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Research Article

ALKBH5 Inhibits Cancer Cell Proliferation in Prostate Cancer through KLF4/TERT Signaling

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N6-methyladenosine (m^6A), as a reversible chemical modification of RNA, is a new type of posttranscriptional gene regulation, which plays an important role in cell differentiation and tumorigenesis, and is also a research hotspot in epigenetic transcriptomics in recent years. The purpose of this study was to discuss the action mechanism of m^6A demethylase ALKBH5 in the occurrence of prostate cancer (PCa). We found that ALKBH5 was lowly expressed in PCa, and the decreased expression of ALKBH5 was responsible for the poor prognosis of prostate carcinomas. Moreover, ALKBH5 downregulated the expression of Krüppel-like factor 4 (KLF4) by reducing its mRNA stability, which reduced the transcriptional activity of KLF4 on the downstream target telomerase reverse transcriptase (TERT) and decreased TERT expression and telomerase activity, eventually inhibiting PCa cell growth. The findings of this study reveal the action mechanism of ALKBH5 in PCa from the perspective of epitranscriptomics, which would provide new ideas for the prevention of PCa.

1. Introduction

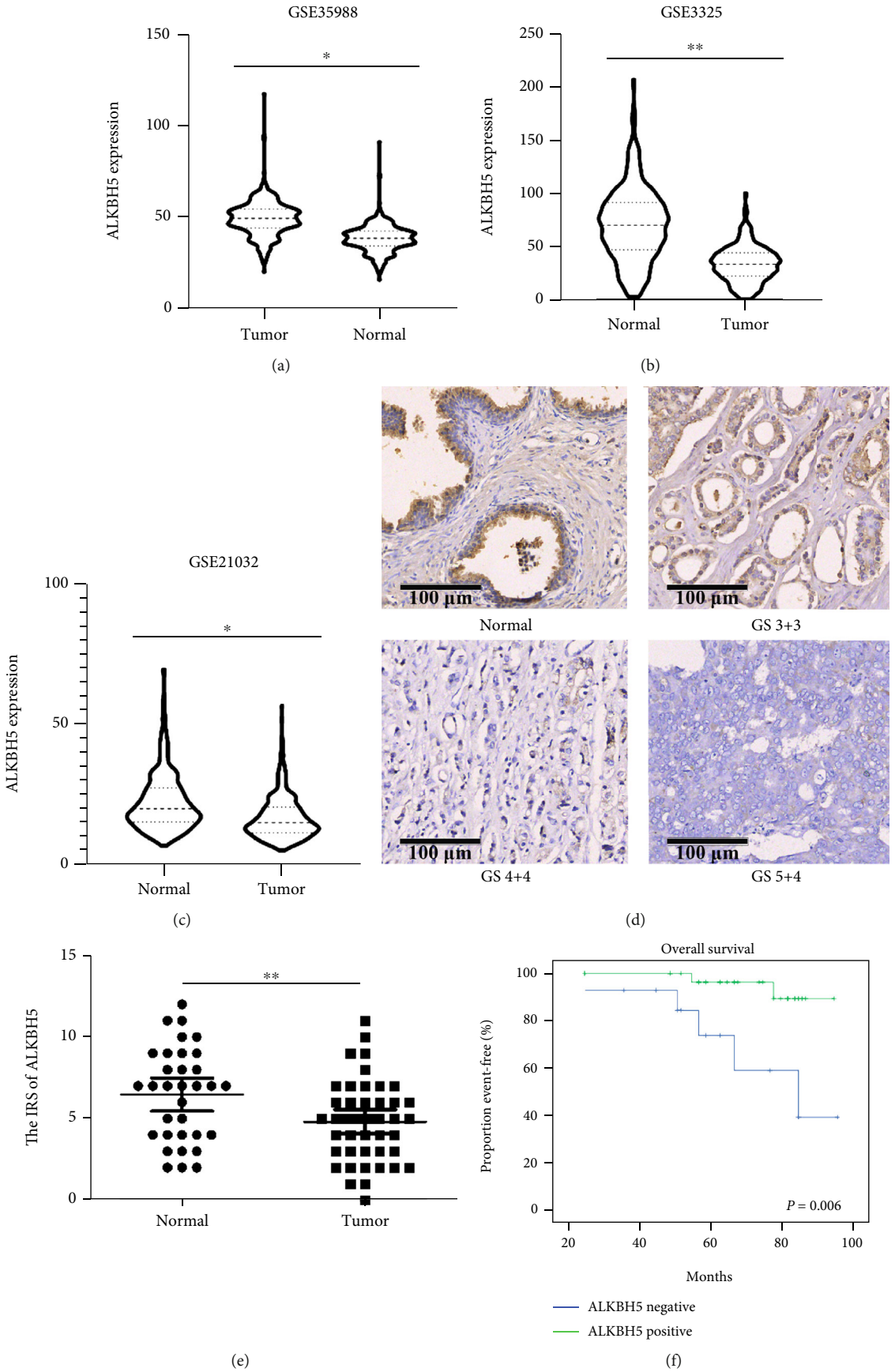
Prostate cancer (PCa) is one of the most common malignant tumors in the urinary system [1]. With growth of the geriatric population, the incidence and mortality of PCa have been rising every year [2]. Research on the molecular mechanism of PCa may help to slow down or even inhibit the malignant progression of PCa [3].

