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ETV4 promotes the progression of gastric cancer through regulating KDM5D

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Abstract. – OBJECTIVE: This study was aimed to investigate the expression characteristics of ETS variant 4 (ETV4) in gastric cancer (GCa), and to further explore whether it promotes the development of GCa by regulating KDM5D.

PATIENTS AND METHODS: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression of ETV4 in 35 pairs of tumor tissue and paracancerous tissue specimens collected from GCa patients, and the interplay between ETV4 expression and clinical indexes, as well as the prognosis of GCa patients, were analyzed. Meanwhile, the expression of ETV4 in GCa cell lines was verified using qRT-PCR assay. Furthermore, ETV4 knockdown model was constructed using lentivirus in GCa cell lines including AGS and BGC-823, and then, the transwell invasion and cell wound healing assays were applied to analyze the effect of ETV4 on the biological function of GCa cells. In addition, an in-depth study of the relationship between ETV4 and KDM5D was conducted.

RESULTS: The results of qRT-PCR showed that the expression level of ETV4 in GCa tissue samples was remarkably higher than that in adjacent tissues, and the difference was statistically significant. Compared with patients with low expression of ETV4, the patients with high ETV4 expression had a higher occurrence rate of lymph node or distant metastasis and a lower overall survival rate. Similarly, the metastasis ability of GCa cells in the ETV4 expression knockdown group (sh-ETV4) was remarkably decreased when compared with the sh-NC group. In addition, qRT-PCR results indicated that the protein expression of KDM5D was significantly increased after the knockdown of ETV4. Therefore, it was demonstrated that ETV4 might be able to regulate the malignant progression of GCa via modulating KDM5D expression. Finally, the results of the cell reverse experiment confirmed that the silence of ETV4 could reverse the malignant progression of GCa induced by the downregulation of KDM5D.

CONCLUSIONS: ETV4 expression was found remarkably elevated in GCa tissues and was significantly associated with the occurrence of lymph node or distant metastasis and poor prognosis. In addition, ETV4 might promote GCa cell metastasis by modulating KDM5D.

Key Words:

ETV4, KDM5D, Gastric cancer (GCa), Metastasis.

Introduction

Gastric cancer (GCa) is the most common malignant tumor of the digestive system clinically, with its mortality rate among the top three in the world[1-3]. In recent years, with the promotion of China's economic and social development and the early screening of cancer, the mortality rate of GCa has remarkably decreased, but the standardized mortality rate in 2018 is still in the third place[4,5]. Due to the lack of a national GCa screening system, the diagnosis of GCa is mostly confirmed in an advanced stage, and the 5-year survival rate after radical gastrectomy is only about 40%, even though the expanded radical gastrectomy fails to significantly improve the postoperative survival rate[6,7]. In order to further improve the curative effect of GCa treatment, the treatment mode of GCa has gradually changed from the radical treatment to the comprehensive. The molecular targeted therapy of GCa is gradually clinically applied. However, how to further improve the therapeutic effect of the molecular targeted therapy for advanced GCa is the current problems[8,9]. Therefore, understanding the mechanism of occurrence and development of GCa is the premise of molecular targeted therapy[9].

ETS variant 4 (ETV4), a member of the ETS family polyomavirus enhancer activator 3 (PEA3) subfamily, has a complex structure and function, and its overexpression can lead to malignant transformation of cells and enhance tumor cell invasion[10,11]. Chen et al[12] have reported that the inhibition of the expression of ETV4 can slow down the proliferation and metastasis of breast cancer cell lines in animal

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experiments; by contrast, the upregulation of its expression can cause the tumorigenic effects on breast tissue. Some reports[13,14] have shown that ETV4 plays a role in promoting cancer in some human tumors, but the specific mechanism still remains elusive, and its role in GCa is unknown.

High invasiveness and metastasis are one of the main features of GCa, which are involved in the regulation of multiple gene expression changes and multiple signal transduction pathways[15,16]. Epithelial-mesenchymal transition (EMT) is one of the key mechanisms for tumor cells to acquire the ability of invasion and metastasis[17,18]. The triggering of EMT in tumor cells is usually accompanied by changes in cell morphology and molecular markers, leading to the destruction of cell polarity[18]. EMT can also cause degradation of some extracellular matrix and basement membrane, so as to effectively damage and destroy the histological barrier that blocks tumor cell invasion, and eventually cause tumor cells to detach from the primary site with complete structure and invade and metastasize to surrounding or distant normal tissues[19,20].

