Analysis of key genes reveal lysine demethylase 5B promotes prostate cancer progression

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Abstract. Prostate cancer (PCa) is one of the most common types of cancer in males globally. However, the molecular mechanisms underlying PCa progression remain largely unclear. In the present study, Gene Expression Omnibus (GEO) datasets and datasets from The Cancer Genome Atlas (TCGA) were used to analyze the expression of lysine demethylase 5B (KDM5B) in PCa. Proliferation, cell cycle and migration assays were used to detect the functional roles of KDM5B. It was found KDM5B was upregulated in PCa tissues by analyzing GEO and TCGA datasets. KDM5B knockdown significantly suppressed proliferation and cell cycle progression in PCa cells. In additional, KDM5B knockdown inhibited PCa cell migration. By analyzing a TCGA dataset, KDM5B was found to be upregulated in patients at N1 stage compared with N0 stage PCa, in patients at T3+T4 stages compared with T2 stage and in patients with Gleason score ≥8 compared with those with score ≤7. Kaplan-Meier analysis revealed that higher expression of KDM5B was associated with shorter biochemical recurrence-free survival and overall survival time in patients with PCa. These results suggest that expression of KDM5B may serve as a biomarker to predict the outcome of PCa.

Introduction

Prostate cancer (PCa) is the second leading cause of male morbidity and mortality in the world (1). In recent years, the incidence rate of PCa has increased dramatically (1,2). In China in 2015, ~603,000 men were estimated to be diagnosed with PCa and 266,000 men died of it (3). One of the most likely causes is that more reliable and effective biomarkers for early diagnosis of PCa are still lacking. Although a large number of studies revealed several key proteins in PCa progression, including androgen receptor (AR), forkhead box A1 (FOXA1) and speckle-type BTB/POZ protein (SPOP) (4-7), the pathogenesis and etiology of PCa is still not well-understood. Therefore, here is an urgent need to identify novel regulators to understand mechanisms underlying PCa carcinogenesis and to serve as biomarkers.

Histone methylation is tightly controlled by histone methyltransferases and histone demethylases, and it is one of the most important types of chromatin post-translational modifications (8). Emerging studies have revealed that histone methylation plays a significant role in transcriptional regulation, maintenance of genome integrity and epigenetic inheritance (9-13). An imbalance between methylation and demethylation is frequently observed in the pathogenesis of human disorders, including cancer (14-17). Lysine demethylase 5B (KDM5B) is a member of the jumonji/AT-rich interaction domain-containing (ARID) family of histone demethylases, and it is also known as JARID1B or PLU-1. KDM5B has been found to be upregulated in squamous cell carcinoma of the head and neck, breast cancer, hepatocellular carcinoma, gastric cancer and glioma (18-24). Previous studies also revealed that KDM5B is upregulated in PCa (25). However, the functional roles of KDM5B in PCa remain largely unknown.

In the present study, the prognostic value of KDM5B in PCa was explored by analyzing 3 independent public datasets. Moreover, experimental validation was performed by investigating the effects of KDM5B on PCa cell proliferation, cell cycle progression and migration. The current study may provide useful information to explore potential candidate biomarkers for the diagnosis of PCa and for predicting prognosis in patients.

Materials and methods

Cell culture. LNCaP, PC-3, 22Rv1, DU145, and WPMY-1 cells were purchased from the American Type Culture Collection. The cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) in an incubator at 37°C with 5% CO_2.

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Cell transfection. Short-interfering RNAs (siRNAs) against KDM5B (siKDM5B-546 and siKDM5B-943) and negative control siRNA (siNC) were purchased from Shanghai GenePharma Co., Ltd. The sequences were as follows: siKDM5B-546 sense, 5'-GCAGUUGUUUGCAAGGAU ATT-3' and antisense, 5'-UAUCCUUGCAAACAACUG CTT-3'; siKDM5B-943 sense, 5'-GCAUCAAGCAAGAAC CUAUTT-3' and antisense, 5'-AUAGGUUCUUGCUUGAUG CTT-3'; and siNC sense, 5'-UUCUCCGAACGUGUCACG UTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfection with the siKDM5Bs or siNC was performed using Lipofectamine 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