Tumorigenesis is the result of the progressive accumulation of multiple cancer-critical gene mutations or epigenetic alterations. As a new epigenetic modification, n6-methyladenosine (m^6A) has attracted increasing attention for its biological role in cancer [4–7]. It is a reversible methylation that occurs at the 6-position nitrogen atom of the RNA molecule and balanced by an interaction between methyltransferases (writers) and demethylases (erasers) [8]. The m^6A sites are identified in the organism by the m^6A-binding proteins (readers) and mediate its function [9]. m^6A modification is involved in the entire process of mRNA metabolism, including mRNA splicing, translation, and storage, thus participating in a variety of biological functions [10–12]. ALKBH5, as the second RNA m6A demethylase [13], and the tumor have the close correlation, while most tumor cell ALKBH5 function weak or flaw [5, 7, 14, 15]. However, its biological significance and underlying regulatory mechanisms in PCa remain elusive.

Here, we demonstrated the relationship between ALKBH5 expression and PCa progression and clarified the molecular mechanisms that ALKBH5 inhibited PCa cell growth although it downregulated KLF4 expression in an m^6A-dependent manner. ALKBH5 may become a biomarker for prognostic prediction and a potential therapeutic target in PCa.

2. Materials and Methods

2.1. Patients and Tumor Tissue Specimens. A total of 45 PCa tissues and 30 adjacent normal tissues were retrieved from



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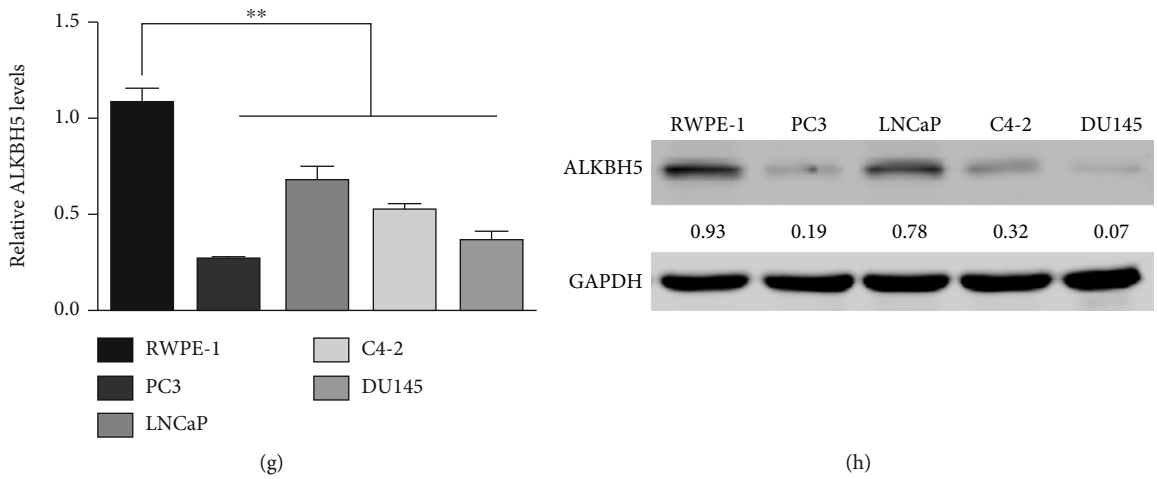


