Metastasis-associated protein 2 (MTA2) promotes the metastasis of esophageal squamous cell carcinoma via EIF4E-Twist feedback loop

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Research

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Abstract

Background

Metastasis-associated protein 2 (MTA2) is frequently amplified in many types of cancers. However, the role and underlying molecular mechanism of MTA2 in esophageal squamous cell carcinoma (ESCC)remain unknown.

Methods

The expression of MTA2 in ESCC tissues and cell lines was detected by RT-qPCR, western blotting and immunohistochemistry. MTS and colony formation were used to analyze proliferation of ESCC cells. Transwell and wound healing assay were utilized to analyze ESCC cells migration and invasion. Xenograft experiments were used to analyze tumor growth and metastasis *in vivo*. Immunofluorescence was applied to test the influence of MTA2 on epithelial-mesenchymal transition (EMT). Microarray was used to test the interaction between MTA2 and EIF4E.Co- Immunoprecipitation(Co-IP) and Chromatin Immunoprecipi- tation (ChIP) assay were utilized to detect the occupation of MTA2 and Twist in the promoter region of *E-cadherin*.

Results

MTA2 was upregulated in ESCC tissues and cell lines. The upregulation of MTA2 in ESCC was correlated with malignant characteristics and poor survival probability of patients with ESCC. Through *in vitro* and *in vivo* experiments, we demonstrated that MTA2 significantly promoted ESCC growth, metastasis and EMT progression. Mechanistically, MTA2 could interact with EIF4E, which positively regulated the expression of Twist, and subsequently promote the malignant progression of ESCC. Moreover, the result of ChIP revealed that MTA2 was recruited to the promoter of *E-cadherin* by Twist to repress the transcription of *E-cadherin*.

Conclusion

MTA2 plays an aggressive role in ESCC metastasis by a novel EIF4E-Twist positive feedback loop, which may provide a potential therapeutic target for the management of ESCC.

Background

Esophageal cancer is one of the least studied and most aggressive malignancies worldwide [1]. The latest data predicted that esophageal carcinoma is the fourth leading cause of cancer-related mortality, and the incidence ranked third in 2015 in China [2].The most prevalent histologic type of esophageal carcinoma is esophageal squamous cell carcinoma (ESCC) [3]. Currently, despite the development of

tumor diagnosis and therapy, the incidence and mortality of esophageal cancer has still not significantly improved, and the overall 5-year survival ranges from 15–25% [4]. Squamous cell carcinoma is a histologic category of epithelial malignancies involving highly capable of metastatic spread and invasion [5], which account for poor outcome of ESCC. However, the molecular mechanism of tumorigenesis and metastasis of ESCC are still poorly understood.

The metastasis-associated (MTA) family was firstly identified by using a differential cDNA screening method [6]. Afterward, MTA protein family was discovered to play an indispensable role in the formation and progression of a wide variety of cancers [7–12].This family mainly consists of three members: MTA1, MTA2 and MTA3. It is reported that MTA2 regulates histone deacetylase activity to control gene transcription, resulting in different types of cell transformations and induce cancer cell invasion and metastasis [13]. MTA2 overexpression has been found in several kinds of human malignant tumors, including gastric cancer [14] colorectal cancer [15], non-small-cell lung cancer [16], and nasopharyngeal carcinoma [17]. Moreover, MTA2 was shown to be associated with early cancer metastasis, and could enhance the motile and anchorage- independent growth phenotypes of ERα-negative breast cancer cells in a retrospective study [18] .Chen et al. also revealed that high MTA2 expression in pancreatic ductal adenocarcinoma serves as an independent biomarker for poor survival [19]. In hepatocellular carcinoma, the overexpression of MTA2 was associated with tumor size and differentiation [20]. However, until now, there have been few reports about the function of MTA2 in the development of ESCC.

Epithelial-mesenchymal transition (EMT) is one of the important mechanisms through which epithelial cell-derived malignant tumor cells obtaining metastatic abilities. During the EMT program, many genes involved in cell adhesion, mesenchymal differentiation, cell migration, and invasion, are transcriptionally altered [21–22]. The best-studied transcriptional modulation during EMT is the repression of the *E-cadherin* gene, which is one of the key regulators of the epithelial phenotype [23]. Accumulating evidence has shown that *E-cadherin* is regulated by specific transcriptional repressors, including Twist/Mi2/ NuRD complex, in which both MTA2 and Twist are important component [24], thus we elucidated the function of MTA2 in the EMT progression.

In this study, we examined the expression of MTA2 in ESCC and analyzed the correlation of MTA2 expression with the clinicopathological parameters and survival rate of the ESCC patients. Furthermore, the gain- and loss-of-function experiments were used to demonstrate that MTA2 downregulation inhibited the proliferation and metastasis of ESCC cells *in vitro* and *in vivo*. Additionally, we find that EIF4E and Twist mediated EMT is the important downstream of MTA2 function. Therefore, our data provide a novel and powerful evidence between MTA2 and ESCC progression and suggest a new therapeutic target for ESCC.

Materials And Methods

Clinical specimens and cell culture

The tissue specimens were collected immediately after surgical resection from patients with ESCC (n = 98) at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) from January 2013 to December 2013. None of the patients underwent chemotherapy and/or radiotherapy before surgery. All tumors and normal tissues were confirmed by experienced pathologists. Precancerous lesions were obtained from endoscopies performed at the Fourth Hospital of Hebei Medical University. Our study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University, and written prior informed consent and approval were obtained from all patients.

The esophageal cancer cell lines Eca-109 and TE-1 were obtained from the Shanghai Institute for Biological Sciences. YES-2, KYSE-30, KYSE-410 and KYSE-510 cell lines were kindly provided by Professor Masatoshi Tagawa (Chiba University, Chiba-ken, Japan). KYSE-150, KYSE-180 and KYSE-450 cell lines were donated by the Zhan Qimin's lab from the Cancer Hospital of Chinese Academy of Medical Sciences (Beijing, China). All cells were maintained in the RPMI 1640 (Gibco, USA) containing 10% FBS (Biological Industries, Israel), 50 μg/mL streptomycin, and 50 U/mL penicillin (Invitrogen, USA) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

qRT-PCR

Total RNA was extracted with the Trizol (Life Technologies, USA) reagent method. cDNA was synthesized from the total RNA using the GoScript™ Reverse Transcription System (Promega, USA) according to the manufacturer's protocol. A SYBR Green PCR kit (Promega,USA) was used in the amplification process with a 7500 Real-time PCR system (Applied Biosystems, USA). The primers used for amplification were listed in Supplementary Table S1. The qRT-PCR results were analyzed with the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control.

Immunohistochemistry (IHC)

Immunohistochemical analysis was performed using the streptavidin-peroxidase (SP) method. The sections were dewaxed and rehydrated with xylene and a series of ethanol concentrations. Antigens were retrieved by boiling under pressure in EDTA buffer (PH = 9.0) for 3 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in deionized water for 20 min and blocked with 1% goat serum for 45 min followed by washing with PBS. The sections were then incubated with primary antibodies against MTA2 (Abcam, UK), Ki-67 and CD31 (ZSBG-BIO, China), E-cadherin, N-cadherin, Vimentin, EIF4E (CST, USA)overnight at 4 °C, followed by incubation with a biotinylated secondary antibody and streptavidin-biotinylated horseradish peroxidase complex (ZSBG-BIO, China), after washing three times (5 min each time). Protein expression was visualized and classified based on the percentage of positive cells and the intensity of staining.

Knockdown and ectopic expression of MTA2 in ESCC cell lines

MTA2 and EIF4E siRNA were purchased from GenePharma(China). After sequencing, the MTA2 siRNA and negative control sequence were inserted into the lentiviral vector hU6-MCS-Ubiquitin- EGFP-IRES, named shNC and shMTA2. MTA2 cDNA was inserted into the eukaryotic lentiviral expression vector pCDH-CMV-MCS-EF1-GFP-CD511B. The CD511B plasmid encoding the full-length MTA2 cDNA sequence and empty vector were transiently transfected into KYSE30 and KYSE510 cell lines with Lipofectamine 2000(Thermo Fisher, USA). The expression of MTA2 in KYSE30 and KYSE510 cell lines was silenced by lentivirus-mediated short hairpin RNA (shRNA) and siRNA. The efficiency of transfection was verified by western blotting.

Cell Proliferation, Migration, And Invasion Assays

Cells were seeded in 96-well plates (2,000 cells per well) and grown in complete medium for 24 h, 48 h, 72 h, and 96 h in a humidified atmosphere at 37 °C with 5% CO_2. For analysis, 20 μL of MTS substrate (Promega, USA) was added to each well. After culturing for 2 h, the absorbance of the wells was measured with a microplate reader (BioTek, USA) at 492 nm. For the colony formation assay, cells were seeded at a density of 1000 cells per well in six-well plates. After culturing for 12 days in an incubator, the cells were fixed with paraformaldehyde (Solarbio, China) and stained with crystal violet (Solarbio, China). The number of colonies was counted under an inverse microscope (Nikon, Japan). For the wound healing assay, 24 h after transfection, scratch wounds were made with 100 μL sterile pipette tips. To remove the disrupted cells, the plates were washed with PBS twice, and photos were taken at 0 h and 36 h, respectively. For the transwell assay, eighty thousand cells in 200 μL serum-free RPMI1640 were seeded in the upper chamber of transwell cell culture inserts(Corning Incorporated, USA) with or without matrigel (BD Biosciences, USA). Complete medium was added to the bottom chamber. After incubation for 20 h, the cells on the upper surface of the membranes were removed with a cotton swab. Then, the membranes were fixed with paraformaldehyde and stained with crystal violet. Cells were observed under a microscope and counted in five fields.