RNA extraction and RT-qPCR. Total RNA was extracted from the cells using the Ultrapure RNA kit (CoWin Biosciences). RT was performed using the SuperQuick RT MasterMix (CoWin Biosciences) according to the manufacturer's protocol. RT-qPCR was performed using the AceQ qPCR SYBR Green Master Mix (Vazyme) according to the manufacturer's protocol. The cycling conditions were as follows: Initial denaturation (2 min at 95˚C) followed by 40 cycles of denaturation (10 sec at 95˚C), annealing (30 sec at 59˚C), elongation (30 sec at 72˚C) and a final extension (30 sec at 72˚C). The PCR primers for mature KDM5B and β-actin were as follows: KDM5B forward, 5'-AGCAGACTGGCATCTGTAAGG-3' and reverse, 5'-GAA GTTTATCAACATCACATGCAA-3'; and β-actin forward, 5'-CCTCTCCCAAGTCCACACAG-3' and reverse, 5'-GGG CACGAAGGCTCATCATT-3'. β-actin was used as internal control. The $2^{-\Delta\Delta Cq}$ method was used to analyze the data (26). Each sample was measured in triplicate.

Western blot analysis. The PCa cells were homogenized and sonicated using Mammalian Protein Extraction Kit (CoWin Biosciences). Protein concentrations were detected using a BCA Protein Quantification kit, according to the manufacturer's protocol. The proteins (50 μg) were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 5% non-fat dry milk for 1 h at room temperature and incubated with specific primary antibodies overnight at 4˚C. The primary antibodies used were as follows: KDM5B (1:1,000; Abnova), ACTB (1:1,000, cat. no. ab8226; Abcam). The secondary antibodies were Goat Anti-Mouse IgG H&L (1:1,000, cat. no. ab205719; Abcam) for ACTB, and Goat Anti-Rabbit IgG H&L (1:1,000, cat. no. ab205719; Abcam) for KDM5B. The blots were detected with an enhanced chemilumi-nescence substrate kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The bands were scanned and quantified by ImageJ v1.47 software (National Institutes of Health).

Cell proliferation assay. Cell proliferation was detected using the Cell Counting Kit-8 (CCK-8) assay (MedChemExpress) in 96-well plates. After transfection, 100 μl/well of cells were added to 96-well plates. CCK-8 reagent was added to each well 2 h before the end of the experiment, and the cells were incubated, and the absorbance was then measured at 450 nm wavelength in a microplate reader.

Cell cycle analysis. Transfected LNCaP and PC-3 cells were collected 48 h post-transfection. Triton X-100 (0.03%) and propidium iodide (50 ng/ml) were used to resuspend cells. After incubation at room temperature for 10 min, the trans-fected cells were examined using a flow cytometer (Beckman Coulter, Inc.). Each sample was measured in triplicate.

Cell migration assay. Cells were treated with siRNAs, including siNC, siKDM5B-546 and siKDM5B-943. Transwell plates were used for the determination of migration ability. Briefly, 700 μl 1640 medium supplemented with 10% FBS was added to the lower chamber of the Transwells. A total of 2,000 cells were resuspended in 100 μl 1640 medium supple-mented with 1% FBS, and then added to the upper chamber of the system. After incubation in an incubator at 37˚C for 3 days, the chambers were removed and unmigrated cells in the upper chamber were wiped with a cotton swab. The migrated cells in the upper chamber were washed twice with PBS and were then fixed with 700 μl methanol for 15 min. Next, the migrated cells were stained with DAPI for 20 min, then washed 3 times with PBS. Cells were imaged with a microscope. Each sample was measured in triplicate.

Statistical analysis. The data are presented as the mean ± SD. Student's t-test or Mann-Whitney U-test were used to compare the difference between the 2 groups of data. Correlation analysis was performed with Spearman's rank correlation. For multiple groups, Statistical analyses were performed using a one-way analysis of variance with the Bonferroni test for post hoc comparisons. Survival analysis was based on the Kaplan-Meier method and the log-rank tests to compare the differences between survival curves. P<0.05 was considered to indicate a statistically significant difference.

Results

KDM5B is upregulated in PCa. The expression levels of KDM5B in PCa and normal samples were previously unknown. In the present study, 3 independent datasets were analyzed, including TCGA, GSE17951 (27) and the Prensner data-sets (28). KDM5B was found to be significantly upregulated in PCa tissues by analyzing TCGA (Fig. 1A). To further validate this result, two additional independent datasets, GSE17951 and Prensner were analyzed, and consistent results were observed (Fig. 1B and C). To further compare KDM5B protein levels in PCa and normal tissues, KDM5B protein expression was analyzed using the Human Protein Atlas. KDM5B protein was upregulated in PCa samples, however, KDM5B was not detected in normal prostate glandular cells (Fig. 1E). KDM5B expression was also detected in cell lines, including LNCaP, PC-3, DU145, and 22Rv1 PCa cells and WPMY-1 noncan-cerous prostate cells. KDM5B was found to be upregulated in PCa cell lines compared to WPMY-1 cells (Fig. 1D).

