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Correlation between HMGB1 expression and drug resistance and prognosis in patients with bladder urothelial carcinoma

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Abstract

Objective This study aimed to explore the association between circulating HMGB1 mRNA expression and clinical pathological characteristics and prognosis in bladder urothelial carcinoma (BUC) patients.

Methods Circulating HMGB1 mRNA expression levels were assessed using real-time fluorescence quantitative PCR, and patients were categorized into low-expression and high-expression groups based on the median value. Follow-up was conducted for 3 years post-surgery, and patients were classified into non-recurrence and recurrence groups. Baseline circulating HMGB1 mRNA expression levels were compared across different clinical pathological characteristics and prognosis groups to evaluate prognostic disparities based on HMGB1 mRNA expression levels. The Western Blot (WB) experiment assesses the expression levels of HMGB1 across various tissue sections and evaluates the inhibitory effects of chemotherapeutic agents on HMGB1. Additionally, after knocking out HMGB1 in vitro cell lines, the cell proliferation is detected.

Results There were no significant differences in HMGB1 mRNA expression levels among patients with varying differentiation grades, lymph node metastasis stage (N stage), primary tumor stage (T stage), or tumor diameter. However, baseline circulating HMGB1 mRNA expression levels were notably lower in surviving patients than in deceased patients. Kaplan–Meier survival analysis revealed a median survival time of 356 weeks in the low-expression group and 259 weeks in the high-expression group. Notably, the low-expression group exhibited significantly prolonged survival compared to the high-expression group (HR = 1.714, 95%CI 1.226, 2.397). WB test showed that the expression level of HMGB1 in tumor tissues of BUC patients was increased, and inhibition of HMGB1 expression could also inhibit the proliferation of tumor cells. Some common chemotherapy drugs can also significantly inhibit the proliferation of BUC cells.

Conclusion HMGB1 is highly expressed in urinary bladder epithelial carcinoma, and chemotherapy drugs can effectively inhibit the expression of HMGB1. Inhibition of HMGB1 expression is helpful to inhibit the proliferation of tumor cells. Circulating HMGB1 mRNA expression levels are closely associated with tumor prognosis, with low-expression patients having a longer survival period.

Keywords Bladder urothelial carcinoma · High mobility group box 1 protein · mRNA · Survival analysis · Drug resistance

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1 Introduction

Bladder carcinoma is one of the most prevalent malignancies affecting the genitourinary system in China, with its frequency progressively escalating in recent times [1]. Among the spectrum of bladder cancers, bladder urothelial carcinoma (BUC), squamous cell carcinoma, and adenocarcinoma are prominent entities [2]. Notably, BUC constitutes the predominant subtype, encompassing over 90% of cases. Alarmingly, within a span of 5 years, approximately 50% to 70% of BUC patients encounter recurrence, with about 10% advancing to an invasive form of the disease, characterized by heightened aggressiveness, and associated with fatal outcomes [3]. Based on the risk stratification, BUC patients can choose surgical treatment, bladder instillation, and follow-up strategies, among which transurethral resection of bladder tumor (TURBT) is the most common surgical approach [4]. Due to the high postoperative recurrence rate in BUC patients, it is recommended that all patients undergo adjuvant bladder instillation therapy after surgery [5]. Currently, the mechanisms of BUC are not well understood, and genetic factors and single nucleotide polymorphisms (SNPs) are key factors in the occurrence and progression of BUC tumors, such as oncogenes or tumor suppressor genes [6].

