Life Sciences 232 (2019) 116614

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The role of SOX18 in bladder cancer and its underlying mechanism in mediating cellular functions

Yin Huaqi, Qin Caipeng, Wang Qiang, Du Yiqing, Xu Tao*

Department of Urology, Peking University People's Hospital, The Second Clinical Medical College of Peking University, Beijing 100044, China

ARTICLE INFO

Keywords:
SOX18
Bladder cancer
Cell invasion
Cell cycle
MMPs

ABSTRACT

Aims: SRY-box 18 (SOX18) is a transcription factor known for its role in regulating cell differentiation and lymphatic and blood vessel development. It has been reported that SOX18 was involved in various diseases, including cancer. This study aimed to explore the significance and biological function of SOX18 in bladder cancer (BCa).

Materials and methods: SOX18 expression in BCa and normal tissues was analyzed by immunohistochemistry, and SOX18 expression in BCa cell lines was quantified by western blotting and quantitative real-time PCR. The role of SOX18 on the proliferation, migration and invasion of BCa cells was explored by CCK-8 and transwell invasion assays in vitro. Cell cycle was measured by flow cytometry assays. Western blotting and qRT-PCR were performed to investigate the potential mechanisms by which SOX18 leads to tumor progression.

Key findings: SOX18 was significantly upregulated in BCa and its expression was associated with clinical features of patients with BCa. Our data demonstrated that SOX18 promoted cell proliferation via accelerating cell cycle and by regulating c-Myc and Cyclin D1, promoted cell invasion via upregulation of MMP-7. Moreover, phosphorylation of c-Met and Akt regulated by SOX18 was identified to be involved in the process of cell migration and invasion, indicating the vital role of SOX18 in the metastasis of BCa.

Significance: Our data demonstrated a cancer-promoting effect of SOX18 in BCa, revealed the potential mechanisms of SOX18 in mediating cellular functions, and indicated that SOX18 may serve as a promising progression and prognostic biomarker and a therapeutic target for BCa.

1. Introduction

Bladder cancer (BCa) is the most common urological cancer and is a global threat. Urothelial cell carcinoma (UCC) is the predominant histological form of this neoplasm [1]. BCas are usually classified as non-muscle invasive and muscle invasive tumors. Muscle-invasive BCa (MIBC) accounts for 30% of BCas and is associated with a high recurrence frequency and a poor prognosis [2]. The standard treatment for BCa is radical cystectomy. Even with recent improvements in treatments, mortality due to MIBC remains high. Studies have demonstrated that a number of molecular therapeutic targets, including PI3K/AKT/mTOR, VEGFR, RTK/RAS and Her2, are potentially beneficial for treating patients with BCa [3–7]. However, the lack of therapeutic target specificity for BCa facilitates the examination of novel molecules involved in tumorigenesis and progression of BCa.

SRY-box 18 (SOX18) is a member of the SOX transcription factor family, which is composed of > 20 family members, sharing a common HMG box [8]. SOX18 is well known for its functional role in regulating cell differentiation and determining cell fate during embryonic development [8,9]. Disruption of the function of SOX18 leads to diseases, including hypotrichosis-lymphedema-telangiectasia [10]. Recently, accumulating evidence suggests that SOX18 is upregulated in various types of human malignant tumors, including ovarian, lung, breast and hepatocellular carcinoma [11–15]. Additionally, previous studies have reported that SOX18 is upregulated in HCC and that upregulated SOX18 expression promotes cell proliferation, migration and invasion and is associated with clinical characteristics. However, the role of SOX18 expression in human BCa remains unknown, and its biological function and potential mechanisms associated with the occurrence and progression of BCa has yet to be elucidated.

In the present study, SOX18 was indicated to be significantly upregulated in BCa tissues and cell lines, and its expression was associated with patients' clinical features. In particular, SOX18 expression was associated with poor outcomes of patients with BCa. In addition, SOX18 promoted the proliferation, migration and invasion of BCa cell lines. Furthermore, expression of molecules associated with tumor

* Corresponding author at: Department of Urology, Peking University People's Hospital, No.11, Xi Zhi Men Nan Street, 100044 Beijing, China.
E-mail address: xutao@pkuph.edu.cn (X. Tao).

https://doi.org/10.1016/j.lfs.2019.116614
Received 27 February 2019; Received in revised form 20 June 2019; Accepted 27 June 2019
Available online 28 June 2019
0024-3205/

progression, including c-Myc, Cyclin D1, Matrix metalloproteinase-7 (MMP-7), phosphorylated (p)-Met (Tyr1349) and p-Akt (Ser473), were reduced or increased following silencing or overexpression of SOX18 in BCa cells. Therefore, the present study demonstrated the cancer-promoting effects of SOX18 in BCa and revealed the potential mechanisms of SOX18 in cell proliferation and tumor metastasis. It also indicated that SOX18 may serve as a promising progression and prognostic biomarker, in addition to a therapeutic target for BCa.

2. Material and methods

2.1. Tissue microarray and specimens

The tissue microarray, consisting of 105 sample spots, including 59 BCa and 46 corresponding normal tissue samples, was purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). A total of 31 BCa specimens were collected from patients diagnosed with BCa, who received a resection at Peking University People's Hospital (Beijing, China). All cases were followed up for 2–96 months. Clinical stage was classified according to American Joint Committee on Cancer. The approval from the Ethical Reviewing Committee of Peking University People's Hospital (Beijing, China) was obtained after written informed consent was provided from all patients prior to inclusion in the study. The clinicopathological information of patients is presented in Table 1.

2.2. Immunohistochemical analysis

The tissue microarray and specimens were dewaxed in xylene, rehydrated in graded alcohol, and subsequently incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxide activity. Following antigen recovery in a 95 °C water bath for 20 min, followed by incubation with 30% normal goat serum for 25 min, the microarray and specimens were incubated with SOX18 rabbit monoclonal antibody (dilution, 1:200; Abcam, Cambridge, MA, USA) at 4 °C overnight. Immunodetection was performed using the Encision ABC Kit (Gene Tech Biotechnology Co., Ltd., Shanghai, China). The microarray and specimens were stained with hematoxylin, dehydrated and mounted. SOX18 immunostaining score and category of SOX18 expression were as previously described [16]. The overall SOX18 staining results were categorized as follows: Low expression, final score < 4; and high expression, final score ≥ 4.

Table 1
Relationship between SOX18 and clinical features of patients with bladder cancer.

Characteristics	Number	Expression of SOX18		P-value
		Low	High	
Pathological grade				0.009
I + II	52(57.8%)	31(34.4%)	21(23.3%)	
III	38(42.2%)	12(13.3%)	26(28.9%)	
Clinical stage				< 0.001
I + II	41(45.6%)	30(33.3%)	11(12.2%)	
III + IV	49(54.4%)	13(14.4%)	36(40.0%)	
Gender				0.467
Male	77(85.6%)	38(42.2%)	39(43.3%)	
Female	13(14.4%)	5(5.6%)	8(8.9%)	
Age (years)				0.833
≥70	45(50%)	22(24.4%)	23(25.6%)	
< 70	45(50%)	21(23.3%)	24(26.7%)	
Size (cm)				0.718
≥4	38(42.2%)	19(21.1%)	19(21.1%)	
< 4	52(57.8%)	24(26.7%)	28(31.1%)	
Lymph node metastasis				0.046
Yes	28(31.1%)	9(10.0%)	19(21.1%)	
No	62(68.9%)	34(37.8%)	28(31.1%)	
Muscle infiltration				0.007
Yes	53(58.9%)	19(21.1%)	34(37.8%)	
No	37(41.1%)	24(26.7%)	13(14.4%)	

2.3. Cell culture and reagent

Two malignant BCa cell lines, including T24 and 5637, and the relatively low-grade malignant cell line RT4, were used in this study. T24, 5637 and RT4 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). RT4 cells were cultured in McCoy's 5A culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the remaining cell lines were cultured in RPMI 1640 culture medium (HyClone; GE Healthcare, Chicago, IL, USA) in a 5% CO_2 humidified atmosphere. All culture medium was supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). For the inhibition of c-Met, the cells were incubated with a selected concentration of cabozantinib (3 μM) for 48 h prior to subsequent experiments.

2.4. Cell transfection

Two small interfering RNAs, including siRNA1 and siRNA2, against SOX18 and corresponding non-silencing control RNA (siNC) were purchased from Shanghai GenePharma Co., Ltd., (Shanghai, China). The sequences of the siRNAs and siNC are presented in Table 2. The siRNAs and siNC were transiently transfected into T24 and 5637 cells using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The efficiency of SOX18 silencing was verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Lentiviruses were packaged by Shanghai Genechem Co., Ltd., (Shanghai, China). The silencing (LV-RNA1) and control sequences (LV-NC) in the lentiviruses were the same as the siRNA1 and siNC sequences, respectively. Lentiviruses containing SOX18 gene sequence and control sequence (NC) were also purchased from Shanghai GeneChem Co., Ltd., (Shanghai, China). T24 or 5637 cell lines were infected with the lentiviruses at a multiplicity of infection of 20 for 48 h. A total of 2 mg/ml-concentration of puromycin (EMD Millipore, Billerica, MA, USA) was subsequently used to screen for stably infected

Table 2
The sequence of gene primer and small interfering RNAs.