Western Blotting

Cells or tumor tissues was lysed with RIPA (Solarbio, China) reagent containing a protease inhibitor. The protein concentration of was determined by using a BCA protein assay kit (Thermo Fisher, USA). Sixty micrograms of protein was fractionated by 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, USA), which were then blocked with 5% skim milk for 1 h at room temperature. Afterwards, the membranes were incubated overnight at 4 °C with primary antibodies. Fluorescent secondary antibodies (Rockland, USA) and an infrared imaging system (LI-COR, USA) were used to visualize the protein bands. The protein levels were normalized to those of GAPDH (Abcam, UK).

Microarray For The Detection Of MTA2-associated Signaling

Total RNA from human KYSE30 ESCC cells, in which MTA2 was stably knocked down, and control KYSE30 cells were isolated and quantified. The RNA integrity was assessed by standard denaturing agarose gel electrophoresis. The expression profiles were determined using a RiboArray™ Custom Array (12 × 90K A10000-1-90) and with an Axon GenePix 4000B scanner. Quantile normalization and the subsequent data processing were performed using the RMA method. The transcript profiling data were deposited in the NCBI Gene Expression Omnibus and are accessible through the GEO series accession number GSE112495.

Co-immunoprecipitation(Co-IP)

Cells were harvested 72 h after transfection, and nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher, USA) following the manufacturer's protocol. A small amount of nuclear protein lysate was prepared for western blotting, and the remaining lysate was incubated overnight at 4 °C with an MTA2 antibody, and rabbit IgG antibody as a negative control. Afterwards, the lysates were pre-cleared with prepared protein A-agarose beads (Thermo Fisher, USA) for 2 h at 4 °C with gentle agitation. After a high-speed centrifugation to remove the supernatant, the beads were washed three times with immunoprecipitation washing buffer and then boiled for 5 min in 2 × SDS loading buffer (Solarbio, China). After centrifugation, the supernatant was used for western blot analysis. The membranes were incubated with a Twist antibody (Abcam, UK).

Chromatin Immunoprecipitation PCR

The chromatin immunoprecipitation (ChIP) experiment was conducted using an EZ-Magna ChIP A/G Kit (Millipore, USA) according to the manufacturer's protocol. The DNA-bound proteins from KYSE30 cells were cross linked in 1% formaldehyde for 10 min at room temperature and quenched in glycine. A rabbit anti-Twist antibody or normal rabbit IgG were used for immunoprecipitation. PCR was performed to detect the binding to the promoter of the E-cadherin gene. The eluted materials from the first IP were used in the re-ChIP and then incubated with an MTA2 antibody .

The primers for the E-cadherin promoter were F, 5'-GCAGGTCCA TAACCCACCTA-3'and R, 5'-CATAGACGCGGTGACCCTCTA-3'.

Immunofluorescence (IF)

KYSE30 cells were treated with si-MTA2 for 24 h, fixed with 4% paraformaldehyde (Solarbio, China) for 30 min, permeabilized with 0.1% Triton X-100(Solarbio, China) for 15 min, and blocked with 5% BSA for 30 min. Then, the cells were incubated with antibodies, including MTA2,E-cadherin□N-cadherin and

Vimentin (CST, USA) for 2 h at room temperature, followed by further incubation at room temperature for 1 h with rabbit IgG (Alexa Fluor 546, green, USA). Nuclear DNA was labeled in blue with DAPI (Beyotime, China). Images were captured by confocal microscopy, with a Zeiss LSM Image Examiner (Carl Zeiss, Germany).

Nude Mouse Xenograft

KYSE30 cells were used in this study to establish stable cell lines via virus transfection that constitutively overexpress the MTA2 protein or MTA2 shRNA. Four-week-old male athymic Balb/C mice (Charles River, China) were randomly divided into two groups. For the tumor formation assay, 1×10^6 KYSE30/shNC or KYSE30/shMTA2 and KYSE30/CD511B or KYSE30/MTA2 cells in 100μL PBS were subcutaneously injected into the right flank of each mouse. Tumor nodules were measured every 7 days after their length exceeded 4 mm, and the volume was calculated with the following formula: $V = (\text{width}^2 \times \text{length})/2$. Xenografts were collected at 6th week for immunohisto- chemical staining and protein extraction.

For the pulmonary metastasis models, 5×10^6 KYSE30/Vector or KYSE30/shMTA2 cells in 200μL PBS were injected via the tail vein. Two months later, all mice were sacrificed. Pulmonary metastases were examined in the gross specimens and with the HE staining of the lung tissues.

Statistical analysis

All observations were confirmed by at least three independent experiments. Statistical analyses were performed using SPSS version 13.0 software. An independent-samples t test, the Mann-Whitney U test and a one-way ANOVA test (LSD post hoc) were used to analyze the quantitative data. The correlation between MTA2 and EIF4E expression was analyzed with Spearman test. Survival analysis was performed by using Kaplan-Meier curves. A chi-square test was performed for the analysis of qualitative data. A *P*-value < 0.05 was considered as statistically significant.

Results

MTA2 overexpression in ESCC tissues correlates with ESCC aggressiveness

We firstly performed an IHC and western blotting analysis to investigate the MTA2 protein expression in normal esophageal squamous epithelium, precancerous lesion tissues and esophageal squamous cell carcinoma tissues. The result showed that the expression of MTA2 was increased in ESCC, compared with precancerous lesion tissue or the corresponding non-tumor tissues (Fig.S1A-B), consistent with our previous result [25].

To investigate the expression level of *MTA2* gene in ESCC malignancy, we measured *MTA2* mRNA expression in 98 human ESCC tissues using reverse transcription and quantitative PCR (RT-qPCR). The results revealed that *MTA2* was highly expressed in ESCC tissues when compared with that in the corresponding non-tumor tissues from the same donor or compared with that in normal esophageal epithelial tissue samples (Fig. 1A and Fig. S1C). Further analysis revealed that the primary tumor invasion depth (Fig. 1B), advanced TNM stage (Fig. 1C), and lymph node metastasis (Figure.1D) were all positively correlated with *MTA2* expression. On the other hand, we divided the samples into an *MTA2* low-expression group and an *MTA2* high-expression group according to the median *MTA2* expression in the ESCC tissues to detect the clinical pathological features of ESCC patients and *MTA2* expression levels. As expected, there were positive correlations between the *MTA2* expression levels and the primary tumor invasion depth, lymph node metastasis, distant metastasis, and TNM stage, while the tumor differentiation grade and tumor size displayed no correlation (Table.1). Furthermore, we examined MTA2 expression in primary tumor tissues and the corresponding lymph–node metastatic tumor tissues from three patients. The results indicated that the expression of MTA2 was much higher in lymph–node metastatic tumor tissues compared to that in primary tumor tissues (Fig. 1E), which strongly suggested that MTA2 might contribute to ESCC malignancy, particularly to metastasis.

Additionally, a prognostic analysis using 79 ESCC tissues with complete follow-up information revealed that high *MTA2* expression in ESCC tissues was associated with reduced overall survival (Fig. 1F). We also evaluate the effect of the *MTA2* expression level on the clinical prognosis of different cancers using the data from the GEPIA (http://gepia.cancer-pku.cn/index.html) and found that high *MTA2* expression was correlated with poor survival probability in mesothelioma (Fig.S1D), adrenocortical carcinoma (Fig.S1E), and liver hepatocellular carcinoma (Fig.S1F). Therefore, we concluded that MTA2 over-expression was associated with ESCC malignancy and poor prognosis.

MTA2 promotes proliferation, migration and invasion of ESCC cells in vitro.

In order to clarify the function of MTA2 in the ESCC cells, we firstly analyzed MTA2 expression in eight ESCC cell lines and a normal esophageal epithelial cell line. Compared with normal esophageal epithelial cell line, MTA2 expression was significantly higher in ESCC cell lines (Fig. S2A). Then MTA2 expression was knocked down in KYSE30 and KYSE510 cells through the transfection of siRNA or shRNA, or was overexpressed through the transfection of the pCDH-MTA2 plasmid (Fig.S2B). Wound healing and transwell assays were performed to explore the impact of MTA2 on the migration and invasion of ESCC cells. As shown in the Fig. 2A, after transfection with siRNA to knockdown the expression of MTA2, the wound healing process was delayed. Moreover, the number of cells penetrating the membrane of the chambers in both the migration and invasion assays was significantly lower in the MTA2 knockdown group compared with that in the control group (Fig. 2B and C). In contrast, the exogenous overexpression of MTA2 facilitated the wound closure of both the KYSE30 and KYSE510 cell lines (Fig. 2D). Transwell migration and Matrigel invasion assays also implied that the overexpression of MTA2 prominently enhanced the migratory and invasive capabilities of both ESCC cell lines compared to those of the controls (Fig. 2E and F).

Moreover, we also found that compared to the controls, MTA2 depletion inhibited the proliferation of KYSE30 and KYSE510 cells based on a MTS assay (Fig.S2C), while MTA2 overexpression promoted the viability of both cell lines (Fig.S2D). The colony formation assay also demonstrated the similar function of MTA2 (Fig.S2E).

Taken together, these data clearly demonstrate that MTA2 plays a carcinogenic role in ESCC by promoting the viability, migration and invasion of ESCC cell lines.

