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Original Article

Expression and clinical significance of BUB1 in esophageal cancer

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Abstract: Objective: To investigate the expression levels, biological functions, and clinical significance of budding uninhibited by benzimidazoles 1 (BUB1) in esophageal squamous cell carcinoma (ESCC). Methods: A total of 50 pairs of ESCC and adjacent normal tissues were collected for analysis. The human normal esophageal epithelial cell line (Het-1A) and the human esophageal cancer cell line (EC109) were cultured. Cells were transfected with pcDNA 3.1+BUB1 and shRNA-BUB1. The effects of BUB1 on cancer cells were assessed using CCK-8, Transwell migration and invasion assays, real-time quantitative PCR, and Western blot. Immunohistochemical staining was performed to analyze BUB1 expression in cancer tissues. Logistic regression analysis was used to evaluate the association between BUB1 expression and clinicopathological parameters. The predictive value of BUB1 expression for tumor differentiation, lymph node metastasis, and clinical stage was assessed using receiver operating characteristic (ROC) curve analysis. Results: BUB1 was highly expressed in cancer tissues and cells. Overexpression of BUB1 promoted the proliferation, migration, and invasion of EC109 cells in vitro, while silencing BUB1 inhibited these processes (all $P<0.05$). The positive expression rate of BUB1 was higher in cancer tissues compared to normal tissues (70% vs. 30%, $P<0.05$). Logistic regression analysis revealed that BUB1 expression was significantly associated with tumor differentiation, lymph node metastasis, and clinical stage (all $P<0.05$). ROC curve analysis showed that the area under the curve (AUC) for BUB1 expression predicting tumor differentiation, lymph node metastasis, and clinical stage was 0.762, 0.789, and 0.831, respectively. Conclusion: BUB1 promotes the proliferation, migration, and invasion of esophageal cancer cells. Increased BUB1 expression is closely associated with tumor differentiation, lymph node metastasis, and clinical stage.

Keywords: Budding uninhibited by benzimidazoles 1, esophageal cancer, proliferation, migration, invasion

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common and malignant cancers, with high morbidity and mortality rates. The overall 5-year survival rate is only 18% [1]. In 2022, ESCC was the sixth most common cancer and the fifth leading cause of cancer-related mortality worldwide [2, 3]. Various treatment options are available for patients, including surgical resection, chemotherapy, and radiotherapy [4]. However, the prognosis remains poor due to postoperative recurrence and early metastasis [5]. The pathogenesis of ESCC is complex, involving mutations in numerous genes, including UBE2B, tumor protein p53, ZNF750, Notch1, FAT1, and NFE2L2 [6, 7]. Recent studies have identified novel molecular and cellular targets, leading to the development of advanced therapies that have improved treatment outcomes and prolonged survival in patients with advanced ESCC [8]. As progress is being made in molecular-targeted therapies, the current study aims to identify a potential novel biomarker for the early diagnosis and prognosis evaluation of ESCC.

Budding uninhibited by benzimidazoles 1 (BUB1) is a mitotic checkpoint serine/threonine kinase characterized by a structural domain that includes a serine or threonine kinase, a protein scaffold, and a tetrapeptide [9]. Studies have shown that BUB1 plays a central role in chromosome alignment, activation of the anaphase-promoting complex/C, and signaling pathways related to the spindle assembly

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checkpoint [10]. Additionally, BUB1 is critical in ensuring the accurate partitioning of chromosomes during the cleavage of daughter cells from the mother cell [11]. As an oncogene, BUB1 has been implicated in various cancers, including breast cancer, hepatocellular carcinoma, pancreatic carcinoma, sarcoma, prostate cancer, and gastric cancer [12, 13]. Some studies have found that increased BUB1 expression is associated with poor prognosis in cancer patients [14, 15]. Other research has demonstrated that BUB1 promotes bladder cancer progression and cell proliferation by modulating the STAT3 signaling pathway [16]. However, the molecular biological role of BUB1 in ESCC remains unclear. This study aims to elucidate the expression and clinical relevance of BUB1 in ESCC and explore how it contributes to disease progression. Real-time quantitative PCR (RT-qPCR), Western blot, and immunohistochemistry were used to assess BUB1 expression in ESCC and adjacent normal tissues. Furthermore, the impact of BUB1 on cancer cell proliferation, migration, and invasion was evaluated in vitro, and its clinical value in ESCC was determined. The results of this study could provide a promising clinical marker and treatment target for ESCC.