High mobility group box-B1 (HMGB1) is a versatile non-histone nuclear protein characterized by its diverse functions contingent upon its intracellular localization [7]. Within the nucleus, HMGB1 serves as a crucial DNA chaperone, facilitating the maintenance of chromosomal structure and functionality. Upon translocation to the cytoplasm, HMGB1 exhibits the capability to induce autophagy by interacting with the BECN1 protein. Following either active secretion or passive release, HMGB1 collaborates with chemotactic factors, cytokines, and growth factors to modulate various cellular processes, including inflammation, immunity, cellular migration, proliferation, metabolic regulation, autophagy, and apoptosis [8, 9]. HMGB1 also plays an important role in tumor metastasis and dissemination. HMGB1 can regulate multiple signaling pathways related to cancer metastasis. For example, HMGB1 can promote the process of epithelial-mesenchymal transition (EMT), which is an important mechanism by which tumor cells migrate from the primary site to other tissues [10]. HMGB1 can also enhance the migration and invasion capabilities of tumor cells, promoting the distant metastasis of tumor cells through the blood or lymphatic system. Furthermore, HMGB1 can regulate the interaction between tumor cells and the external microenvironment, facilitating the adaptation and survival of tumor cells in a new environment [11]. High expression of HMGB1 has been confirmed in tumor tissues such as lung cancer, breast cancer, cervical cancer, and BUC, and it is closely associated with tumor cell proliferation, migration, apoptosis, autophagy, and tumor tissue angiogenesis [12]. However, the relationship between HMGB1 expression and the prognosis of BUC is not yet clear.

This study included postoperative BUC patients who received combined gemcitabine bladder instillation chemotherapy in our hospital to explore the relationship between HMGB1 expression and prognosis. The proliferation and migration of tumor cells were observed by knockout of HMGB1 in vitro.

2 Materials and methods

2.1 Study population

This study comprised 228 individuals diagnosed with BUC who underwent surgical resection and received pathological confirmation from January 2019 to January 2023. The levels of HMGB1 mRNA were quantified using real-time fluorescence quantitative PCR. According to the median value, patients were stratified into low-expression and high-expression groups. Subsequently, a 3-year follow-up period post-surgery categorized patients into non-recurrence and recurrence groups. The study involving patients was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (Ethics Number: 2024-127). Informed consent was obtained from BUC patients. This investigation adhered to the principles outlined in the declaration of Helsinki. BUC patients were recruited from Zhejiang Provincial People's Hospital between January 2019 and January 2021 for this study.

2.2 Inclusion and exclusion criteria

Inclusion criteria: (1) Age between 18 and 80 years. (2) Pathologically confirmed diagnosis of urothelial carcinoma. (3) Planned to undergo transurethral resection of bladder tumor. (4) ECOG performance status score of 0–2. (5) Expected survival of at least 3 months.

Exclusion criteria: (1) Presence of other malignant tumors. (2) History of previous treatments for urothelial carcinoma, such as surgery, chemotherapy, or immunotherapy. (3) Significant abnormalities in cardiac, pulmonary, hepatic, or renal function, insufficient compensatory function, or presence of severe systemic diseases.

2.3 Cell line

Human bladder cancer cell lines T24 and 5637 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). SV-HUC-1 cells were grown in F12K medium, T24 cells were grown in McCoy medium, and 5637 and EJ cells were grown in the smallest RPMI-1640 medium (Gibco, Gaithersburg, MD, USA). Incubate at 37 °C under non-ionic conditions (5% CO_2, 95% O_2).

2.4 Assessment of circulating HMGB1 mRNA levels

To quantify HMGB1 mRNA expression in peripheral blood, we employed real-time fluorescence quantitative PCR [13]. Specifically designed primer sequences targeting human HMGB1 and the reference gene GAPDH were utilized, formulated using Primer-BLAST and Oligo 7 tools (Table 1).

2.5 Data collection

Baseline information, including patient demographics (gender, age), clinical parameters (body mass index, chronic disease history, family history, smoking history), and pathological characteristics (tumor diameter, tumor stage, tumor location, number of tumors, hydronephrosis, lymph node metastasis), were collected from the medical records of the enrolled patients. After surgical resection, patients were followed up for a duration of 3 years. The primary endpoint was tumor recurrence leading to disease-specific mortality, which was determined based on clinical and imaging assessments.

2.6 Western blot

Cells were lysed for 30 min using RIPA lysis buffer, followed by centrifugation at 12,000 rpm for 10 min. The quantity of total protein was assessed by the BCA protein assay kit (P0012, Beyotime, China). The protein samples were separated on a 10% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Millipore, Shenzhen, China). The membranes were blocked with 5% bovine serum albumin (BSA) in TBST buffer for 1 h and then incubated with primary antibodies (1:1000, ABCAM, USA) at 4 °C overnight. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Santa Cruz, USA) for 1 h at room temperature. Protein bands were detected with enhanced chemiluminescence (ECL), and the relative protein quantity was determined using ImageJ software (National Institutes of Health, USA) [14].

