BZW1 Promotes Cell Proliferation in Prostate Cancer by Regulating TGF-β1/Smad Pathway

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Abstract

Background Recently, basic leucine zipper and the W2 domain-containing protein 1 (BZW1) is reported to be implicated in tumor progression. However, the role of BZW1 in prostate cancer remains unknown. This study is aimed to investigate the expression of BZW1 and its influence on cell proliferation in prostate cancer.

Methods The expression levels of BZW1 were measured in 136 cases of prostate cancer and matched adjacent non-cancerous prostate tissues by quantificational real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC). Then, the effect of BZW1 on cell proliferation was further explored.

Results QRT-PCR analysis shown that the mRNA levels of BZW1 in prostate cancer were significantly greater compared with those in matched adjacent non-cancerous prostate tissues (P<0.001). IHC results shown the high-expression rate of BZW1 in prostate cancer and matched adjacent non-cancerous prostate tissues were 68.4% and 32.4%, and the difference was statistically significant (P<0.001). BZW1 high-expression significantly correlated with T stage, lymph node metastasis, prostate specific antigen (PSA) and Gleason score (P<0.05). Patients with BZW1 high-expression presented unfavorable prognosis compared with those with BZW1 low-expression (P=0.002). In addition, CCK-8 and Colony formation assays revealed that BZW1 over-expression significantly promoted cell proliferation in *vitro*. Tumor xenograft shown BZW1 knockdown significantly inhibited tumor growth in vivo. Moreover, BZW1 overexpression activated the TGF-β1/Smad1/Smad3 pathway.

Conclusion BZW1 over-expression predicts poorer prognosis and promotes cell proliferation in prostate cancer by regulating TGF-β1/Smad pathway.

Background

Prostate cancer (PCa) is one of the most prevalent urological cancers in men worldwide [1–3]. The incidence of PCa is increased worldwide in recent years [4–5]. In the USA and Europe, PCa accounts for the second most leading cause of cancer-related mortality [6–7]. Although the testing of PSA was generally used in the early diagnosis, the incidence and mortality rate of PCa are still increasing [8–10]. Therefore, identifying new potential markers of prostate cancer is crucial for the early diagnosis and clinical therapy.

BZW1 is a member of the bZIP superfamily of transcription factors, which contains C-terminal W2-type HEAT domains [11–12]. BZW1 plays a key role in the cell cycle by regulating histone H4 gene transcription [13]. Moreover, BZW1 promotes cell proliferation in salivary mucoepidermoid carcinoma and lung adenocarcinoma [14–16]. BZW1 overexpression is an independent prognostic marker in lung adenocarcinoma [16]. However, the biological function of BZW1 in prostate cancer remains unknown.

Here, the expression of BZW1 and its clinical significance in prostate cancer were investigated. Moreover, the effect of BZW1 expression on cell proliferation and its potential mechanism were explored.

Methods

Patients and samples

A total of 136 prostate cancer and matched adjacent non-cancerous prostate tissues were enrolled in this study. All patients received surgery resection during 2010 to 2018, while no patient treated with chemotherapy or radiotherapy before surgery. Clinicalpathological characters were obtained from hospitalized records. The follow-up time was ranged from 8 to 64 months. This study was supported by the ethics committee of Sun Yat-sen University (No. Z81771573) and performed in accordance with the Declaration of Helsinki. The informed consent was signed by each patient.

Cell Culture And Transfection

Human prostate cancer cell lines (PC3, Du145 and LnCap) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RPMI-1640 (Gibco) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) at 37 °C in a humidified incubator with 5% CO_2. Cell transfection was performed by Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific, Inc, USA) according to the manufacturer's instructions. Then, cells were cultured with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) supplemented with puromycin (0.5 μg/mL). BZW1 down-regulation was performed by transferring shRNA lentivirus vectors (Genepharma, Suzhou, China) and BZW1 over-expression was performed by transferring recombinant lentivirus vectors (Genepharma, Suzhou, China). Non-target shRNA (Genepharma, Suzhou, China) lentivirus vectors were used as the negative control (NC).