Therefore, we aimed to investigate the relationship between ETV4 and the malignant progression and clinical characteristics of GCa, and analyze the correlation between the expression of ETV4 in GCa tissues and existing pathological indicators, so as to explore whether ETV4 mediates the invasion and metastasis of gastric cancer through the regulation of KDM5D and other related EMT pathways, so as to provide a new target for the diagnosis and treatment of this tumor.

Based on this, we analyze the relationship between ETV4 and malignant progression and clinical characteristics of GCa, and the correlation between the expression of ETV4 in GCa tissues and the existing pathological indicators. We want to explore whether ETV4 mediates the invasion and metastasis of GCa through the regulation of KDM5D and other related EMT pathways and provide a new target for the diagnosis and treatment of GCa.

Patients and Methods

Patients and GCa Samples

In this work, 35 pairs of GCa tissue specimens, and their corresponding adjacent ones were selected from surgically treated GCa cases and then stored at -80°C. The collection of the clinical specimens was approved by the Ethics Committee of Zhangzhou Affiliated Hospital of Fujian Medical University, and patients and their families had been fully informed that their specimens would be used for scientific research. All patients participating in this investigation signed an informed consent.

Cell Lines and Reagents

Human GCa cell lines (AGS, BGC-823, SGC-7901) and immortalized normal gastric mucosal epithelial cell line (GES-1) were purchased from American American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) high glucose medium containing 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C, in a 5% CO_2 incubator. The cells were passaged with 1% trypsin + ethylenediaminetetraacetic acid (EDTA) for digestion when they reached 80%-90% confluence.

Transfection

The negative control group (sh-NC) and the lentivirus containing the ETV4 knockdown sequence (sh-ETV4) were purchased from Shanghai Jima Company (Shanghai, China). The cells were plated in 6-well plates and grown to a cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After 48h, the cells were collected for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Transwell Cell Migration and Invasion Assay

After 48 h of transfection, the cells were trypsinized and resuspended in serum-free medium. After cell counting, the diluted cell density was adjusted to 2.0×10^5/ml, and the transwell chamber containing Matrigel and no Matrigel was placed in a 24-well plate. 200 μL of the cell suspension was added in the upper chamber, and 500 μL of a medium containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) was added to the lower chamber. After incubation in a 37°C incubator for 48 h, the chamber was removed, fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet for 15 min. Subsequently, the cells were washed with phosphate-buffered saline (PBS), and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

Cell Wound Healing

The cells after transfection for 48 h were digested, centrifuged, and resuspended in the medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of the cells plated was set to 50,000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, the cells were gently rinsed with PBS for 2-3 times and observed again after 24 h incubation with low-concentration serum medium (such as 1% FBS).

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and the total RNA was extracted. The initially extracted RNA was treated with DNase I to remove the genomic DNA and purify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, Real-Time PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions, and the PCR reaction was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicate wells were repeated for each sample, and the assay was repeated twice. The Bio-Rad PCR instrument (Hercules, CA, USA) was used to analyze and process the data with the software iQ5 2.0. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers were as follows: ETV4: Forward: 5'-GATGAAAGCCGGATACTTGGAC-3'; Reverse: 5'-TTCGCGCAAGCTCCCATTT-3'; KDM5D: Forward: 5'-CAAGACCCGCTTGGCTACATT-3'; Reverse: 5'-TTGGACGCGAGGAGTAAATCT-3'; GAPDH: Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Western Blot

The transfected cells were lysed using PRO-PREP™ lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 g for 15 min at 4°C. The total protein concentration was calculated by the HCCA Protein Assay Kit (Pierce, Rockford, IL, USA). Rabbit anti-human monoclonal antibodies against E-cadherin, N-cadherin, Vimentin, MMP-9, KDM5D, and ETV4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from GenScript (Nanjing, China). GAPDH was used as the internal reference control. The protein samples were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), and blocked with 5% skim milk powder for 1 h at room temperature. The primary antibodies were added for incubation overnight at 4°C shaker. In the next day, the membrane was rinsed 3 times with Tris-Buffered Saline and Tween (TBST) and incubated with the second antibody for 1 h at room temperature. After that, the protein samples on the membrane were finally semi-quantitatively analyzed by alpha SP image analysis software.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism5 V5.01 software (La Jolla, CA, USA). The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment, and the data were shown as averaged ± standard deviation. There were three levels of $p<0.05$, $p<0.01$, and $p<0.001$ at the significance level, and $p<0.05$ was considered statistically significant.

Results

ETV4 Was Highly Expressed in GCa Tissues and Cell Lines

The expression of ETV4 in 35 pairs of GCa tumor tissue and adjacent normal tissue specimens was detected by qRT-PCR, and the results showed that ETV4 expression was higher in the former than in the latter (Figure 1A). In addition, the expression of ETV4 in patients with distant metastasis was found remarkably higher than that those without (Figure 1B). Meanwhile, ETV4 was also found highly expressed in GCa cell lines compared with the normal gastric cell line (GES-1), suggesting that ETV4 might act as a cancer-promoting gene in GCa (Figure 1C).