2.7 Cell-viability assay

Cell viability was assessed using a CCK-8 kit, following the manufacturer's protocol. Neurons were plated in 96-well plates at an initial density of 1×10^4 cells per well and cultured for 24 h. Cell treatment was conducted as per experimental

Table 1 Primer sequences

	Upstream	Downstream	Length (bp)
HMGB1	5′-TGCAGATGACAAGCA GCCTT-3′	5′-GCTGCATCAGGCTTTCCTTT-3′	102
GAPDH	5′-TGCCAAATATGATGA CATCAAGAA-3′	5′-GGAGTGGGTGTCGCTGTTG-3′	121

protocols. Subsequently, 10 µl of CCK-8 reagent was added to each well and incubated for 1 h. The optical density (OD) at 450 nm for each well was measured using a microplate reader. This procedure was repeated a minimum of three times. Cell viability was calculated with the formula: cell viability (%) = (OD of the experimental group / average OD of the control group) × 100% [15].

2.8 Transwell assay

Transwell chambers were used to investigate cell migration. The BUC 5637 and T24 cells were starved for 24 h. After digestion and centrifugation, the cells were resuspended in serum-free medium, and the cells were counted under a microscope. The cell concentration was adjusted to 1 × 105 cells/ml, and a 100 µl/well cell suspension was placed in the bottom of a 24-well plate. We added 600 µl of 30% FBS medium to each well and then added the cell suspension to an MTS 96-well plate. We added 5000 cells to each well and measured the absorbance at OD570 as a metastasis reference. After incubation at 37 °C for 24 h, the cells in the internal compartment were removed with a cotton swab. The migrated cells were fixed in methanol and stained with a 1% crystal violet solution. Under a light microscope, three randomly selected 200 × fields of view were counted, and images were captured [16].

2.9 Statistical analysis

Data analysis was conducted using SPSS version 23.0. Continuous variables were presented as mean ± standard deviation (mean ± SD), and group comparisons were performed using independent t-tests. Categorical variables were described as frequencies or percentages and compared using chi-square tests. To identify independent factors associated with postoperative disease-free survival, Lasso regression and Cox regression analyses were utilized. A column line graph model was constructed using the R programming language. The predictive performance of the model was assessed using the concordance index (C-index), while the predictive accuracy was evaluated through receiver operating characteristic (ROC) curve analysis, calibration curve analysis, and decision curve analysis. A significance level of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Patient characteristics

Table 2 summarizes the baseline characteristics of the included patients. The mean age of the patients was (62.45 ± 9.07) years, with 138 male and 90 female individuals. The average body mass index (BMI) was (21.46 ± 1.59) kg/m^2. The mean tumor diameter was (3.39 ± 1.40) cm. Among the patients, 59 had high-grade tumors, while 169 had intermediate to low-grade tumors. Lymph node metastasis was observed in 31 patients (N1–2), whereas 197 patients had no lymph node involvement (N0). The primary tumor stage (T stage) was classified as Ta in 37 cases and T1–T4 in 191 cases. Forty-nine patients had a history of alcohol consumption, 118 had a smoking history, and 39 had a family history of bladder urothelial carcinoma. The relative expression level of HMGB1 was (4.17 ± 1.13).

3.2 Comparative analysis of HMGB1 mRNA expression levels across various differentiation grades

Figure 1 illustrates the comparison of HMGB1 mRNA expression levels across different grades of differentiation. In the high-grade group, the expression level of HMGB1 mRNA was measured at (4.07 ± 1.18), whereas in the intermediate to low-grade group, it was (4.20 ± 1.12). No statistically significant variance in HMGB1 mRNA expression levels was observed among patients with differing grades of differentiation ($p > 0.05$).

3.3 Comparison of HMGB1 mRNA expression levels among different tumor stages

The comparison of HMGB1 mRNA expression levels among different tumor stages is presented in Fig. 2. The expression level of HMGB1 mRNA in the N0 stage was (4.11 ± 1.36), while in the N1-2 stage, it was (4.18 ± 1.10). For the Ta stage, HMGB1 mRNA expression level was (4.16 ± 1.13), and for the T1-4 stage, it was (4.21 ± 1.16). There was no significant difference in HMGB1 mRNA expression levels among different T and N stages (all $P > 0.05$).