QRT-PCR

Total RNA was extracted from fresh tissues and cells by using TRIzol Reagent (Life Technologies). According to the manufacture's protocol, the RNA was reversed into cDNA using a PrimeScript RT kit (Takara, Dalian, China). Real-time quantitative PCR was performed by SYBR1 Premix Ex Taq (DDR041A, TaKaRa, Dalian, China). Primers for BZW1 were 5'-ACTGGTGTTCTTCTGGCTAA-3' (Forward) and 5'-GTGCTCATTACACTTGACCA-3' (Reverse). The primer sequence for GAPDH were 5'-CTGAACGGGAAGCTCACTGG-3' (Forward) and 5'-TGAGGTCCACCACCCTGTTG-3' (Reverse). GAPDH was considered as the internal control and each test was repeated three times. The relative expression level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Proteins were extracted by radioimmunoprecipitation assay (RIPA) Lysis buffer (Abcam, Cambridge, MA, USA) and quantified by bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). 40 μg of protein per sample was separated onto 10% SDS-PAGE gels and transferred onto polyvinyl difluoride (PVDF) filter membrane (Bio-Rad, Hercules, CA, USA). Then, PVDF membranes were blocked with 5% non-fat dried milk for 2 h, and incubated with anti-BZW1 (1: 1000, ABcam Corp, USA), anti-Smad1 (1: 1000, ABcam Corp, USA), anti-p-Smad1 (1: 1000, ABcam Corp, USA), anti-Smad3 (1: 1000, ABcam Corp, USA), anti-p-Smad3 (1: 1000, ABcam Corp, USA), anti-TGF-β1 (1: 2000, ABcam Corp, USA) and anti-GAPDH (1: 2000, ABcam Corp, USA), respectively at 4 ℃ overnight. Finally, membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit). Signals were developed using an enhanced chemiluminescence reaction kit (Applygen Technologies, Beijing, China).

Immunohistochemistry (ihc)

Tissue sections (Two-micrometre-thick) were deparaffinized with xylene and alcohol. Antigen retrieval was completed by sodium citrate buffer (pH 6.0). Then, sections were incubated with rabbit polyclonal BZW1 antibody (Abcam Corp, USA) (diluted 1: 100) overnight at 4 °C. Sections were washed with Phosphate-Buffered Saline (PBS) and incubated with biotin-labeled secondary antibodies for 30 minutes at room temperature. Signals were detected by diaminobenzidine tetrahydrochloride (DAB). PBS substituted with primary antibody was regarded as negative controls. The positive controls were performed by lung adenocarcinoma with positive expression of BZW1 [15]. The scores were calculated by the number of positive cells and staining intensity. Percentage of cells was recorded as 1 (< 25%), 2 (25%-50%) and 3 (> 51%). Staining intensity was recorded as blank (0), weak (1, +), moderate (2, ++) and strong (3, +++). Total score of each section was ranged from 0 to 9. Based on the semiquantitative analysis, BZW1 expression was scored as low-expression (< 4) and high-expression (≥ 4) [15].

Cell counting kit-8 (CCK-8) analysis

CCK-8 assay was used to evaluate the cell proliferation. Cells were transferred and seeded in 96-well plates (1.0×10^3 cell/well) for 5 days. Then, cells were cultured with 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) for 3 h, and the optical density of each well was examined by a microplate reader (BioTek, Winooski, VT, USA) at 450 nm. Each test was repeated in triplicate at the same condition.

Colony formation analysis

Tansfected cells (1.0×10^3 cell/well) were seeded into 6-well plates. After 2 weeks, cells were obtained and fixed with 100% methanol. Then, cells were incubated with 0.1% crystal violet and stained with hemocytometer for counting. Each experiment was repeated in triplicate at the same condition.

Tumor Xenograft In Vivo

Balb/c nude mice (4 weeks old) were purchased from Animal Center and kept on specific pathogen-free conditions in accordance with institutional guidelines. To evaluate the effect of BZW1 on tumor growth, 6×10^6 prostate cancer cells with shRNA lentivirus vectors were subcutaneously injected into the flank regions of legs (5 mice per group). Negative controls (NC) were treated with prostate cancer cells with non-target shRNA lentivirus vectors. After 2 weeks, tumor size and tumor weight were calculated.

Statistical analysis

All data was characterized as mean ± standard deviation, and analyzed by SPSS software (version 19.0; SPSS, Chicago, IL, USA). The mRNA expression levels of BZW1 between prostate cancer and matched adjacent non-cancerous tissues were analyzed by paired *t*-test. The correlation between BZW1 expression and clinicalpathological characters was evaluated by Chi-square test (χ^2). Survival analysis was performed by using the Kaplan-Meier method with log-rank test. Hazard Ratio (HR) was evaluated using the Cox's proportional hazards model. $P < 0.05$ was considered as statistical significance.

