Original Article

Expression profile, regulatory mechanism and prognostic potential of MBNL2 in esophageal squamous cell carcinoma

Shenglai Zhang[1#], Xiaoqin Chu[2#], Yan Zhang[1#], Jianwei Qiu[1]^, Liuhong Pan[1], Liugen Gu[1], Haifeng Kang[1], Lin Wang[1]

[1]Department of Gastroenterology, The Second Affiliated Hospital of Nantong University, Nantong, China; [2]Department of Gastroenterology, Hai'an People's Hospital, Hai'an, China

Contributions: (I) Conception and design: X Chu, J Qiu; (II) Administrative support: Y Zhang, S Zhang; (III) Provision of study materials or patients: Y Zhang, S Zhang, L Pan, X Chu; (IV) Collection and assembly of data: S Zhang, X Chu, Y Zhang; (V) Data analysis and interpretation: Y Zhang, L Wang, H Kang, L Gu, L Pan; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Jianwei Qiu, MM. Department of Gastroenterology, The Second Affiliated Hospital of Nantong University, No. 666 Shengli Road, Nantong 226001, China. Email: ppfoxs@126.com.

Background: It remains to refresh the understanding about the pathogenic mechanism of esophageal squamous cell carcinoma (ESCC). This study aimed to profile the expression of muscleblind like protein 2 (MBNL2), as well as its associations with ESCC behaviors.

Methods: Bioinformatic tools were used to mine The Cancer Genome Atlas (TCGA) database for the expression data of MBNL2 in ESCC. The expression of MBNL2 in tissue microarray of 179 ESCC patients was determined by immunohistochemistry (IHC), and the relationship of MBNL2 with patients' clinical and pathological characteristics was analyzed. The expression of MBNL2 was tested in fresh ESCC and adjacent normal tissues *in vitro*. Experiments about cellular invasion, migration and proliferation were performed to detect the impacts of silencing MBNL2 on the biological behaviors of ESCC, and the positive results were checked *in vivo*.

Results: In the TCGA database, the expression of MBNL2 in ESCC was higher than that in adjacent tissues ($P<0.05$). The protein level of MBNL2 in the tissue microarray of 179 ESCC patients was positively correlated with tumor stage and lymph node metastasis, and negatively correlated with the prognosis of patients. The expression of MBNL2 was significantly upregulated in five fresh ESCC tissues, compared to that in adjacent tissues. In functional experiments, knocking down MBNL2 significantly inhibited the migration and invasion of ESCC cell lines KYSE150 and Eca109, but had no significant effect on their proliferation. Finally, silencing MBNL2 inhibited the epithelial-mesenchymal transition (EMT) of ESCC cells, as evidenced by the upregulation of E-cadherin, the downregulation of Snail and Slug.

Conclusions: MBNL2 is highly expressed in ESCC and associated with its Tumor Node Metastasis (TNM) stage, lymph node metastasis and prognosis. MBNL2 may promote ESCC progression through facilitating EMT.

Keywords: Muscleblind like protein 2 (MBNL2); esophageal squamous cell carcinoma (ESCC); epithelial-mesenchymal transition (EMT); prognosis

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^ ORCID: 0009-0008-3442-8183.

Introduction

According to the 2020 Global Cancer Report (1), esophageal squamous cell carcinoma (ESCC) ranks seventh in the prevalences and sixth in the mortalities of all cancers globally. Besides, the prevalence of ESCC is rising gradually, especially among the young. ESCC is not easy to be found in the early stage, and once diagnosed, it has often invaded the trachea and great vessels in the middle to late stages. Surgery, chemotherapy and radiotherapy can retard the progression of ESCC, but the 5-year survival rate is still less than 15% (2,3). It is urgent to dig deeper into the pathogenesis of ESCC and quest for potential molecular therapeutic targets.

Muscleblind like protein (MBNL), an RNA binding protein, can regulate tissue-specific alternative splicing, messenger RNA (mRNA) stability and trafficking, alternative polyadenylation, as well as microRNA (miRNA) biogenesis (4-8). MBNL is encoded by three genes: *MBNL1*, *MBNL2*, and *MBNL3* (9,10). *MBNL1* and *MBNL2* are ubiquitously expressed, whereas *MBNL3* is predominantly in the placenta (10). This gene encodes a CCCH-type (C3H-type) zinc finger protein that modulates alternative splicing of pre-mRNAs (11). C3H-type zinc finger is a highly conserved domain that uses zinc to combine three cysteine residues (C) and one histidine residue (H) to form a stable finger structure, which has maintained high stability throughout evolution. The MBNL family plays important roles in the differentiation of embryonic stem (ES) cells, as well as in neuronal differentiation. MBNL2, a negative regulator of alternative splicing, is differentially expressed between ES cells and other cell types (12). MBNL2 may also regulate the splicing pattern of cancer cells to promote tumorigenesis (13,14). As the deepening of cancer science research, its role in cancer is bidirectionally regulated. MBNL2 is upregulated in renal cell carcinoma (RCC), and facilitates M2 polarization to inhibit the MBNL2/Bcl-2/beckin 2-mediated autophagy and secretion of C-C motif chemokine 1, thus promoting the growth and metastasis of RCC cells (15). In hepatocellular carcinoma (HCC), high expression of MBNL2 is associated with a smaller tumor volume and a lower tumor stage, and MBNL2-positive liver cancer patients have a higher 5-year overall survival rate. Overexpression of MBNL2 inhibits the growth and invasion HCC cells and HCC in non-obese diabetes/server combined immune-deficiency (NOD/SCID) mice (16,17). We have identified that the expression of MBNL2 is abnormally high in ESCC tissues through The Cancer Genome Atlas (TCGA) database analysis. Our preliminary immunohistochemistry has confirmed that the expression of MBNL2 in ESCC tissue exceeds that in adjacent cancer tissue, which is consistent with the database analysis results.