Table 2 Baseline characteristics of the included patients

Variables	Total (mean, n)	SD, %
n	228	
Age (years old)	62.45	9.07
Sex, n (%)		
Male	138	60.53
Female	90	39.47
BMI (kg/m^2)	21.46	1.59
Diameter (cm)	3.39	1.40
Differentiation, n (%)		
High	59	25.88
Low-medium	169	74.12
N stage, n (%)		
N0	197	86.40
N1, 2	31	13.60
T stage, n (%)		
T1–4	191	83.77
Ta	37	16.23
Drink, n (%)		
No	179	78.51
Yes	49	21.49
Smoke, n (%)		
No	110	48.25
Yes	118	51.75
History, n (%)		
No	189	82.89
Yes	39	17.11
HMGB1	4.17	1.13

SD standard deviation

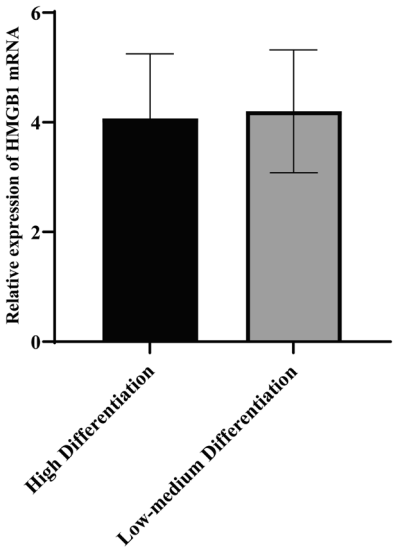

Fig. 1 Comparison of HMGB1 mRNA expression levels in different degrees of differentiation

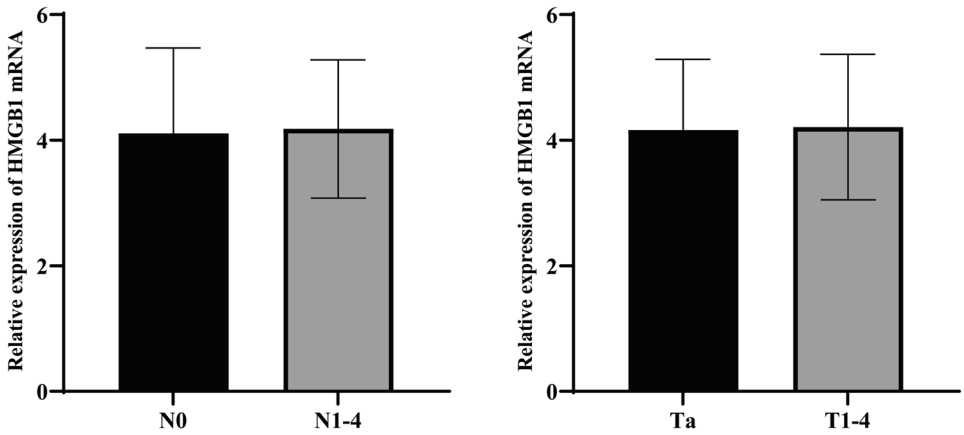

Fig. 2 Comparison of HMGB1 mRNA expression levels in different tumor stages

3.4 Correlation between tumor diameter and HMGB1 mRNA expression

The relationship between tumor diameter and HMGB1 mRNA expression was explored using Pearson's linear regression, as shown in Fig. 3. There was no significant correlation between tumor diameter and HMGB1 mRNA expression ($P > 0.05$).

3.5 Comparison of HMGB1 mRNA expression in different prognoses

During the 3-year follow-up after surgery, 84 patients did not experience a recurrence, while 144 patients had a recurrence. The 3-year survival rate was 36.84%. The baseline HMGB1 mRNA expression level in patients without recurrence was (3.75 ± 1.10), while in patients with recurrence, it was (4.42 ± 1.08). The baseline circulating HMGB1 mRNA expression