The knockdown of KDM5B inhibits PCa cell proliferation. The roles of KDM5B on the proliferation of PCa cells was then evaluated. siRNAs against KDM5B (siKDM5B-546 and siKDM5B-943) were designed to knockdown KDM5B expression. LNCaP and PC-3 cells were transfected with siNC or siKDM5B siRNAs. At 48 h post-transfection, both the mRNA and protein levels of KDM5B were significantly suppressed in both siKDM5B groups compared with the
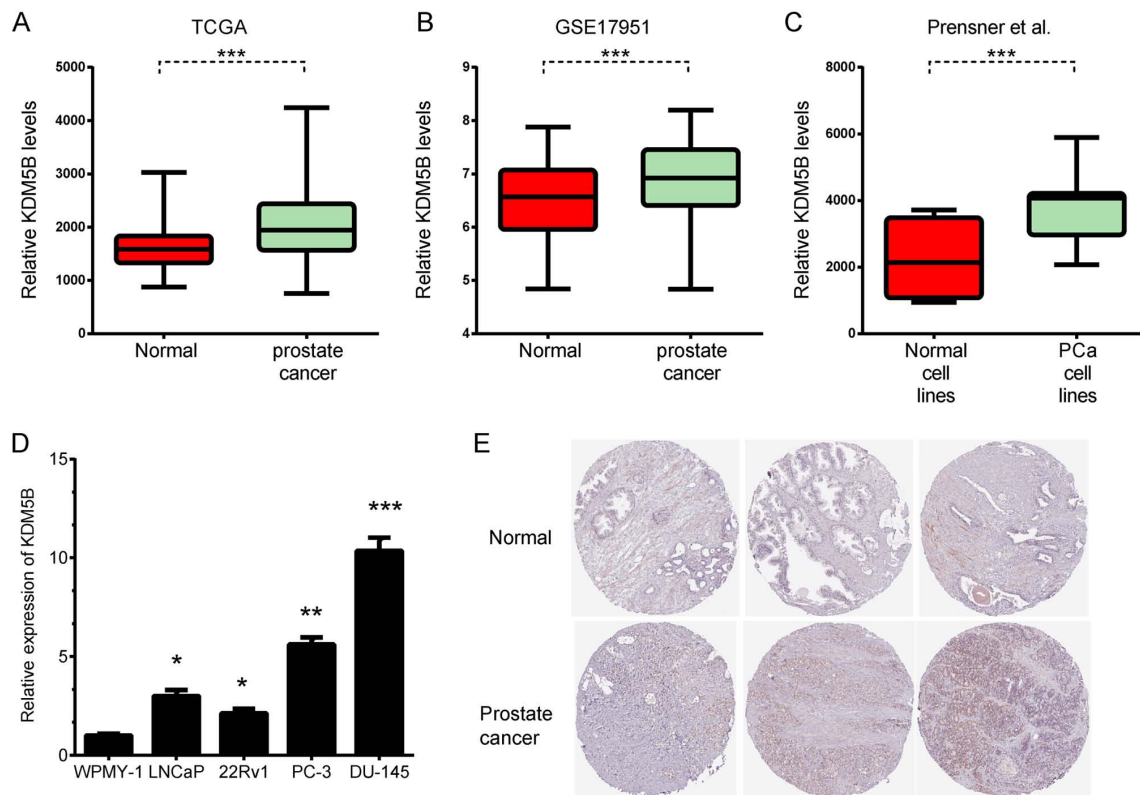