Materials and methods

ESCC tissue sample

Fifty paired samples of ESCC and corresponding adjacent normal tissues (at least 5 cm away from the cancerous tissues) were collected from patients who underwent surgery between January 2023 and December 2024 at the Second Affiliated Hospital of Shandong First Medical University. ESCC tissues were confirmed by histological examination. These tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Written informed consent was obtained from the patients before enrollment. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Shandong First Medical University (Approval number: No. 2020-067).

Cell culture

Human ESCC cell line EC109 and normal human esophageal epithelial cell line Het-1A were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. EC109 and Het-1A cells were cultured in DMEM (FBS, Gibco, USA) at 37°C and 5% CO_2 in a humidified incubator. The medium was supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin (Solarbio, China).

Cell transfection

Cells (1×10^5/well) were seeded in 6-well culture plates and incubated overnight at 37°C until they reached approximately 70% confluency. The medium was then replaced with serum-free medium. Full-length cDNA encoding human BUB1 was obtained from human whole blood via RT-PCR. The BUB1 cDNA was cloned into the pcDNA 3.1 vector (Takara Bio, Inc., Japan) according to the manufacturer's instructions. The pcDNA 3.1+BUB1 construct was used to transfect EC109 and Het-1A cell lines. Additionally, ShRNA-BUB1 was used to knockdown BUB1, with the following sequences:

shRNA control: antisense: 5'-CCGGCAAACTTTGTATGCCCGCT TTCTCGAGAAAGCGGGCATACAAAGTTTGTTTTTG-3', sense: 5'-AATTCAAA AACAAACTTTGTATGCCCGCTTTCTCGAGAAAGCGGGCAT ACAAAG TTTG-3'. ShRNA-BUB1: antisense: 5'-CCGGGAATTTC AATTGGGTTCTAA GCTCGA GCT TAG AACCCAATTGAAATTCTTTTTG-3', sense: 5'-AATTCAAA AAGAATT TCAATTGGGTTCTAAGCTCGAGCTTAGAACCCAATTGAAATTC-3'. shRNA control and shRNA-BUB1 were transfected into cells for 6 hours using Lipofectamine (Invitrogen, USA) according to the manufacturer's instructions. Afterward, the cells were incubated with medium containing 10% FBS for 24 hours, then harvested, and knockdown efficiency was verified.

Transwell migration and invasion assays

For the migration assay, 200 µL of serum-free medium containing 2×10^4 EC109 cells were added to the upper chamber of a Transwell insert with an 8.0 µm pore size membrane. The lower chamber contained 400 µL of DMEM supplemented with 20% FBS. After 24 hours of incubation, the migrated cells were fixed and stained with a 95% hematoxylin solution mixed with ethanol. The number of stained cells was counted to evaluate the migration index, and images were captured using an inverted microscope (Nikon, Tokyo, Japan).

For the invasion assay, transwell inserts were pre-coated with a 1:30 dilution of Matrigel. The remaining procedures were the same as those in the migration assay. After incubation, the invasive cells were detected using the same methods as in the migration assay.

CCK-8 assays

A total of 1×10^3 cells/well were seeded into 96-well plates and cultured in medium with 10% FBS. At 0, 24, 48, 72, and 96 hours after incubation, 10 μL of CCK-8 solution was added to each well and incubated for 2 hours at 37°C with 5% CO_2. Absorbance was measured at 450 nm after the 2-hour incubation.

RT-qPCR analysis

Total RNA was extracted from cell lines and tissues using TRIzol reagent (Thermo Fisher Scientific, Inc., Invitrogen, USA), and cDNA was synthesized using a reverse transcription kit (Takara Bio, USA). SYBR® Premix Ex Taq (Takara Bio, Inc.) was used for qPCR. The PCR thermocycling conditions were as follows: pre-denaturation at 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 12 seconds, annealing at 59°C for 40 seconds, and extension at 72°C for 45 seconds. The relative mRNA expression level of BUB1 was calculated using the $2^{-\Delta\Delta Cq}$ method. The primer sequences for RT-qPCR were:

BUB1 forward primer: 5'-TGGGAAAGATACATAAGTGGGT-3' and reverse primer: 5'-AGGGGATGACAGGGTTCCAAT-3'; GAPDH forward primer: 5'-AATC-CCATCACCATCTTCCA-3', reverse primer: 5'-TTTCTTCCTGGGCTGCTTAT CTGG-3'.