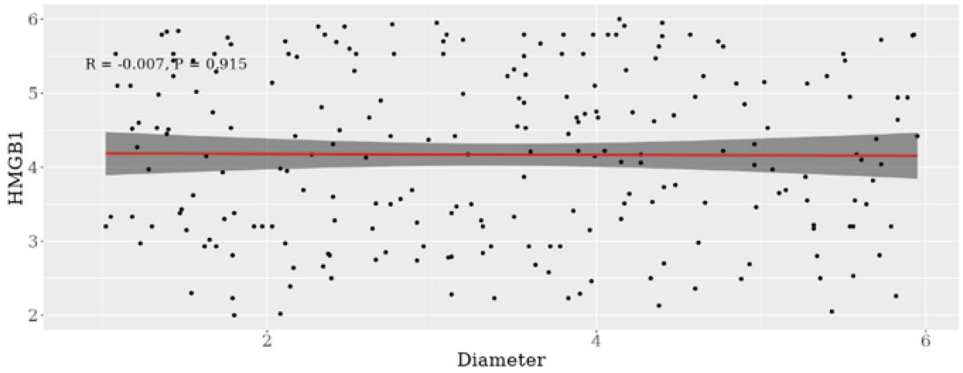

Fig. 3 Correlation between tumor diameter and HMGB1 mRNA expression

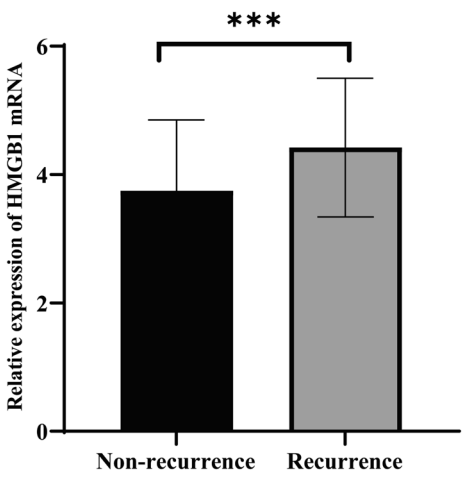

Fig. 4 Comparison of HMGB1 mRNA expression levels in different prognostic groups

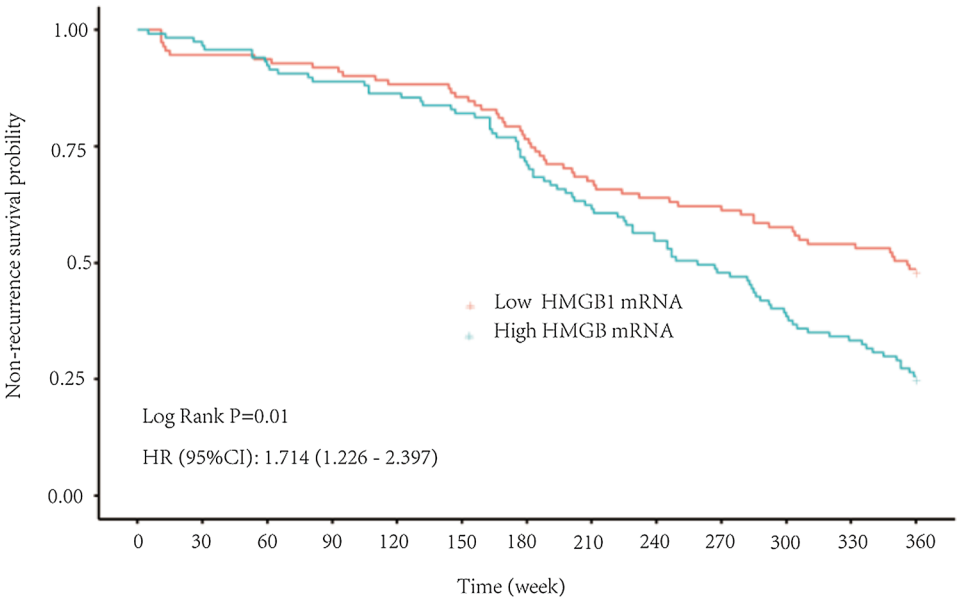

Fig. 5 Recurrence-free survival curves based on different levels of HMGB1 mRNA expression

Table 3 Median recurrence-free survival time

Variables	N	Events	Median (95%CI)	Rate/1000 (person-years)	Log rank P value
HMGB1 median					0.001
Low	111	57	356.00 (292.00–NA)	158.33	
High	117	87	259.00 (226.00–299.00)	324.63	

level was significantly lower in patients without recurrence compared to those who experienced recurrence. The results are shown in Fig. 4.