Figure 1. KDM5B is upregulated in prostate cancer. KDM5B is upregulated in (A) TCGA, (B) GSE17951 and (C) Prensner datasets. ***P<0.001. (D) Expression of KDM5B mRNA in WPMY-1, LNCaP, 22RV1, DU145 and PC-3 cells. (E) Protein levels of KDM5B in normal prostate and prostate cancer samples by analyzing the Human Protein Atlas. *P<0.05, **P<0.01, ***P<0.001 vs. WPMY-1. KDM5B, lysine demethylase 5B; TCGA, The Cancer Genome Atlas.
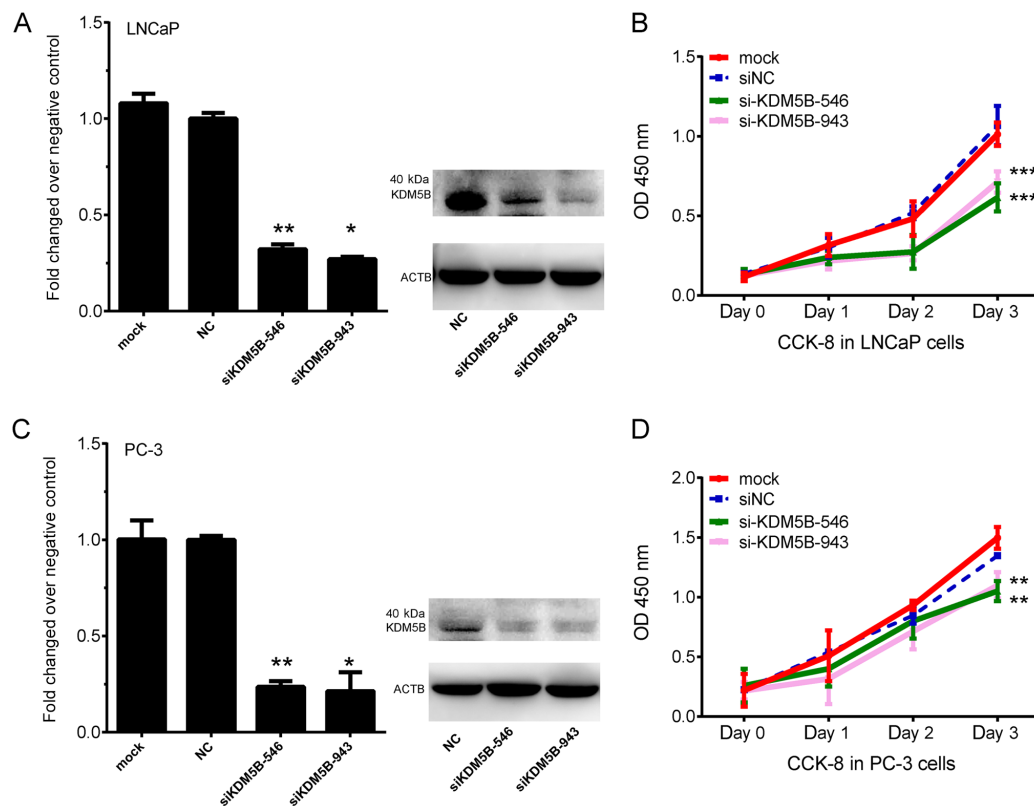