Epithelial-mesenchymal transition (EMT) is an evolutionarily conservative developmental process, which is committed to cancer invasion and metastasis. Expressed in epithelial cells, E-cadherin can form tight junctional barriers between cells to maintain epithelial cell polarity, stability and integrity. When normal epithelial mucosal cells are damaged, the expression of E-cadherin decreases or even disappears. Snail protein is the earliest and strongest transcription inhibitor found in the EMT pathway (18). Snail can downregulate E-cadherin transcriptional activity, inhibit its expression, and promote distant metastasis of tumors by binding to E-box elements in E-cadherin promoter region (19). And it could break down the junctions between epithelial cells, thus depriving their polarity, enhancing their mobility, and then allowing tumor cells to penetrate mucosal barrier and acquire abilities to invade and metastasize. Slug, a member of the zinc finger protein family, has a structure similar to Snail, and contains 4–6 zinc fingers that form up a highly conserved carboxyl terminal and a variable amino terminal; Slug has been proved as an important regulatory factor in the EMT process (20). By binding to the E-box structure of the target gene, Slug inhibits the expression of E-cadherin, thereby promoting the formation of EMT. In a study of pancreatic cancer (21), inhibiting the expression of Slug reduces the ability of pancreatic cancer cells to spread. In this study, we investigated the expression of E-cadherin, Snail, and Slug

Highlight box

Key findings

- Muscleblind like protein 2 (MBNL2) may promote esophageal squamous cell carcinoma (ESCC) progression related to epithelial-mesenchymal transition (EMT).

What is known and what is new?

- MBNL2 contributes to the development of several types of cancer.
- This study confirmed that MBNL2 in ESCC is associated with its Tumor Node Metastasis (TNM) stage, lymph node metastasis and prognosis, and MBNL2 may be a potential therapeutic target for ESCC.

What is the implication, and what should change now?

- This study shows that MBNL2 is significantly involved in the metastasis and prognosis of esophageal cancer. Thus, MBNL2 may be a promising target for the treatment of ESCC.

and explored the possible mechanism of MBNL2 on the occurrence of EMT in ESCC.

Mounting evidence has shown the close relationship between MBNL2 and ESCC occurrence (22). However, the regulatory mechanism of MBNL2 in ESCC remains not fully understood, and its prognostic value has not been evaluated, both of which might are investigated in the present study. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1933/rc).

Methods

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of The Second Affiliated Hospital of Nantong University (No. 2021KT136) and individual consent for this retrospective analysis was waived.

Collection and storage of tissue samples

RNA-sequencing (RNA-seq) data of the expression profiles in 94 ESCC samples were downloaded from the TCGA database (http://www.cancer.gov/about-nci/organization/ccg/research/structual-genomics/tcga). RNA-seq data of the expression profiles in 516 normal esophageal samples were downloaded from the Genotype-Tissue Expression database (GTEX, USCS Xena, http://xena.ucsc.edu/) at the cancer genomics analysis platform. All data were standardized.

A total of 200 paraffin sections of ESCC tissues (44 female, 135 male; age from 47 to 82 years) and 50 of adjacent tissues (14 female, 36 male; age from 50 to 82 years) were collected from The Second Affiliated Hospital of Nantong University between October 2010 and May 2017. Five pairs of fresh ESCC and adjacent (≥5 cm outer of tumor margin) tissue samples were collected from The Second Affiliated Hospital of Nantong University in 2022. The samples were immediately placed in dry Eppendorf tubes (EP tubes) and stored at −80 ℃. The samples of all patients had been diagnosed by pathologists, and no radiotherapy or chemotherapy had been conducted at the time of sampling. Excluded were those with (I) incomplete clinical data or pathological paraffin specimens; (II) anticancer treatment before surgery for patients; (III) other malignant tumors.

Cell culture and lentivirus infection

KYSE150 and Eca109 ESCC cell lines were purchased from Shanghai Yuchi Biotechnology Co., Ltd., and cultured in RPMI 1640 medium (Invitrogen, Carlasbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlasbad, CA, USA) and 1% penicillin (Invitrogen, Carlasbad, CA, USA) and streptomycin (New Cell & Molecular Biotech Co., Ltd., Suzhou, China) at 37 ℃ with 5% CO_2. *MBNL2* gene was silenced by lentiviral short hairpin RNA (shRNA) (Shanghai Genechem Co., Ltd., Shanghai, China) infection to construct a stable transgenic strain. The ESCC cells were infected by the lentivirus in the complete medium for 16 h at 37 ℃, followed by incubation in fresh complete medium for additional 48 to 72 h. The transfection efficiency was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting (WB).

Cell counting kit 8 (CCK-8) assay

The cells were washed with phosphate buffer saline (PBS), resuspended in RPMI 1640, seeded into 96-well plates at a concentration of 2,000 cells/well, and incubated at 37 ℃ for 24 h. Next, CCK-8 reagent (DOJINDO, Kyushu Island, Japan) was added to the wells according to the manufacturer's instructions. Enzyme-linked immunosorbent assay (Biotek, Beijing, China) was performed to detect the absorbance value at 450 nm. The above steps were repeated at 48 and 72 h afterward.

Wound healing migration assay

The ESCC cells (5×10^6/mL) in exponential growth were isolated and seeded into 127 6-well tissue culture plates. When a confluent monolayer was formed, the monolayer was scratched vertically with the tip of a 100 μL sterile pipette. Then, the medium was gently washed three times with PBS, and the medium replaced with fresh RPMI-1640 medium. At 0, 24 and 48 h, a microscope (20×, Olympus, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan) was placed at three randomly selected positions to photograph the cells in migration. The images were analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA). The wound width was measured to represent cell migration.

Transwell invasion assay

The invasion model was established by using 24-well

Table 1 Primers for quantitative real-time polymerase chain reaction of *MBNL2* and *β-actin*

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
β-actin	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC
MBNL2	CCTTTACCAAAGAGACAAGCAC	ATTATCTGTTGGAATGAACGCG

MBNL2, muscleblind like protein 2.

Matrigel Invasion Chambers (pore size, 8 μm; Corning, Tewksbury, MA, USA). Every 2×10^5 ESCC cells were added into 100 μL of serum-free medium, and seeded onto the upper chambers. The lower chambers were filled with complete culture medium. After 24 h, the cells on the surfaces of the upper chambers were scraped off. Then, the cells having invaded were washed with PBS once, fixed with 4% paraformaldehyde for 20 to 30 min, washed with PBS twice, and stained with crystal violet staining solution for 15 min at room temperature. Cell images were captured under a microscope. Five visual fields were randomly selected from each group and analyzed by ImageJ.