MTA2 promotes the growth and metastasis of transplanted tumors in vivo.

To further examine the oncogenic activity of MTA2 in tumor progression in vivo, we generated animal models by subcutaneously injecting KYSE30/shMTA2 or KYSE30/MTA2 cells into nude mice. Both the control and KYSE30/MTA2 groups formed tumors after injection, and the tumor formation rate in the KYSE30/shMTA2 group was 80% (4/5). The growth rate and average tumor weight of the xenografts were both lower in the MTA2 knockdown group than in the control group. (Fig. 3A). Moreover, the expression levels of both the cell proliferation marker Ki-67 and the tumor angiogenesis marker CD31 were significantly reduced in the MTA2 knockdown group (Fig. 3B). However, the tumors that were derived from MTA2 overexpressed cells were significantly larger than those in the control group (Fig. 3C).As expected, MTA2 overexpression promoted the expression of Ki-67 and CD31 in the xenografts (Fig. 3D).

On the other hand, the influence of MTA2 expression on ESCC metastasis was evaluated. KYSE30/shNC and KYSE30/shMTA2 cells were injected into the tail vein of nude mice. As shown in the figure, though no visible metastasis was found in either group, histologic analysis showed that many pulmonary metastatic foci was formed in the lung of mice in the control group, while no pulmonary tumor nodules were found in the MTA2 knockdown group (Fig. 3E). These results strongly suggest that MTA2 promoted the growth and metastasis of esophageal carcinoma *in vivo*.

MTA2 Promotes Epithelial-mesenchymal Transition In ESCC Cells

There is strong evidence that EMT is involved in the different stages of tumor metastasis and promotes a malignant phenotype in tumors. To further investigate the mechanism behind the MTA2-mediated promotion of metastasis in ESCC cells, the expression of molecules that are associated with the transition from an epithelial to a mesenchymal phenotype was examined. The results indicated that compared to the controls, the expression of N-cadherin, Vimentin, MMP2,MMP9 and ZEB1 was suppressed, and the expression of E-cadherin and ZO-1 was

upregulated when the expression of MTA2 was silenced in both KYSE30 and KYSE510 cells (Fig. 4A). Likewise, compared to the controls, in MTA2-overexpressing cells, the expression levels of N-cadherin, Vimentin, MMP2, MMP9 and ZEB1 was significantly increased, whereas those of E-cadherin and ZO-1was decreased (Fig. 4B).

Furthermore, this change was further confirmed by examination the subcellular presence of proteins using IF staining. As shown in the Fig. 4C, compare to the controls, MTA2 deficiency repressed the expression of N-cadherin and Vimentin, while the MTA2-knockdown group exhibited increased E-cadherin staining at the cell membrane.

We also analyzed E-cadherin, N-cadherin and Vimentin expression in xenograft tumor tissues to explore whether MTA2 promoted EMT transformation *in vivo*. The result was consistent with the findings *in vitro* (Fig. 4D). All these data prompted us to hypothesize that the MTA2-mediated metastasis of ESCC might be linked to the promotion of EMT.

EIF4E Is A Target Of MTA2

Moreover, to further examine the molecular mechanism that underlies the pro-tumorigenic role of MTA2, we conducted a gene expression microarray analysis of KYSE30 cells that were depleted of MTA2 and of control cells and 93 genes with significant changes in expression (fold change > 2, $P < 0.05$, GSE112495) were identified (Fig. 5A). For microarray analysis result of MTA2 knockdown, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the most significantly overrepresented pathways involved in RNA transport, Rap1 signaling pathway and TGF-β signaling pathway(Fig. 5B).The mRNA levels of six representative genes (three upregulated and three downregulated) were confirmed by qRT-PCR, as shown in Fig. 5C. Among these genes, we found that EIF4E expression was consistently and significantly decreased in ESCC cells with depletion of MTA2 compared to that in controls. Then we detect the expression of *EIF4E* in ESCC tissues from 60 patients in our study, and the results showed that *EIF4E* expression was significantly increased in ESCC tissues as compared to that in the matched para-cancerous tissues (Fig. 5D). Further analysis revealed that *EIF4E* expression was positively correlated with primary tumor invasion depth (Fig. 5E), lymph node metastasis (Fig. 5F) and TNM stage (Fig. 5G). Moreover,the Kaplan- Meier survival analysis was then conducted and the result demonstrated that higher *EIF4E* levels in patients were correlated with shorter overall survival than that in patients with low *EIF4E* levels (Fig. 5H). According to the GEPIA analysis (http://gepia.cancer-pku.cn/index.html), high *EIF4E* expression was correlated with poor survival probability in glioma patients (Fig.S3A), liver hepatocellular carcinoma patients (Fig.S3B) and lung adenocarcinoma patients (Fig.S3C).These results suggested that EIF4E overexpression was an important prognosis factor of various types of cancer, including ESCC.

The MTA2-mediated promotion of malignancy in ESCC is mediated by EIF4E

Next we will evaluate whether EIF4E are important factor of MTA2 function. Firstly, our result showed that knockdown of MTA2 decreased the expression of EIF4E, while overexpression of MTA2 exerts contrast function (Fig. 6A). Consistently, decreased EIF4E was detected in tumor tissues in knockdown MTA2 group (Fig. 6B). Furthermore, a positive correlation between the expression of *EIF4E* and *MTA2* was found in the human ESCC tissues in our study (Fig. 6C), which was consistent with the data from the GEPIA (http://gepia.cancer-pku.cn/index.html)(Fig.S3D). Thus, we focused on the role of EIF4E in the promotion of malignancy in ESCC. Transwell assays were performed to investigate whether the cell migration and

invasion ability was influenced by EIF4E.The downregulation of EIF4E with siRNA inhibited both the migration and invasion capacities of the cell lines (Fig. 6D). After EIF4E overexpression, the migration and invasion ability of both cell lines was dramatically increased (Fig. 6E). We next conducted a rescue experiment by reintroducing EIF4E expression in stable MTA2-silenced KYSE30 cells. As expected, the inhibition of the migration and invasion that was caused by MTA2 knockdown was abrogated as a result of the forced expression of EIF4E (Fig. 6F). Therefore, our findings firstly revealed that EIF4E was a major downstream mediator of MTA2-induced metastatic activity.

MTA2 promotes epithelial-mesenchymal transition in ESCC via the regulation of EIF4E

The aforementioned results indicated that the interaction of MTA2 and EIF4E promotes ESCC metastasis. Therefore, we were interested in determining whether EIF4E was involved in the MTA2-mediated regulation of EMT in ESCC. Firstly, we examined the effect of EIF4E on EMT transformation in ESCC. The expression of E-cadherin and ZO-1 evaluated by western blotting was significantly reduced, and the expression of N-cadherin, Vimentin, MMP2 and MMP9 was elevated after EIF4E overexpression in both ESCC cell lines (Fig. 7A). Accordingly, knocking down EIF4E with siRNA demonstrated the opposite result (Fig. 7B). Furthermore, we found that the overexpression of EIF4E was able to rescue the loss of N-cadherin and Vimentin induced by MTA2 absence. In contrast, the expression of E-cadherin was decreased after EIF4E reintroduction compared with that in the MTA2 knockdown group (Fig. 7C). However, knocking down EIF4E with siRNA in both cell lines, which were ectopically overexpressing MTA2, generated the opposite result (Fig. 7D). These data indicated that the inducible expression of EIF4E mediated, at least in part, the promotion of EMT by MTA2 in ESCC.

MTA2 and EIF4E-Twist formed a positive feedback to repress E-cadherin expression

Twist, a basic helix–loop–helix (bHLH) transcription factor, which recognizes the canonical E-box (CANNTG) to regulate gene transcription, plays a critical role in metastasis. MTA2 is the vital component of proteins complex Twist/Mi2/NuRD, in which Twist is the most important transcription factor that regulated EMT [24]. In our study, the forced expression of Twist prominently enhanced the migratory and invasive capabilities of KYSE30 and KYSE510 cells (Fig. 8A). Furthermore, compared to those of the controls, cells with enhanced expression of Twist inhibited the E-cadherin and ZO-1 levels significantly, whereas N-cadherin and Vimentin exhibited the opposite trend (Fig. 8B). Thus, we were interested in determining whether Twist participates in the MTA2-mediated regulation of EMT in ESCC. Co-IP with the extraction from MTA2-overexpressing KYSE30 cells followed by immunoblotting with a Twist antibody verified that Twist was co-precipitated with MTA2 (Fig. 8C). Additionally, this interaction was confirmed with endogenously expressed proteins in KYSE30 cells (Fig. 8D). To investigate whether *E-cadherin* is a transcriptional target of Twist, we analyzed by bioinformatics the promoter of the *E-cadherin* gene and found several putative Twist consensus binding sites (Fig. 8E). Furthermore, ChIP assay with anti-Twist followed by RT-PCR and qPCR validated the occupancy of Twist at the *E-cadherin* promoter. Furthermore, the immuno- precipitated materials associated with the anti-Twist complex were subjected to re-ChIP using an antibody against MTA2. The results showed that MTA2 was also enriched at the promoter of *E-*

cadherin, with IgG as a negative control (Fig. 8F). Taken together,above results all showed that MTA2 was recruited by Twist to the promoter of *E-cadherin* and repress its transcription.