Figure 2. Knockdown of KDM5B inhibits PCa cell proliferation. (A) mRNA and protein expression of KDM5B after transfection with mock, siNC, siKDM5B-546 and siKDM5B-943 in LNCaP cells. (B) Knockdown of KDM5B inhibits LNCaP cell proliferation. (C) mRNA and protein expression of KDM5B after transfection with mock, siNC, siKDM5B-546 and siKDM5B-943 in PC-3 cells. (D) Knockdown of KDM5B inhibits PC-3 cell proliferation. *P<0.05, **P<0.01, ***P<0.001 vs. NC. KDM5B, lysine demethylase 5B; PCa, prostate cancer; si, small interfering; NC, negative control; OD, optical density; CCK-8, Cell Counting Kit-8.
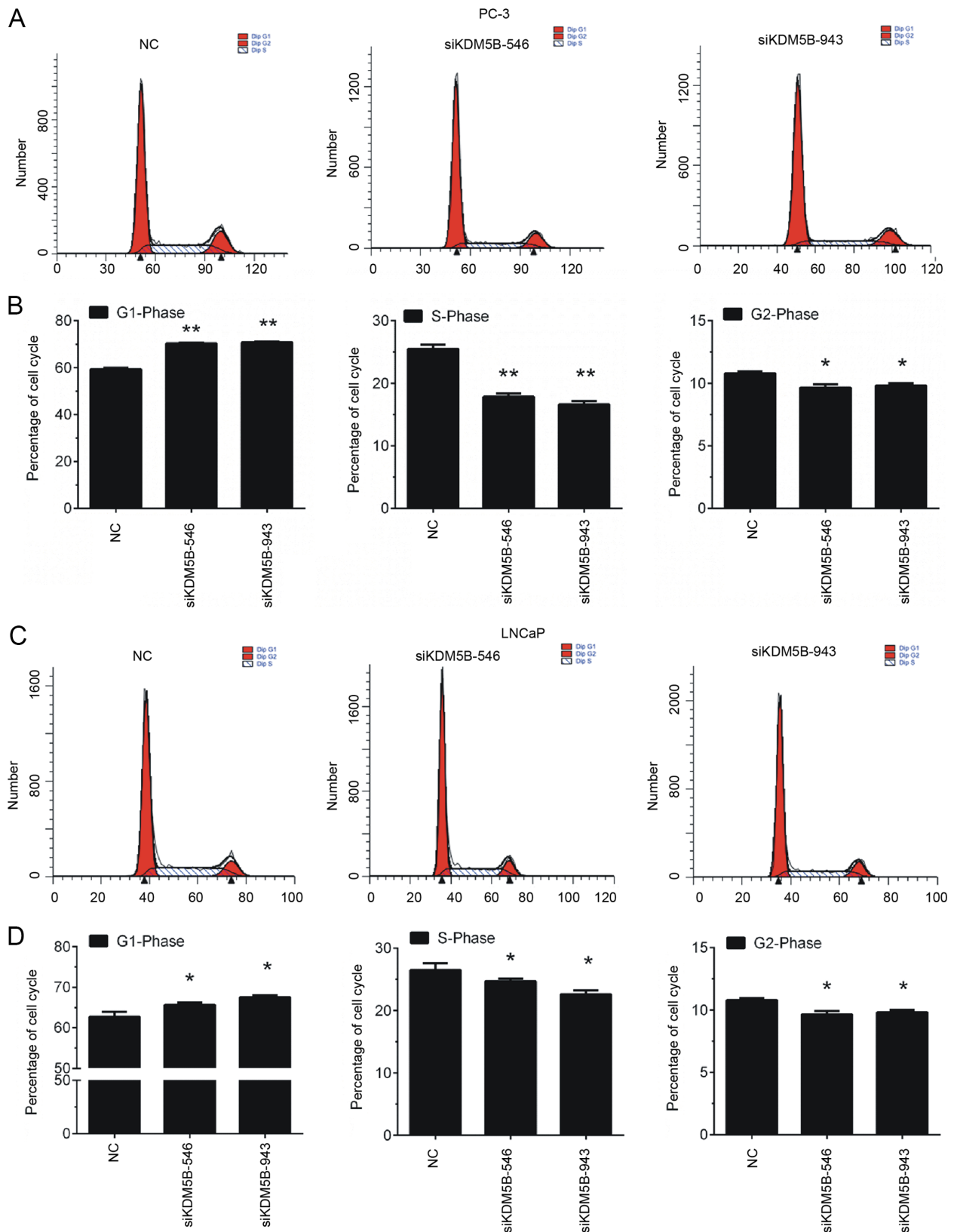
Figure 3. Knockdown of KDM5B induced prostate cancer cell cycle arrest at the G_1 phase. (A and B) Knockdown of KDM5B induced PC3 cell cycle arrest at the G_1 phase. (C and D) Knockdown of KDM5B induced LNCaP cell cycle arrest at the G_1 phase. $^*P<0.05$, $^{**}P<0.01$ vs. NC. KDM5B, lysine demethylase 5B; si, small interfering; NC, negative control.