Western blotting analysis

Cells or homogenized tissues were lysed in RIPA lysis buffer (Solarbio, China) containing Protease Inhibitor Cocktail (Roche, USA). The lysates were centrifuged at 10,000 g for 10 minutes at 4°C. The total protein concentration was determined using a BCA Protein Detection Kit (Solarbio, China). Proteins were separated using 10% SDS-PAGE, then transferred to 0.45 μm PVDF membranes (GE Healthcare Life Sciences, Germany). The membranes were incubated in 5% fat-free milk for 1 hour at room temperature, followed by overnight incubation with primary antibodies against BUB1 (1:800 dilution, ab195268; Abcam, USA) and GAPDH (1:1000 dilution, 5174; Cell Signaling Technology, Inc., USA) at 4°C. Afterward, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:6000 dilution, 7074; Cell Signaling Technology, Inc., USA) for 2 hours at room temperature. Protein bands were visualized using enhanced chemiluminescent reagents and a Gel Doc XR system (Bio-Rad Laboratories, Inc., USA).

Immunohistochemical method

Surgically resected esophageal cancer and adjacent non-cancerous tissues were fixed in formaldehyde, paraffin-embedded, and sectioned. The sections were deparaffinized and subjected to antigen retrieval. After blocking with 5% bovine serum albumin (BSA) for 1 hour, sections were incubated overnight at 4°C with anti-BUB1 antibody (1:100 dilution, ab195268; Abcam, USA). Sections were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:600 dilution, ab6759; Abcam, USA) for 2 hours at room temperature. After incubation, sections were treated with the substrate for chromogenic reaction, followed by counterstaining with hematoxylin, dehydration in graded alcohols, vitrification, and sealing. The sections were examined under a light microscope to assess BUB1 protein expression, graded according to the following criteria [17, 18]:

No positive staining or positive cell count ≤ 10% was (-), 11-30% was (+), 31-50% was (++), and ≥51% was (+++). (-) indicated negative expression, and (+) to (+++) indicated positive expression.

Statistical methods

Clinical data were analyzed using SPSS version 24.0. Continuous data are presented as mean ± standard deviation (SD), and comparisons were made using the independent t-test. Categorical data are expressed as percentages or cases, with comparisons between groups performed using the χ^2 test. Further analysis of variables such as degree of differentiation, metastasis stage, and lymph node metastasis was conducted using multiple logistic regression models with the forward likelihood ratio (LR) method to identify risk factors associated

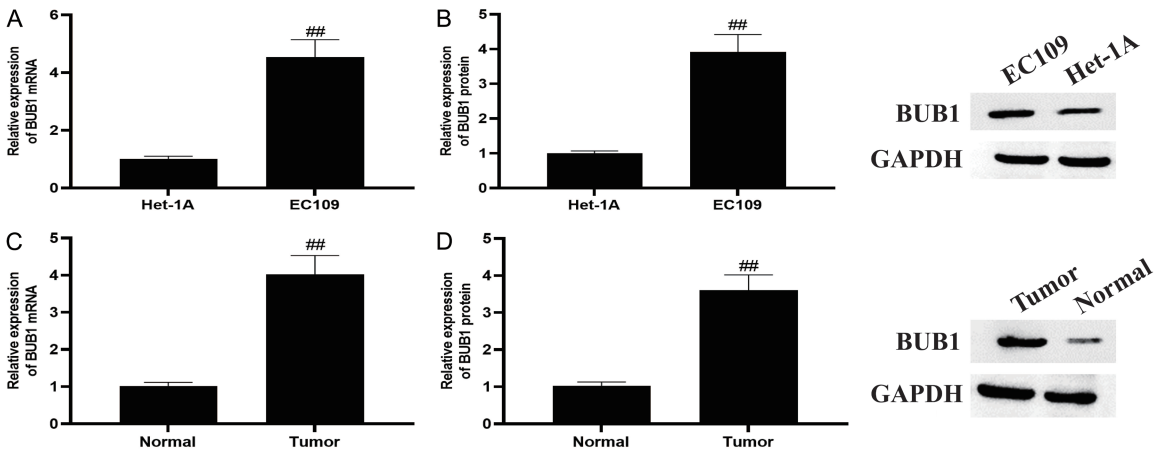

Figure 1. The expression of BUB1 in esophageal cancer cell lines and tumor tissues. A: BUB1 mRNA expression in Het-1A and EC109 cell lines. B: BUB1 protein expression in Het-1A and EC109 cell lines. C: BUB1 mRNA expression in normal and esophageal cancer tissues. D: BUB1 protein expression in normal and esophageal cancer tissues. ##P<0.01 vs Het-1A or Normal.

with BUB1 expression in esophageal cancer patients. Logistic regression analysis was performed as described in previous studies [19]. Receiver operating characteristic (ROC) curve analysis was used to evaluate the predictive ability of BUB1 expression for these variables, with P<0.05 considered statistically significant.