To further investigate the effect of the association between MTA2 and Twist on the EMT in ESCC, we also reintroduced Twist to stable MTA2- silenced KYSE30 cells. The rescue experiment showed that the ectopic overexpression of Twist reversed the increase in E-cadherin and ZO-1 levels caused by MTA2 depletion, while the expression of N-cadherin and Vimentin was elevated after Twist reintroduction compared with that in the MTA2 knockdown group (Fig. 8G). We also found that compared to the controls, the expression of Twist was suppressed when the expression of EIF4E was silenced. On the contrary, the expression level of Twist was significantly increased in the EIF4E- overexpressing cells (Fig. 8H), which was consistent with previous report [26]. Collectively, these results indicated that MTA2 and EIF4E-Twist formed a positive feedback to regulate the development of ESCC.

Discussion

MTA2 was firstly described as a novel protein that was closely related to the metastasis-associated protein MTA1 in 1998[27]. The aberrant expression of MTA2 was first reported in cervical cancer, and its expression was related to rapid cell division [28]. In the present study, we found that MTA2 overexpression frequently occurred in ESCC and related with advanced TNM stage. Moreover, high MTA2 expression in ESCC tissues was associated with a poor prognosis. Functionally, MTA2 markedly promoted cell viability and metastasis via enhanced EMT progression.

Increasing evidence suggests that MTA2 plays an oncogenic role in the development of malignant carcinoma. For example, MTA2 over expression was associated with enhanced proliferation in nasopharyngeal carcinoma [17] and lung cancer [29].The knockdown of MTA2 could suppress the proliferation of human glioma cells *in vitro* and *vivo* [30]. MTA2 has been shown to influence cell growth by affecting IL-11, BAX, BCL-2 and CyclinD1 [31–32]. According to Luo, et al, MTA2 modulated p53-mediated cell growth and apoptosis by reducing p53 acetylation and expression [33]. Our present data demonstrated that MTA2 had lower basal expression in normal ESCC tissues (Figure S1A). This further demonstrates an important role of MTA2 in the carcinogenesis of ESCC. Our functional data for MTA2 suggested that the down regulation of MTA2 decreased the viability of ESCC cells *in vitro* and *in vivo*. Thus, the mechanism through which MTA2 promoted proliferation in ESCC requires further investigation. Nevertheless, the *in vivo* experiment from our study revealed that the promotion of angiogenesis should be considered.

In lung cancer, almost 80% of the cells in distant metastases were MTA2-positive [32]. MTA2 depletion inhibited the invasion of human glioma cells in *vitro* and in *vivo* [30]. Consistently, the overexpression of MTA2 was positively correlated with TNM stage and the lymphatic invasion of ESCC patients in our study. Our gain- and loss-of-function experiments further confirmed that MTA2 increased the migratory capacity of ESCC cells *in vitro* and *in vivo*. In breast cancer, the activation of the Rho pathway by MTA2 overexpression enhanced the motility of breast cancer cells [18]. The regulation of the cytoskeleton

through the modulation of CD24 and MYLK might be one of the mechanisms by which MTA2 participates in gastric cancer cell invasion [14]. The process of cancer invasion and metastasis is complex, and the highly conserved EMT program has been shown to be involved in the dissemination of cancer cells [21]. Furthermore, MTA2 promotes the metastasis of non-small-cell lung cancer through the inhibition of the cell adhesion molecules Ep-CAM and E-cadherin [16]. However, whether MTA2 can affect the EMT phenotype of ESCC has remained unclear. In the present study, expression analysis of a set of EMT markers was conducted to explore whether MTA2 is involved in the EMT progression. The result showed that, compared to the controls, MTA2 depletion suppressed the expression of mesenchymal markers, and upregulated the expression of epithelial markers. In MTA2 overexpressing cells, we observed the loss of epithelial markers, while the expression of mesenchymal markers was strongly induced. These data indicated that the MTA2- mediated promotion of metastasis in ESCC relies, at least partly, on EMT.

To further examine the molecular mechanism that underlies the pro-EMT role of MTA2 in ESCC, we performed the transcriptome analysis in MTA2 knockdown cells. We found that the different expressed genes are mostly involved in cancer-associated signaling pathways, such as Rap1 and TGF-β signaling pathway, which are associated with metastasis and EMT process. Among these genes, we found that eukaryotic initiation factor 4E (EIF4E) expression was consistently and significantly decreased in ESCC cells with depletion of MTA2 compared to that in controls. EIF4E has been shown to be elevated in esophageal cancer [34]. A previous study indicated that EIF4E plays a crucial role in EMT and tumor metastasis [35–36]. Although this protein is needed for the translation of all cap-dependent mRNAs, elevated EIF4E expression selectively and preferentially enhances the translation of mRNAs that are linked to malignant transformation and metastasis [37]. According to Pettersson et al, the inhibition of EIF4E reduced breast cancer cell metastasis by suppressing TGFβ-induced EMT [38]. The phosphorylation of EIF4E promoted EMT and prostate tumor cell metastasis via the translational control of SNAIL and MMP-3[35]. In our present study, the introduction of exogenous EIF4E promoted ESCC cell migration and invasion as well as the EMT phenotype compared to those of the controls. Furthermore, the rescue experiment indicated that EIF4E was involved in MTA2-mediated regulation of EMT in ESCC. However, further research should be conducted to investigate the precise regulatory mechanism of MTA2 on EIF4E.

Twist, which is a member of the basic helix-loop-helix (bHLH) transcription factor family, is overexpressed in various types of human cancers, including ESCC [39–40]. In present study, our data also indicated that the expression of Twist was suppressed when EIF4E was silenced, while EIF4E overexpression increased the Twist level. According to the previous report [26], activation of EIF4E promotes metastatic progression via translation of several EMT-associated mRNAs such as Twist. EIF4E binds to the 5'7-methylguanosine cap of mRNAs and recruits to the EIF4F complex (EIF4G, EIF4A and EIF4E), expanding mRNA second structure to expose the translation initiation codon and enabling translation[41]. We will further investigate the detailed interaction mechanism of EIF4E and Twist in ESCC. A number of studies have shown that Twist plays an essential role in tumor metastasis by inducing EMT [42–43]. The loss of E-cadherin appears to be necessary for EMT [44]. Twist has been shown to silence E-cadherin transcription by binding to the E-box motifs in its promoter. MTA2, as a central component of the NuRD complex, which

functions primarily in gene repression, was described to be recruited by Twist for inhibition of E-cadherin expression in HEK-293 cells [24]. The result of ChIP followed by PCR in our study demonstrated that MTA2 was recruited by Twist to bind to the promoter of E-cadherin to repress its expression. The rescue experiment further confirmed that the promotion of the EMT program in ESCC by MTA2 was ascribed, at least in part, to the interaction with Twist and the subsequent E-cadherin suppression.

Conclusions

Collectively, our findings illustrate that the aberrant expression of MTA2 promotes proliferation, invasion, and metastasis of human ESCC cells through the regulation of EMT, which is mainly dependent on the EIF4E-Twist positive feedback loop. These discoveries reinforce the hypothesis that MTA2 plays an essential and aggressive role in ESCC metastasis by promoting the EMT program, and MTA2 may be the putative target for the treatment of ESCC metastasis.

Abbreviations

MTA2:Metastasis-associated protein 2;ESCC:Esophageal squamous cell carcinoma; EMT: Epithelial mesenchymal transition;EIF4E: Eukaryotic initiation factor 4E; ChIP: Chromatin immunoprecipitation; NuRD: Nucleosome remodeling and histone deacetylase; PVDF: Polyvinylidene fluoride; GEO: Gene expression omnibus; GEPIA: Gene expression profiling interactive analysis; HE: Hematoxylin and eosin; KEGG: Kyoto encyclopedia of genes and genomes; IP: Immunoprecipitation.

Declarations

Acknowledgment

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Author contributions

Baoen Shan and Lianmei Zhao conceived and designed the experiments; Suli Dai, Sisi Wei, Xiaoya Li performed the experiments; Huilai Lv contributed to the sample collection; Cong Zhang and Ming Ma analyzed the data; Yueping Liu contributed to the pathological identification; Suli Dai wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during the current study are available in the Gene Expression Omnibus Database (Accession: GSE112495).