Results

BZW1 is highly expressed in prostate cancer and correlated with clinicalpathological characters

To evaluate the expression of BZW1, the mRNA levels of BZW1 were detected in 136 cases of prostate cancer and matched adjacent non-cancerous prostate tissues by qRT-PCR. Results shown the mRNA levels of BZW1 was significantly greater in prostate cancer compared with those in matched adjacent non-cancerous prostate tissues (Fig. 1A, $P < 0.001$). Then, the protein expression levels of BZW1 was detected by IHC. As shown in Fig. 1B-E, positive staining of BZW1 was mainly located in the cell cytoplasm as brown and yellow. Semiquantitative analysis shown that the high-expression rate of BZW1 in prostate cancer and non-cancerous prostate tissues were 68.4% and 32.4%, and the difference was statistically significant (Table 1, $P < 0.001$).

Table 1
BZW1 expression was detected in PCa and non-cancerous prostate tissues by IHC

Types	N	BZW1		*P* value
		low-expression	high-expression	
PCa tissues	136	43 (31.6)	93 (68.4)	< 0.001
Non-cancerous prostate tissues	136	92 (67.6)	44 (32.4)	

To investigate the clinical significance of BZW1 in prostate cancer, the correlation between BZW1 expression and clinicalpathological characters was investigated. Results shown BZW1 high-expression was significantly correlated with T stage, lymph node metastasis, PSA and Gleason score ($P < 0.05$,

Table 2). However, no significant correlation was observed in BZW1 expression with age and smoke history ($P > 0.05$, Table 2). Furthermore, Kaplan-Meier analysis revealed that patients with BZW1 high-expression presented unfavorable prognosis than those with BZW1 low-expression (Fig. 2, P = 0.002). T stage, lymph node metastasis, PSA and Gleason score rather than age and smoke history were significantly correlated with overall survival time ($P < 0.05$, Table 3). Moreover, multivariate Cox regression analysis shown that BZW1 expression, T stage, lymph node metastasis, PSA and Gleason score were independent prognostic factors for overall survival in prostate cancer (Table 4, $P < 0.05$).

Table 2
BZW1 expression correlated with clinicalpathological characters in PCa

Clinicalpathological characters	N	BZW1		*P* value
		low-expression	high-expression	
Age (years)				
≤ 60	60	24	36	0.067
> 60	76	19	57	
Smoke history				
Negative	79	29	40	0.058
Positive	57	14	43	
T stage				
T1-T2	65	34	31	< 0.001
T3	71	9	62	
Lymph node metastasis				
Negative	70	33	37	< 0.001
Positive	66	10	56	
PSA (ng/ml)				
< 10	34	18	16	0.003
≥ 10	102	25	77	
Gleason Score				
< 8	73	34	39	< 0.001
≥ 8	63	9	54	

Table 3
Survival analysis was performed by Kaplan-Meier method

Variables	N	Mean survival time (Month, 95% CI)	*P* value
BZW1 expression			
Low-expression	43	56 (51–61)	0.002
High-expression	93	44 (40–48)	
Age (years)			
≤ 60	60	48 (43–53)	0.969
> 60	76	47 (42–52)	
Smoke history			
Negative	79	47 (42–52)	0.439
Positive	57	49 (44–54)	
T stage			
T1-T2	65	55 (51–59)	< 0.001
T3	71	41 (37–45)	
Lymph node metastasis			
Negative	70	54 (50–58)	< 0.001
Positive	66	41 (37–45)	
PSA (ng/ml)			
< 10	34	55 (49–61)	0.024
≥ 10	102	45 (41–49)	
Gleason Score			
< 8	73	55 (51–59)	< 0.001
≥ 8	63	38 (34–42)	

Table 4
Prognostic factors were analyzed by Cox's proportional hazards model

Variables	Hazard rate	95% CI	*P* value
BZW1 (High-expression VS Low-expression)	3.394	1.658–6.947	0.001
Age (≤ 60 years VS > 60 years)	0.686	0.385–1.223	0.202
Smoke history (Positive VS Negative)	0.582	0.327–1.039	0.067
T stage (T3 VS T1-T2)	2.878	1.614–5.132	< 0.001
PSA (≥ 10 ng/ml VS < 10 ng/ml)	2.096	1.056–4.163	0.034
Lymph node metastasis (Positive VS Negative)	2.636	1.516–4.584	0.001
Gleason Score (≥ 8 VS < 8)	3.235	1.857–5.635	< 0.001