siNC group (P<0.05 and P<0.01; Fig. 2A and C). Additionally, knockdown of KDM5B inhibited proliferation of LNCaP and PC-3 cells (P<0.001 and P<0.001; Fig. 2B and D).

Knockdown of KDM5B induces PCa cell cycle arrest in the G1 phase. Furthermore, the effects of KDM5B on cell cycle progression of LNCaP and PC-3 cells were detected by flow
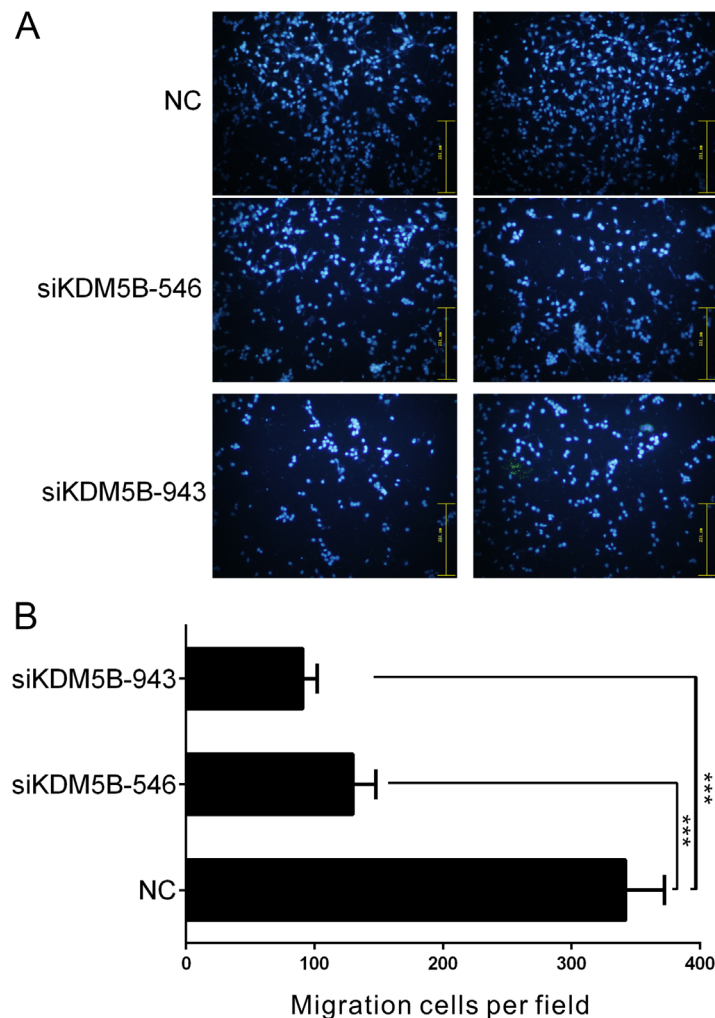
Figure 4. Knockdown of KDM5B inhibits migration of prostate cancer cells *in vitro*. (A and B) Knockdown of KDM5B inhibited cell migration in PC-3 cells. Each experiment was performed in triplicate. n=3. Bars represent the mean ± SD. Scale bar, 251 μm. ***$P<0.001$. KDM5B, lysine demethylase 5B; si, small interfering; NC, negative control.

cytometry. The findings revealed that knockdown of KDM5B in LNCaP and PC-3 cells induced a significant increase in the proportion of cells in G1 phase, however, KDM5B knockdown decreased the proportion of cells in S and G2/M phase ($P<0.05$ and $P<0.01$; Fig. 3).

The knockdown of KDM5B inhibits the migration of PCa cells in vitro. The number of PC-3 cells that migrated through the filter of the Transwell chambers was used to estimate the migratory ability of the cells (Fig. 4). Compared with the scrambled siRNA-treated control group, the number of migrating cells was decreased by 2.74- and 3.91-fold in PC-3 cells treated with siKDM5B-546 and siKDM5B-943, respectively ($P<0.001$; Fig. 4).

KDM5B expression is associated with clinical variables in patients with PCa. The association between KDM5B expression and the clinicopathological characteristics of patients with PCa was investigated. As presented in Fig. 5, KDM5B expression was significantly upregulated in N1 stage PCa samples compared to N0 samples (Fig. 5A), in T3/T4 PCa samples compared to T2 samples (Fig. 5B). Moreover, we analyzed the correlation between KDM5B expression and Gleason score in

patients with PCa. The results showed that the higher expression levels of KDM5B significantly correlated to the higher Gleason score in PCa patients (Fig. 5C-D).

In addition, Kaplan-Meier analysis was performed to determine whether KDM5B expression was associated with biochemical recurrence (BCR)-free survival and overall survival in patients with PCa by analyzing the TCGA dataset. In order to divide all PCa samples into groups based on the high and low expression of KDM5B, the cut-off value was calculated using the Cutoff Finder (http://molpath.charite.de/cutoff/) (29). In TCGA analysis, the BCR-free survival and overall survival rates were higher in the KDM5B-low compared with the KDM5B-high patients (Fig. 5E and F). These results indicate that KDM5B expression may serve as a biomarker to predict the prognosis of patients with PCa.