WB analysis

The protein levels of MBNL2, E-cadherin, Snail and Slug in ESCC cells were detected by WB. According to the instructions of the Bicinchoninic Acid Assay (BCA) kit (Beyotime Biotechnology, Shanghai, China), the total protein was collected from cells. Rat-anti MBNL2 (Santa Cruz, California, USA, 1:500), E-cadherin (1:1,000, Cell Signaling Technology, Danvers, MA, USA), Snail (1:1,000, Wanlei Biotechnology Company, Shenyang, China), Slug (1:1,000, Wanlei Biotechnology Company, Shenyang, China) and their corresponding secondary antibodies were applied. Gray values were analyzed by ImageJ.

qRT-PCR assay

Total RNA was extracted from the tissues using TRIzol (Invitrogen, Grand Island, NY, USA) according to the standard TRIzol method. For each sample, first-strand complementary DNA (cDNA) was synthesized from every 0.5 μg of RNA by using PrimeScript™ RT Master MIX (Takara Biotechnology, Takara, Japan). qRT-PCR was performed on an ABI 7500 RT-PCR (BIO-LAB, Beijing, China) by using 2× SYBR Green qPCR Mix (Beyotime Biotechnology, Shanghai, China). *Table 1* shows the primers for FABP4 and the internal control GAPDH. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence staining

Staining was performed according to instructions of the Substance P (SP) kit. The primary antibody was MBNL2 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The slides were deparaffinized and rehydrated using routine methods, and then boiled in ethylene diamine tetraacetic acid (EDTA) retrieval solution to retrieve antigenicity. The non-specific blocking solution was incubated at room temperature for 15 min and then washed. The slides were incubated with primary antibody (1:100) overnight at 4 ℃, incubated with goat serum for 40 min at room temperature, and then with secondary antibody at room temperature for 40 min. Then, horseradish peroxidase (HRP)-labeled streptavidin was added and the slides were incubated for 10 min at room temperature. Diaminobenzidine (DAB) chromogenic reagent was introduced. The expression of MBNL2 was calculated using the proportion score and intensity score by two senior pathologists. The intensity was scored as follows (A): 0, negative; 1, weak expression; 2, moderate expression; and 3, strong expression. The proportion of positive cells was scored as follows (B): 0, 0% positive cells; 1, 1–25% positive cells; 2, 26–50% positive cells; and 3, 51–100% positive cells. The total score was calculated as A × B. A total score less than 3 was regarded as a low level of expression, otherwise as a high level of expression.

Statistical analysis

All experiments were repeated for three times or more, and data were expressed as mean ± standard deviation and subjected to the SPSS software (IBM SPSS statistics, Version 23.0) and R software 4.0.5. The Mann-Whitney *U* test was conducted to determine the statistical significance of continuous data. The Chi-squared test, contingency table or Fisher exact test was employed to determine the statistical significance of categorical data. The Kaplan-Meier survival curves are used to plot survival curves. The R.utils package, rjson package and XML package were used to read the original data downloaded

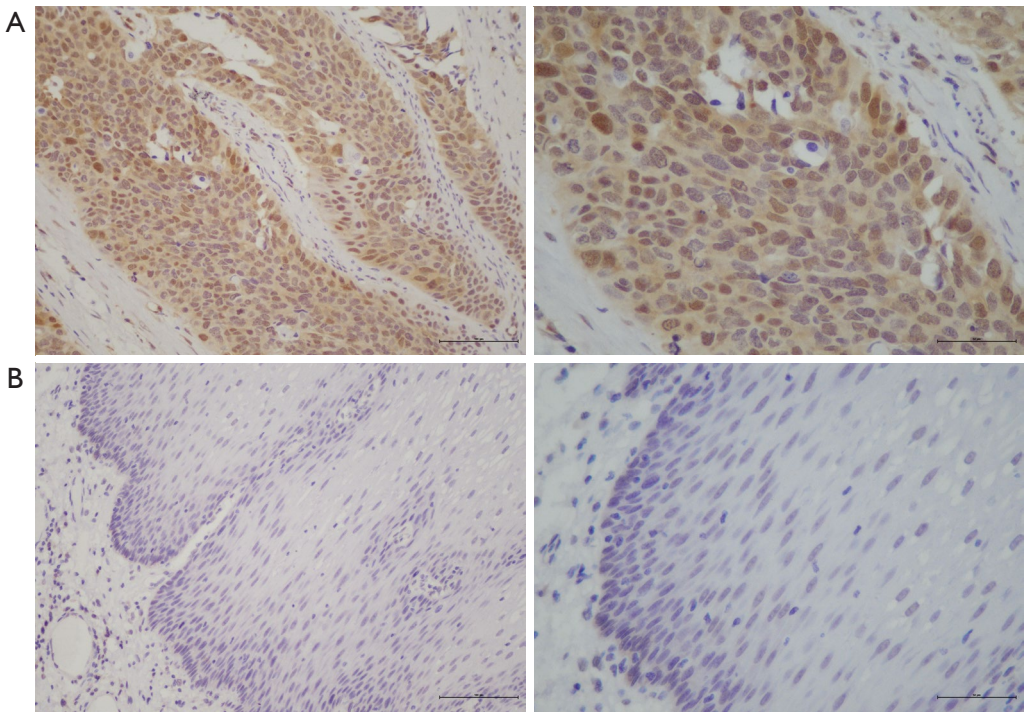


Figure 1 Immunohistochemical analyses of MBNL2 expression. (A) Positive expression in esophageal squamous cell carcinoma; (B) positive expression in normal adjacent tissues. Scale bar =100 μm. Magnification ×200, ×400. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2.

Table 2 Expression of MBNL2 protein in ESCC and normal adjacent tissues

Variables	N	MBNL2, n (%)		χ^2	P value
		Low expression	High expression		
ESCC	50	36 (72.00)	14 (28.00)	20.873	<0.001
Normal adjacent tissues	179	64 (35.75)	115 (64.25)		

ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2.

from the TCGA database. The Survminer package was used to calculate the best intercept value for grouping. The data were analyzed by GraphPad Prism 8.0. The image was analyzed by ImageJ. A P value <0.05 was considered statistically significant in all analyses.

Results

Positive correlation of MBNL2 expression with clinical ESCC characteristics

The expression of MBNL2 protein in ESCC tissues was significantly higher than that in the normal adjacent tissues (*Figure 1A,1B*). In normal adjacent tissue samples, the high expression rate of MBNL2 protein was 64.25% (115/179) and the low expression rate was 35.75% (64/179). In ESCC tissue samples, the high expression rate of MBNL2 protein was 28% (14/50) and the low expression rate was 72% (36/50) (*Table 2*). The expression of MBNL2 in ESCC was significantly higher than that in normal adjacent tissues (χ^2=20.873, P<0.001). In ESCC tissue samples, the high expression rate of MBNL2 protein in III + IV Tumor Node Metastasis (TNM) stages was significantly higher than that in I + II stages (χ^2=18.165, P<0.001). Similarly, the high expression rate of MBNL2 protein in the tissues with lymph

node metastasis was higher than that in the tissues without (χ^2=14.866, P<0.001). The high expression rate of MBNL2 protein was correlated with lymph node metastasis and TNM stage, but not correlated with age (P=0.59), gender (P>0.99), smoking history (P=0.41), drinking history (P=0.13), tumor location (P=0.46) and tumor size (P=0.53), distant metastasis (P=0.16), differentiation (P=0.07), nerve invasion (P=0.33), and vascular tumor thrombus (P=0.43) (*Table 3*).