BZW1 promotes cell proliferation in prostate cancer by regulating TGF-β1/Smad pathway

To explore the biological functions of BZW1 in prostate cancer, the influence of BZW1 expression on cell proliferation was investigated. As shown in Fig. 3A, BZW1 was highly expressed in PC3 cells, but was relatively lower in DU145 and LnCap cells. Thus, BZW1 expression was knocked down in PC3 cells and over-expressed in LnCap cells. QRT-PCR and Western blot analysis shown BZW1 down-regulation and up-regulation were successfully performed (Fig. 3B and 3C). Then, cell proliferation was evaluated by CCK-8 and Colony formation assays *in vitro*. CCK-8 assay showed that BZW1 knockdown significantly inhibited the proliferation in PC3 cells (Fig. 3D), while BZW1 over-expression significantly promoted the proliferation in LnCap cells. The colony number in cells with BZW1 down-regulation was significantly decreased, while was significantly increased in cells with BZW1 over-expression (Fig. 4A). To further investigate the effect of BZW1 expression on cell proliferation, xenografts *in vivo* was performed. Results showed that the size and weight of tumor with BZW1 knockdown were significantly decreased compared with those in NC groups (Fig. 4B and 4C).

To explore the potential mechanism of BZW1, TGF-β1/Smad pathway was investigated. As shown in Fig. 4D, the expression of TGF-β1 was significantly decreased with the knockdown of BZW1, while was significantly up-regulated by BZW1 over-expression. The protein expression levels of Smad1 and Smad3 were unchanged regardless of whether the BZW1 expression interference, while BZW1 down-regulation resulted in the decrease of p-Smad1 and p-Smad3.

Discussion

BZW1, as one of transcription factors, encode a N-terminal bZIP domain and a C-terminal nucleotide binding domain [11–12]. The proteins and transcription factors of bZIP domain play crucial roles in cancer development [17–18]. Recently, BZW1 over-expression was observed in mucoepidermoid

carcinoma [14] and lung adenocarcinoma [16]. However, the role of BZW1 in prostate cancer remains unknown.

Here, the expression and clinical significance of BZW1 was investigated in prostate cancer. Results shown that the mRNA and protein levels of BZW1 in prostate cancer were significantly higher compared with those in non-cancerous tissues. These data indicated that BZW1 overexpression was associated with the formation of prostate cancer, which was consistent with the observations in mucoepidermoid carcinoma [14] and lung adenocarcinoma [16]. BZW1 over-expression might be helpful for the diagnosis of prostate cancer. In addition, BZW1 over-expression significantly correlated with T stage, lymph node metastasis, PSA and Gleason score, suggesting that BZW1 over-expression was connected with the progression of PCa. Moreover, survival analysis shown that BZW1 as well as T stage, lymph node metastasis, PSA and Gleason score were associated with patients' survival, which could be as independent prognostic factors in PCa. Moreover, Chiou et al [16] reported BZW1 over-expression was an independent poor prognosis marker in lung adenocarcinoma.

Subsequently, the influence of BZW1 expression on cell proliferation and its potential mechanism were explored. Results shown BZW1 down-regulation significantly suppressed cell proliferation, while BZW1 over-expression significantly promoted cell proliferation in *vitro*. Xenografts assays further validated that BZW1 knockdown significantly inhibited the proliferation of prostate cancer *in vivo*. To explore the potential mechanism of BZW1, TGF-β1/Smad pathway was investigated. It is well known that TGF-β/Smad pathway is involved in cell proliferation [19–21]. Results shown that BZW1 over-expression activated the TGF-β1/Smad pathway. Thus, these data indicated that BZW1 over-expression promoted cell proliferation, which was connected with the TGF-β1/Smad pathway.

Conclusion

These data indicate that BZW1 is highly expressed in prostate cancer and correlates with tumor progression and prognosis. Moreover, BZW1 overexpression promotes cell proliferation by regulating TGF-β1/Smad pathway.

Declarations

Not applicable.

Declarations

Authors' contributions

KL conceived and designed the study. TCL and ZLS performed the analysis. JYW and CTX coordinated the statistical analyses of results. KL and TCL wrote the manuscript. All authors read and approved the manuscript. All authors consent for publication.