Name	Primer/interfering sequence
SOX18	F: 5′-TTCGACCAGTACCTCAACTGC-3′
	R: 5′-GACATGGAACCAAACATACACG-3′
Cyclin B1	F: 5′-TGGTGTCACTGCCATGTTTATTG-3′
	R: 5′-CCATCTGTCTGATTTTTGTCTTAG-3′
Cyclin D1	F: 5′-CGCCCCACCCCTCCAG-3′
	R: 5′-CCGCCCAGACCCTCAGACT-3′
Cyclin E1	F: 5′-GCCTTGTATCATTTCTCGTC-3′
	R: 5′-GCTGTCTCTGTGGGTCTG-3′
c-Myc	F: 5′-TGCCCATTTGGGGACACTTC-3′
	R: 5′-TACGGCTGCACCGAGTCGT-3′
MMP-2	F: 5′-CCAAAGTAAACAGCAAGAGAACC-3′
	R: 5′-GCCACAATAAAGCAACAAAGCAAC-3′
MMP-3	F: 5′-GAGTGTGGATTCTGCCATTGAAA-3′
	R: 5′-GCAAAGGAGATCATTATGTTCAGCC-3″
MMP-7	F: 5′-AAACTCCCGCGTCATAGAAAT-3′
	R: 5′-TCCCTAGACTGCTACCATCCG-3′
MMP-9	F: 5′-AAACTCCCGCGTCATAGAAAT-3′
	R: 5′-TCCCTAGACTGCTACCATCCG-3′
GAPDH	F: 5′-CACCCACTCCTCCACCTTTG-3′
	R: 5′-CCACCACCCTGTTGCTGTAG-3′
siRNA1	F: 5′-GGGUUACAUUUUUGAAGCATT-3′
	R: 5′-UGCUUCAAAAAUGUAACCCTT-3′
siRNA2	F: 5′-CUCUCUCAUACGCGUGUAUTT-3′
	R: 5′-AUACACGCGUAUGAGAGAGTT-3′
siNC	F: 5′-UUCUCCGAACGUGUCACGUTT-3′
	R: 5′-ACGUGACACGUUCGGAGAATT-3′

cells. The infection efficiency was verified by RT-qPCR and western blot analysis.

2.5. RNA isolation and RT-qPCR

Total RNA was extracted from the cells using an RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocols. Complementary DNA (cDNA) was synthesized using a Fast Quant RT kit (Tiangen Biotech Co.), according to the manufacturer's protocols, and stored at −20 °C. RT-qPCR was performed using a KAPA SYBR FAST Universal q-PCR kit (Kapa Biosystems; Roche Diagnostics, Indianapolis, IN, USA) on an iQ5 real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was amplified as an internal control. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. The primers are listed in Table 2.

2.6. Western blot analysis

Preparation of whole cell proteins and western blot analysis was performed as described previously [17]. The membranes were incubated with primary antibodies at 4 °C overnight. The primary antibodies were as follows: SOX18 rabbit monoclonal antibody (1:1000 dilution; Abcam), Cyclin B1/D1/E1 rabbit monoclonal antibodies (1:1000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), MMP-2/3/7/9 rabbit monoclonal antibodies (1:1000 dilution; Cell Signaling Technology, Inc.), c-Myc rabbit monoclonal antibody (1:1000 dilution; Cell Signaling Technology, Inc.), Akt/p-Akt (Ser473) rabbit monoclonal antibody (1:1000 dilution; Cell Signaling Technology, Inc.), Met/p-Met (Tyr1349) rabbit monoclonal antibody (1:1000 dilution; Cell Signaling Technology, Inc.), GAPDH rabbit monoclonal antibody (1:2000 dilution; Cell Signaling Technology, Inc.). Goat anti-rabbit IgG-HRP antibody (1:2000 dilution; Cell Signaling Technology, Inc.) was used as the secondary antibody, and GAPDH was used as a loading control.

2.7. CCK-8 assays

Cells (2×10^3) of a 100 μl volume were seeded into 96-well plates. The absorbance rate of cells was measured at a wavelength of 450 nm at different time points (0, 24, 48, 72 and 96 h). At each time point, 10 μl of CCK-8 reagent was added to each well to stain the cells. Following incubation for 2 h at 37 °C, the absorbance at 450 nm was measured to assess cell proliferation. All experiments were performed in triplicate.

2.8. Tumor xenograft model in nude mice

All procedures for the animals operation were approved by the Peking University Institutional Animal Care and Use Committee. Twelve BALB/c nude mice (female, 4–6 weeks old, weighing 18–25 g) were divided into two groups. A total of 5×10^6 T24 cells infected with LV-RNA1/LV-NC in 150 μl Col-Tgel (Bioruo, Beijing, China) randomly implanted subcutaneously into nude mice on Day 0. The mice were fed in a germ-free environment with regulated temperature and humidity. The tumor volumes were measured every 5 days and calculated as the length × width^2 × 0.5. Five weeks later, the mice were sacrificed by breaking their cervical vertebra on Day 35, and the tumors were removed, weighed and photographed.

2.9. Transwell invasion assays

Cell migration and invasion were analyzed using Transwell invasion assays without or with Matrigel. Briefly, 4×10^4 cells in 200 μl of serum-free medium were transferred into the upper chamber of a 24-well Transwell or Matrigel chamber with 8-μm pores (Corning Inc., Corning, NY, USA). The lower chamber contained 600 μl of medium supplemented with 10% fetal bovine serum as the chemoattractant. Following incubation for 24 h, cells on the upper membrane surface were removed with cotton swabs, and cells that migrated or invaded through the filter were fixed with 4% paraformaldehyde for 25 min and stained with 1% hematoxylin for 25 min. The number of migrated or invaded cells was calculated in 5 random-selected fields, and each condition was analyzed in triplicate.

2.10. Flow cytometry assay

Cells were harvested, washed with ice-cold PBS and pelleted. The cell cycle was determined by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) following staining with propidium iodide (MultiSciences (Lianke) Biotechnology Corporate Limited, Hang Zhou, China), according to the manufacturer's protocol.

2.11. Statistical analysis

Statistical analyses were carried out using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) statistical software packages. The association between SOX18 expression and patients characteristics was analyzed using the χ^2 test. Data were expressed as the mean ± standard error of the mean, and comparisons between two groups were performed using Student's *t*-test. Two-way analysis of variance was used to analyze the dataset containing multiple groups. Overall survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multiple analyses were carried out using the COX regression model. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. SOX18 is upregulated in human BCa

In order to detected SOX18 expression level in BCa, the expression of SOX18 was first investigated in a panel of BCa cell lines. Western blot analysis and RT-qPCR analyses revealed the upregulated expression of SOX18 protein and mRNA levels in 2 malignant BCa cell lines compared with the relatively low-grade cell line (Fig. 1A and B). In order to further analyze SOX18 expression differences between BCa tissues and normal tissues, immunohistochemistry in tissue microarray and samples was performed. Immunohistochemical analysis indicated that positive staining of SOX18 was for the majority of cases mainly in the cell nuclei. In addition, SOX18 protein expression in human BCa tissues was higher compared with those in normal tissues (Fig. 1C). The SOX18 staining score in 46 pairs of cancer and corresponding normal tissues, included in the tissue microarray assay, were statistically analyzed, in order to gain insight into SOX18 expression level differences. As indicated in Fig. 1D, 37 (80%) spots exhibited a higher SOX18 staining score compared with corresponding spots, while only 3 (7%) spots exhibited a lower SOX18 staining score compared with corresponding spots. No differences in SOX18 staining score were indicated in 6 (13%) pairs of spots. In addition, statistical analysis indicated that the rate of high SOX18 expression was 52.2% (47/90) in BCa tissues and 23.9% (11/46) in normal tissues (Fig. 1E). These data suggested elevated SOX18 expression levels in human BCa.

3.2. SOX18 expression is associated with the clinicopathological characteristics of patients with BCa

Subsequently, it was further investigated whether SOX18 expression was associated with the clinicopathological characteristics of patients with BCa. As presented in Table 1, there were no significant differences between SOX18 expression and age, sex and tumor size ($P = 0.833$, $P = 0.467$ and $P = 0.718$, respectively). However, the rate of high SOX18 expression was increased in cancer tissues with positive lymph node metastasis, higher histological grade (III + IV) and clinical stage