Results

BUB1 expression

The expression of BUB1 in ESCC cell lines and patient tissues was evaluated. BUB1 expression levels were higher in EC109 cells compared to the Het-1A cell line (**Figure 1A** and **1B**). Furthermore, RT-qPCR and Western blot analysis of 30 paired normal and cancerous esophageal tissue samples revealed a significant increase in BUB1 expression in cancerous tissues (**Figure 1C** and **1D**).

Immunohistochemical results

Positive BUB1 protein expression appeared as yellow or brownish-yellow granules, predominantly in the cytoplasm, with occasional presence in the nucleus. The staining was also observed in some lymphocytes and small blood vessels in the mesenchyme. As shown in **Figure 2**, in adjacent normal tissues, BUB1 protein expression was categorized as 7 cases (+), 2 cases (++), and 1 case (+++). In contrast, in esophageal cancer tissues, BUB1 expression was observed in 5 cases (+), 10 cases (++), and 20 cases (+++). The high expression rate of BUB1 in adjacent normal tissues was 20.0% (10/50), while in esophageal cancer tissues, it was 70.0% (35/50). Statistically significant differences were found between the two groups (χ^2=25.250, P<0.001).

Effects of BUB1 expression on cell proliferation

As shown in **Figure 3**, CCK-8 analysis revealed that, starting from day 2, cell proliferation was significantly higher in the pcDNA 3.1+BUB1 group compared to the pcDNA 3.1 vector group (P<0.05). In contrast, cell proliferation in the shRNA-BUB1 group was significantly lower than that in the shRNA+control group (P<0.05).

Effects of BUB1 expression on cell migration and invasion

As shown in **Figure 4**, migration and invasion assays demonstrated that the average number of migrated (P=0.019) and invaded cells (P=0.023) was significantly reduced in the shRNA-BUB1 group compared to the control group. Conversely, the average number of migrated (P=0.016) and invaded cells (P=0.020) in the

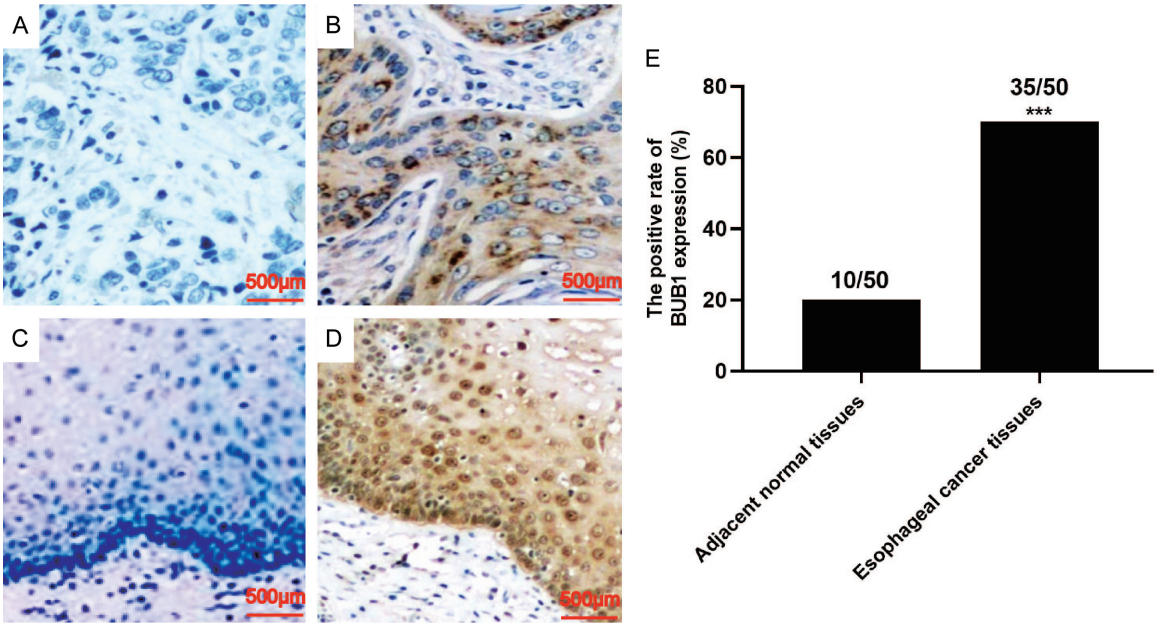

Figure 2. Immunohistochemical results of BUB1 protein expression in esophageal cancer tissues and adjacent normal tissues (×200). A: The representative image of negative BUB1 expression in esophageal cancer tissues; B: The representative image of positive BUB1 expression in esophageal cancer tissues; C: The representative image of negative BUB1 expression in adjacent normal tissues; D: The representative image of positive BUB1 expression in adjacent normal tissues; E: The comparison of the positive rate of BUB1 expression between esophageal cancer tissues and adjacent normal tissues. ***P<0.001 vs adjacent normal tissues.