ETV4 Expression Was Correlated With Lymph Node, Distance Metastasis and Overall Survival in GCa Patients

According to the ETV4 mRNA expression, the above-collected tissue samples were divided into

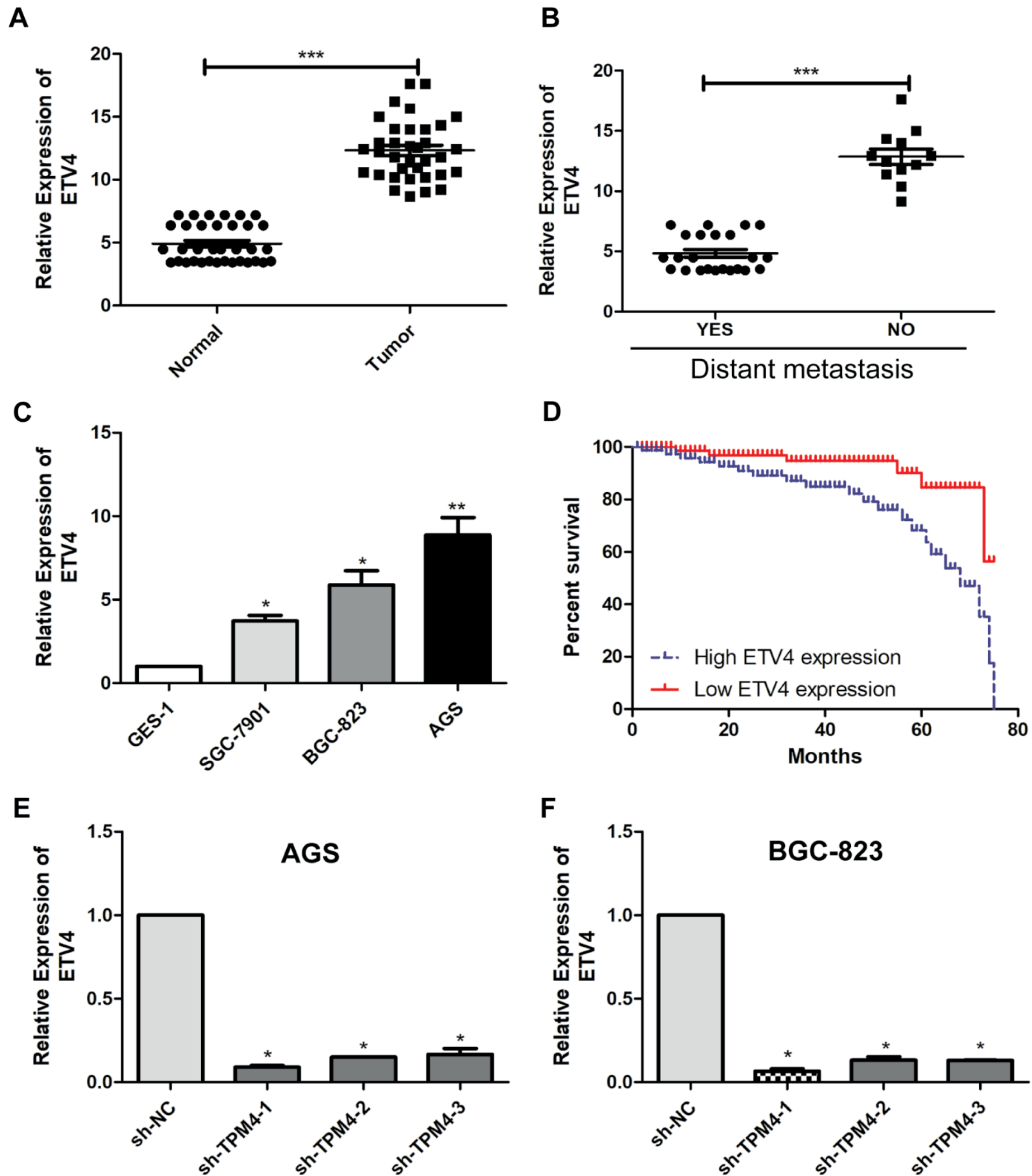

Figure 1. ETV4 is highly expressed in gastric cancer tissues and cell lines. **A,** qRT-PCR was used to detect the difference in the expression of ETV4 in gastric cancer tissues and adjacent tissues. **B,** qRT-PCR was used to detect the difference in the expression of ETV4 in tissue samples from patients with gastric cancer with distant metastasis. **C,** qRT-PCR was used to detect the expression level of ETV4 in gastric cancer cell lines. **D,** Based on the Kaplan-Meier survival curve of gastric cancer patients with ETV4 expression, the prognosis of patients with high expression was found significantly worse than that of the low expression group. **E,** qRT-PCR was used to verify the interference efficiency of ETV4 after transfection of the ETV4 knockdown vector in AGS gastric cancer cell line. **F,** qRT-PCR was used to verify the interference efficiency of ETV4 after the transfection of ETV4 knockdown vector in BGC-823 cell line. Data are mean ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$.