Discussion

In recent decades, studies focused on exploring the functions of several key proteins, such as AR, SPOP, and FOXA1 in PCa (4-7), however, the molecular mechanisms underlying PCa progression remain largely unclear. Following the application of high-throughput screening techniques like microarray
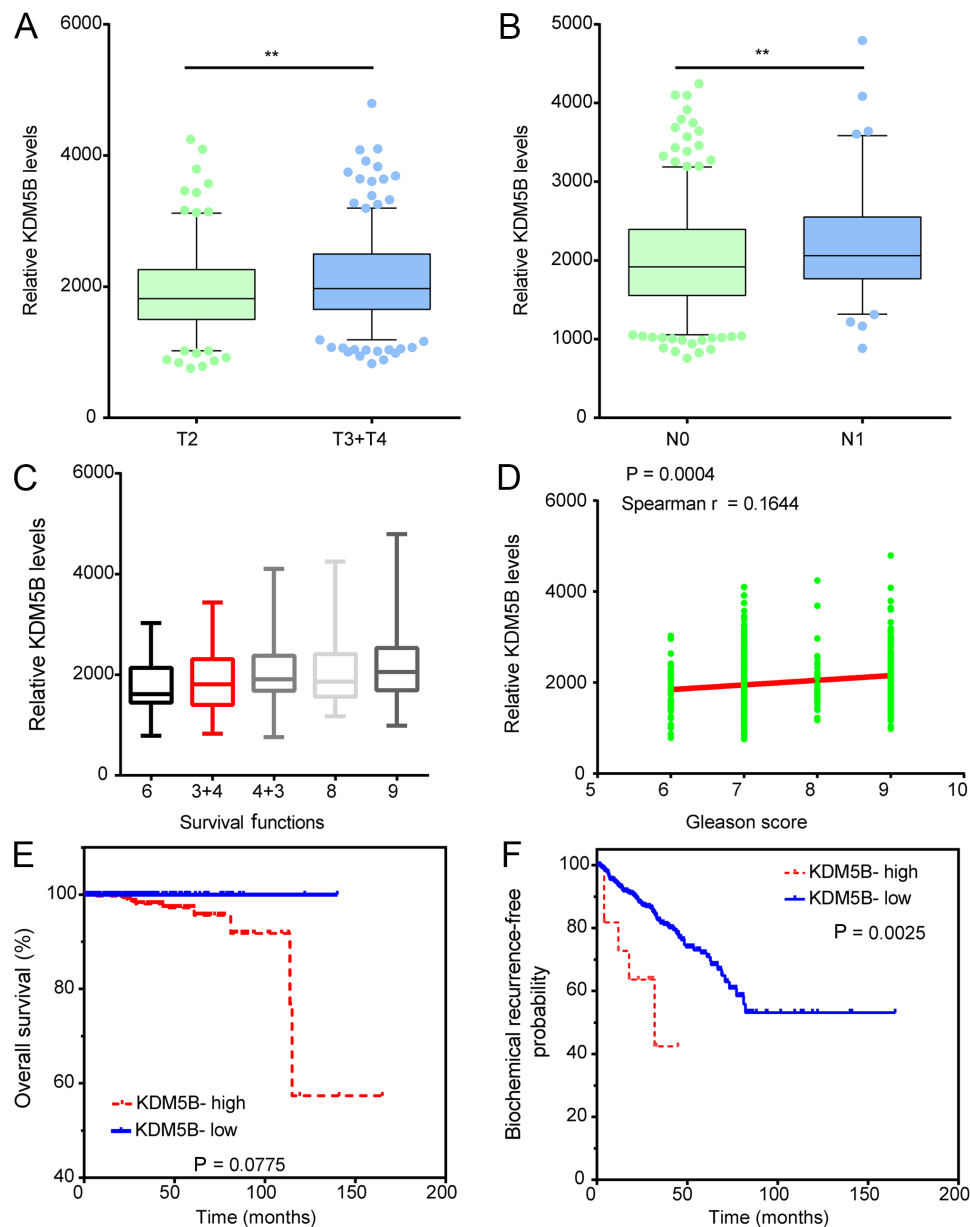
Figure 5. KDM5B expression is associated with PCa clinical variables. (A) KDM5B expression was upregulated in T3/4 prostate cancer compared with T2 prostate cancer. (B) KDM5B expression was upregulated in N1 prostate cancer compared with N0 prostate cancer. (C) KDM5B expression was upregulated in Gleason Score 7, 8 and 9 PCa samples compared with Gleason Score 6 PCa. (D) Higher expression levels of KDM5B significantly correlated to the higher Gleason score in patients with PCa using Spearman's rank correlation analysis. Comparing with patients with low KDM5B expression, the (E) overall survival rates and (F) biochemical recurrence-free survival rates were lower in patients with high KDM5B expression in The Cancer Genome Atlas dataset. Survival analysis was based on the Kaplan-Meier method and the log-rank tests to compare the differences between survival curves. **$P<0.01$. KDM5B, lysine demethylase 5B; PCa, prostate cancer.