Figure 1: Expression level and clinical significance of ALKBH5 in PCa. (a–c) The expression levels of ALKBH5 in normal prostate tissues and prostate tumor tissues were analyzed based TCGA database (GSE21032, GSE35988, and GSE3325). (d) The expression of ALKBH5 in clinical PCa tissues was analyzed by IHC. (e) IRS of ALKBH5 in PCa tissues. (f) COX regression model was established to analyze the OS of patients ($P = 0.006$). (g, h) The expression of ALKBH5 in PCa cell lines. $^{*}P < 0.05$ and $^{**}P < 0.01$.

PCa patients who underwent radical prostatectomy at The First Affiliated Hospital of University of South China between January 2016 and December 2020 (age at onset from 42 to 85 years, 66.4 ± 9.01). None of the patients received endocrine therapy or chemoradiotherapy. Tissue samples obtained from surgery were stored in liquid nitrogen.

2.2. Cell Culture. Human prostate epithelial cell line RWPE-1 and PCa cell lines LNCaP, DU145, and PC3 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The RWPE-1 cells were cultured in keratinocyte serum-free medium (Gibco, Grand Island, NY) and 1% penicillin and streptomycin combination (KeyGEN BioTECH, Nanjing, China). PCa cell lines were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin.

2.3. Transfection. siRNAs targeting ALKBH5, YTHDF1/2, and KLF4 were synthesized by Shanghai GenePharma (Shanghai, China). The siRNA sequences are summarized in Supplementary Table S1. For ALKBH5 overexpression, its coding sequence obtained by PCR (sequence shown in Supplementary Table S2) was cloned into the pCDNA3.1 (+) vector [16].

2.4. Western Blotting. Western blotting was performed as previously described by our laboratory [16]. The primary antibodies used were as follows: ALKBH5 (ab#195377, Abcam, Cambridge, UK), KLF4 (ab#214666, Abcam), TERT (ab#32020, Abcam), and GAPDH (ab#8245, Abcam).