high expression and low expression group. At the same time, the relationship between the expression of ETV4 and the age, sex, pathological stage, lymph node, or distant metastasis of GCa patients were analyzed. As shown in Table I, the high expression of ETV4 is positively correlated with lymph node or distant metastasis, but not with age, gender, and pathological stage. In addition, the relevant fol-

low-up data were collected to explore the association between ETV4 expression and the prognosis of GCa patients. As a result, the Kaplan-Meier survival curves revealed that the high expression of ETV4 was remarkably associated with poor prognosis of GCa; namely, the higher the expression level of ETV4, the worse the prognosis ($p<0.05$; Figure 1D).

Knockdown of ETV4 Inhibited Cell Metastasis in GCa

To investigate the influence of ETV4 on GCa cell biological functions, the ETV4 knockdown lentiviral vector was constructed. After transfecting the vector in AGS and BGC-823 cell lines, the qRT-PCR assay was performed to verify the interference efficiency, and the difference was shown statistically significant (Figures 1E, 1F). After the knockdown of ETV4, the transwell and cell wound healing assays were performed; as a result, the invasion and migration abilities, as well as the crawling capacity of GCa cells was found remarkably decreased when compared with the sh-NC group (Figures 2A, 2B).

Downregulation of ETV4 Decreased the Expression of EMT Signaling Pathway

In order to further explore the ways in which ETV4 promotes the malignant progression of GCa, after the downregulation of ETV4, we examined the expression of the key proteins in the EMT pathway by Western blot method. The result revealed that the silence of ETV4 resulted in a significant increase in the expression of EMT-related key proteins, including E-cadherin and KDM5D. By contrast, the expression of other proteins, including N-cadherin, Vimentin, and MMP-9 was found remarkably decreased (Figure 3A).

KDM5D Was Lowly Expressed in GCa Tissues and Cell Lines

Subsequently, qRT-PCR was used to detect the differential expression of KDM5D in GCa tumor samples and adjacent ones, and the results indicated that KDM5D expression was lower in the former than in the latter (Figure 3B). In addition, the Kaplan-Meier survival curves revealed that the low expression of KDM5D was significantly associated with poor prognosis of GCa, indicating that, the lower the KDM5D expression, the worse the prognosis ($p <0.05$; Figure 3C). Therefore, we subsequently detected the expression of ETV4 along with KDM5D in GCa tissues using qRT-PCR; as a result, the expression levels of the above two genes were shown negatively correlated (Figure 3D). Furthermore, after the knockdown of KDM5D, ETV4 protein, and mRNA expression levels were both found conspicuously increased (Figures 3E, 3F).

ETV4 Modulated KDM5D Expression in Human GCa Cells

ETV4 was downregulated in GCa cell lines on the basis of the silence of KDM5D to investi-

Table I. Association of ETV4 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	ETV4 expression Low (%)	ETV4 expression High (%)	*p*-value)
Age (years)				0.105
<60	13	9	4	
≥60	22	9	13	
Gender				0.238
Male	18	11	7	
Female	17	7	10	
T stage				0.122
T1-T2	23	14	9	
T3-T4	12	4	8	
Lymph node metastasis				0.042
No	26	16	10	
Yes	9	2	7	
Distance metastasis				0.003
No	23	16	7	
Yes	12	2	10	