Negative correlation of MBNL2 expression with ESCC prognosis

Kaplan-Meier survival curve showed that the survival rate was lower in patients with high MBNL2 expression than in those with low MBNL2 expression (P=0.006). This indicates a significant correlation between the expression of MBNL2 and the prognosis of ESCC (*Figure 2*).

High expression of MBNL2 in ESCC

The RNA-seq data about the expression profiles of 94 ESCC samples from the TCGA database and 516 normal esophageal samples from GTEx database were analyzed by bioinformatic tools. The results showed that MBNL2 was significantly overexpressed in ESCC, with a level higher than that in normal esophageal tissues (P<0.001) (*Figure 3*).

The expression of MBNL2 in five pairs of frozen ESCC tissues and their adjacent tissues was detected by qRT-PCR and WB. The results showed that MBNL2 was highly expressed at both mRNA and protein levels in the former (*Figure 4*).

The mRNA levels of MBNL2 in human esophageal cancer cells (Eca109) and TE-1 cell lines were significantly higher than those in normal esophageal epithelial cells Het-1A (P<0.001). The protein levels of MBNL2 in KYSE150 and Eca109 cell lines were higher than those in Het-1A cell line (*Figure 5*).

Silencing MBNL2 inhibited the invasion and migration but did not change the proliferation of ESCC cells

We transfected KYSE150 and Eca109 cells with shRNA lentiviral particles, and found that ShMBNL2#1, ShMBNL2#2, and ShMBNL2#3 silenced MBNL2 expression in KYSE150 and Eca109 cell lines by qRT-PCR (*Figure 6A,6B*). The silencing efficiency of MBNL2 in KYSE150 and Eca109 cell lines was verified by WB (*Figure 6C,6D*). ShMBNL2#2 and ShMBNL2#3 were selected to construct stable transformants on KYSE150 cell line (*Figure 6E*), and ShMBNL2#1and ShMBNL2#3 on Eca109 cell line (*Figure 6F*). The stable transformant was used in the following experiments.

The wound healing assay was conducted to measure the migration of ESCC cells. In KYSE150 and Eca109 cells, the wound width at 24 h after scratching in control group was 71% and 78% of those in shMBNL2-1 and shMBNL2-2 groups, respectively (*Figure 7A*, P<0.05), suggesting that silencing MBNL2 inhibited the migration of ESCC cells. After MBNL2 silencing, the numbers of transmembrane ESCC cells in shMBNL2-1 and shMBNL2-2 groups were significantly lower than that in the control group (t=6.901, P=0.002; t=7.419, P=0.002) (*Figure 7B,7C*), implying that silencing MBNL2 suppressed the invasion of ESCC cells. After MBNL2 silencing, no significant difference in proliferation were observed in shMBNL2-1 and shMBNL2-2 groups, compared to the control group (*Figure 7D*, P>0.05), suggesting that silencing MBNL2 had no significant effect on the proliferation of ESCC cells.

MBNL2 enhanced EMT in ESCC cells

EMT has been confirmed as a key mechanism underlying tumor metastasis (23). To explore the relationship between MBNL2 and EMT, the expressions of EMT-related protein markers, such as Snail, E-cadherin, and Slug, were determined by WB. After silencing MBNL2, the gray value of E-cadherin in KYSE150 cells was about 1.5 times, and those of Snail and Slug were about 76% and 50% of the values in control cells (*Figure 8A*, P<0.05). After silencing MBNL2, the gray value of E-cadherin in Eca109 cells was about 1.5 times, and those of Snail and Slug were about 68% and 80% of the values in control cells (*Figure 8B*, P<0.05). Overall, these results indicated that MBNL2 enhanced EMT to promote the migration and invasion of ESCC cells.

Discussion

In the present study, we verified the positive correlation of MBNL2 protein expression with ESCC behaviors, its negative correlation with ESCC prognosis; besides, MBNL2 promoted the invasion and migration of ESCC cells through enhancing EMT. These findings may have added valuable information on ESCC pathogenesis. MBNL2 undertakes a carcinogenic role in RCC (15). MBNL2 acts as a suppressor during the occurrence of liver

Table 3 Relationship between MBNL2 protein and pathology of ESCC

Variable	N	MBNL2, n (%)		χ^2	P value
		Low expression	High expression		
Gender				0.009	>0.99
Female	44	16 (36.4)	28 (63.6)		
Male	135	48 (35.6)	87 (64.4)		
Age (years)				0.352	0.59
<60	43	17 (39.5)	26 (60.5)		
≥60	136	47 (34.6)	89 (65.4)		
Smoking history				0.832	0.41
Yes	58	18 (31.0)	40 (69.0)		
No	121	46 (38.0)	75 (62.0)		
Drinking history				2.502	0.13
Yes	37	9 (24.3)	28 (75.7)		
No	141	54 (38.3)	87 (61.7)		
Location				0.740	0.46
Upper	8	4 (50.0)	4 (50.0)		
Middle and lower	171	60 (35.1)	111 (64.9)		
Tumor size (cm)				0.408	0.53
≤4	98	33 (33.7)	65 (66.3)		
>4	81	31 (38.3)	50 (61.7)		
Lymph node metastasis				14.866	<0.001
No	97	47 (48.5)	50 (51.5)		
Yes	82	17 (20.7)	65 (79.3)		
Distant metastasis				Fisher's exact test	0.16
No	170	63 (37.1)	107 (62.9)		
Yes	9	1 (11.1)	8 (88.9)		
TNM stage				18.165	<0.001
I + II	105	51 (48.6)	54 (51.4)		
III + IV	74	13 (17.6)	61 (82.4)		
Differentiation				3.338	0.07
I	36	16 (44.4)	20 (55.6)		
II	106	39 (36.8)	67 (63.2)		
III	37	9 (24.3)	28 (75.7)		
Nerve invasion				1.348	0.33
No	143	54 (37.8)	89 (62.2)		
Yes	36	10 (27.8)	26 (72.2)		
Vascular tumor thrombus				0.735	0.43
No	145	54 (37.2)	91 (62.8)		
Yes	34	10 (29.4)	24 (70.6)		

ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; TNM, Tumor Node Metastasis.