3.6 Survival analysis based on circulating HMGB1 mRNA expression

Patients were stratified into two cohorts according to the median HMGB1 mRNA expression level: the low-expression group (< 4.17, n = 111) and the high-expression group (≥ 4.17, n = 117). Kaplan–Meier survival curves were utilized to evaluate the disparity in recurrence-free survival between these groups, as depicted in Fig. 5 and summarized in Table 3. The median recurrence-free survival duration in the low-expression group was 356.00 (292.00–NA) weeks, whereas in the high-expression group, it stood at 259.00 (226.00–299.00) weeks. Remarkably, the low-expression group demonstrated markedly superior recurrence-free survival compared to their high-expression counterparts (HR = 1.714, 95% CI 1.226, 2.397).

3.7 Downregulating the expression of HMGB1 can significantly impede the proliferation and migration of tumor cells

WB assay confirmed the high expression of HMGB1 in BUC tissue and tumor basal layer (Fig. 6A, B). In *vitro* cell experiments, we found that HMGB1 was highly expressed in T24 cell lines and 5637 cell lines compared with the control group (Fig. 6C–F). By injecting chemotherapy drugs (pharmorubicin, pirarubicin, gemcitabine), we found that the migration of tumor cells was significantly inhibited, and the migration of tumor cells was also significantly inhibited after inhibiting the expression of HMGB1 (Fig. 6G–J). In order to measure the effects of HMGB1 on T24 cells and 5637 cells viability, CCK-8