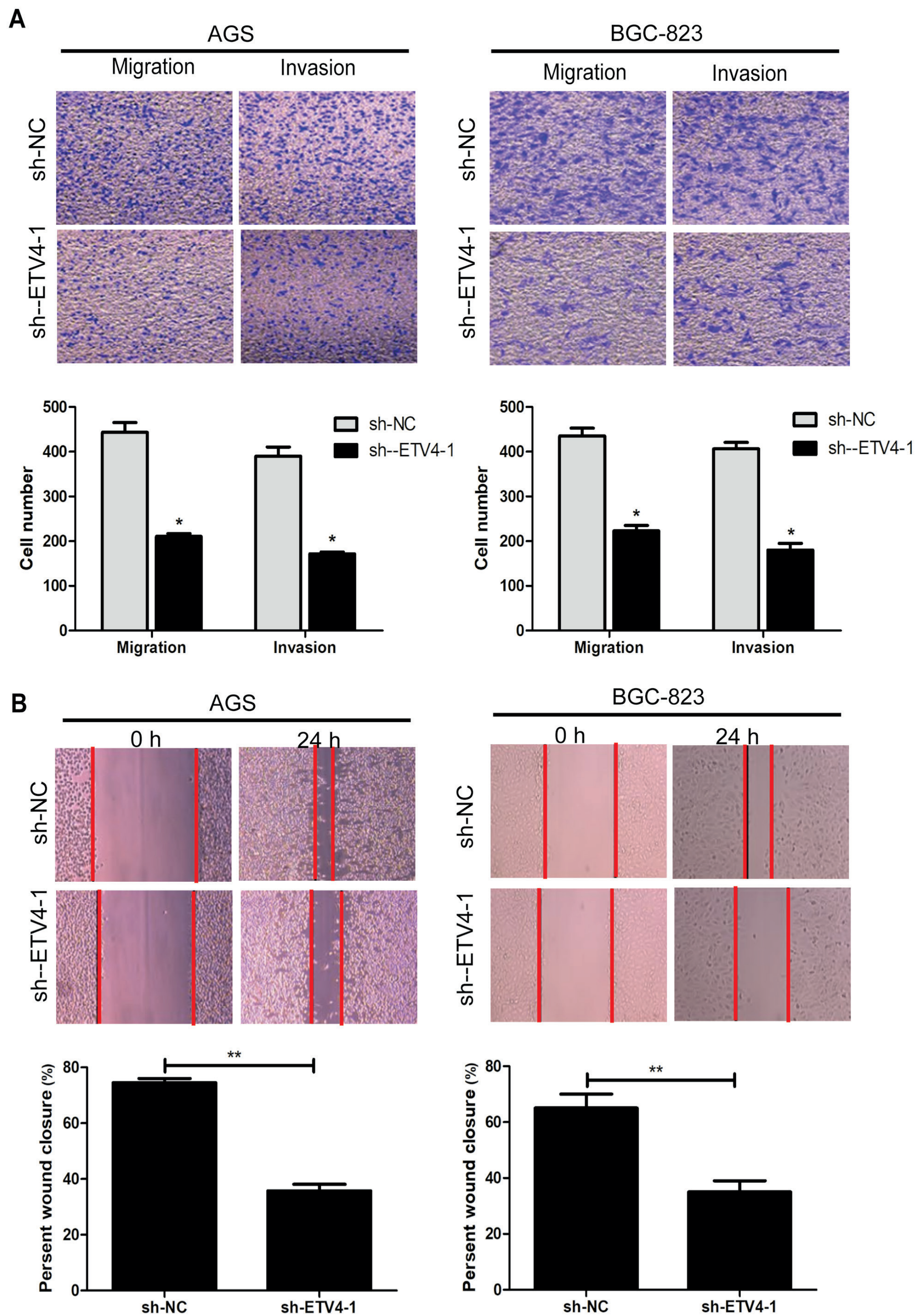

Figure 2. Knockdown of ETV4 inhibits the metastasis of gastric cancer cells. **A,** The transwell migration assay was used to detect the ability of the cells to invade and migrate to AGS and BGC-823 gastric cancer cell lines after the knockdown of ETV4 (magnification: 20×). **B,** The cell scratch assay was used to detect the cell crawling ability of AGS and BGC-823 gastric cancer cell lines after the knockdown of ETV4 (magnification: 20×). Data are mean ± SD, *$p < 0.05$, **$p < 0.01$.