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Availability of data and materials

The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was reviewed and approved by the internal review boards (IRB) of the involved institutions: Sun Yat-sen University. (No. Z81771573).

All eligible subjects signed an informed consent to participate in this study.

Consent for publication

Not applicable.

Competing interests

All author declare they have no financial disclosures and conflicts of interest.

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Figures

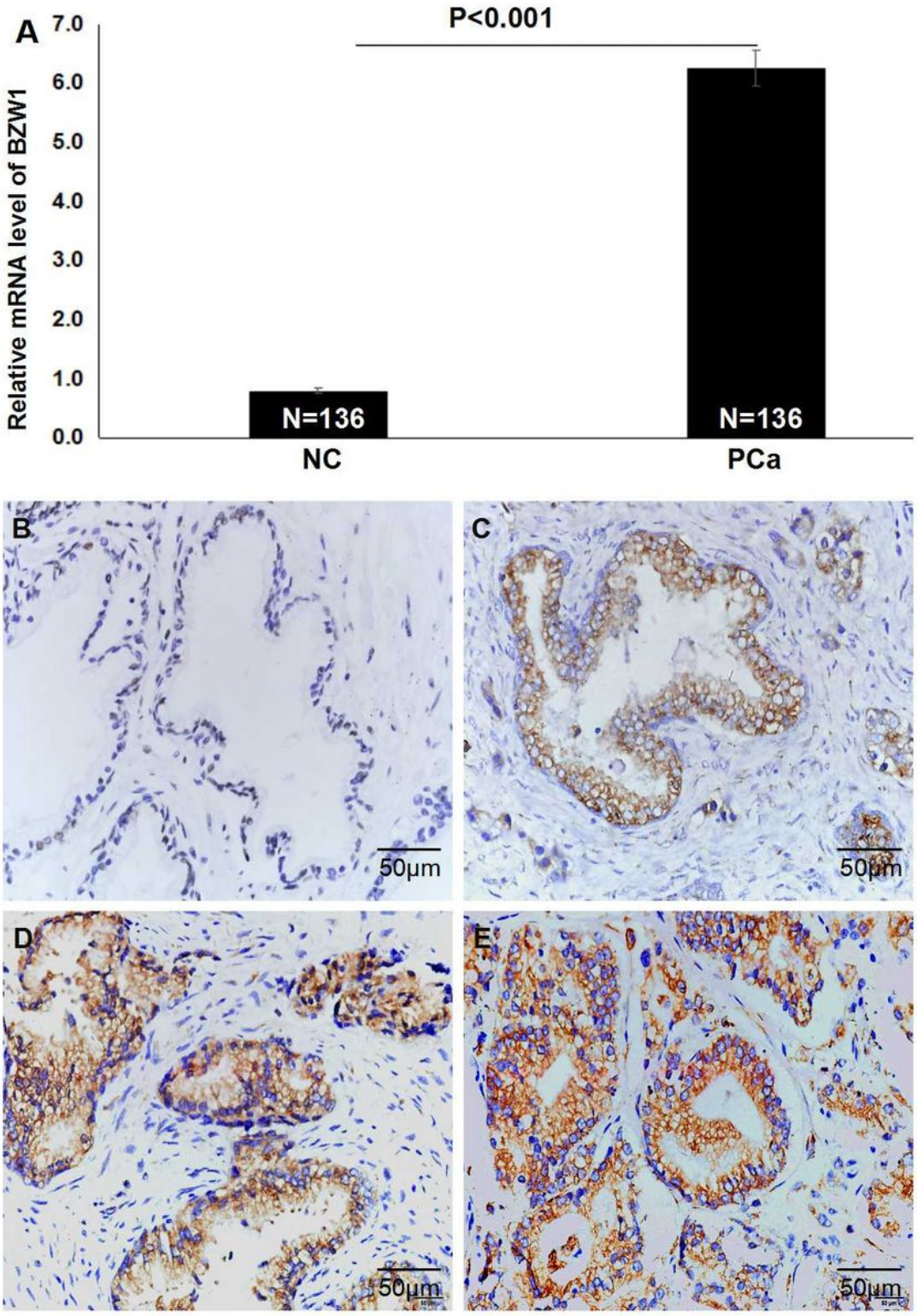

Figure 1

The expression levels of BZW1 were detected in prostate cancer. A: qRT-PCR revealed that the mRNA levels of BZW1 were higher in PCa compared with those in NC. (NC: non-cancerous prostate tissues). B: BZW1 low-expression in non-cancerous prostate tissues by IHC (Score=0). C: BZW1 low-expression in PCa by IHC (Score=2). D: BZW1 high-expression in PCa by IHC (Score=4). E: BZW1 high-expression in PCa by IHC (Score=6).

