoptosis, including pro-apoptotic Bax and caspase-3 and anti-apoptotic Bcl2 (25,26) were evaluated. METTL3 inhibition upregulated Bax and caspase-3, and downregulated Bcl-2 in ESCC cells, consistent with the apoptosis assay results. Cancer cell migration and invasion are two key processes of tumour metastasis (27,28); since METTL3 upregulation was associated with ESCC progression in the clinical samples, the function of METTL3 in regulating ESCC cell migration and invasion was also studied *in vitro*. Gene silencing of METTL3 significantly inhibited the migratory and invasive ability of ESCC cells, suggesting that METTL3 had a promoting role in ESCC migration and invasion *in vitro*.

It has been well established that the PI3K/AKT signalling pathway serves a crucial role in most aspects of tumour growth and metastasis, and the inactivation of this signalling pathway could effectively inhibit the malignant phenotypes of ESCC cells (29,30). Wang *et al* (29) reported that the long non-coding RNA, growth-arrest specific 5, suppressed ESCC cell viability and migration through inactivation of the PI3K/AKT signalling pathway. He *et al* (31) demonstrated that circRNA VRK serine/threonine kinase 1 inhibited ESCC progression and radioresistance through regulating the expression of microRNA-624-3p, in addition to the activity of the PI3K/AKT signalling pathway. The present study reported that METTL3 knockdown significantly reduced the phosphorylation levels of PI3K and AKT, indicating that the PI3K/AKT signalling pathway was inactivated. Similarly, knockdown of METTL3 inhibited the expression and phosphorylation of proteins involved in the PI3K signaling pathway in lung cancer cells (16). These findings suggested that the PI3K/AKT signalling pathway may serve an important role in METTL3-mediated effects in ESCC cells. One limitation of this study is that the clinical sample number was small and more patients should be included in future studies. Moreover, the relationship between METTL3 and AKT should be studied using clinical samples in future studies.

In conclusion, it was demonstrated that upregulation of METTL3 promoted ESCC progression and that inhibition of METTL3 significantly suppressed the malignant phenotype of ESCC cells, at least in part, through downregulating

PI3K/AKT signalling. This study may help elucidate the molecular mechanism underlying ESCC progression.

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Availability of data and materials

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

Authors' contributions

WH designed the study, wrote and revised the manuscript. WL, HL, CZ, MZ and BZ performed all the experiments and the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Second Xiangya Hospital (Changsha, China). Written informed consent was obtained from all patients.

Patient consent for publication

All participants provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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