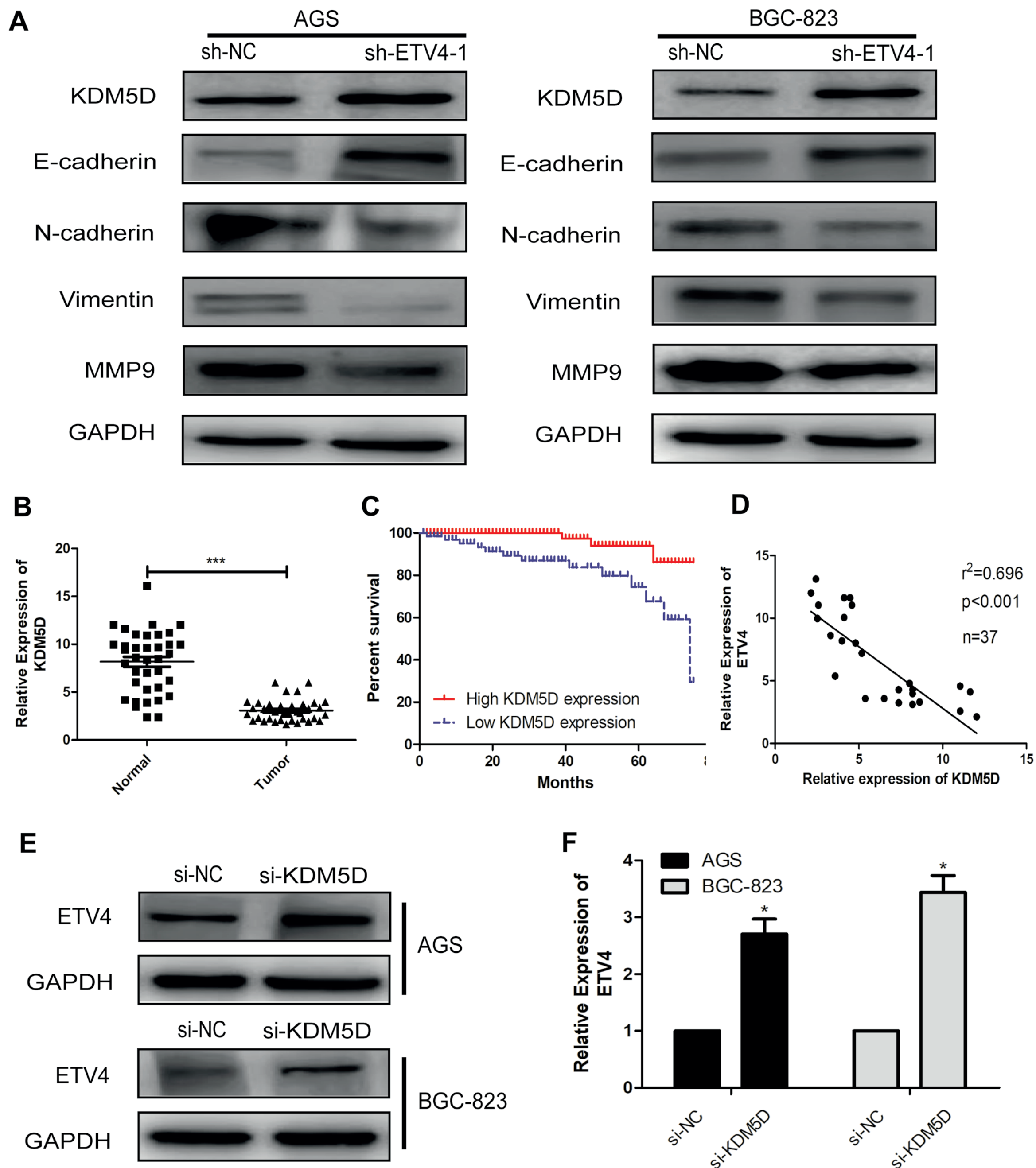

Figure 3. Interaction of ETV4 and KDM5D in gastric cancer. **A,** Western blotting was used to verify the expression levels of KDM5D, E-cadherin, N-cadherin, Vimentin, and MMP-9 proteins after interference with ETV4 in AGS and BGC-823 cell lines. **B,** qRT-PCR was used to detect the differential expression of KDM5D in gastric cancer tissues and adjacent tissues. **C,** The Kaplan-Meier survival curve of gastric cancer patients was shown based on KDM5D expression; the prognosis of patients with low expression was significantly worse than that of the high expression group. **D,** There was a significant negative correlation between the expression levels of ETV4 and KDM5D in gastric cancer tissues. **E,** Western blotting verified the interference efficiency of ETV4 after the transfection of the KDM5D knockdown vector in AGS and BGC-823 gastric cancer cell lines. **F,** qRT-PCR verified the interference efficiency of ETV4 after transfection of the KDM5D knockdown vector in AGS and BGC-823 gastric cancer cell lines. Data are mean ± SD, $*p < 0.05$, $***p < 0.001$.