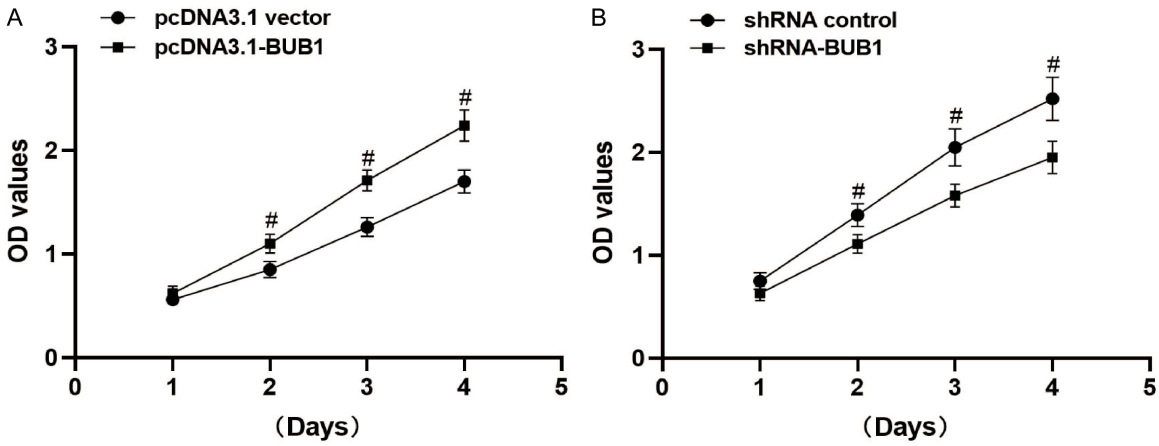

Figure 3. The cell proliferation was detected by CCK-8 analysis. A: The effects of BUB1 overexpression. B: The effects of BUB1 low-expression. *P<0.05 vs pcDNA3.1 vector or shRNA control.

pcDNA 3.1+BUB1 group was significantly higher compared to the pcDNA 3.1 vector group.

Multiple Logistic regression analysis of the relationship between clinicopathological parameters and BUB1 expression

Multiple logistic regression analysis was performed using variables with a *P* value < 0.05 in the univariate analysis (**Table 1**). Stepwise regression was applied, with BUB1 expression as the dependent variable and independent variables including degree of differentiation, lymph node metastasis, and clinical stage. The assignments of independent variable in multiple logistic regression analysis were listed in **Table 2**. The results (**Table 3**) showed HR with 95% CI for degree of