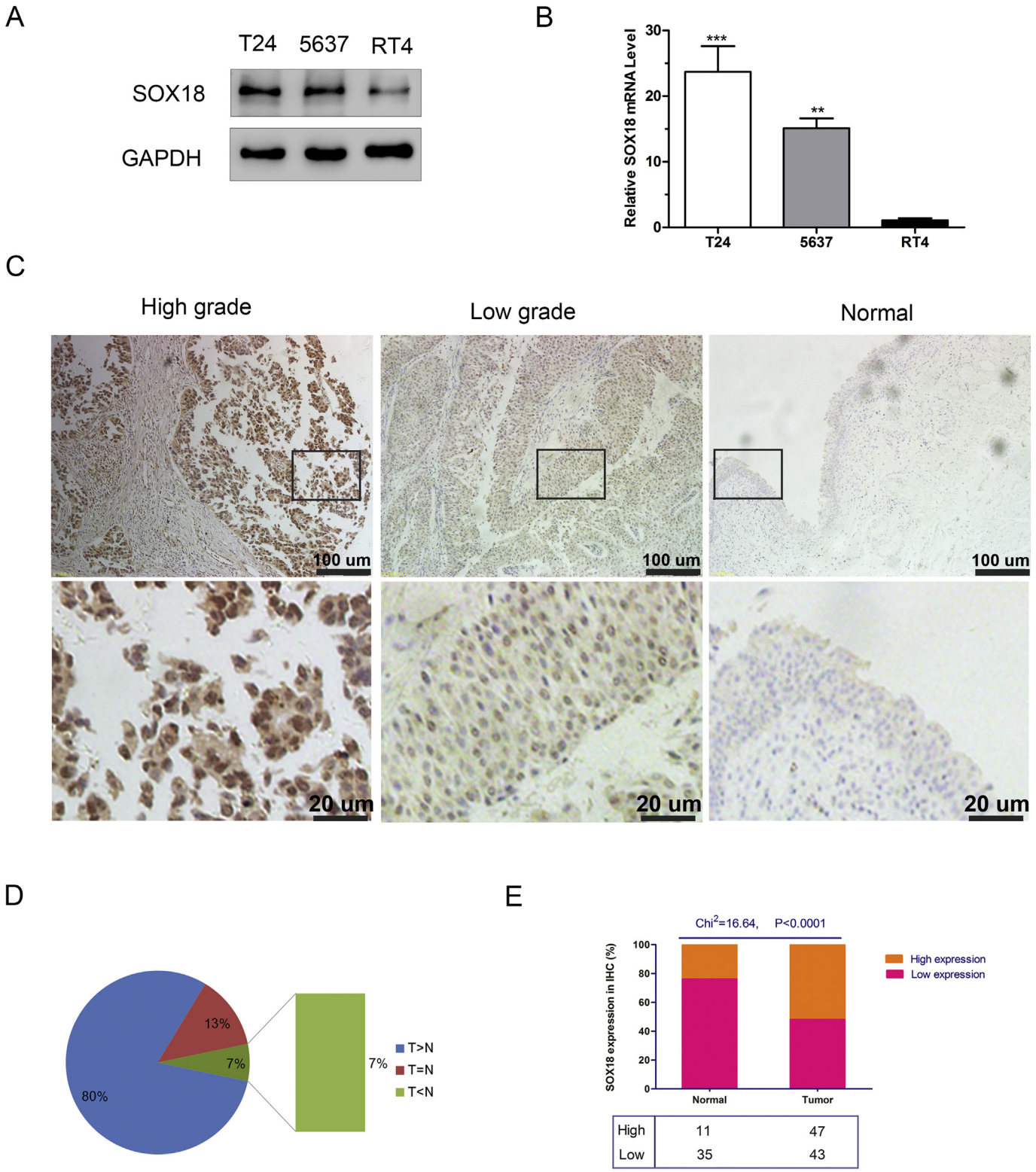

Fig. 1. SOX18 was overexpressed in BCa. SOX18 (A) protein and (B) mRNA expression levels in malignant cell lines, including T24 and 5637, and relatively low-grade cell line RT4. GAPDH was used as a loading control. (C) Immunohistochemical staining of SOX18 protein expression in BCa and normal tissues (magnification, x100 and x400). (D) Distribution of SOX18 staining score in 46 pairs of BCa and corresponding normal tissues. (E) Quantification and percentage of low and high SOX18 expression in BCa and normal tissues. T, cancer; N, normal. ***, ** and * represented $P < 0.0001$, $P < 0.001$ and $P < 0.05$, respectively.

(III + IV), and muscle infiltration ($P = 0.046$, $P = 0.009$, $P < 0.001$ and $P = 0.007$, respectively), which indicated an association between SOX18 expression and adverse clinical characteristics of patients with BCa.

In addition, the overall survival of patients with high SOX18 expression was significantly poorer compared with those with low SOX18 expression as indicated by the Kaplan-Meier survival analysis, and mortality incidence in the high SOX18 expression group was increased

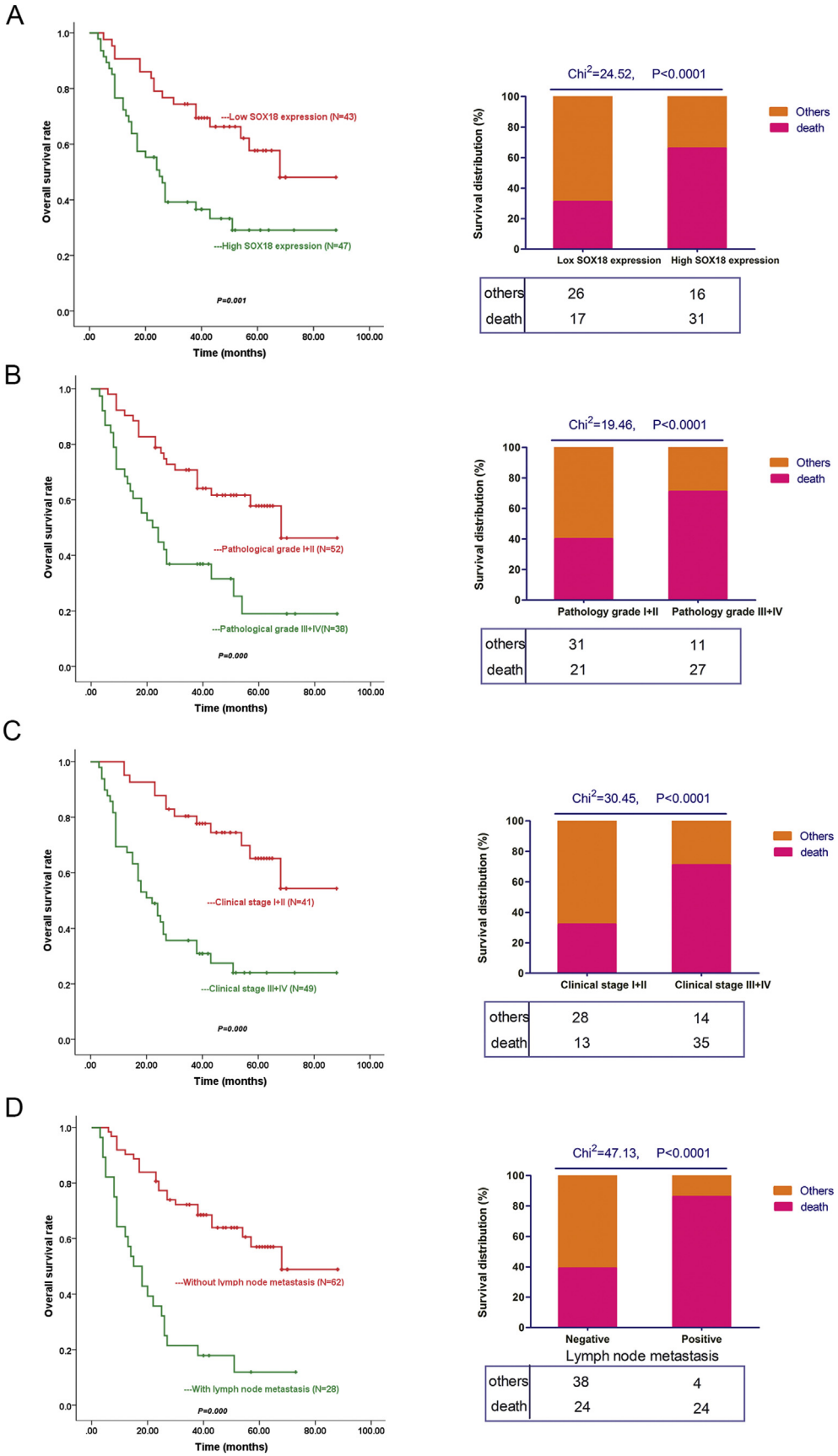

Fig. 2. Patients with high SOX18 expression displayed poor overall survival rate compared with those with low SOX18 expression. (A) Kaplan-Meier curves and survival distribution of patients in the high and low SOX18 expression groups. (B) Kaplan-Meier curves and survival distribution of patients in the different pathological grade groups. (C) Kaplan-Meier curves and survival distribution of patients in the different clinical stage groups. (D) Kaplan-Meier curves and survival distribution of patients with and without lymph node metastasis.

Table 3
Univariable and multivariable analysis of BCa survival using Cox's proportional hazards model.

Clinical features	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥70/ < 70 years)	1.016	0.601–1.872	0.839		Not included	
Gender (male/female)	1.3	0.607–2.786	0.5		Not included	
Tumor size (≥3/ < 3 cm)	0.686	0.389–1.211	0.194		Not included	
Grade (III/I + II)	2.657	1.493–4.728	0.001	1.566	0.75–2.754	0.151
Stage (III + IV/I + II)	3.994	2.092–7.624	0.000	1.887	0.849–2.887	0.135
SOX18 (high/low)	2.548	1.4–4.637	0.002	1.437	0.75–2.754	0.274
Lymph node metastasis (yes/no)	4.199	2.355–7.487	0.000	2.267	1.129–4.551	0.021

compared with the low SOX18 expression group during the 7-year follow-up period (Fig. 2A). Furthermore, the overall survival and mortality incidence in different clinicopathological groups were analyzed to validate the survival rate of patients participating in the survival analysis. Higher histological grade (III + IV), higher clinical stages (III + IV) and positive lymph node metastasis were associated with the poor overall survival rate and the higher mortality incidence in patients with BCa (Fig. 2B, C and D).

According to the aforementioned analysis, it was speculated that high SOX18 expression, high histological grades, high clinical stages and positive lymph node metastasis were risk factors for the prognosis of patients with BCa, which were further confirmed by univariate analysis. However, the multivariate analysis revealed that only lymph node metastasis could serve as an independent risk factor for prognosis of patients with BCa (Table 3), despite the fact that the data of the present study demonstrated the participation of SOX18 in the development of BCa.