and small RNA sequencing, a series of studies identified genes associated with PCa progression. For example, a study by Taylor *et al* (30) and TCGA groups performed integrative genomic analysis of human PCa. In the present study, a comprehensive analysis of PCa-related genes was performed by using 3 public datasets, TCGA and GSE17951, and KDM5B was found to be upregulated in PCa samples.

KDM5B was included in this hub-network. The functional roles of KDM5B in PCa remain largely unknown. Previous studies had observed that dysregulation of KDM5B was associated with cancer progression. KDM5B was found to be upregulated in squamous cell carcinoma of the head and neck, breast cancer, hepatocellular carcinoma, gastric cancer

and glioma (18-24). These studies suggested that KDM5B may serve as a diagnostic and therapeutic target for cancers. In the present study, the function of KDM5B was explored in PCa cells. KDM5B was found to act as an oncogene in PCa cells, as knockdown of KDM5B significantly inhibited cell proliferation, cell cycle progression, and migration. To the best of our knowledge, this is the first study to reveal the effects of KDM5B on the biological functions of PCa cells.

Prostate-specific antigen testing is the most widely used biomarker for patients with PCa, but its efficacy is limited by low specificity. Of note, several recent studies revealed that Low serum total testosterone level and Body mass index could serve as a predictor of upstaging and upgrading in low-risk

prostate cancer patients. For example, Ferro *et al* reported that low serum total testosterone levels as a predictor of upstaging and upgrading in low-risk prostate cancer patients meeting the inclusion criteria for active surveillance (31). And de Cobelli *et al* revealed that Body mass index was associated with upstaging and upgrading in patients with low-risk prostate cancer who met the inclusion criteria for active surveillance (32). Moreover, the expression levels of multiple protein coding genes or non-protein coding genes were also revealed to be associated with the progression and prognosis of patients with PCa, such as PHI, PCA3, sarcosine, and Urotensin II receptor. For example, de Cobelli *et al* showed Urotensin II receptor on preoperative biopsy is associated with upstaging and upgrading in prostate cancer (33). Sreekumar and his colleges found sarcosine levels in PCa samples were associated with the progression of cancer (34). However, lacking reliable and effective biomarkers for PCa diagnosis remained to be one of the biggest challenges in PCa treatment was lacking reliable and effective biomarkers for PCa diagnosis. In the current study, KDM5B was evaluated as a potential biomarker for PCa. By analyzing public datasets, KDM5B was found to be upregulated in PCa compared with normal samples, in T3/T4 PCa samples compared with T2 samples, in N1 stage samples compared to N0 samples and in Gleason score, ≥8 samples compared to Gleason score ≤7 samples. Moreover, overall survival rates were higher in patients with low expression of KDM5B compared with those with high expression. These results indicate that KDM5B expression may serve as a biomarker of PCa. We also realized that the combined analysis of KDM5B levels and other potential biomarkers, such as low serum total testosterone level, Body mass index, PHI, PCA3, sarcosine, and Urotensin II receptor levels, in PCa samples using clinical samples could strength the clinical importance of KDM5B in PCa.

In conclusion, 3 public datasets were analyzed to identify differentially expressed genes in PCa. A total of 3,834 genes were found to be dysregulated in PCa. Bioinformatic analysis revealed that these DEGs were associated with cell cycle, translation, and metabolic pathways. PPI network analysis revealed that KDM5B was a key regulator in PCa progression. Knockdown of KDM5B in PCa cells significantly inhibited proliferation, cell cycle progression and migration. In addition, KDM5B was upregulated in PCa tissues and associated with PCa clinical variables. High expression of KDM5B was associated with worse prognosis in patients with PCa. Given these results, KDM5B may be a potential therapeutic target for PCa.

Authors' contributions

Conception and design of the study was conducted by ZY and JXX. Development of methodology was conducted by ZY, JXX, DPF and JK. ZY, JXX, DPF and JK performed the analysis and interpretation of data, and wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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