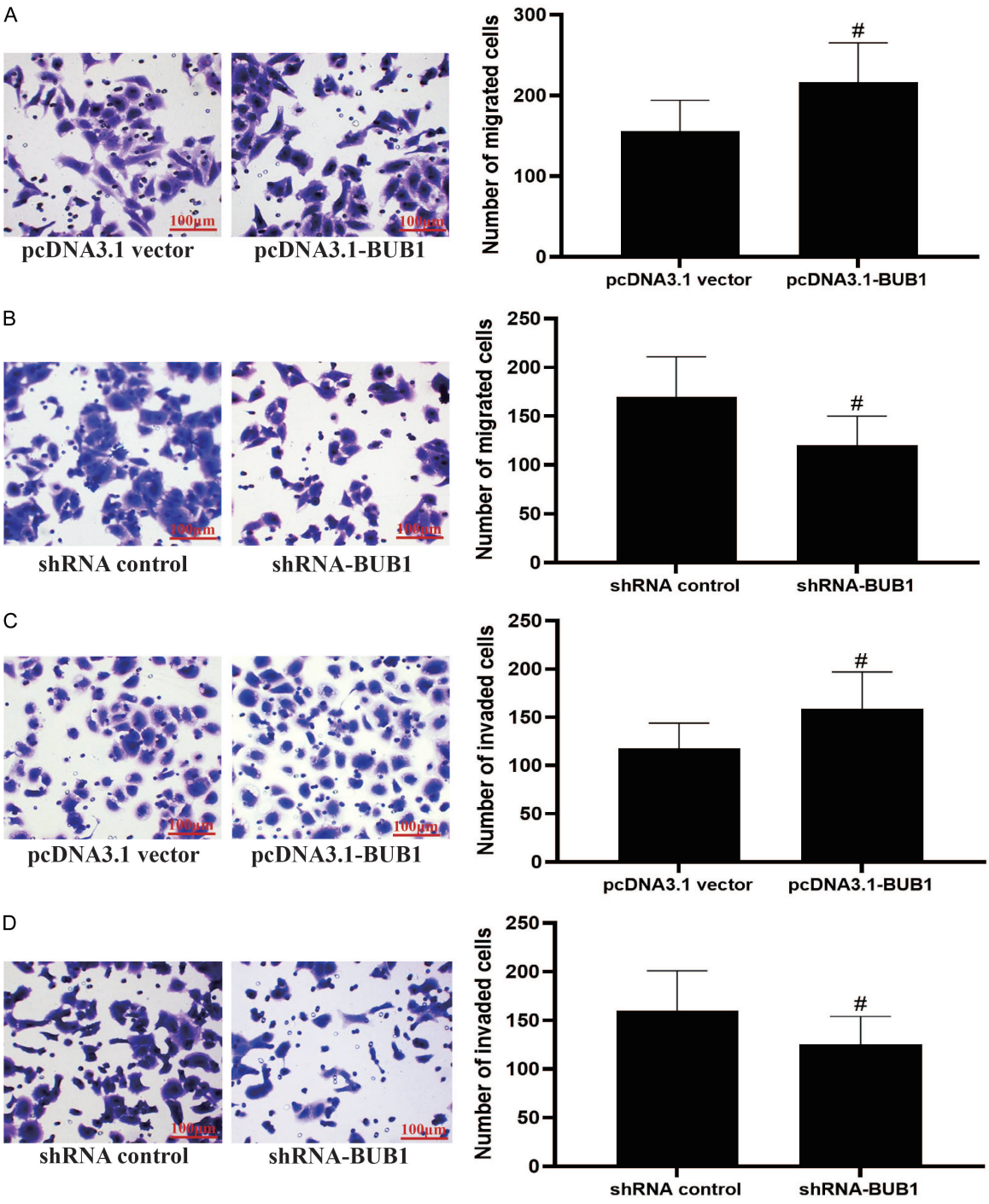

Figure 4. The cell migration and invasion were detected by Transwell migration and invasion assays. A: Transwell migration assays of BUB1 overexpression (×200). B: Transwell migration assays of BUB1 low-expression (×200). C: Transwell invasion assays of BUB1 overexpression (×200). D: Transwell invasion assays of BUB1 low-expression (×200). *P<0.05 vs pcDNA 3.1 vector or shRNA control.

differentiation, lymph node metastasis, and clinical stage as 3.108 (1.351-7.006), 3.794 (1.829-7.882), and 2.847 (1.371-6.023), respectively. These results suggest that degree of differentiation, lymph node metastasis, and clinical stage are independent risk factors influencing BUB1 expression in esophageal cancer.

Table 1. Univariate regression analysis for the relationship between BUB1 protein expression and clinicopathological features

Parameters	Cases (N)	Positive rate (%)	χ^2 value	*P* value
Gender			1.023	0.312
Male	38	73.68 (28/38)		
Female	12	57.14 (7/12)		
Age (years)			0.244	0.621
≥60	26	73.08 (19/26)		
<60	24	66.67 (16/24)		
Degree of differentiation			5.534	0.019
Well and moderately	31	58.06 (18/31)		
Poorly	19	89.47 (17/19)		
Lymph node metastasis				
Yes	21	90.48 (19/21)	7.229	0.007
No	29	55.17 (16/29)		
Clinical stage			4.163	0.041
I and II	38	62.16 (23/37)		
III and IV	12	92.31 (12/13)		
Infiltrating depth			0.095	0.758
None-reach ectoptygma	25	68.0 (17/25)		
Reach ectoptygma	25	72.0 (18/25)		
Location of Tumor			0.661	0.416
Superior and middle segment	29	65.52 (19/29)		
Inferior segment	21	76.20 (16/21)		
Tumor size (cm)			0.397	0.529
≥3.5	30	73.33 (22/30)		
<3.5	20	65.0 (13/20)		

Table 2. The assignments of independent variable in multiple logistic regression analysis