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interest

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Table

Table 1
Correlation between MTA2 expression and clinicopathological features in 98 ESCC patients

Parameters	Total	Expression level of MTA2		P value[a]
		Low	High	
Age/year				
< 60	41	19	22	0.539
≥ 60	57	30	27	
Gender				
Male	76	36	40	0.333
Female	22	13	9	
Differentiation				
Well	37	15	22	0.145
Poor	61	34	27	
TNM				
T				
T1	14	11	3	0.006**
T2	21	11	10	
T3	52	23	29	
T4	11	4	7	
N				0.024*
N0	57	34	23	
N1	41	15	26	
M				0.022*
M0	94	49	44	
M1	5	0	5	
Stage				0.001**

* P < 0.05; **P < 0.01

[a] Chi-squared test results

Parameters	Total	Expression level of MTA2		P value[a]
		Low	High	
□	9	5	4	
□	48	34	14	
□	36	10	26	
□	5	0	5	
Lymphatic invasion				0.026*
Negative	51	31	20	
Positive	47	18	29	
Tumor size(cm^3)				0.075
≤ 5	16	8	8	
□5 and ≤ 10	21	15	6	
□10	61	26	35	
* P < 0.05; **P < 0.01				
[a] Chi-squared test results				

Figures

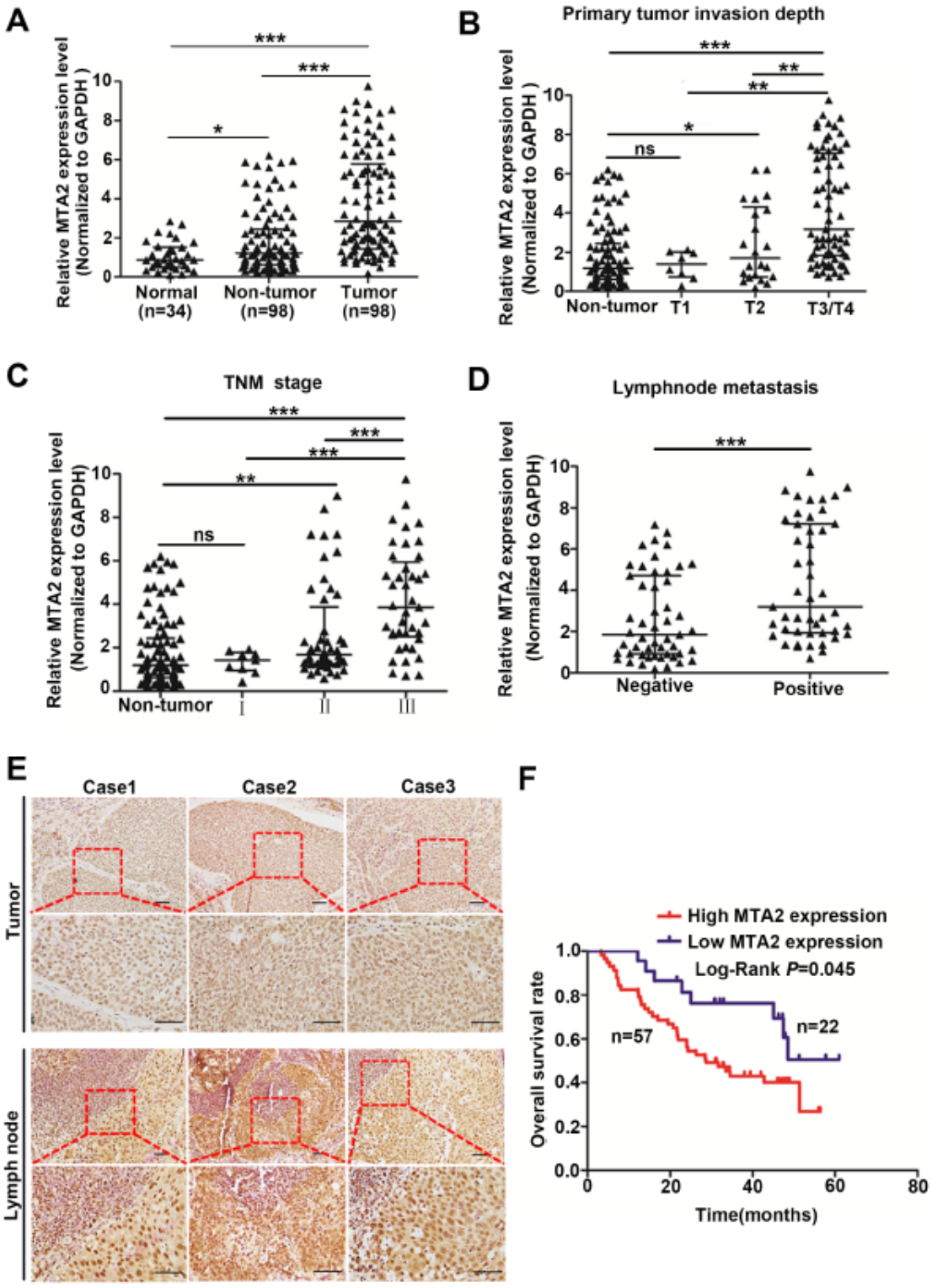


Figure 1

MTA2 overexpression in ESCC tissues correlates with ESCC aggressiveness. (A) Expression of MTA2 was analyzed by qRT-PCR in esophageal squamous cell carcinoma tissues (n=98), adjacent non-tumor tissues (n=98), and normal esophageal epithelial tissues (n=34). GAPDH was set as internal control. (B) The correlation between MTA2 expression and primary tumor invasion depth.T1: muscularis mucosae, T2: muscularis propria, T3/T4: tunica fibrosa and surrounding tissues. (C) The correlation between MTA2

expression and TNM stage. Different TNM stage of ESCC according to the latest UICC (Union for International Cancer Control) and AJCC (American Joint Committee on Cancer). (D) The correlation between MTA2 expression and lymph node metastasis. Negative: no lymph node metastasis, positive: with lymph node metastasis. (E) Expression of MTA2 was analyzed by IHC in primary tumor tissues and the corresponding lymph node metastatic tumors tissues from three patients. (F) The effect of the MTA2 expression level on clinical prognosis was analyzed by Kaplan–Meier survival analysis. *P < 0.05, **P < 0.01, ***P < 0.001

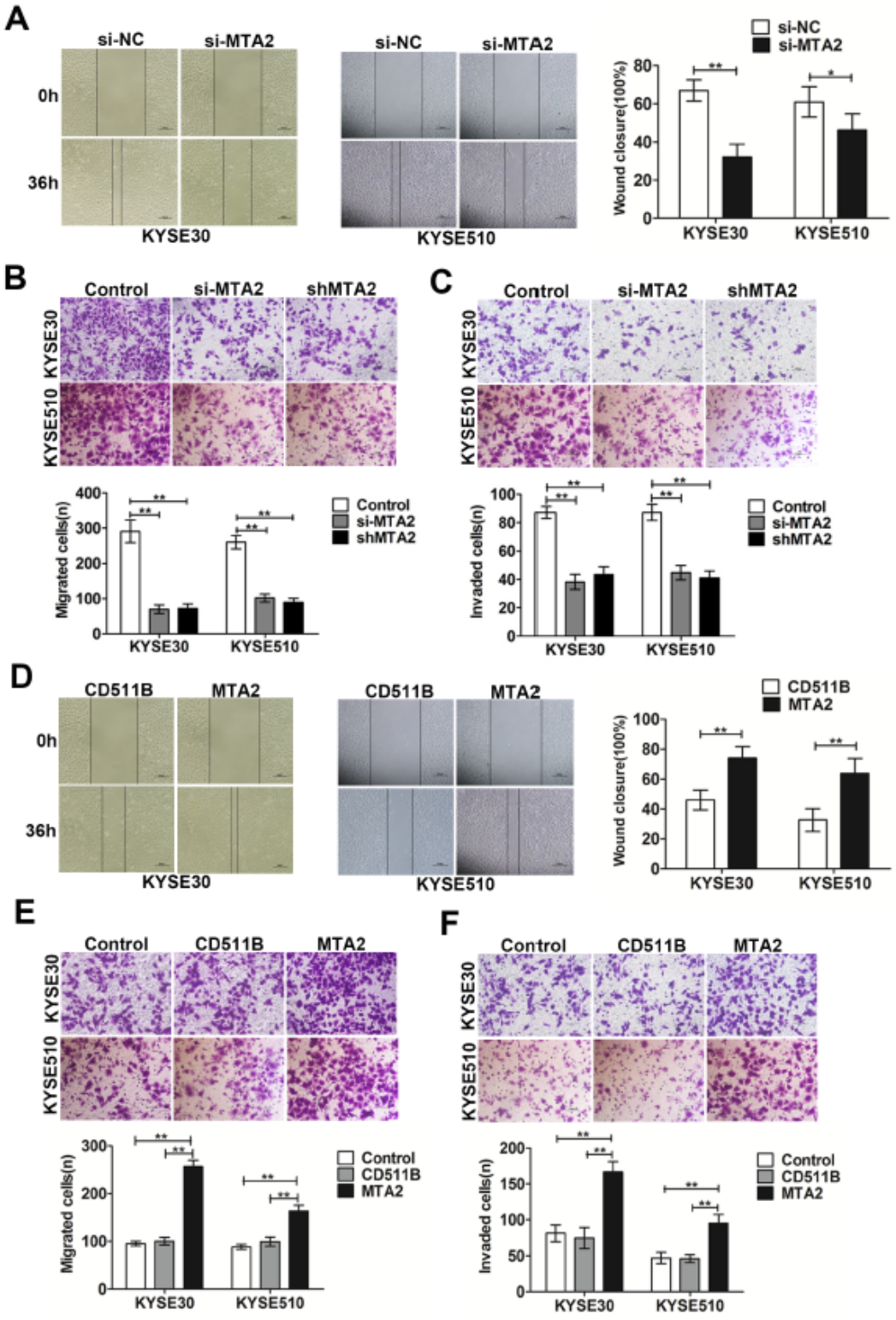
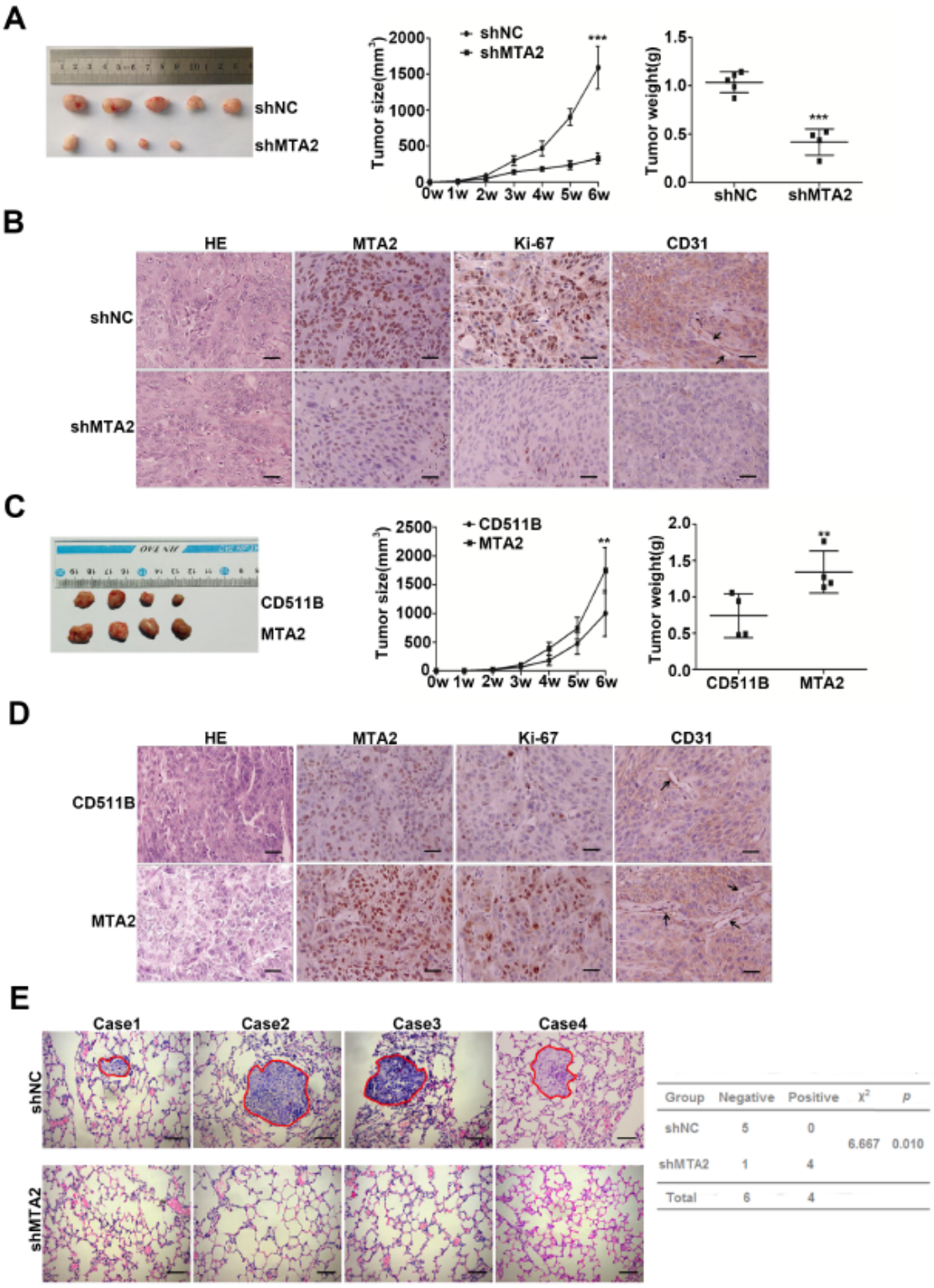