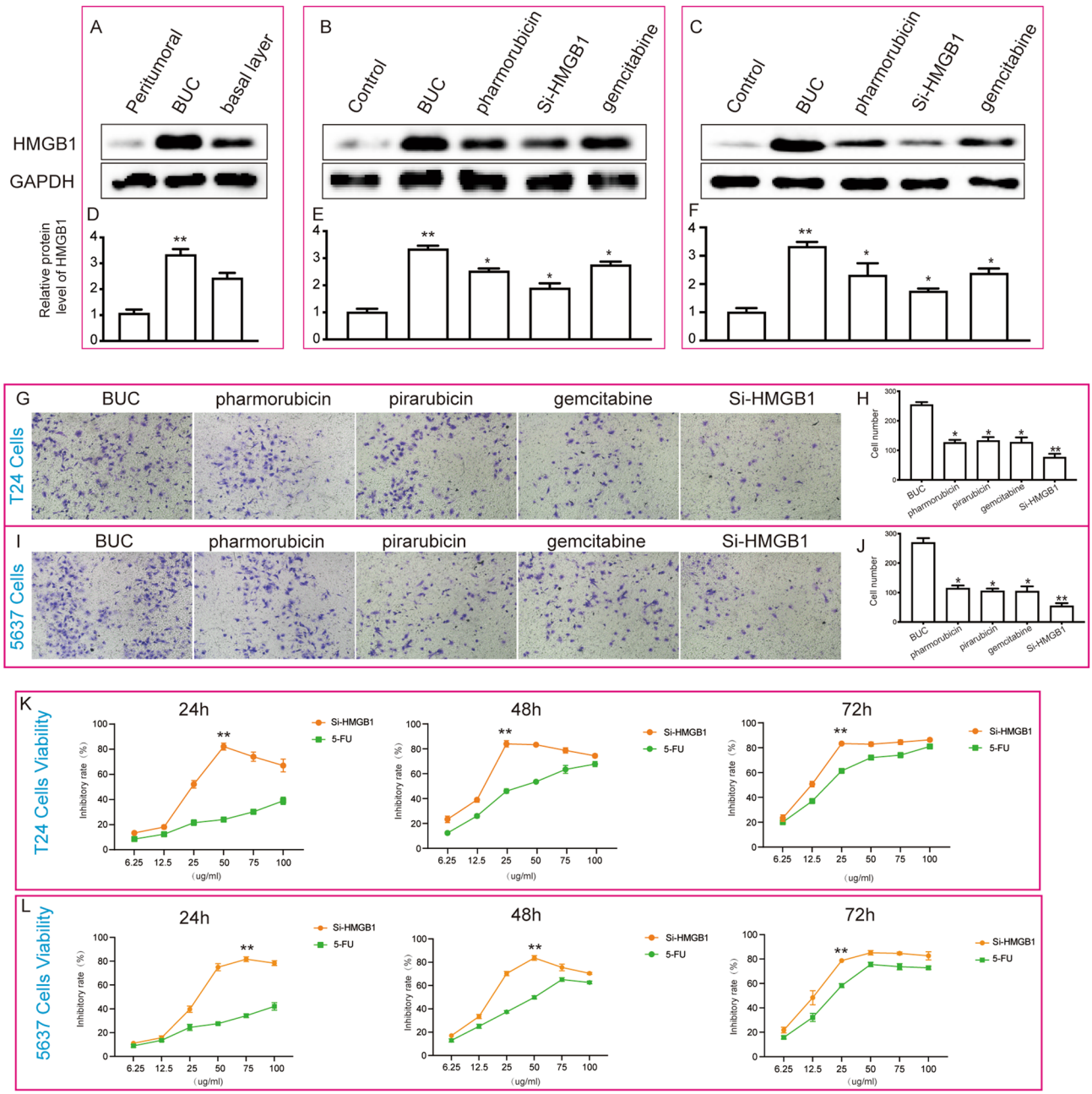

Fig. 6 Inhibition of HMGB1 expression can effectively inhibit the development of BUC. **A**-**F** , representative western blot images and quantitative analyses of HMGB1, mean values for the peritumoral and control group were normalized to 1.0.; **G**- **J**, after transfection of T24 and 5637 cells, cross-well migration were performed to detect cell migration capabilities. The number of cells in 5 random areas was counted in units of 200 × magnification; **K** , viability of T24 cells with different concentrations of Si-HMGB1 treatment in 24, 48, and 72 h were measured by CCK-8 assay; **L** , viability of 5637 cells with different concentrations of Si-HMGB1 treatment in 24, 48, and 72 h were measured by CCK-8 assay. *$p < 0.01$ and $p < 0.05$

assay was used. Furthermore, 5- fluorouracil (5-FU) was selected as a positive control. The results were represented in Fig. 6K, L which revealed that the inhibition of Si-HMGB1 on T24 cells/5637 cells viability was significantly higher than 5-FU with the same concentration and time. Compared with the control group, T24 cells/5637 cells viability in

Si-HMGB1-treated group was significantly decreased, indicating that Si-HMGB1 significantly inhibited T24 cells/5637 cells viability in a time- and concentration-dependent manner.

4 Discussion

In this investigation, we scrutinized the expression patterns of HMGB1 mRNA in individuals diagnosed with BUC, aiming to unravel potential associations with diverse clinical and pathological characteristics. Furthermore, our inquiry delved into the impact of varied HMGB1 mRNA expression levels on recurrence-free survival. Surprisingly, our findings unveiled an absence of notable correlations between HMGB1 mRNA expression levels and critical factors such as lymph node stage, tumor stage, or tumor diameter. Notably, individuals devoid of recurrence exhibited markedly lower baseline HMGB1 mRNA expression levels in contrast to their recurrent counterparts. Moreover, employing Kaplan–Meier survival analysis, we unveiled a substantial discrepancy in survival outcomes, where patients harboring low HMGB1 mRNA expression showcased notably enhanced survival rates relative to those characterized by high expression levels.

Emerging data indicate that the interplay of genetic predisposition, familial aspects, and environmental elements might exert influence over the susceptibility and occurrence of BUC [17]. A comprehensive meta-analysis encompassing 18 investigations spanning 11 distinct cancer types, such as gastric cancer, colorectal cancer, hepatocellular carcinoma, and others, unveiled a noteworthy association between HMGB1 overexpression and unfavorable overall survival (OS) as well as progression-free survival (PFS). This finding underscores the potential of HMGB1 as a prognostic determinant and a plausible biomarker for cancer prognosis [18].