Independent variable	Variable Declaration	Assignments
Degree of differentiation	categorical variable	Poorly = “1”, Well and moderately = “0”
Lymph node metastasis	categorical variable	Yes = “1”, No = “0”
Clinical stage	categorical variable	II-III stage = “1”, I-II stage = “0”

Table 3. Multiple logistic regression analysis for BUB1 protein expression and clinicopathological features

Parameters	β	SE	Wald	P	HR (95% CI)
Degree of differentiation	1.037	0.398	7.102	0.003	3.108 (1.351-7.006)
Lymph node metastasis	1.329	0.368	12.904	0.002	3.794 (1.829-7.882)
Clinical stage	1.058	0.405	7.714	0.006	2.847 (1.371-6.023)

Note: SE: standard error; HR: hazard ratio.

ROC curve results of BUB1 expression predicting degree of differentiation, lymph node metastasis, and clinical stage in ESCC

As shown in **Figure 5**, ROC curve analysis revealed that the AUC for BUB1 expression in esophageal cancer tissues for predicting the degree of differentiation, lymph node metastasis, and clinical stage were 0.762, 0.789, and 0.831, respectively, indicating moderate predictive efficacy.

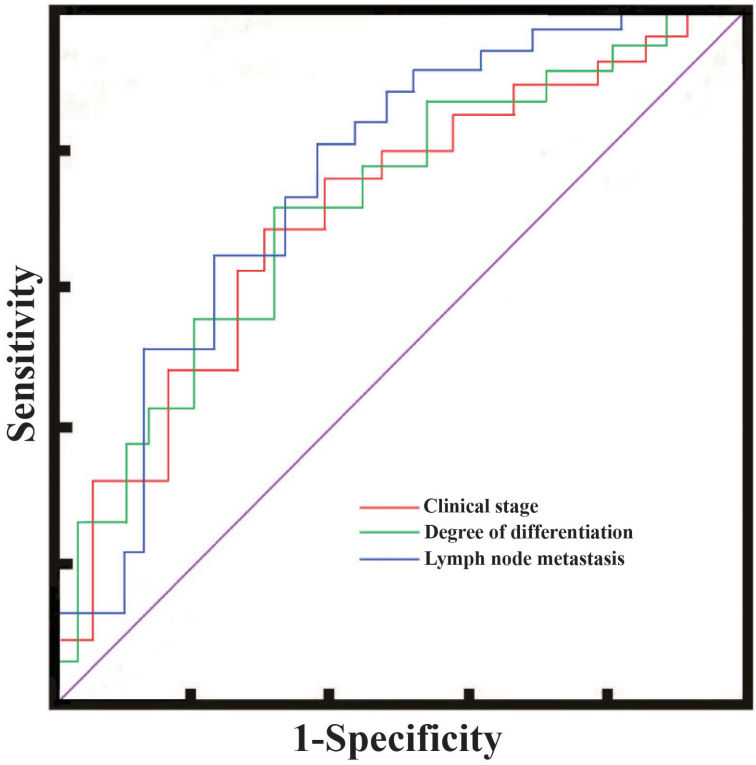

Figure 5. ROC curve showed that the expression of BUB1 predicted the degree of differentiation, lymph node metastasis and clinical stage in patients with esophageal cancer. Note: ROC: relative operating characteristic.

Discussion

Abnormal expression and mutations of BUB1 are associated with aneuploidy and various types of tumors, including pancreatic ductal adenocarcinoma and breast cancer [20]. Several studies have reported that BUB1 is significantly upregulated in various cancers, such as liver and gastric cancer, and is linked to poor prognosis [21, 22]. However, BUB1 appears to serve different roles depending on the type of cancer. In patients with endometrial carcinoma [23] or gastric adenocarcinoma [24], increased BUB1 expression is associated with a favorable prognosis, while in ovarian cancer [25], high BUB1 expression correlates with poor prognosis.

In the present study, we found that BUB1 expression was significantly higher in esophageal cancer tissues compared to normal tissues. A similar trend was observed in EC109 cell lines, which exhibited higher BUB1 expression compared to Het-1A cells. Cell proliferation is a crucial aspect of cellular function, and uncontrolled proliferation in cancer cells leads to tumor development and progression. BUB1 has been identified as an important gene associated with hepatocellular carcinoma [26]. Another study demonstrated that KIF4A knockdown suppressed ovarian cancer progression by regulating BUB1 [27]. Furthermore, BUB1 acts as a positive regulator of gastric cancer cell proliferation by activating the TRAF6/NF-κB/FGF18 pathway through METTL3-mediated m6A methylation [28].