Figure 2

MTA2 promotes ESCC migration and invasion in vitro. (A) Wound healing assay were performed in KYSE30 and KYSE510 cells transfected with siRNA. The distance of wound healing was measured and calculated as a percentage of the distance at 0h. Original magnification, ×100. (B, C) Transwell assays were performed in control and MTA2-depleted KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in five different fields. Original magnification, ×200. (D) Wound healing assay were performed in KYSE30 and KYSE510 cells transfected with different vectors. The distance of wound healing was measured and calculated as a percentage of the distance at 0h. Original magnification, ×100. (E, F) Transwell assays were performed in control and MTA2- overespressed KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in five different fields. Original magnification, ×200. *P < 0.05, **P < 0.01



Group	Negative	Positive	χ^2	p
shNC	5	0	6.667	0.010
shMTA2	1	4		
Total	6	4		

Figure 3

MTA2 promotes the growth and metastasis of transplanted tumors in vivo. (A, C) KYSE30 cells stably transfected with different plasmids or empty vectors were subcutaneously injected into BALB/c nude mice. Representative images of mice from different treatment groups 6 weeks after injections were showed. Tumor growth and tumor weight were analyzed. (B, D) Representative immunohistochemical images showing the intensity of Ki-67 and CD31 expression (brown) in xenografted tumors from different

groups. Original magnification, ×400. (E)KYSE30 cells stably transfected with shMTA2 plasmid or empty vector were slowly injected into BALB/c nude mice via tail vein. Representative images of HE staining of lung metastasis. Original Magnification, ×200. **P < 0.01, ***P < 0.001

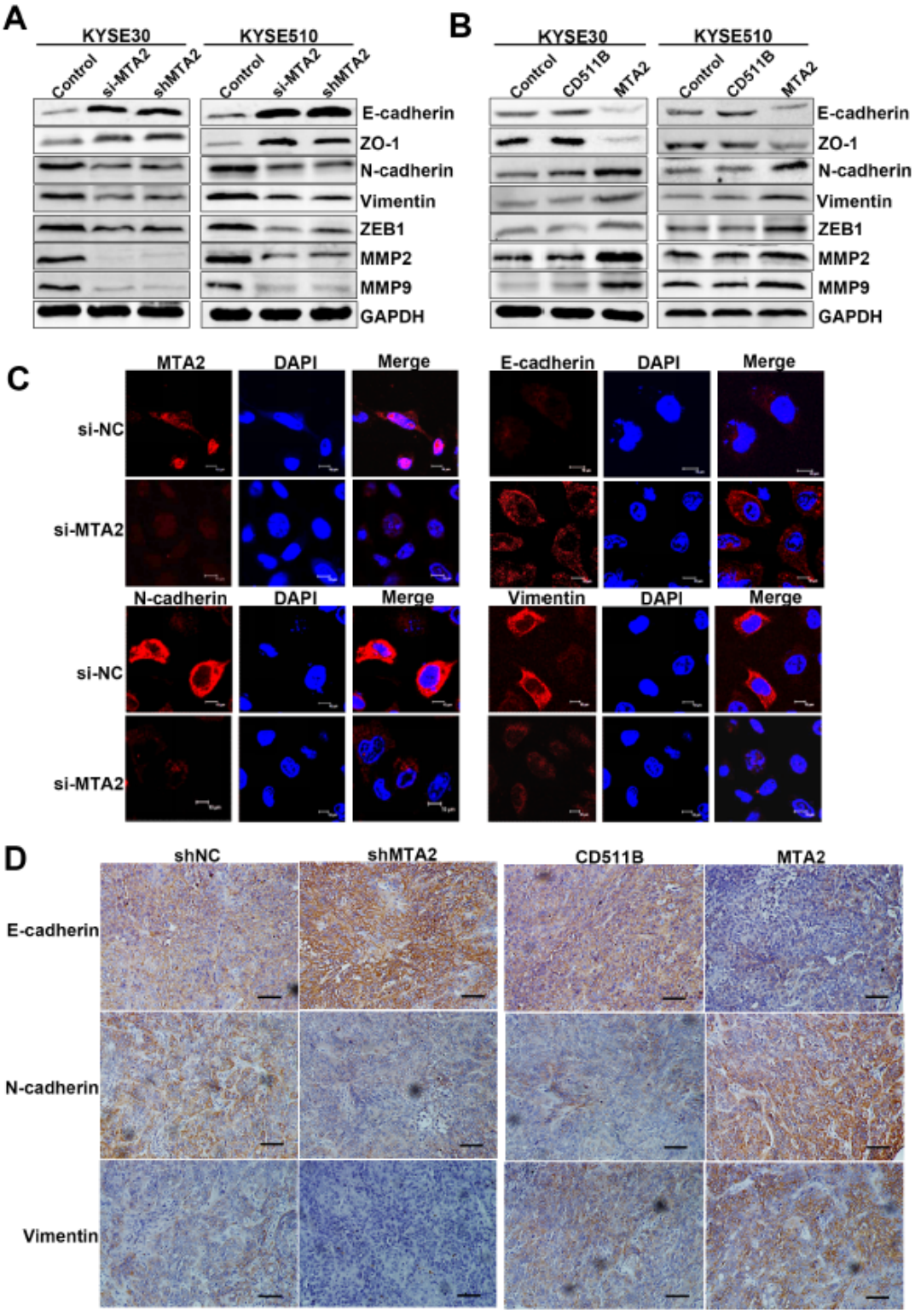


Figure 4

MTA2 promotes epithelial-mesenchymal transition in ESCC cells. (A, B)Western blot analyzed the expression of EMT markers in MTA2- depleted or MTA2-overexpressed KYSE30 and KYSE510 cells. (C)

Immunofluorescence staining for EMT markers in KYSE30 cells transfected with siRNAs. (D) The expression of epithelial marker E-cadherin and the mesenchmal markers N-cadherin and Vimentin in mice tumor tissues was examined by IHC. Original magnification, ×400.