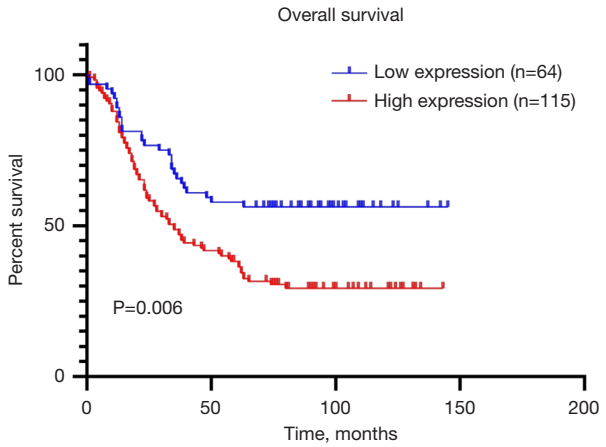


Figure 2 Relationship between MBNL2 expression and the overall survival rate of ESCC patients. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2.

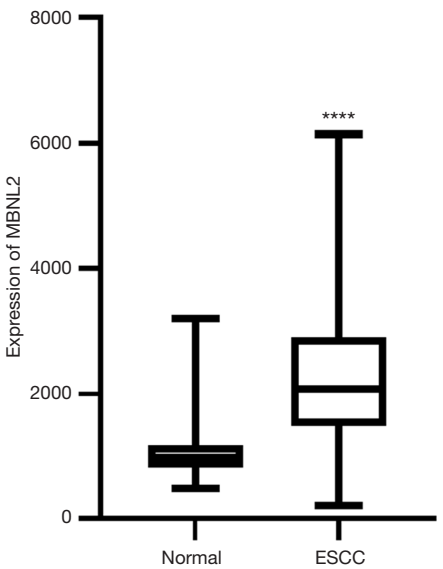


Figure 3 Expression of MBNL2 RNA-seq expression in ESCC tissue samples from TCGA database. ****, P<0.0001. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; RNA-seq, RNA-sequencing; TCGA, The Cancer Genome Atlas.

cancer (16). Here, we reported the close implication of MBNL2 in ESCC, implying that MBNL2 may be an active player in various cancers.

Our previous analysis of TCGA database has shown that MBNL2 is significantly overexpressed in ESCC. Therefore, we detected the expression of MBNL2 in 179 ESCC patients by immunohistochemistry. The results

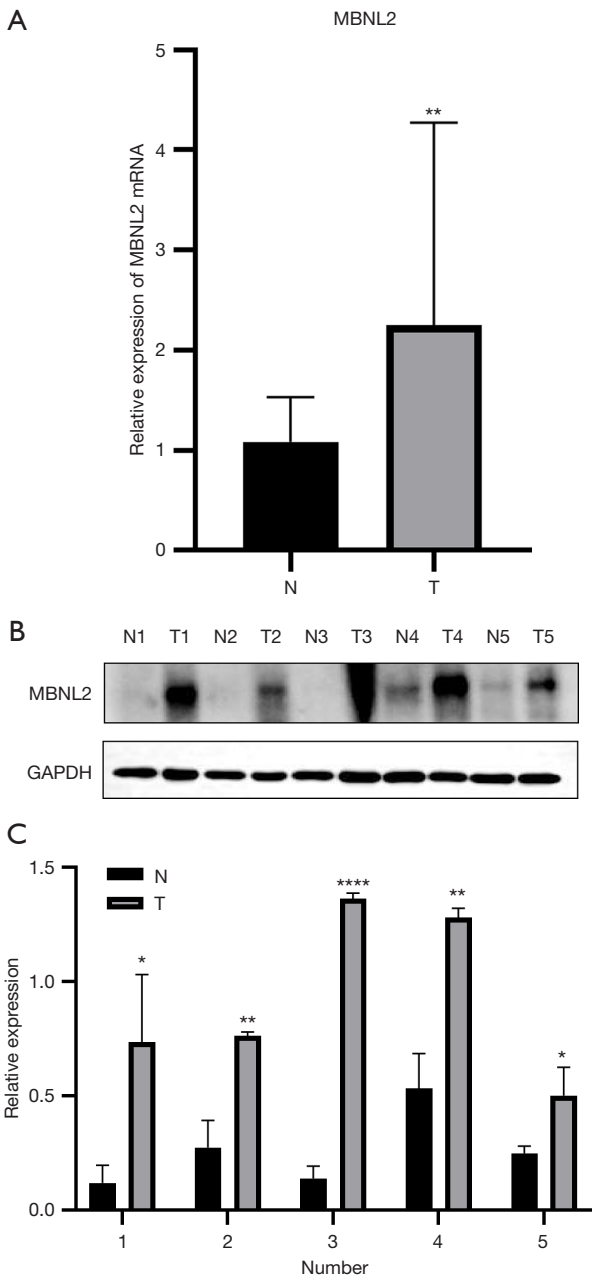


Figure 4 Higher expression of MBNL2 in five pairs of fresh ESCC than in adjacent normal tissues. (A) The mRNA levels of MBNL2 in ESCC and adjacent normal tissues were determined by qRT-PCR; (B) the protein levels of MBNL2 in ESCC and adjacent normal tissues were detected by Western blot; (C) ImageJ software was used to analyze the statistical results of gray scales of B image. *, P<0.05; **, P<0.01; ****, P<0.0001. T1, T2, T3 means esophageal squamous cell carcinoma tissues. N1, N2, N3 means adjacent normal tissues. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction.