3.3. SOX18 promotes BCa cell proliferation and migration in vitro, and tumor growth in vivo

To examine the role of SOX18 in BCa cells, functional cellular experiments were performed using siRNA-mediated silenced SOX18 and lentivirus-mediated overexpressed SOX18 in T24 and 5637 cells. The efficiency of SOX18 silencing or overexpression was determined by western blot analysis and RT-qPCR (Fig. 3A). As shown in Fig. 3B, CCK-8 assays indicated that cell proliferation was significantly suppressed in SOX18-silenced cells, indicating the proliferation-promoting role of SOX18 in BCa cells. This was further confirmed by overexpressed SOX18 cells with improved proliferative capacity. In addition, knocking down SOX18 reduced the tumor volume and weigh in nude mice (Fig. 3C). Taken together, these data suggested that SOX18 is crucial for maintaining BCa cell proliferation and tumor growth.

The influence of SOX18 on cellular migration was subsequently investigated using Transwell invasion assays without Matrigel. The number of migrated cells on the bottom of the cell culture insert in the SOX18-silenced groups was decreased compared with that in the corresponding control groups (Fig. 3D), while upregulation of SOX18 demonstrated a reverse effect (Fig. 3E). The data of the present study demonstrated the important role of SOX18 in accelerating the migration ability of BCa cells, indicating that SOX18 might be responsible for the progression of cancer metastasis.

3.4. SOX18 induces the G_1 to S phase transition via upregulation of c-Myc and Cyclin D1

To examine the proliferation-promoting role of SOX18, flow cytometry assays were performed to analyze the cell cycle distribution, which was suggested to be associated with cell proliferation. The results indicated that SOX18-silenced cells exhibited an increase in the G_0/G_1 phase population, in addition to a decrease in the S phase population compared with the control (Fig. 4A). In contrast, upregulation of SOX18 expression slightly decreased the percentage of cells in G_0/G_1 phase and increased the percentage of cells in the S phase (Fig. 4B). However, no differences were indicated in the population of cells in G_2/M phase among groups (Fig. 4A and B). These results indicated that SOX18 could facilitate BCa cell cycle transition from G_1 to S phase, resulting in rapid cell proliferation.

To elucidate the potential mechanisms behind SOX18-mediated cell cycle progression, mechanistically studies using western blot analysis and RT-qPCR analysis were performed. As c-Myc and Cyclin complex are the main molecules associated with cell cycle, the present study investigated whether these molecules changed in accordance with SOX18 expression. As indicated in Fig. 4C, the knockdown and overexpression of SOX18 reduced and induced the expression of c-Myc and Cyclin D1, respectively, at a protein level involving G_1 to S phase transition [18,19], while neither changes of Cyclin B1 nor cyclin E1 were measured. The effect of SOX18 on c-Myc and Cyclins was further verified by RT-qPCR on an mRNA level (Fig. 4D). According to the aforementioned results, it was demonstrated that SOX18 facilitates BCa cell proliferation by accelerating cell cycle progression via upregulation of c-Myc and Cyclin D1.

3.5. SOX18 enhances the invasiveness of BCa cells via upregulating MMP-7 expression

Statistical analyses of IHC staining score revealed the association of high SOX18 expression with advanced clinical stage and positive lymph node metastasis. This suggests that SOX18 may be implicated with BCa cell invasiveness, which is strongly associated with invasion depth, lymph node and distant metastases. Therefore, the effect of SOX18 on invasion was investigated using Matrigel-coated Transwell invasion assays. Genetic ablation of SOX18 in T24 and 5637 cells by siRNAs significantly reduced the numbers of invasive cells on the bottom of the cell culture insert compared with that by siNC (Fig. 5A). SOX18 upregulation enhanced the invasiveness of BCa cells (Fig. 5B), which in combination with the aforementioned results indicates the precise role of SOX18 in increasing the invasive ability of BCa cells. Therefore, knockdown of SOX18 expression is a promising target for inhibiting progression of BCa metastasis.

Taking into consideration that the main constituent of Matrigel is a mixture of gelatinous protein similar to the extracellular matrix, which has the ability to prevent cell invasion [20], the present study investigated whether the upregulation of SOX18 enhanced the invasive ability of BCa cells via the degradation of Matrigel. Previous studies demonstrated that MMPs are members of a family that is known to promote cell invasion, through degradation of the extracellular matrix [21], therefore making them the optimal candidates for investigation. As indicated in Fig. 5C and D, both protein and mRNA expression levels of MMP-7 were decreased and increased following silencing and upregulation of SOX18, respectively. Our results indicated that SOX18 may enhance the invasiveness of BCa cells by promoting the transcription and translation of MMP-7 gene. The effect of SOX18 on other members of MMPs, including MMP-2, MMP-3 and MMP-9, was also assessed,

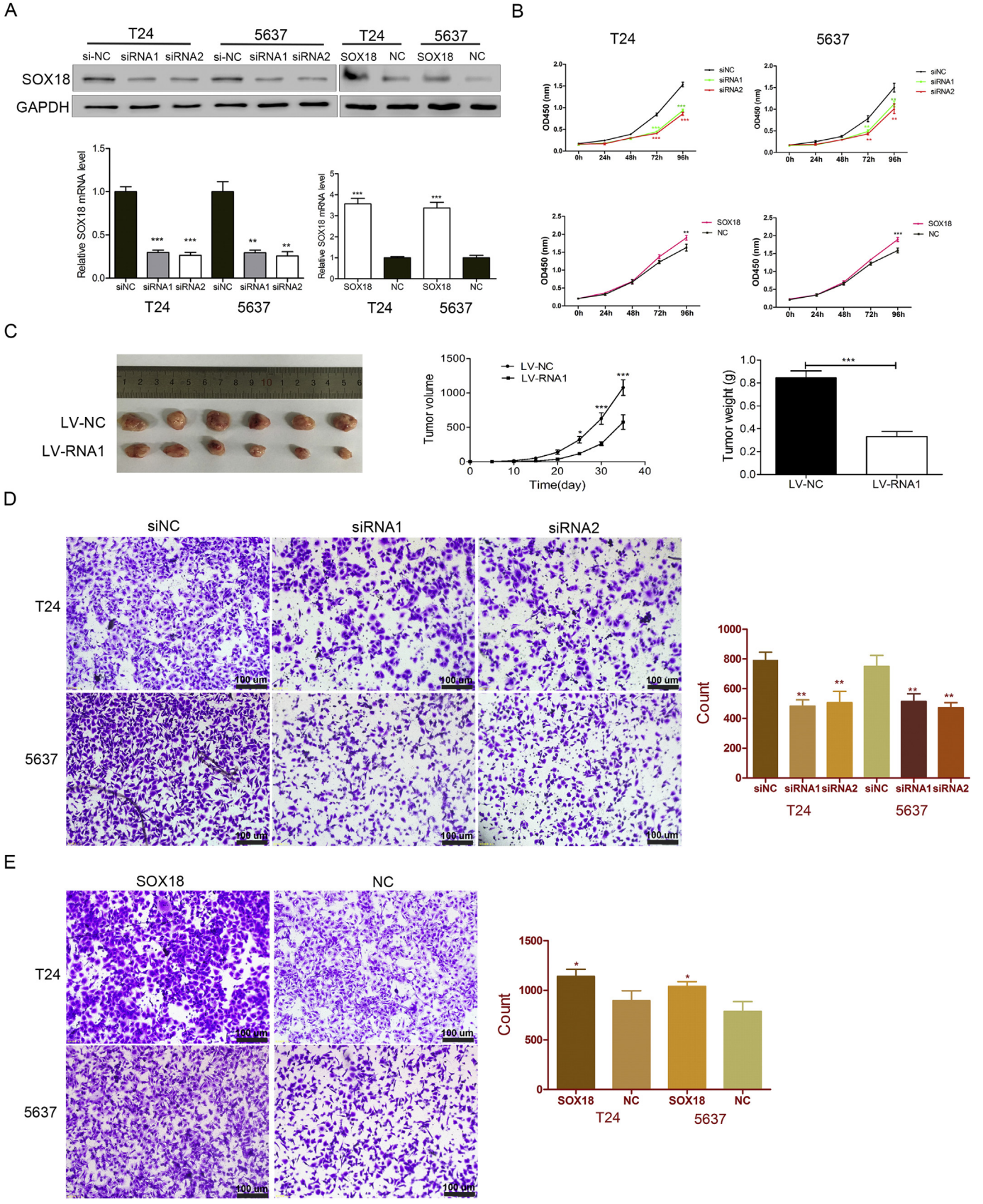

Fig. 3. SOX18 promoted the proliferation and migration of BCa cells. (A) SOX18 protein and mRNA expression levels in SOX18-silenced and SOX18-overexpressed cells. (B) CCK-8 assays indicated the absorbance of SOX18-silenced and SOX18-induced cells at an optical density of 450 nm decreased and increased, respectively. (C) Compared with LV-NC groups, the tumor size and weight were both decreased in LV-RNA1 groups. (D) Number of migrated T24 and 5637 cells was decreased in SOX18 knockdown groups. (E) Number of migrated T24 and 5637 cells was increased in SOX18 overexpression groups. ***, ** and * represented $P < 0.0001$, $P < 0.001$ and $P < 0.05$, respectively.