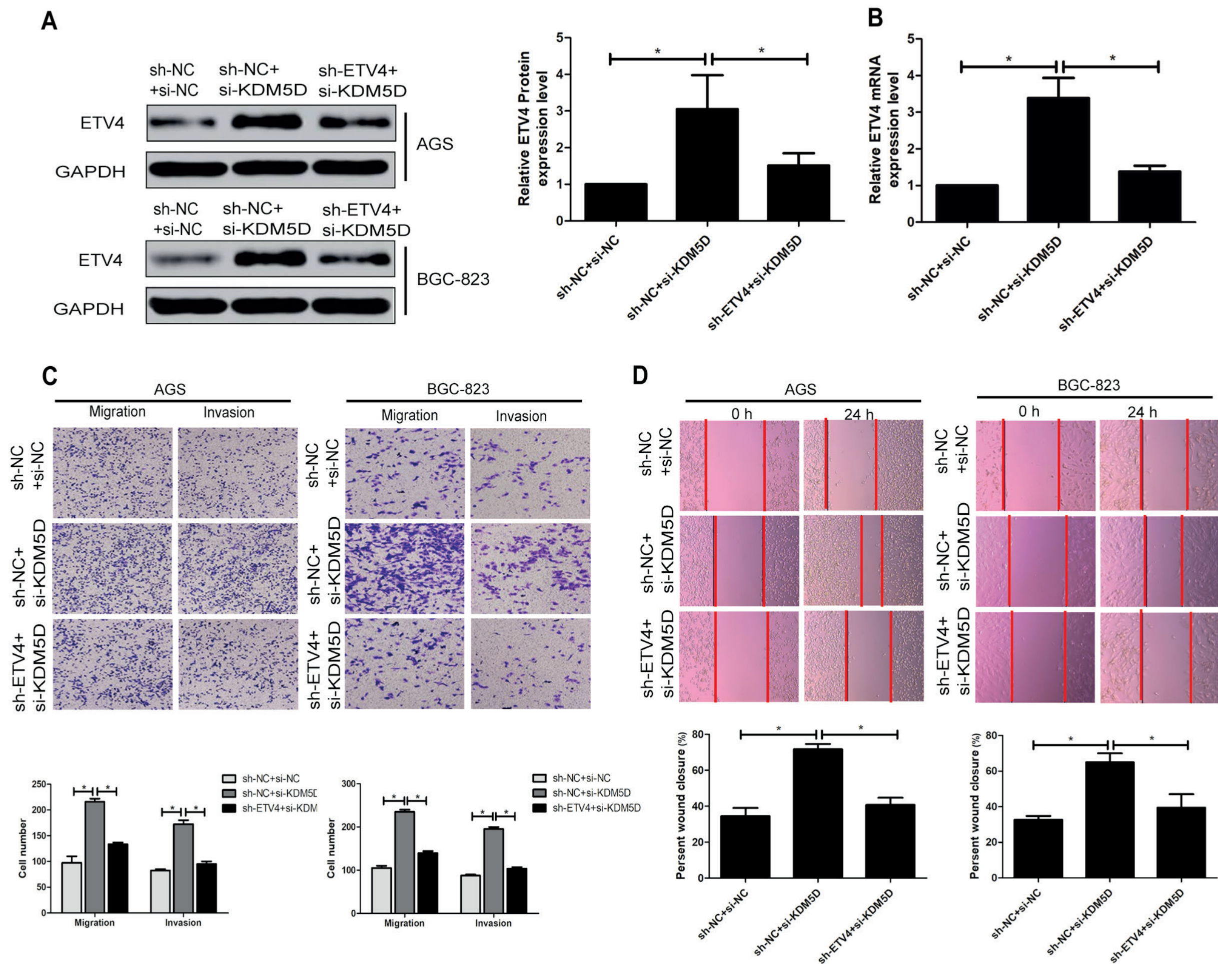

Figure 4. ETV4 regulates KDM5D to promote the malignant progression of gastric cancer. **A,** The expression level of ETV4 protein after the co-transfection of ETV4 and KDM5D was detected by Western blotting. **B,** The mRNA expression level of ETV4 after the co-transfection of ETV4 and KDM5D was detected by qRT-PCR. **C,** The transwell migration assay was used to detect the invasion and migration ability of gastric cancer cells after the co-transfection of ETV4 and KDM5D in AGS and BGC-823 cell lines (magnification: 20x); **D,** The cell scratch assay was performed to detect the crawling ability of gastric cancer cells after the co-transfection of ETV4 and KDM5D in AGS and BGC-823 cell lines (magnification: 20x). Data are mean ± SD, $*p < 0.05$.

gate the interaction between ETV4 and KDM5D in GCa cells. qRT-PCR and Western Blot assays were respectively performed to examine the expression levels of ETV4 in each treatment group (Figures 4A, 4B). Subsequently, the transwell assay and reverse cell experiments were performed. As a result, the silence of ETV4 was found to be capable of reversing the effect of KDM5D on migration ability in GCa cell lines (Figures 4C, 4D).

Discussion

Previous research methods[7] can only detect one or several genes at a time, and it is difficult to explain the whole process of tumor occurrence, invasion, and metastasis. Gene chip is one of the most effective techniques to detect the differential expression profile of tumor genes, which is of great significance to explore the molecular mechanism of the occurrence, development, invasion, and metastasis of gastric cancer, and to look for molecular markers and gene therapy targets for the early diagnosis and prognosis of gastric cancer[8,9].