To explore the impact of BUB1 on the proliferative capacity of esophageal cancer cells, CCK-8 assays were performed in our study and showed that cell proliferation was significantly enhanced in the pcDNA 3.1+BUB1 group compared to the control group. Conversely, cell proliferation was significantly reduced in the shRNA-BUB1 group. These results suggest that BUB1 promotes the proliferative capacity of esophageal cancer cells.

Invasion and metastasis are key features of malignant tumors. BUB1 is highly expressed in anaplastic thyroid carcinoma, and downregulation of BUB1 expression reduces the migration and invasive ability of these cells [29]. Zhang et al. found that BUB1 knockdown reduced the migration and invasion of esophageal cancer cells [30]. Huang et al. reported that BUB1 inhibited migration and invasion in osteosarcoma cells by blocking the PI3K/Akt and ERK signaling pathways [31]. In this study, Transwell assays demonstrated that the average number of migrated cells in the pcDNA 3.1+BUB1 group was significantly higher than that in the control group, while migration was significantly reduced in the shRNA-BUB1 group. This finding further indicates that BUB1 promotes the migration of esophageal cancer cells. Similarly, the Transwell invasion assay revealed that the number of invasive cells was significantly lower in the shRNA-BUB1 group, while it was significantly higher

in the pcDNA 3.1+BUB1 group, confirming that BUB1 enhances the invasive potential of esophageal cancer cells. These findings align with previous studies on other cancer types.

Immunohistochemical staining was used in this study to detect BUB1 protein expression in 50 ESCC tissues and 50 adjacent normal tissues. The results showed that the positive expression rate of BUB1 in cancer tissues was 70.0%, while in adjacent normal tissues it was 30.0%. The expression of BUB1 was significantly higher in ESCC tissues, with a similar positive expression rate observed in invasive breast cancer [32] and pancreatic ductal adenocarcinoma [33]. Additionally, the results revealed that as the degree of differentiation of ESCC decreased, BUB1 expression increased. Furthermore, BUB1 expression was associated with the presence of lymph node metastasis and tumor stage. Higher levels of BUB1 expression were observed in ESCC tissues with lymph node metastasis or advanced stage, indicating that BUB1 is linked to differentiation, clinical stage, and lymph node metastasis in ESCC. Aberrant expression of BUB1 can result in defects in the spindle checkpoint, leading to chromosomal instability and tumor formation.

BUB1 is a protein kinase, although its mechanism of action is not yet fully understood. Some researchers have suggested that BUB1 is preferentially localized at the centromere, where it may serve as a platform for the localization of other components. Upon induction by spindle inhibitors, BUB1 undergoes rapid self-phosphorylation and subsequently phosphorylates BUB3. BUB1 plays a crucial role in the phosphorylation of specific proteins in the spindle checkpoint pathway [34] and in the activation of the spindle checkpoint process. One study [35] proposed that normal expression of BUB1 ensures proper chromosome segregation, and defects in BUB1 function can lead to perinatal embryonic death or the development of malignant tumors. Another study [36] revealed that reduced expression of BUB1 and the tumor suppressor gene p53 impairs the p53-mediated apoptosis pathway, resulting in the accumulation of aneuploid cells. As a spindle checkpoint protein, abnormal expression of BUB1 disrupts spindle checkpoint function, losing its ability to monitor the mitotic process. When spindle-chromosome attachment is incomplete or chromosome separation is abnormal, cells fail to arrest at metaphase due to spindle checkpoint dysfunction and directly progress to anaphase. This results in the formation of aneuploid cells, ultimately leading to tumor development. Thus, BUB1, as a key component of the spindle checkpoint, plays a critical role in the development of ESCC. Assessing BUB1 expression levels may assist in evaluating the biological behavior and clinical prognosis of ESCC.

In conclusion, this study demonstrated that BUB1 promotes the proliferation, migration, and invasion of esophageal cancer cells. Additionally, positive BUB1 expression in esophageal cancer was associated with tumor differentiation, clinical stage, and lymph node metastasis. These findings provide a deeper understanding of BUB1's role in tumor development and highlight BUB1 as a potential target for the diagnosis and treatment of esophageal cancer. However, this study has several limitations: it is a single-center study with a small sample size, lacks subgroup comparisons, does not include long-term follow-up data, and does not address the associated mechanisms. Future research with a controlled, multicenter design, larger sample size, and long-term follow-up is necessary to confirm these results.

Disclosure of conflict of interest

None.

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