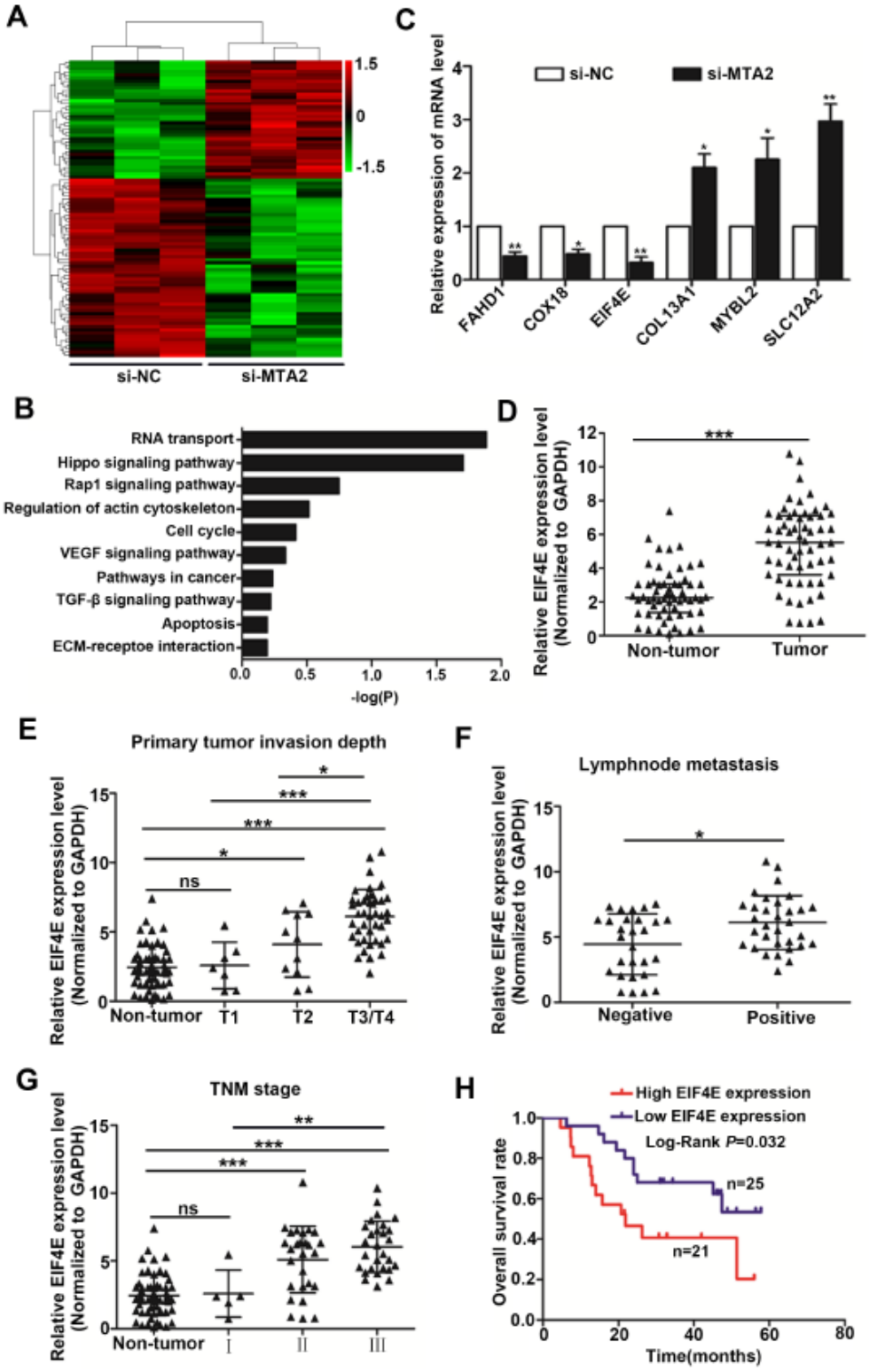


Figure 5

EIF4E was a target of MTA2. (A)Heatmap representation of the fold change in gene expression as determined by transcriptome analysis in MTA2-silenced cells and control cells (KYSE30). (B) KEGG

pathway analysis of MTA2-regulated genes. Pathways showing enrichment are presented. (C) The expression of cancer related genes after MTA2 knockdown was detected by RT-qPCR. (D)Expression of EIF4E was analyzed by qRT-PCR in esophageal squamous cell carcinoma tissues (n=60) and adjacent non-tumor tissues (n=60). GAPDH was set as internal control. (E)The correlation between EIF4E expression and primary tumor invasion depth based on the qRT-PCR data .T1: muscularis mucosae, T2: muscularis propria, T3/T4: tunica fibrosa and surrounding tissues. (F)The correlation between EIF4E expression and lymph node metastasis based on the qRT-PCR data was analyzed. Negative: no lymph node metastasis, positive: with lymph node metastasis. (G) The correlation between EIF4E expression and TNM stage based on the qRT-PCR data. Different TNM stage of ESCC according to the latest UICC (Union for International Cancer Control) and AJCC (American Joint Committee on Cancer). (H) The effect of the EIF4E expression level on clinical prognosis was analyzed by Kaplan–Meier survival analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

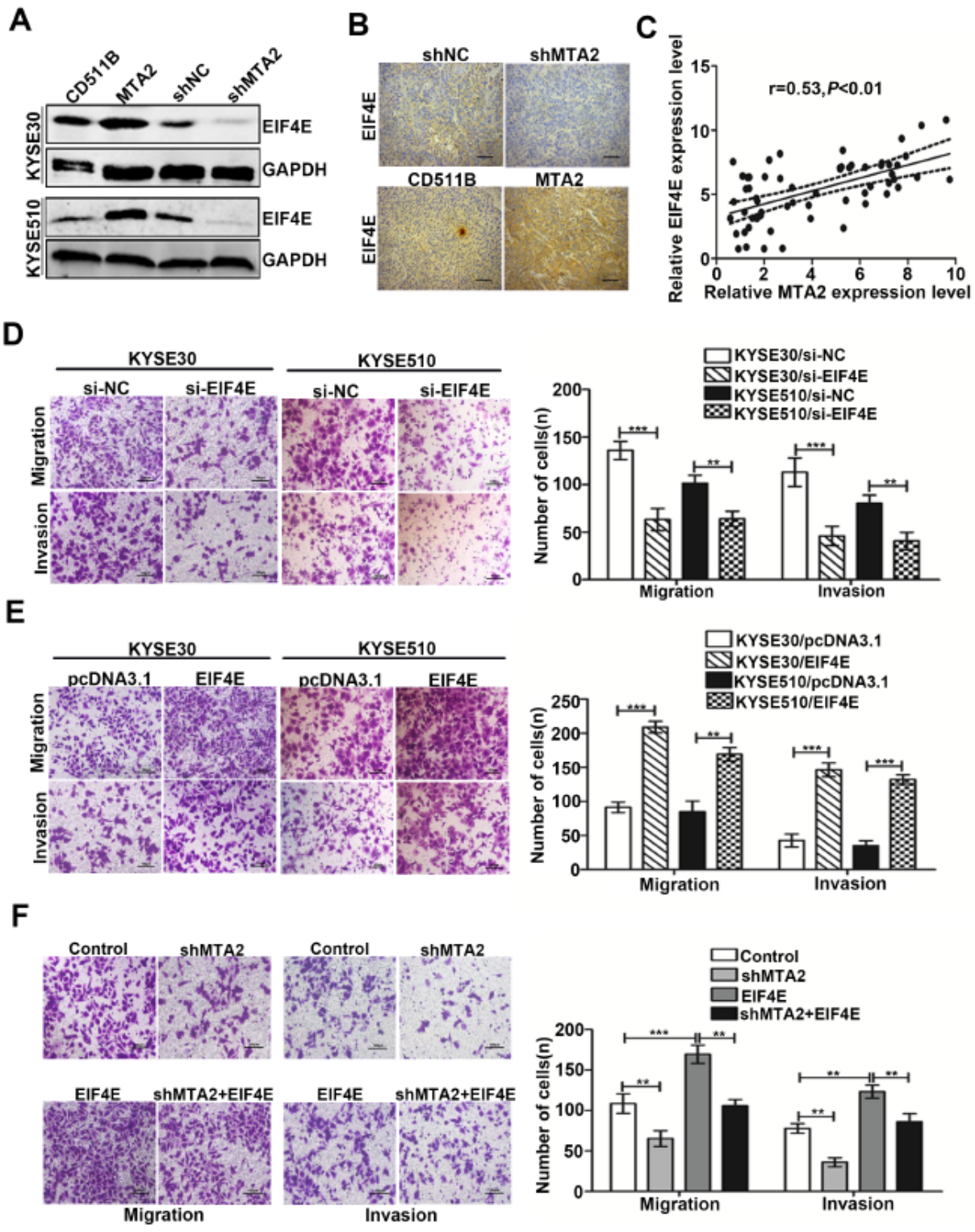


Figure 6

The MTA2-mediated promotion of malignancy in ESCC is mediated by EIF4E. (A) Western blot assay was performed to examine the expression of EIF4E after MTA2 knockdown or overexpressed in KYSE30 and KYSE510 cells. (B) The expression of EIF4E in mice tumor tissues from different treat groups was examined by IHC. (C) The correlation between MTA2 and EIF4E expression in ESCC tissues was analyzed by Spearman test. (D) Transwell assays were performed in control and EIF4E-knockdown KYSE30 and

KYSE510 cells. The numbers of cells that migrated or invaded were counted in five different fields. Original magnification, ×200. (E) Transwell assays were performed in control and EIF4E-over expressed KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in five different fields. Original magnification, ×200. (F)Reinforce of EIF4E expression reversed the MTA2 downregulation-reduced migration and invasion of KYSE30 and KYSE510 cells. **P < 0.01, ***P < 0.001