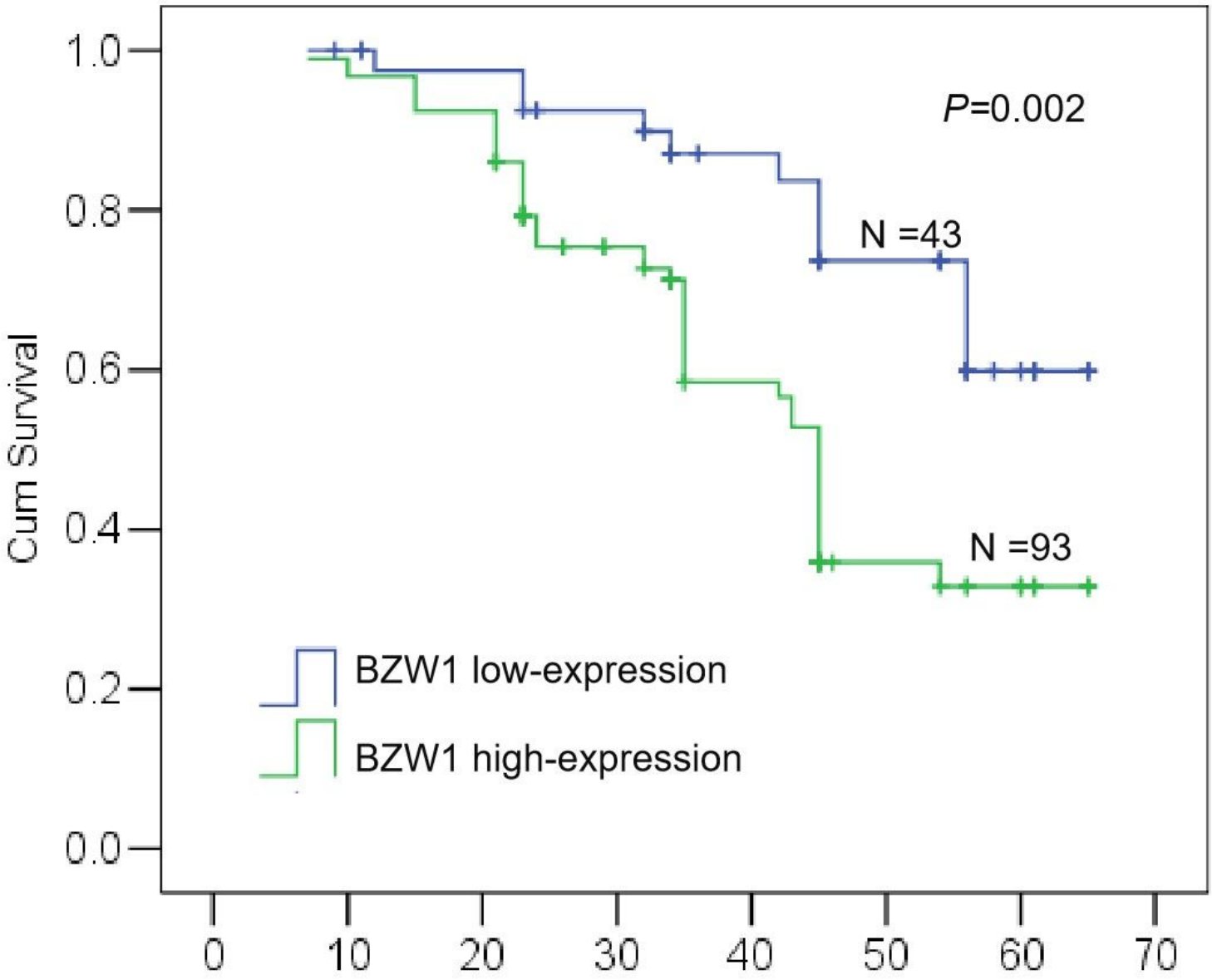

Figure 2

Kaplan-Meier survival analysis shown patients with BZW1 over-expression had unfavorable survival.