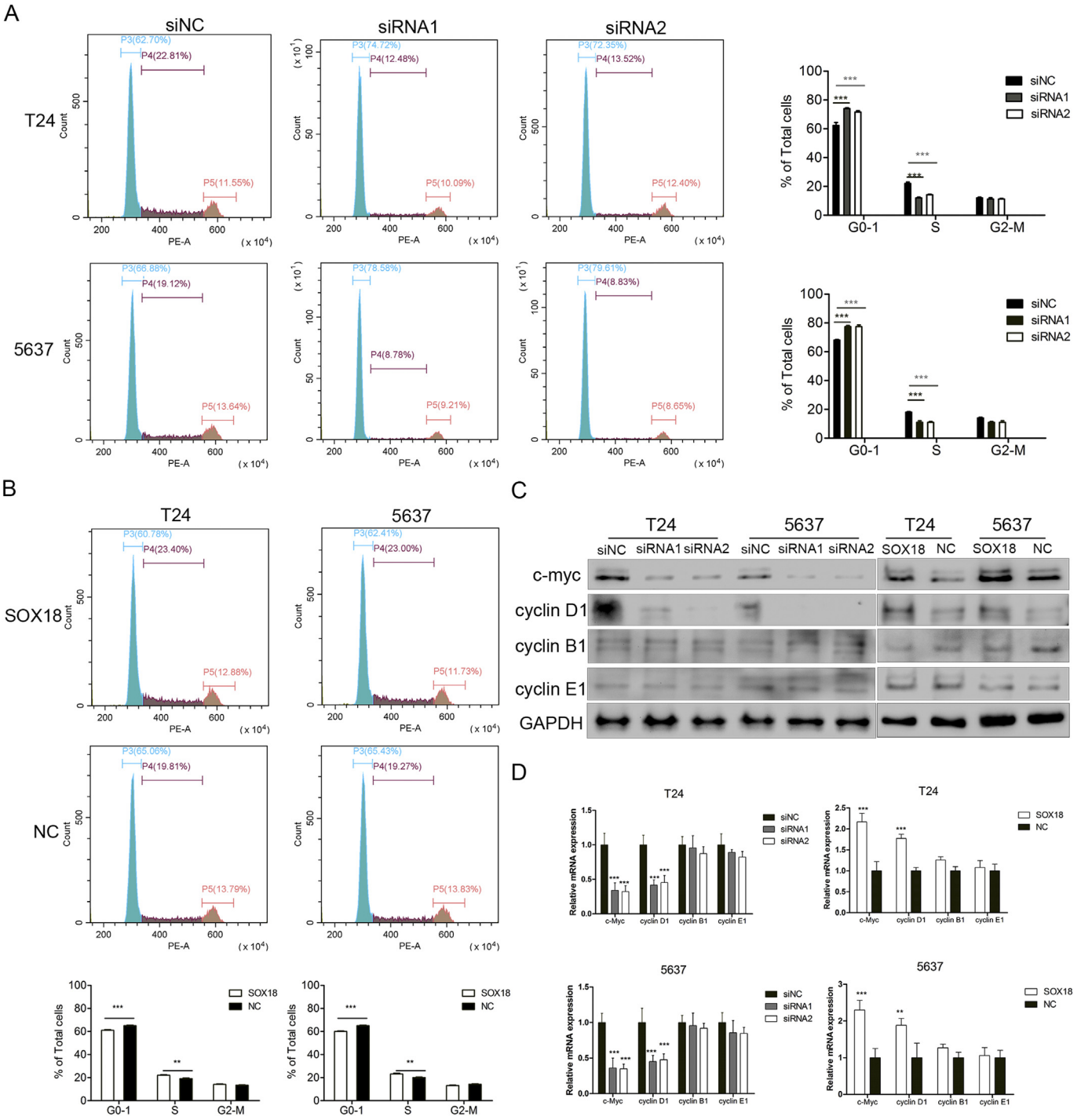

Fig. 4. SOX18 induced the G_1 to S phase transition via regulation of c-Myc and Cyclin D1. (A) Knockdown of SOX18 decreased the percentage of cells in G_1 phase and increased the percentage of cells in S phase, indicating an inhibition of cell cycle in G_1 and S phase transition. (B) Cell cycle transition from G_1 to S phase induced by SOX18 was further analyzed in SOX18-overexpressed cells. (C) Western blot analysis of c-Myc, Cyclin D1, Cyclin B1 and Cyclin E1 proteins in SOX18-silenced and SOX18-induced cells. (D) The mRNA expression levels of c-Myc, Cyclin D1, Cyclin B1 and Cyclin E1. BCa. ***, ** and * represented $P < 0.0001$, $P < 0.001$ and $P < 0.05$, respectively.

however, no changes were detected (Fig. 5C and D). Therefore, our data demonstrated that SOX18 may increase the invasive ability of BCa cells via promoting MMP-7 expression, and not MMP-2/3/9 expression.

3.6. Pharmacological inhibition of c-Met reduces malignant phenotype of BCa cells mediated by SOX18 through the c-Met/Akt signaling axis

To further examine the underlying mechanisms for increased capabilities of biological behaviors through SOX18 overexpression in BCa cells, the expression levels of various molecules associated with tumorigenesis and metastasis were subsequently evaluated. Following SOX18 silencing or upregulation, the phosphorylation levels of c-Met (p-Met Tyr1349) and Akt (p-Akt Ser473) were decreased and increased, respectively (Fig. 6A). As a receptor tyrosine kinase, c-Met was reported to be activated and enhanced the proliferation, motility and invasion of BCa cells via activating a number of downstream signals, including

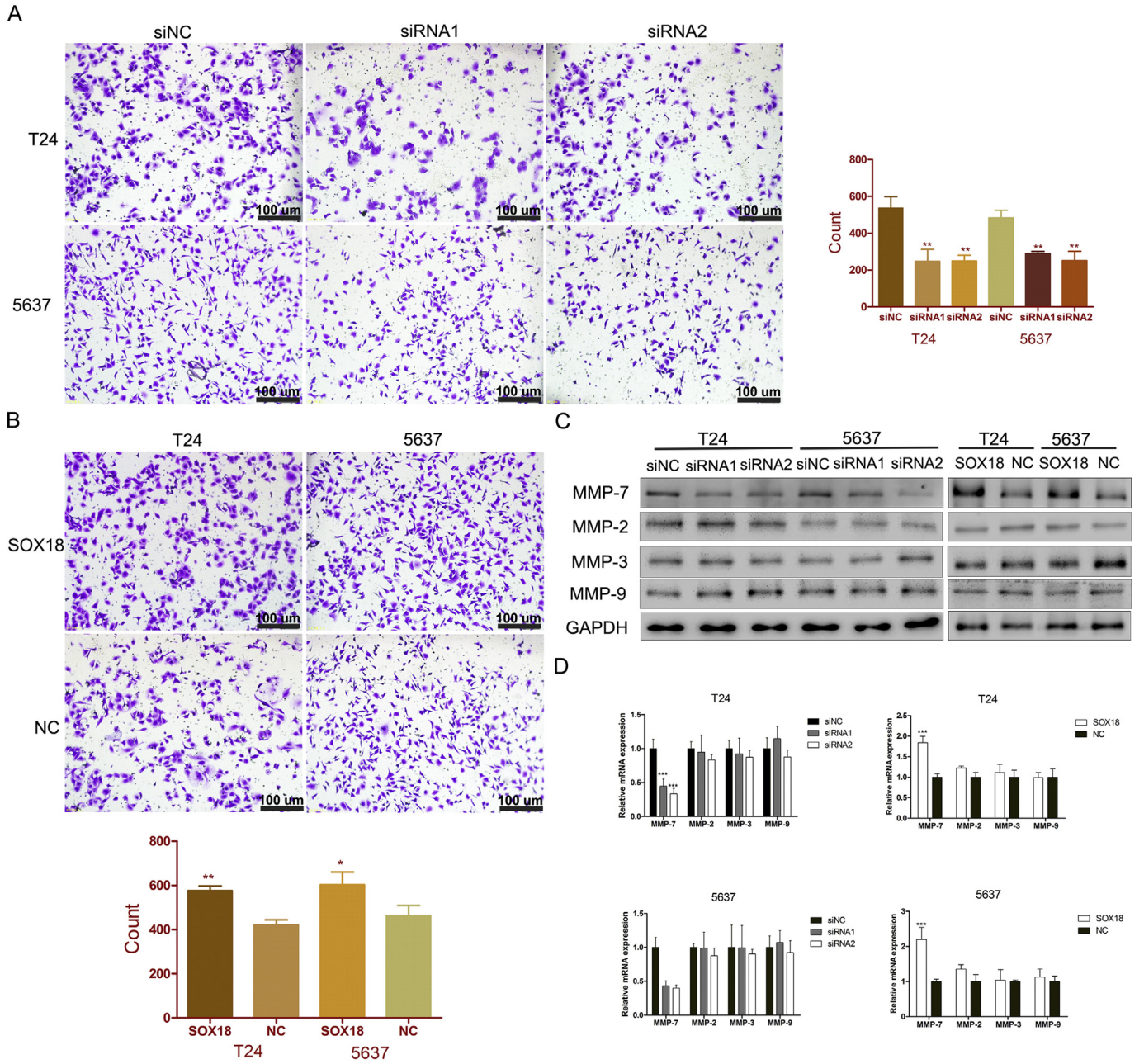

Fig. 5. SOX18 enhanced the invasiveness of BCa cells via promoting MMP-7 expression. (A) Number of invaded T24 and 5637 cells was decreased in SOX18 knockdown groups. (B) Number of invaded T24 and 5637 cells was increased in SOX18 overexpression groups. (C) The protein expression level of MMP-7, MMP-2, MMP-3 and MMP-9 in SOX18-silenced and SOX18-induced cells. (D) Analysis of MMP-7, MMP-2, MMP-3 and MMP-9 mRNA expression levels in SOX18-silenced and SOX18 induced cells. ***, ** and * represented $P < 0.0001$, $P < 0.001$ and $P < 0.05$, respectively.