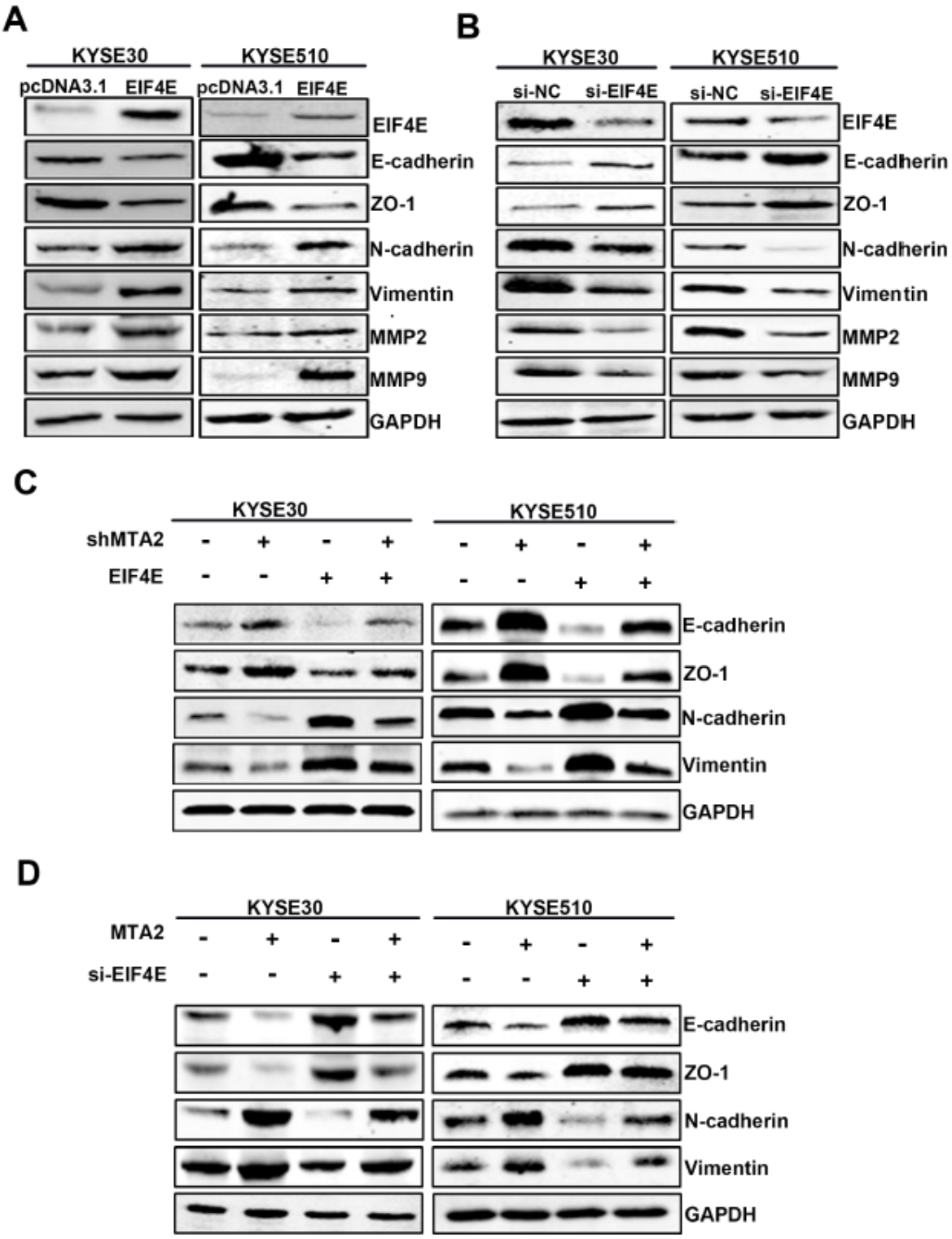


Figure 7

MTA2 promotes epithelial-mesenchymal transition in ESCC via the regulation of EIF4E. (A) Western blot analyzed the expression of EMT markers in EIF4E- overexpressed KYSE30 and KYSE510 cells. (B) Western blot analyzed the expression of EMT markers in EIF4E- depleted KYSE30 and KYSE510 cells. (C) Overexpression of EIF4E reversed the effect of MTA2-down regulation on EMT transformation. (D)Knocking down EIF4E expression reversed the effect of MTA2-up regulation on EMT transformation.

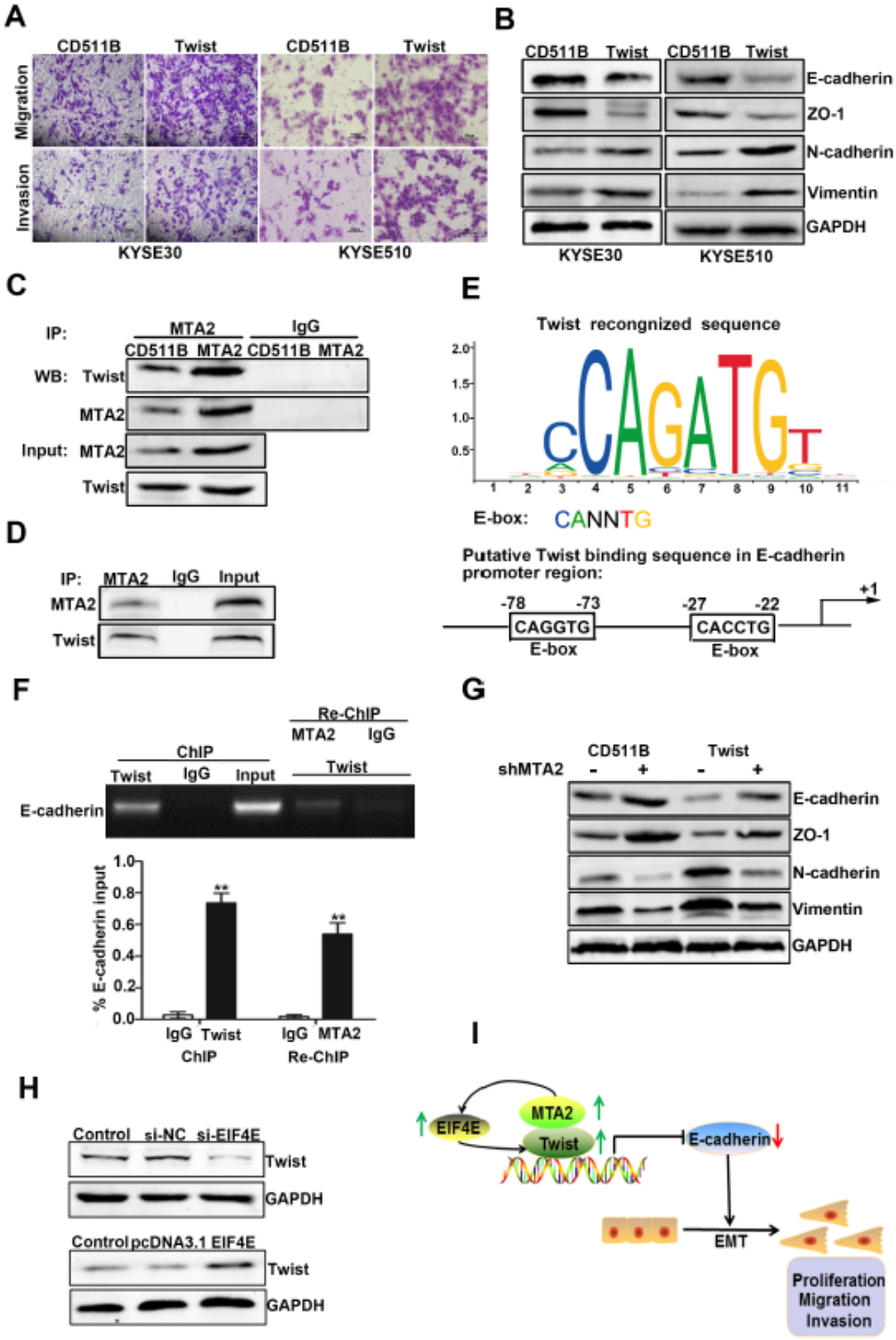


Figure 8

MTA2 was recruited by Twist to repress E-cadherin expression. (A) Transwell assays were performed in control and Twist-overexpressed KYSE30 and KYSE510 cells. The numbers of cells that migrated or invaded were counted in five different fields. Original magnification, ×200. (B) Western blot analyzed the expression of EMT markers in Twist- overexpressed KYSE30 and KYSE510 cells. (C) Coimmunoprecipitation assays were performed in control and MTA2- overexpressed KYSE30 cells. IgG was used as a negative control. (D) Endogenous MTA2 and Twist were coimmunoprecipitated from KYSE30 cells and the bound endogenous MTA2 and Twist were examined by immunoblotting. (E) Twist recognized consensus site was identified in the promoter region of E-cadherin using a bioinformatics website (http:// jaspar.genereg. net/matrix/MA1123.1/). The number represents the nucleotide position relative to the transcription start site (+1). (F) ChIP-re-ChIP assays were performed in KYSE30 cells to examine the recruitment of Twist and MTA2 onto E-cadherin promoter. (G) The rescue experiment with western blot analyzed the expression of EMT markers in KYSE30 cells. (H)The effect of EIF4E on the expression of Twist was analyzed by western blot. *P< 0.05, **P< 0.01.

Supplementary Files

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