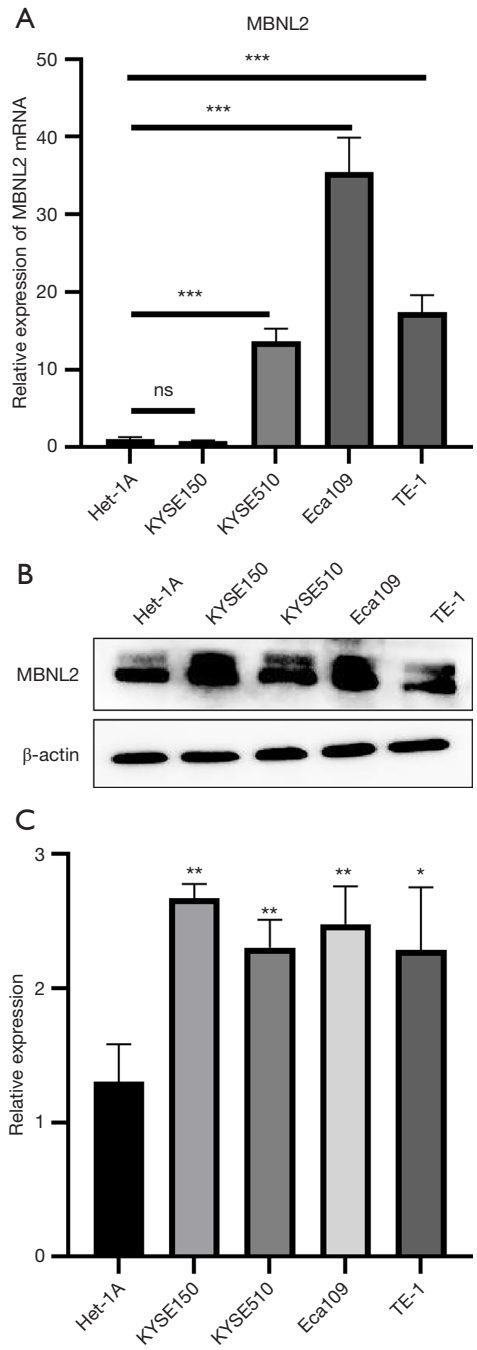


Figure 5 Expression of MBNL2 in normal esophageal epithelial cells and ESCC cell lines. (A) The mRNA levels of MBNL2 in ESCC cell lines were determined by qRT-PCR; (B) the protein levels of MBNL2 in ESCC cell lines were detected by Western blot; (C) ImageJ software was used to analyze the statistical results of gray scales of image in (B). ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction.

showed that the high expression rate of MBNL2 protein was 64.25% (115/179) in ESCC tissue samples, and 28.00% (14/50) in adjacent tissue samples. We detected the mRNA and protein levels of MBNL2 through qRT-PCR and WB. The results showed that compared with those in normal adjacent tissues, the mRNA and protein levels of MBNL2 in ESCC tissues were significantly increased (P<0.05). All these are consistent with the database analysis results. Lee *et al.* (16) have found that MBNL2 promotes the growth and metastasis of RCC. In our study, MBNL2 overexpression in ESCC tissues was significantly correlated with TNM stage and lymph node metastasis (P<0.05). However, there was no significant correlation between MBNL2 expression and some other clinical pathological features, such as age, gender, smoking history, and alcohol consumption history, suggesting that MBNL2 might play a specific role in ESCC infiltration and metastasis.

Zhang *et al.* (17) have reported that a high MBNL2 expression is associated with a poor prognosis of HCC. Our study showed that ESCC patients with a high MBNL2 expression had a significant decrease in postoperative 5-year survival. We speculate that MBNL2 may be a factor associated with the poor prognosis of ESCC, and an independent prognostic biomarker.

Our study investigated the effect of silencing MBNL2 on the biological behaviors of ESCC cells *in vitro*. Wound healing and Transwell assays showed that silencing MBNL2 inhibited the migration and invasion of KYSE150 and Eca109 cells (P<0.05). The results of the CCK-8 assay showed that silencing MBNL2 had no significant effect on the proliferation of ESCC cells (P>0.05). Consistent with the results in ESCC tissues, the high expression rate of MBNL2 among ESCC patients in III + IV TNM stages was significantly higher than that in stages I + II. To further verify its correlation with ESCC metastasis, further *in vivo* experiments are needed.

We further conducted mechanistic studies involving MBNL2 and EMT. We detected the expression of EMT markers E-cadherin, Snail and Slug after silencing MBNL2 through WB.

We found that after silencing MBNL2, the expression of E-cadherin protein significantly increased, while the expression of Snail and Slug proteins decreased, suggesting that MBNL2 may, at least partially, act on EMT to promote the invasion and metastasis of ESCC. The study has shown that MBNL2 potently regulates the migration and invasion of breast and lung cancer cells by means of PI3K/AKT-mediated EMT (24). MBNL2 also exerts a tumor-