phosphorylation of Akt [22,23]. Therefore, it was inferred that SOX18 partly induced malignant phenotype of BCa cells by activating c-Met/Akt through phosphorylation. To verify our hypothesis, cabozantinib, a multiple inhibitor of c-Met, which have been applied to cancer therapy and exhibit a positive clinical effect on tumor growth and bone metastasis was used [24,25], in order to suppress the activation of c-Met in SOX18-upregulated T24 and 5637 cells. The changes of malignant phenotype were subsequently analyzed. It has been previously reported that cell proliferation will be inhibited when the concentration of cabozantinib reaches a specific value. Therefore, one of the concerns of the present study was the effect of cabozantinib on the migration and invasion of BCa cells rather than cell proliferation. In order to avoid the impact caused by cell proliferation on cell migration and invasion, the maximum concentration (3 μM) of cabozantinib was selected, which could have no effect on cell proliferation. Following incubation with cabozantinib, the migration ability of SOX18-overexpressed cells was decreased (Fig. 6D). In addition, the inhibitory effect of cabozantinib on the invasion of SOX18-overexpressed cells was also identified (Fig. 6C). Therefore, our results demonstrated that SOX18 could promote BCa cell migration and invasion via the regulation of the c-Met/Akt signaling pathway.

4. Discussion

Recently, the role of transcriptional deregulation of SOX18 in human diseases, including cancers was identified by an increasing number of studies. Studies demonstrated that SOX18 was significantly associated with clinicopathological features of patients with cancers

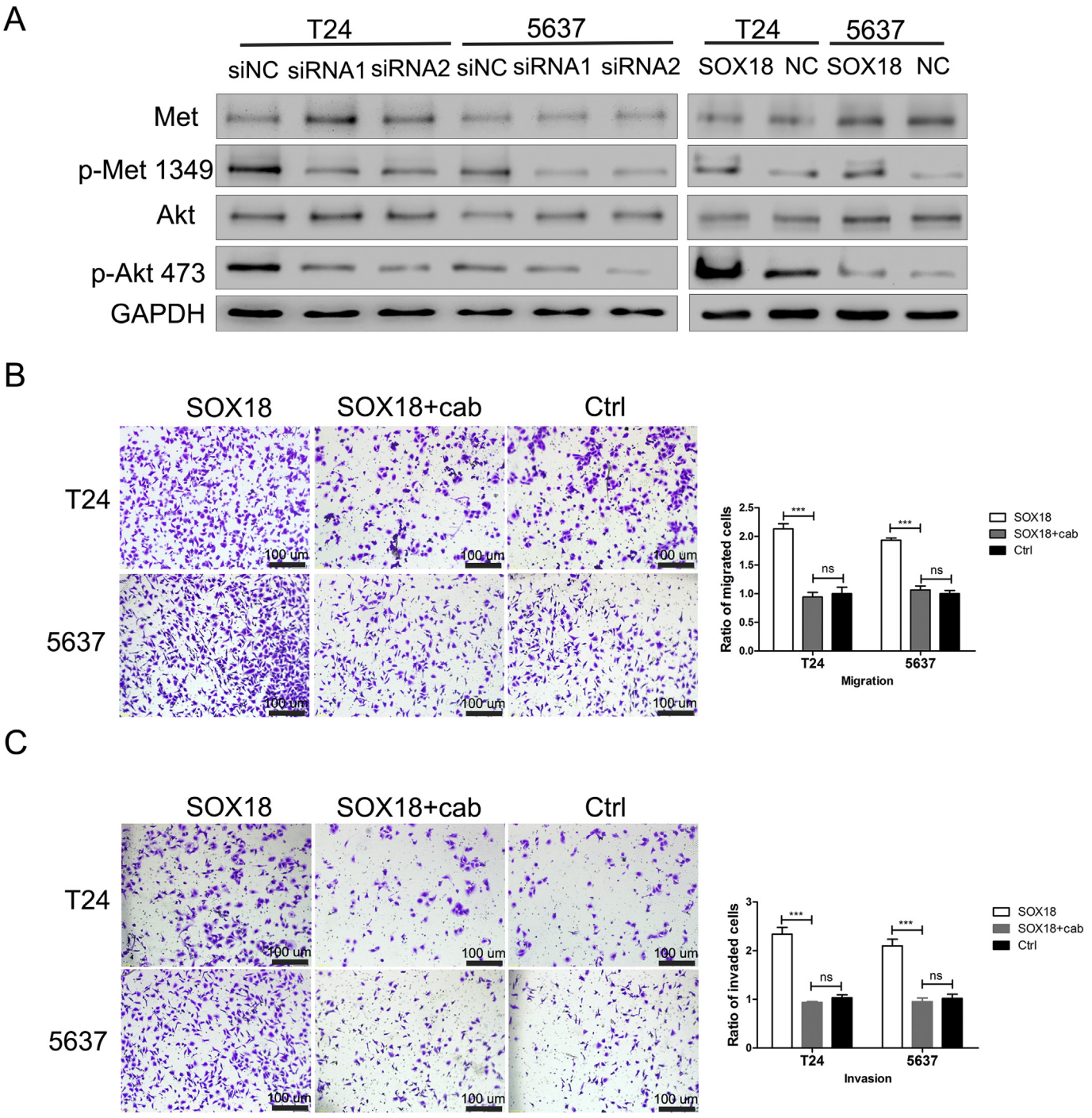

Fig. 6. Pharmacological inhibition of c-Met reduced migration and invasion of BCa cell lines, by SOX18 through phosphorylation of c-Met and Akt. (A) Knockdown and overexpression of SOX18 reduced and induced protein levels of p-Met (Tyr1349) and p-Akt (Ser473), respectively, while no expression changes of total c-Met and Akt were identified. (B) Inhibition of c-Met phosphorylation using c-Met inhibitor cabozantinib reduced cell migration induced by SOX18. (C) c-Met inhibitor reduced cell invasion induced by SOX18. The concentration of cabozantinib used was 3 μM. Ctrl, negative control + cabozantinib. ***, ** and * represented $P < 0.0001$, $P < 0.001$ and $P < 0.05$, respectively.

and the malignant behaviors of cancer cells, however little was known about SOX18 in BCa. The present study reports a high expression of SOX18 in BCa. Our data indicated that the rate of high SOX18 protein expression in BCa tissues was 52.2%, while that in normal tissues was 23.9%, which suggested high SOX18 expression in BCa. Association analyses revealed an association between high SOX18 expression and poor clinical characteristics of BCa, including lymph node metastasis, histological grade, clinical stages, and muscle infiltration, suggesting that SOX18 promoted BCa progression. Furthermore, Kaplan-Meier survival analysis revealed poor prognoses in patients with high SOX18 expression, which indicated that SOX18 may serve as a predictor of survival for patients with BCa. Despite the fact that multivariate analysis denied the possibility of SOX18 as an independent prognostic factor for BCa, overexpression of SOX18 remained a vital factor in its development and progression. However, only 90 cases were analyzed in our study, and further study should include a larger sample size and extend follow-up time to produce solid conclusions.

To examine the role of SOX18 on cell proliferation, cellular

functional assays were performed using SOX18-silenced and SOX18-overexpressed T24 and 5637 cells. Silencing and overexpression of SOX18 resulted in an obvious decrease and increase in the proliferation rate of cells, indicating that SOX18 could accelerate cell proliferation of BCa, which was consistent with findings of previous reports [14,26]. Uncontrolled cell proliferation serves an important role in tumorigenesis and tumor progression. Previously, it has been reported that aberrant cell cycle was associated with the proliferation of BCa cells and its occurrence [27]. In the present study, cell cycle transition from G_1 to S phase was accelerated in SOX18-transduced cells and blocked in SOX18-silenced cells, indicating that SOX18 could enhance BCa proliferation by regulating cell cycle progression. Cell cycle is a complicated and elaborate process controlled by various regulatory proteins, including c-Myc and Cyclins [28,29]. C-myc has been reported to be overexpressed in multiple cancers and served as a key regulator in the cell cycle [30]. Studies have demonstrated that overexpressed c-Myc may activate the transcription of Cyclin D1 by targeting the promoter of Cyclin D1, which is a vital molecule that forms a complex with Cyclin-dependent kinase 4 (CDK4), and subsequently serves as a regulator of the G_1/S restriction checkpoint [31]. Therefore, Cyclin D1 is also a key regulator of the cell cycle and facilitates cell proliferation by promoting cell cycle transition from G_1 to S phase [32]. Cyclin D1 was reported to be upregulated and involved in cell proliferation in various cancers, including human BCa [33–37]. Therefore, abnormality of c-Myc and Cyclin D1 could alter cell cycle progression. Following the silencing or overexpression of SOX18 in BCa cells, the mRNA and protein expression levels of c-Myc and Cyclin D1 were decreased or increased subsequently, while no Cyclin B1 and Cyclin E1 changes associated with the different phases in cell cycle were observed [38,39]. This mechanistic analysis accurately explained the SOX18-mediated alterations in the cell cycle distribution using flow cytometry. Therefore, our results indicated that SOX18 promotes BCa cell proliferation by facilitating the cell cycle transition from G_1 to S phase by upregulating the expression of Cyclin D1, however not of Cyclin B1 or Cyclin E1. Cancer has been previously defined as a cell cycle disease, however inhibitors cell cycle-associated molecules, including Cyclin D1 and c-Myc display little significance in a clinical setting [40]. Furthermore, transcriptional factor SOX18 may be a potential substitute target for cell cycle. Therefore, the underlying mechanism of c-Myc and cyclin D1 regulation by SOX18 requires further investigation.