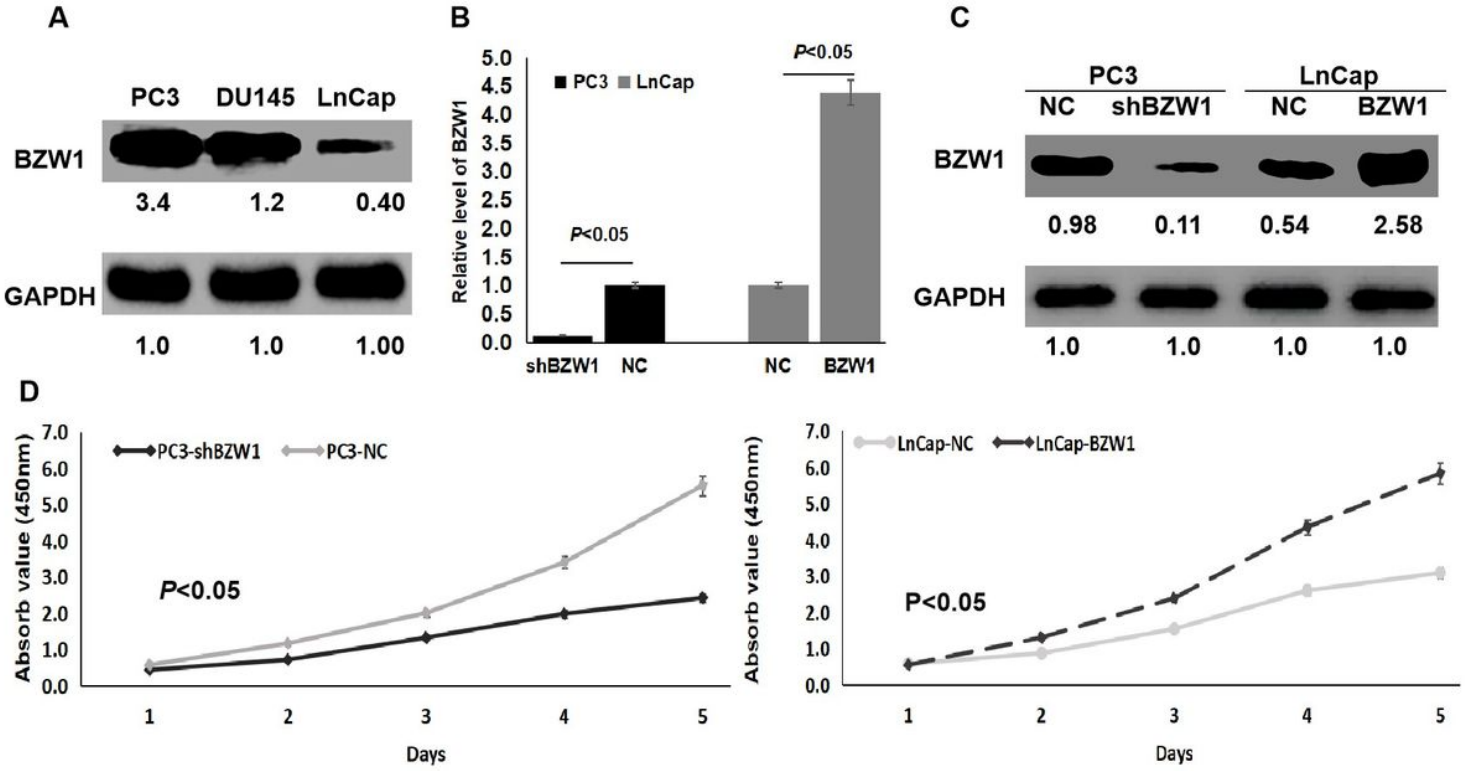

Figure 3

BZW1 overexpression promoted cell proliferation in vitro. A: The protein expression of BZW1 was detected in prostate cancer cell lines by Western blot. B: qRT-PCR analysis revealed that BZW1 down-regulation and up-regulation were successfully performed. C: Western blot analysis revealed that BZW1 down-regulation and up-regulation were successfully performed D: CCK-8 assay shown BZW1 down-regulation significantly inhibited cell proliferation, while BZW1 overexpression significantly promoted cell proliferation. (Full-length blots/gels are presented in Supplementary Figure)