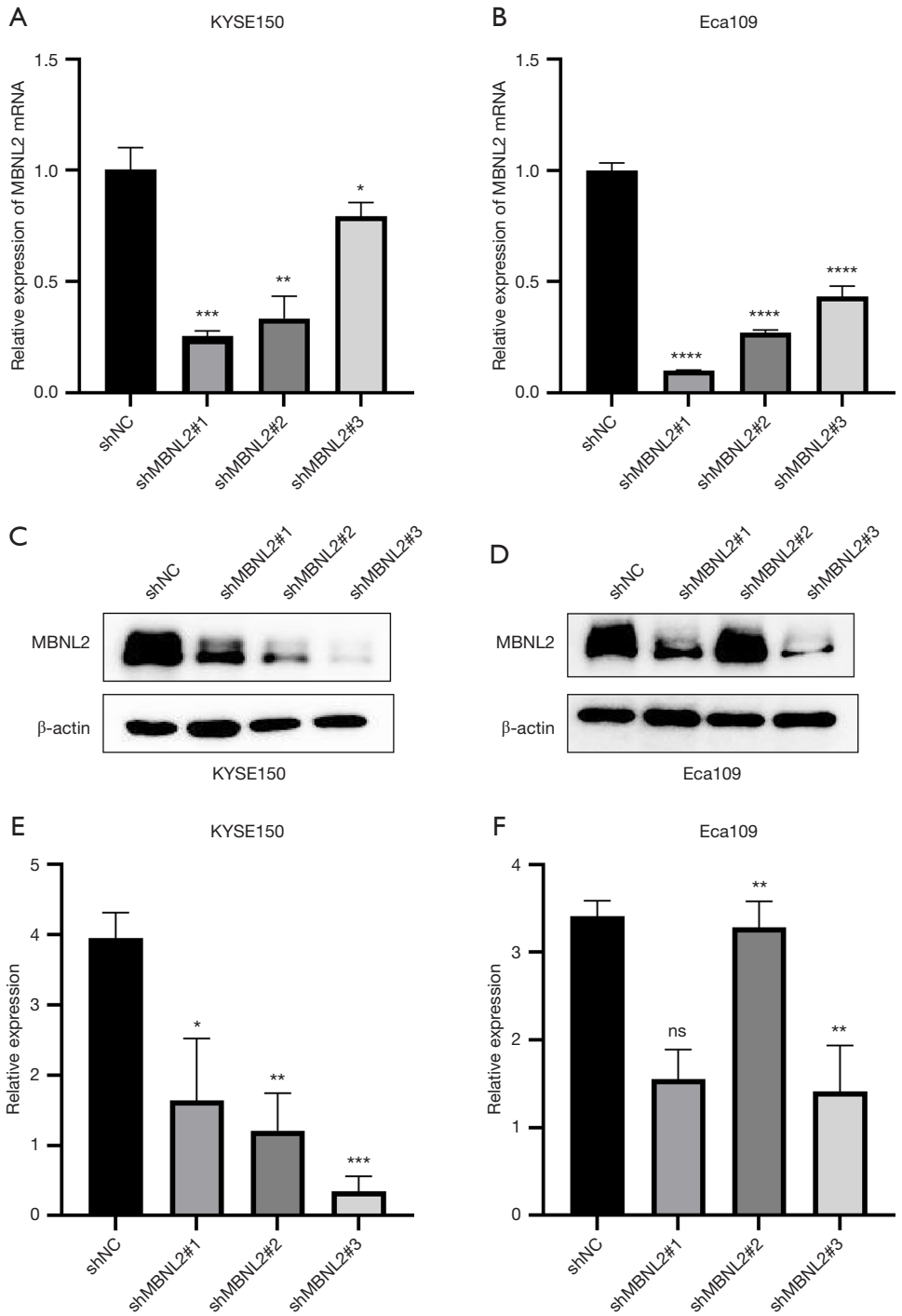
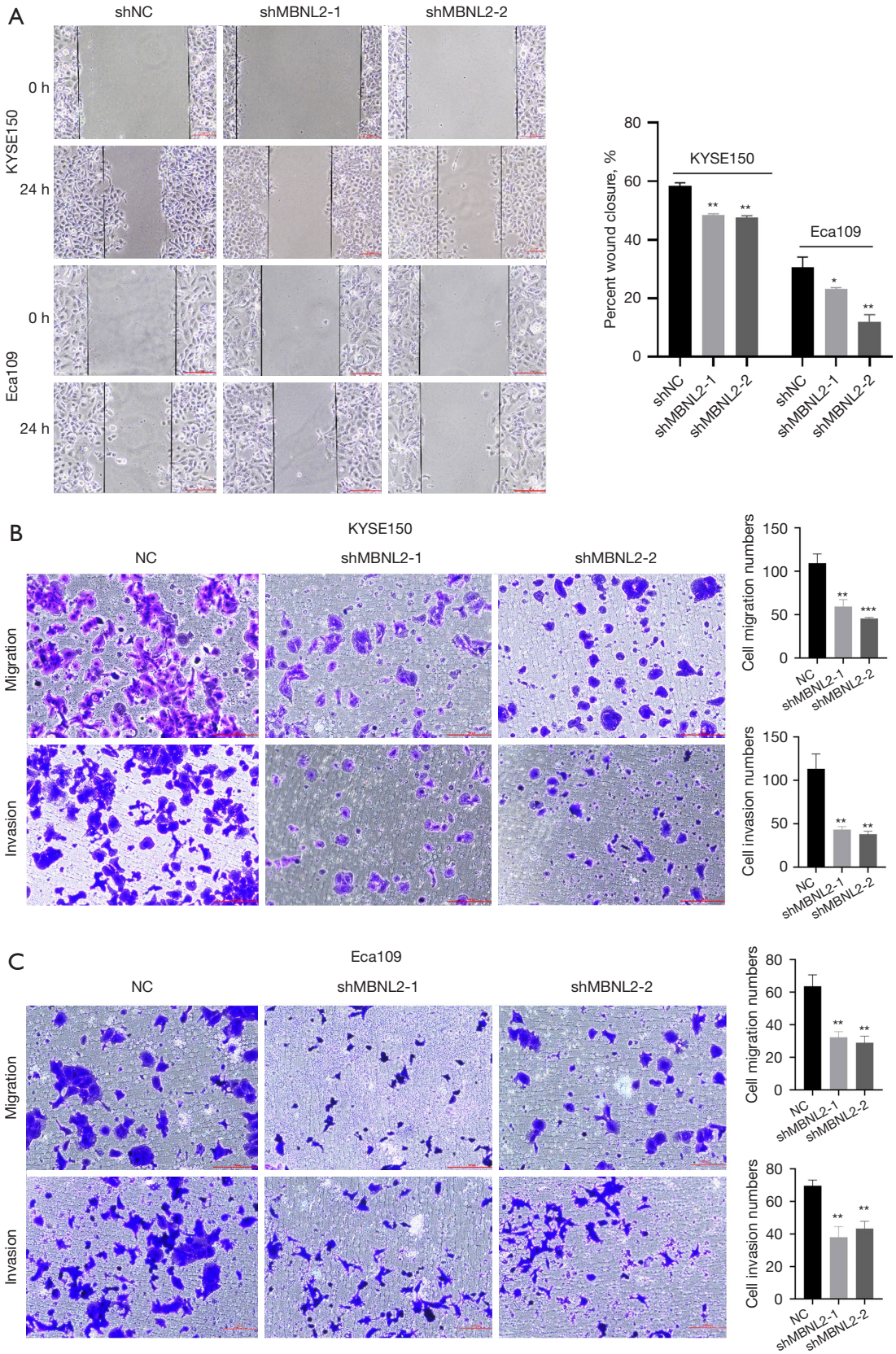


Figure 6 Expression of MBNL2 at mRNA and protein levels. (A) MBNL2 silencing efficiency in KYSE150 cell line was determined by qRT-PCR; (B) MBNL2 silencing efficiency in Eca109 cell line was determined by qRT-PCR; (C) expression of MBNL2 protein in KYSE150 cell line after silencing MBNL2 gene was determined by WB; (D) expression of MBNL2 protein in Eca109 cell line after silencing the MBNL2 gene was determined by WB; (E) ImageJ software was used to analyze the statistical results of gray scales of image in (C); (F) ImageJ software was used to analyze the statistical results of gray scales of image in (D). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; mRNA, messenger RNA; NC, negative control; sh, short hairpin RNA; qRT-PCR, quantitative real-time polymerase chain reaction; WB, Western blotting.