In the development and progression of BCa, muscle infiltration depth and lymph node metastasis of BCa indicated a poor prognosis. This provided further insight to the metastatic mechanisms of BCa and may be useful for inhibiting infiltration and lymph node metastasis. MMPs are members of a family that is known to promote cell invasion through degradation of the extracellular matrix [41–43]. Inhibition of the expression of MMPs may impact the invasive ability of cancer cells. Previous studies have reported that MMPs were overexpressed in human BCa and could enhance BCa invasion and metastasis [44–46]. In the present study, the knockdown or overexpression of SOX18 resulted in a reduced or increased invasion ability of T24 and 5637 cells, respectively, indicating that SOX18 facilitates the invasion of BCa cells. In addition, the expression of MMP-7 was reduced at an mRNA and protein expression level following SOX18 silencing, and increased following SOX18 overexpression. This result suggests that SOX18 overexpression may lead to increased invasiveness in bladder cells by MMP-7, whose overexpression has been reported in tumors, in addition to facilitating the invasiveness of cancer cells [47]. The expression of other MMPs was also detected; however no changes in expression levels were indicated. Despite the fact that there is, to the best of our knowledge, a lack of direct evidence, our findings demonstrated that SOX18 upregulated the expression of MMP-7, however not of MMP-2/9, to manage the invasion and metastasis of BCa cells. In terms of the mechanisms by which SOX18 regulates MMP-7, a previous study demonstrated that SOX18 bound to the promoter, and activated the transcription of MMP-7 in endothelial cells [48]. However, it is unclear whether SOX18 regulates MMP-7 via similar mechanisms in BCa cells, and further studies are required to determine the mechanisms by which SOX18 regulates MMP-7 in these cells.

Disease progression in BCa is a multi-step progress and is associated with various factors. Akt is a vital regulator in multiple cellular functions, and phosphorylation of Akt (p-Akt) contributed to cell proliferation, migration and invasion [3]. Phosphorylation of Akt could be stimulated by a series of upstream signaling molecules, including activated EGFR and c-Met, and subsequently activated downstream signal transduction in cellular process [49]. It has been reported that phosphorylation of Akt accompanied with p-Met that is induced by long-term exposure to angiogenesis inhibitors in renal cell carcinoma (RCC) cells, may contribute to the resistance of targeted therapy in RCC [50]. In the present study, we observed that the phosphorylation of Akt at Ser 473 (p-Akt Ser473) and c-Met at tyr1349 (p-Met Tyr1349) was decreased and increased following SOX18 silencing and overexpression. Considering the fact that Akt could be activated by phosphorylation of c-Met, we hypothesized that SOX18 increased the phosphorylation of Akt by promoting the activation of c-Met. In addition, our data indicated that inhibition of c-Met activation by cabozantinib may reduce the migrated and invasive abilities enhanced by SOX18. This in turn suggests that overexpression of SOX18 may promote the migration and invasion ability of BCa cells through c-Met/Akt signaling transduction. c-Met was reported to be overexpressed in various types of cancer and is responsible for the malignant phenotype of cancer cells, including BCa. As a multiple inhibitor targeting c-Met and other receptor tyrosine kinases, cabozantinib has been applied to cancer therapy and has exhibited positive clinical effects. It has been reported that cabozantinib was approved by the FDA for use in patients with advanced RCC subsequent to anti-angiogenic therapy [51]. The overall survival, progression-free survival and objective response rate of patients treated with cabozantinib was improved compared with that in patients treated with everolimus [52]. On the basis of c-Met overexpression in BCa, cabozantinib was a promising treatment modality for BCa. Data in the present study demonstrated that cabozantinib could decrease the migration and invasion of BCa cells enhanced by SOX18 overexpression, which indicated the pharmacological inhibition of cabozantinib on SOX18-induced malignant phenotype, which further confirmed the potential of cabozantinib in BCa treatment. In addition, our data suggested that SOX18 may promote cellular migration and invasion, through c-Met/Akt signaling transduction, indicating a synergistic effect when targeting SOX18 and cabozantinib in BCa treatment. Therefore, understanding the association of SOX18 and c-Met in BCa is crucial. However, the precise mechanisms by which SOX18 increased activation of c-Met require further investigation. In addition, transcriptional factor SOX18 became the optimal target for investigation, due to the fact that the application on c-Met and other tyrosine kinase inhibitors to various types of cancers resulted in the incidental resistance to the aforementioned.

5. Conclusion

The present study indicated the overexpression of SOX18 in BCa, and the role of SOX18 as an indicator of poor clinical features and prognosis for patients with BCa. Functional and mechanistic assays demonstrated that SOX18 promotes cell proliferation via accelerating cell cycle, by regulating c-Myc and Cyclin D1. In addition MMP-7, p-Met (Tyr1349) and p-Akt (Ser473) were identified to be involved in the migration and invasion of BCa cells, enhanced by SOX18. Comprehensive and thorough understanding of the accurate role and underlying mechanism of transcriptional factor SOX18 in BCa is required, in order to contribute to the development of novel therapeutic strategies for cancer, in addition to assisting in the transition from research to its application in a clinical setting.

Declaration of Competing Interest

The author declares no conflicts of interest.

Acknowledgments

We thank Pathologists Wang Gongwei and Zhao Chenglong in Pathologic Section of Peking University People's Hospital for their technology supports of immunohistochemistry and pathologic score. This work was supported by the National Natural Science Foundation of China (No.31671469) and Beijing Municipal Natural Science Foundation (No.7152149).