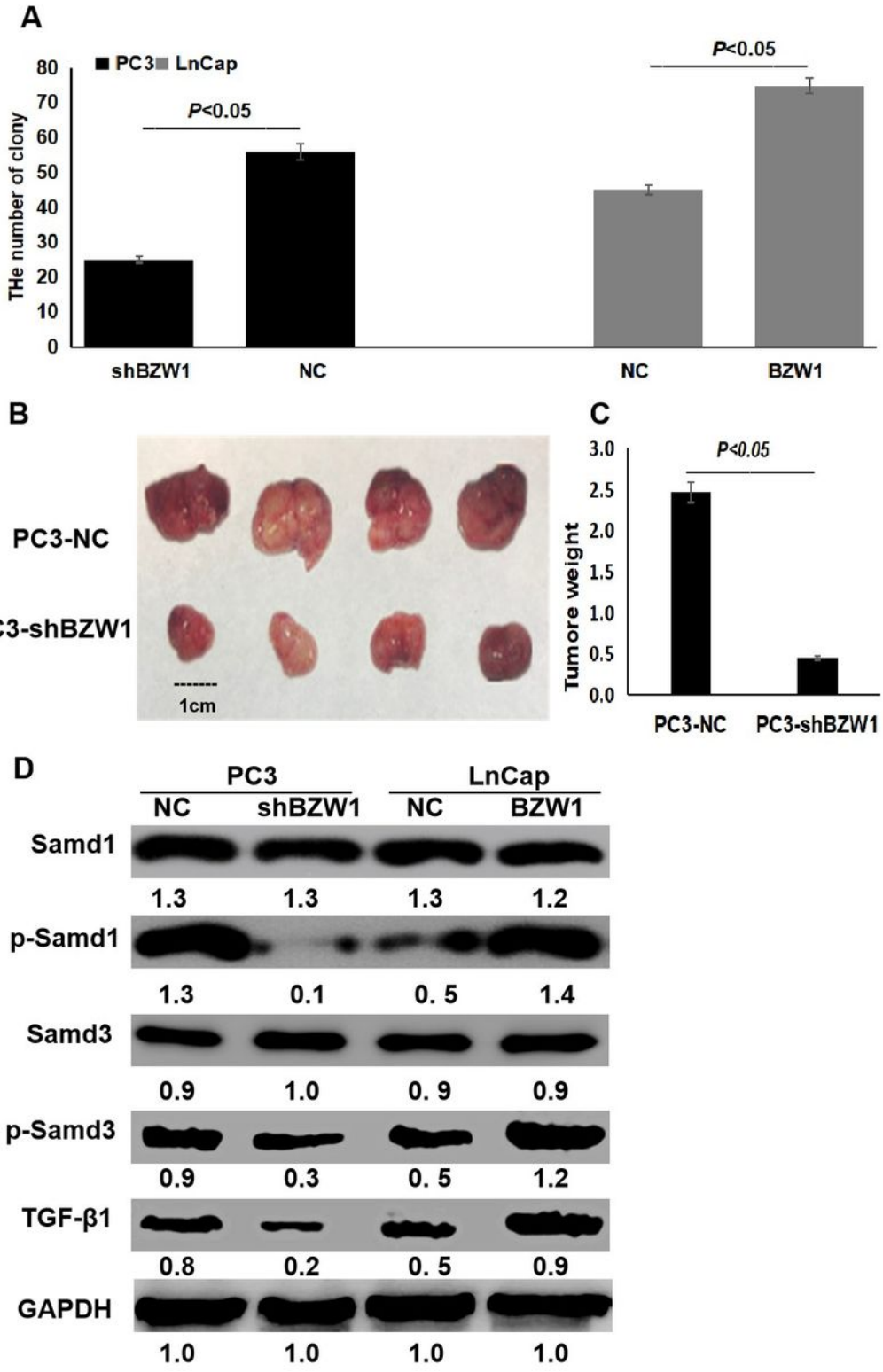

Figure 4

BZW1 overexpression promoted cell proliferation in vivo. A: BZW1 overexpression promoted colony formation. B: Xenografts assay indicated tumor size was decreased by down-regulating the expression of BZW1. C：Xenografts assay indicated tumor weight was significantly reduced by down-regulating the expression of BZW1. D: BZW1 over-expression activated the TGF-β/Smad pathway. (Full-length blots/gels are presented in Supplementary Figure)

Supplementary Files

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