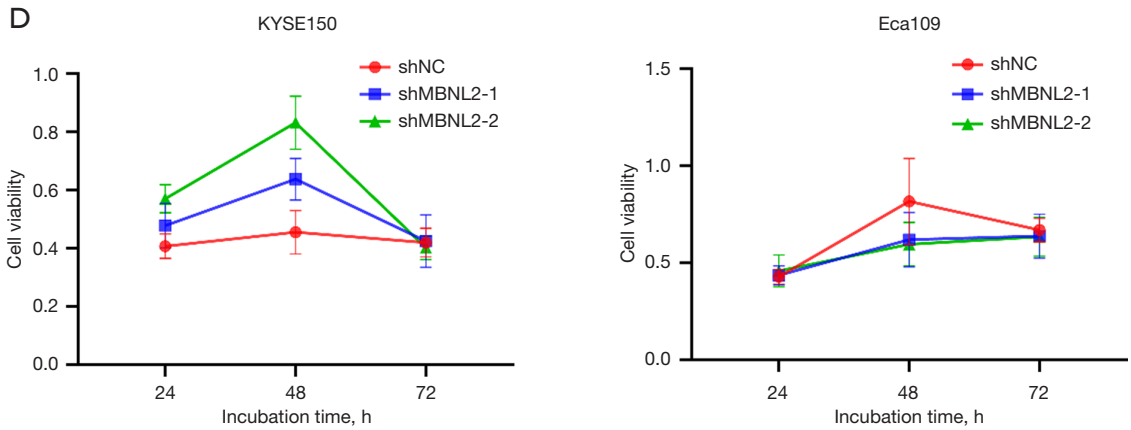


Figure 7 Silencing MBNL2 inhibited the invasion and migration, but did not change the proliferation of ESCC cells. (A) The migration of ESCC cells with MBNL2 silencing was determined by the wound healing assay (scale bar =100 μm); (B) the effects of MBNL2 silencing on the migration and invasion of KYSE150 cell were determined by Transwell assay (violet crystal staining; scale bar =100 μm); (C) the effects of MBNL2 silencing on the migration and invasion of Eca109 cell were determined by Transwell assay (violet crystal staining; scale bar =100 μm); (D) the proliferation of ESCC cells was determined by EdU assay. *, P<0.05; **, P<0.01; ***, P<0.001. EdU, 5-ethynyl-2'-deoxyuridine; ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; NC, negative control; sh, short hairpin RNA.

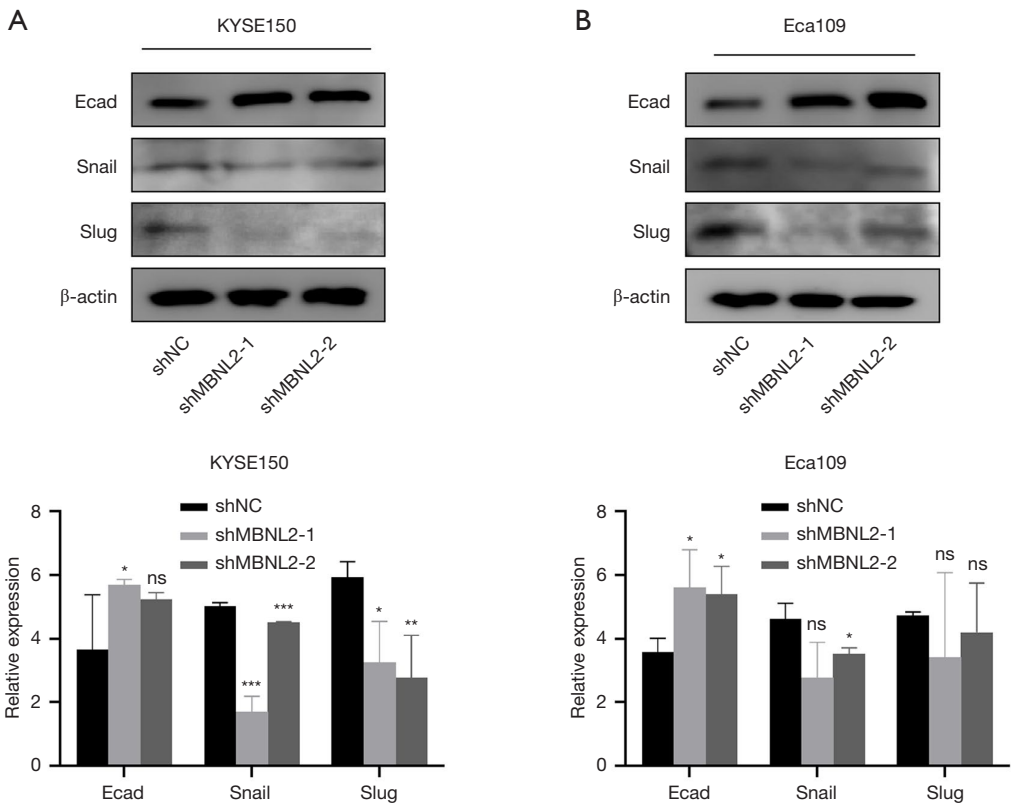


Figure 8 MBNL2 enhanced epithelial-mesenchymal transition in ESCC cells. Western blot was performed to detect the protein levels of E-cadherin, Snail and Slug, and the band densitometry analysis was carried out. All the samples were prepared in triplicate, and all experiments were repeated for at least three times. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. EdU, 5-ethynyl-2'-deoxyuridine; ESCC, esophageal squamous cell carcinoma; MBNL2, muscleblind like protein 2; NC, negative control; sh, short hairpin RNA.

suppressing function through the miR-182-MBNL2-akt-emt signaling pathway (22).

In summary, MBNL2 is significantly overexpressed in ESCC tissue, and its overexpression is significantly correlated with a higher TNM stage, lymph node metastasis, as well as a poor prognosis. Silencing MBNL2 can inhibit the migration and invasion of ESCC cells, but has no significant effect on proliferation.

Conclusions

The present finding is about the high expression of MBNL2 in ESCC and the correlation between MBNL2 and the clinical pathology and metastasis of ESCC. We hypothesize that MBNL2 may manipulate tumor invasion and metastasis of ESCC, at least in part, via the EMT pathway. However, there are limitations in this study. The sample size of this study is relatively small, which can easily cause statistical errors. In the future, experimental and clinical studies with larger sample size should be performed to verify our clinical results and the potential of MBNL2 on the treatment of ESCC.

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None.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1933/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1933/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of The Second Affiliated Hospital of Nantong University (No. 2021KT136) and individual consent for this retrospective analysis was waived.

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