References

[1] R. Rossi, M. Lichtner, F. Iori, et al., Dendritic cells in blood and urine samples from bladder cancer patients undergoing BCG immunotherapy[J], Arch. Ital. Urol. Androl. 85 (4) (2013) 157–163.
[2] J.C. Park, D.E. Citrin, P.K. Agarwal, et al., Multimodal management of muscle-invasive bladder cancer[J], Curr. Probl. Cancer 38 (3) (2014) 80–108.
[3] K. Wu, J. Fan, L. Zhang, et al., PI3K/Akt to GSK3beta/beta-catenin signaling cascade coordinates cell colonization for bladder cancer bone metastasis through regulating ZEB1 transcription[J], Cell. Signal. 24 (12) (2012) 2273–2282.
[4] P.K. Kopparapu, S.A. Boorjian, B.D. Robinson, et al., Expression of VEGF and its receptors VEGFR1/VEGFR2 is associated with invasiveness of bladder cancer[J], Anticancer Res. 33 (6) (2013) 2381–2390.
[5] M.H. Hussain, G.R. Macvicar, D.P. Petrylak, et al., Trastuzumab, paclitaxel, carboplatin, and gemcitabine in advanced human epidermal growth factor receptor-2/neu-positive urothelial carcinoma: results of a multicenter phase II National Cancer Institute trial[J], J. Clin. Oncol. 25 (16) (2007) 2218–2224.
[6] G. Xia, S.R. Kumar, D. Hawes, et al., Expression and significance of vascular endothelial growth factor receptor 2 in bladder cancer[J], J. Urol. 175 (4) (2006) 1245–1252.
[7] N. Oka, S. Tanimoto, R. Taue, et al., Role of phosphatidylinositol-3 kinase/Akt pathway in bladder cancer cell apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand[J], Cancer Sci. 97 (10) (2006) 1093–1098.
[8] Z.Y. She, Yang W X. SOX family transcription factors involved in diverse cellular events during development[J], Eur. J. Cell Biol. 94 (12) (2015) 547–563.
[9] A. Sarkar, K. Hochedlinger, The sox family of transcription factors: versatile regulators of stem and progenitor cell fate[J], Cell Stem Cell 12 (1) (2013) 15–30.
[10] A. Irrthum, K. Devriendt, D. Chitayat, et al., Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia[J], Am. J. Hum. Genet. 72 (6) (2003) 1470–1478.
[11] G. Wang, Z. Wei, H. Jia, et al., Knockdown of SOX18 inhibits the proliferation, migration and invasion of hepatocellular carcinoma cells[J], Oncol. Rep. 34 (3) (2015) 1121–1128.
[12] A. Jethon, B. Pula, M. Olbromski, et al., Prognostic significance of SOX18 expression in non-small cell lung cancer[J], Int. J. Oncol. 46 (1) (2015) 123–132.
[13] B. Pula, C. Kobierzycki, D. Solinski, et al., SOX18 expression predicts response to platinum-based chemotherapy in ovarian cancer[J], Anticancer Res. 34 (8) (2014) 4029–4037.
[14] J. Zhang, Y. Ma, S. Wang, et al., Suppression of SOX18 by siRNA inhibits cell growth and invasion of breast cancer cells[J], Oncol. Rep. 35 (6) (2016) 3721–3727.
[15] B. Pula, M. Olbromski, A. Wojnar, et al., Impact of SOX18 expression in cancer cells and vessels on the outcome of invasive ductal breast carcinoma[J], Cell Oncol. (Dordr.) 36 (6) (2013) 469–483.
[16] B. Han, L. Luan, Z. Xu, et al., Clinical significance and biological roles of CRKL in human bladder carcinoma[J], Tumour Biol. 35 (5) (2014) 4101–4106.
[17] M. Milivojevic, I. Petrovic, N. Kovacevic-Grujicic, et al., Construction and functional analysis of novel dominant-negative mutant of human SOX18 protein[J], Biochemistry (Mosc) 78 (11) (2013) 1287–1292.
[18] Y.J. Jeong, H.S. Hoe, H.J. Cho, et al., Suppression of c-Myc enhances p21(WAF1/CIP1) -mediated G1 cell cycle arrest through the modulation of ERK phosphorylation by ascochlorin[J], J. Cell. Biochem. 119 (2) (2018) 2036–2047.
[19] L. Zhou, F. Wu, W. Jin, et al., Theabrownin inhibits cell cycle progression and tumor growth of lung carcinoma through c-myc-related mechanism[J], Front. Pharmacol. 8 (2017) 75.
[20] J. Virga, C.D. Szemcsak, J. Remenyi-Puskar, et al., Differences in extracellular matrix composition and its role in invasion in primary and secondary intracerebral malignancies[J], Anticancer Res. 37 (8) (2017) 4119–4126.
[21] K.H. Shen, J.H. Hung, C.W. Chang, et al., Solasodine inhibits invasion of human lung cancer cell through downregulation of miR-21 and MMPs expression[J], Chem. Biol. Interact. 268 (2017) 129–135.
[22] H.K. Byeon, H.J. Na, Y.J. Yang, et al., c-Met-mediated reactivation of PI3K/AKT signaling contributes to insensitivity of BRAF(V600E) mutant thyroid cancer to BRAF inhibition[J], Mol. Carcinog. 55 (11) (2016) 1678–1687.
[23] S. Shibasaki, S. Tsunemi, S. Kitano, et al., Differential regulation of c-Met signaling pathways for synovial cell function[J], Springerplus 3 (2014) 554.
[24] C. Lee, Y.M. Whang, P. Campbell, et al., Dual targeting c-met and VEGFR2 in osteoblasts suppresses growth and osteolysis of prostate cancer bone metastasis[J], Cancer Lett. 414 (2018) 205–213.
[25] S. Previdi, F. Scolari, R. Chila, et al., Combination of the c-Met inhibitor tivantinib and zoledronic acid prevents tumor bone engraftment and inhibits progression of established bone metastases in a breast xenograft model[J], PLoS One 8 (11) (2013) e79101.
[26] Y. Jia, B. Cao, Y. Yang, et al., Silencing NKD2 by promoter region hypermethylation promotes gastric cancer invasion and metastasis by up-regulating SOX18 in human gastric cancer[J], Oncotarget 6 (32) (2015) 33470–33485.
[27] J. Liberal, A. Carmo, C. Gomes, et al., Urolithins impair cell proliferation, arrest the cell cycle and induce apoptosis in UMUC3 bladder cancer cells[J], Investig. New Drugs 35 (6) (2017) 671–681.
[28] J. Chen, X. Li, Q. Cheng, et al., Effects of cyclin D1 gene silencing on cell proliferation, cell cycle, and apoptosis of hepatocellular carcinoma cells[J], J. Cell. Biochem. 119 (2) (2018) 2368–2380.
[29] Y. Ciribilli, P. Singh, R. Spanel, et al., Decoding c-Myc networks of cell cycle and apoptosis regulated genes in a transgenic mouse model of papillary lung adenocarcinomas[J], Oncotarget 6 (31) (2015) 31569–31592.
[30] L.J. Tang, Y. Li, Y.L. Liu, et al., USP12 regulates cell cycle progression by involving c-Myc, cyclin D2 and BMI-1[J], Gene 578 (1) (2016) 92–99.
[31] S. Jirawatnotai, S. Sharma, W. Michowski, et al., The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis[J], Cell Cycle 13 (18) (2014) 2889–2900.
[32] J.T. Lin, H.Y. Li, N.S. Chang, et al., WWOX suppresses prostate cancer cell progression through cyclin D1-mediated cell cycle arrest in the G1 phase[J], Cell Cycle 14 (3) (2015) 408–416.
[33] B. Ren, W. Li, Y. Yang, et al., The impact of cyclin D1 overexpression on the prognosis of bladder cancer: a meta-analysis[J], World J. Surg. Oncol. 12 (2014) 55.
[34] O. Barbash, P. Zamfirova, D.I. Lin, et al., Mutations in Fbx4 inhibit dimerization of the SCF(Fbx4) ligase and contribute to cyclin D1 overexpression in human cancer [J], Cancer Cell 14 (1) (2008) 68–78.
[35] Y. He, O.E. Franco, M. Jiang, et al., Tissue-specific consequences of cyclin D1 overexpression in prostate cancer progression[J], Cancer Res. 67 (17) (2007) 8188–8197.
[36] J.M. Pattison, V. Posternak, M.D. Cole, Transcription factor KLF5 binds a cyclin E1 polymorphic intronic enhancer to confer increased bladder cancer risk[J], Mol. Cancer Res. 14 (11) (2016) 1078–1086.
[37] S.F. Shariat, R. Ashfaq, A.I. Sagalowsky, et al., Correlation of cyclin D1 and E1 expression with bladder cancer presence, invasion, progression, and metastasis[J], Hum. Pathol. 37 (12) (2006) 1568–1576.
[38] B. Lohberger, A. Leithner, N. Stuendl, et al., Diacerein retards cell growth of chondrosarcoma cells at the G2/M cell cycle checkpoint via cyclin B1/CDK1 and CDK2 downregulation[J], BMC Cancer 15 (2015) 891.
[39] F. Wang, X.D. Fu, Y. Zhou, et al., Down-regulation of the cyclin E1 oncogene expression by microRNA-16-1 induces cell cycle arrest in human cancer cells[J], BMB Rep. 42 (11) (2009) 725–730.
[40] C.C. Mills, E.A. Kolb, V.B. Sampson, Recent advances of cell-cycle inhibitor therapies for pediatric cancer[J], Cancer Res. 77 (23) (2017) 6489–6498.
[41] N. Liu, B. Zhou, G. Zhu, Potential role of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in regulation of matrix metalloproteinases (MMPs) expression in periodontal diseases[J], Med. Sci. Monit. 22 (2016) 1936–1938.
[42] W.J. Wang, X.H. Yu, C. Wang, et al., MMPs and ADAMTSs in intervertebral disc degeneration[J], Clin. Chim. Acta 448 (2015) 238–246.
[43] G.K. Gilcy, G. Kuttan, Evaluation of antiangiogenic efficacy of Emilia sonchifolia (L.) DC on tumor-specific neovessel formation by regulating MMPs, VEGF, and proinflammatory cytokines[J], Integr. Cancer Ther. 15 (4) (2016) P1–P12.
[44] Z. Ou, Y. Wang, L. Liu, et al., Tumor microenvironment B cells increase bladder cancer metastasis via modulation of the IL-8/androgen receptor (AR)/MMPs signals [J], Oncotarget 6 (28) (2015) 26065–26078.
[45] E. Wieczorek, E. Reszka, Z. Jablonowski, et al., Genetic polymorphisms in matrix metalloproteinases (MMPs) and tissue inhibitors of MPs (TIMPs), and bladder cancer susceptibility[J], BJU Int. 112 (8) (2013) 1207–1214.
[46] J.E. Nutt, G.C. Durkan, J.K. Mellon, et al., Matrix metalloproteinases (MMPs) in bladder cancer: the induction of MMP9 by epidermal growth factor and its detection in urine[J], BJU Int. 91 (1) (2003) 99–104.
[47] S. Basu, R. Thorat, S.N. Dalal, MMP7 is required to mediate cell invasion and tumor formation upon Plakophilin3 loss[J], PLoS One 10 (4) (2015) e123979.
[48] M. Hoeth, H. Niederleithner, R. Hofer-Warbinek, et al., The transcription factor SOX18 regulates the expression of matrix metalloproteinase 7 and guidance molecules in human endothelial cells[J], PLoS One 7 (1) (2012) e30982.
[49] S. Wang, X. Wang, J. Li, et al., c-Met, CREB1 and EGFR are involved in miR-493-5p inhibition of EMT via AKT/GSK-3beta/Snail signaling in prostate cancer[J], Oncotarget 8 (47) (2017) 82303–82313.
[50] L. Zhou, X.D. Liu, M. Sun, et al., Targeting MET and AXL overcomes resistance to sunitinib therapy in renal cell carcinoma[J], Oncogene 35 (21) (2016) 2687–2697.
[51] N.M. Tannir, G. Schwab, V. Grunwald, Cabozantinib: an active novel multikinase inhibitor in renal cell carcinoma[J], Curr. Oncol. Rep. 19 (2) (2017) 14.
[52] R.J. Motzer, B. Escudier, T. Powles, et al., Long-term follow-up of overall survival for cabozantinib versus everolimus in advanced renal cell carcinoma[J], Br. J. Cancer 118 (9) (2018) 1176–1178.