The etiology of gastric cancer is complex, and there is no clear conclusion on the pathogenesis except for the helicobacter pylori[2-5]. Currently, the people's understanding of the pathogenesis, etiology, biological behavior, and treatment of gastric cancer still has great limitations[1,5,6]. The study of

the molecular mechanisms contributes to the early diagnosis, accurate assessment, treatment, and prognosis of GCa, thereby improving the survival rate of patients[4-6]. The occurrence, development, invasion, and metastasis of tumors are a multi-event process, influenced by a variety of biomolecules and regulated by signaling pathways[6,7]. Previous research methods[7] can only detect one or several genes at a time, and it is difficult to interpret the whole process of tumorigenesis, infiltration, and metastasis. Gene chip is one of the most effective techniques for detecting differential expression profiles of tumor genes, which is of great significance for exploring the molecular mechanism of gastric carcinogenesis, invasion, and metastasis, and searching for molecular markers and gene therapy targets for the early diagnosis and prognosis of GCa[8,9].

ETS was first discovered in the gene sequence of the avian myeloablative virus E26 by the Molecular Oncology Laboratory of the National Cancer Institute of Frederick, USA[10-12]. As a member of the ETA family of the PEA3 subfamily, ETV4 was also known as the adenovirus ElA enhancer-binding protein (ElAF)[11]. It was found that ETV4, the gene was expressed at low levels in normal tissues of various organs in human embryonic and adult stages[12-14]. In the comparative analysis of normal tissues and cancer tissues of GCa, ETV4 mRNA level was detected to show a significant difference between the two tissues, and ETV4 may play a key role in the genetic regulation of gastric cancer.

In this work, ETV4 knockdown of GCa cell lines established by lentivirus was demonstrated to promote the production of malignant progression of gastric cancer through a series of cellular functional experiments. Next, it was found that ETV4 expression in tumor tissues of gastric cancer patients was significantly higher than that in adjacent tissues and was positively correlated with the incidence of lymph node or distant metastasis and poor prognosis of gastric cancer, suggesting that ETV4 may play a pro-cancer role in this cancer. In order to further investigate the effect of ETV4 in the metastatic ability of GCa cell lines. The transwell, migration, invasion, and cell wound healing assays were conducted. The results showed that, after the downregulation of ETV4, the invasion, migration, and crawling ability of gastric cancer cell lines were significantly reduced compared with the sh-NC group, indicating that ETV4 knockdown could inhibit the metastasis ability of gastric cancer cell lines. The results of this report provide a theoretical basis for revealing the mechanism of GCa development. Of course, the specific molecular mechanism of signal transduction needs further investigations.

EMT refers to the biological process in which epithelial cells transform into mesenchymal phenotype after being stimulated by external signals[15]. In this process, the epithelial cells lose their polarity, lose the tight adhesion between cells, and the expression of epithelial markers is downregulated or missing. Meanwhile, they gradually acquire the morphological characteristics of the interstitial cells and the expression of interstitial markers, and the ability of movement and migration of cells is enhanced[16,17]. The most significant molecular event in EMT process was a decrease in the E-cadherin expression and an increase in the expression of N-cadherin, Vimentin, and MMP9, which can induce EMT process[18,19]. Therefore, we examined the expression of these EMT-related classical markers in GCa, clarified their role in the prognosis, and tried to select the key molecules for screening patients with a high risk of metastasis, so as to provide effective auxiliary support for the judgment, metastasis prediction, and individualized treatment of GCa[19,20]. However, until now, the relevant mechanism of EMT in the development and progression of GCa has not been fully explored. KDM5D is a regulatory molecule in the EMT process, and bioinformatics suggests its targeted regulation with ETV4. In addition, the silence of ETV4 was found to be able to result in a significant increase in KDM5D expression, thereby regulating EMT signaling pathway to promote the development of GCa. At the same time, the result of the cell reverse experiment demonstrated that the silence of ETV4 could reverse the malignant progression of GCa caused by the knockdown of KDM5D, suggesting that the transcriptional activity of the gene locus where ETV4 is located may be regulated by the key protein KDM5D. In summary, the above findings indicated that ETV4 might promote the invasion and migration ability of GCa cells by altering the KDM5D expression.

Conclusions

We demonstrated that the expression of ETV4 was remarkably increased in the proliferation of GCa and was conspicuously associated with lymph node or distant metastasis, as well as the poor prognosis of GCa patients. In addition, ETV4 may promote the metastasis of GCa *via* regulating KDM5D.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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