Yang et al. found that in 53% of cases of UBC, HMGB1 protein was found to be overexpressed, showing a significant correlation with both tumor grade and stage. Moreover, according to Kaplan–Meier survival analysis, HMGB1 expression exhibited a significant association with reduced disease-free survival and overall survival rates [19]. Wang et al. conducted immunohistochemical analysis of HMGB1 in 64 UBC patients and found that HMGB1 was overexpressed in tumor tissues compared to normal tissues and was associated with clinical stage, pathological grade, and angiogenesis [20]. The survival outcomes of these studies are consistent with the results of this study. However, this study did not find differences in HMGB1 mRNA expression among clinical and pathological stages and grades. This discrepancy may be due to the different detection methods used. This study employed real-time fluorescence quantitative PCR to detect HMGB1 mRNA expression in peripheral blood, which is more convenient than detecting in tumor tissues but may have relatively lower concentrations in circulation.

HMGB1 is a nuclear protein that acts as a chromatin-binding factor and is present in the cell nucleus of both cancer cells and normal cells [17]. The relationship between HMGB1 and poor prognosis in tumors is not yet clear but is associated with various factors. HMGB1 has a dual regulatory effect on the immune function of the body. It can act as a protective factor, activating immune cells and enhancing immune function to exert an anti-tumor effect [21]. However, it can also bind to TIM-3 on the surface of infiltrating immature dendritic cells in the tumor microenvironment (TME), stimulating their immune inhibitory potential and causing these cells to differentiate into myeloid-derived suppressor cells, exerting a pro-tumor effect [22]. In addition, HMGB1 can inhibit tumor cell apoptosis by upregulating the expression of the B lymphoma-2 gene family members. Chai et al. found through ultrastructural analysis that depletion of HMGB1 also reduced the amount of autophagosomes in tumor cells, indicating that HMGB1 has a certain regulatory effect on tumor cell autophagy [23]. Furthermore, HMGB1 can increase the expression of vascular endothelial growth factors and attract macrophages, which in turn produce many effective angiogenic cytokines and growth factors [24].

Although our clinical analysis indicated that the elevation of HMGB1 mRNA levels in blood circulation correlates with a poor prognosis for patients, this finding necessitates further validation at the molecular level. WB experiments confirmed the upregulation of HMGB1 expression in tumor tissues, suggesting its significant role in bladder urothelial carcinoma (BUC) progression. Additionally, increased HMGB1 expression was observed in vitro across various cell lines. We selected Epirubicin and gemcitabine for injection and discovered that these two chemotherapeutic agents effectively suppressed HMGB1 expression; consequently, migration assays demonstrated that chemotherapy drugs significantly inhibited tumor cell migration. Following the downregulation of HMGB1 expression, BUC cell mobility decreased markedly. These findings indicate that targeting HMGB1 could substantially impede tumor cell development. Ultimately, through cellular viability assays, we established that reducing HMGB1 levels effectively curtails tumor cell proliferation. Collectively, these results underscore the close association between HMGB1 and BUC pathogenesis, suggesting that inhibiting its expression may represent a promising therapeutic strategy for managing BUC.

Although this article has conducted a certain study on the prognosis of HMGB1 in BUC patients, it also has certain limitations. The study included a small number of patients and did not explore specific molecular mechanisms. In addition, patient information was not summarized more accurately. In the final experiment, we did not conduct animal model tests, and we did not have a better explanation of relevant upstream and downstream molecules. We will pay more attention to this content in future work.

5 Conclusion

Circulating HMGB1 mRNA expression level is closely related to the prognosis of BUC patients, and patients with low HMGB1 expression have longer survival. HMGB1 is highly expressed in tumor tissues, and HMGB1 can promote the proliferation and migration of tumor cells. Chemotherapy drugs can inhibit the expression of HMGB1, and inhibition of HMGB1 can effectively inhibit the proliferation of BUC cells. This goal is expected to lead to new treatment options for patients with BUC and change their long-term prognosis.

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Author contributions All the authors had full access to all the data of the study, who were responsible for data integrity and accuracy. JJL worked on study concept. ZC were responsible for data acquisition. MX wrote the manuscript. LH participated in the design, administrative, technical, and material support. TLY worked on supervision.

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

Declarations

Ethics approval and consent to participate The present study was approved by the Ethics Committee of Zhejiang provincial people's hospital. The prostate cancer patients and healthy controls all provided informed consent. This investigation was conducted based on the principles of the declaration of Helsinki.

Consent for publication All authors have read the manuscript and approved for publication.

Competing interests The authors declare no competing interests.

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