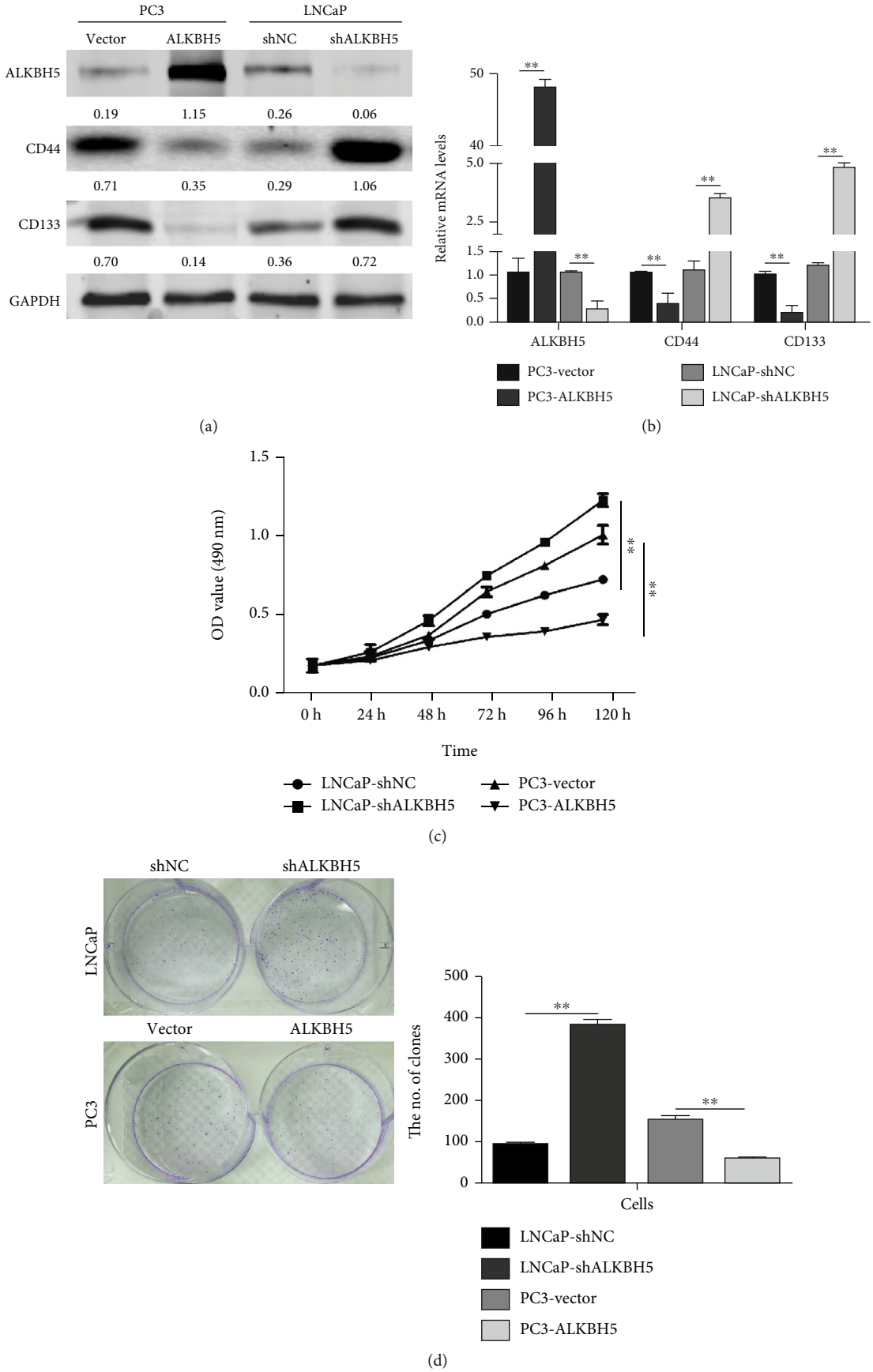
2.5. Immunohistochemistry (IHC). Clinical PCa or xenograft tumor tissues were taken by paraffin imbedding and cut. The expression levels of ALKBH5 and KLF4 in tissues were detected according to our previous IHC procedure [17]. The results were expressed as the immunoreactivity score (IRS), which was the percentage of positive cell score multiplied by the staining intensity score.

2.6. Quantitative Real-Time PCR (qRT-PCR). Total RNA from cells was extracted by TRIzol/chloroform/isopropanol method. RNA was reverse transcribed into cDNA according to the introduction of PrimeScript RT Reagent Kit (Takara, Japan). PCR amplification and quantification were performed using cDNA as a template according to the PrimeScript RT Master Mix (Perfect Real Time) kit (Takara, Japan). PCR primer sequences are shown in Supplementary Table S2. Formula $2^{-\Delta\Delta CT}$ was used to process and analyze the data.

2.7. MTS Assay. 2000 cells were seeded in 96-well plates, and 10 μL MTS was added to the medium at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively. The absorbance of each well was measured at 490 nm wavelength, and the cell growth curve was drawn according to the absorbance.

2.8. Nude Mouse Xenograft Experiment. Sixteen 4-week-old immunodeficient nude mice were housed in the Laboratory Animal Center of the University of South China. PCa cell lines were digested with trypsin, counted, and prepared into cell suspension at a concentration of 5×10^7 cells/mL. Then, 100 μL of the cell suspension was inoculated in the underarm of each nude mouse. The tumorigenesis of nude mice and the size and volume of transplanted tumors were recorded. Eight weeks after inoculation, the mice were humanely sacrificed and calculated the volume and weight of the transplanted tumor.

2.9. RNA-Binding Protein Immunoprecipitation (RIP). RIP-qPCR was used to verify the interaction between YTHDF1 protein and KLF4 transcripts. Briefly, according to the manufacturer's instructions, cells were resuspended with lysis buffer, and magnetic beads coated with anti-YTHDF1 or anti-IgG were incubated with the cell suspension at 4°C



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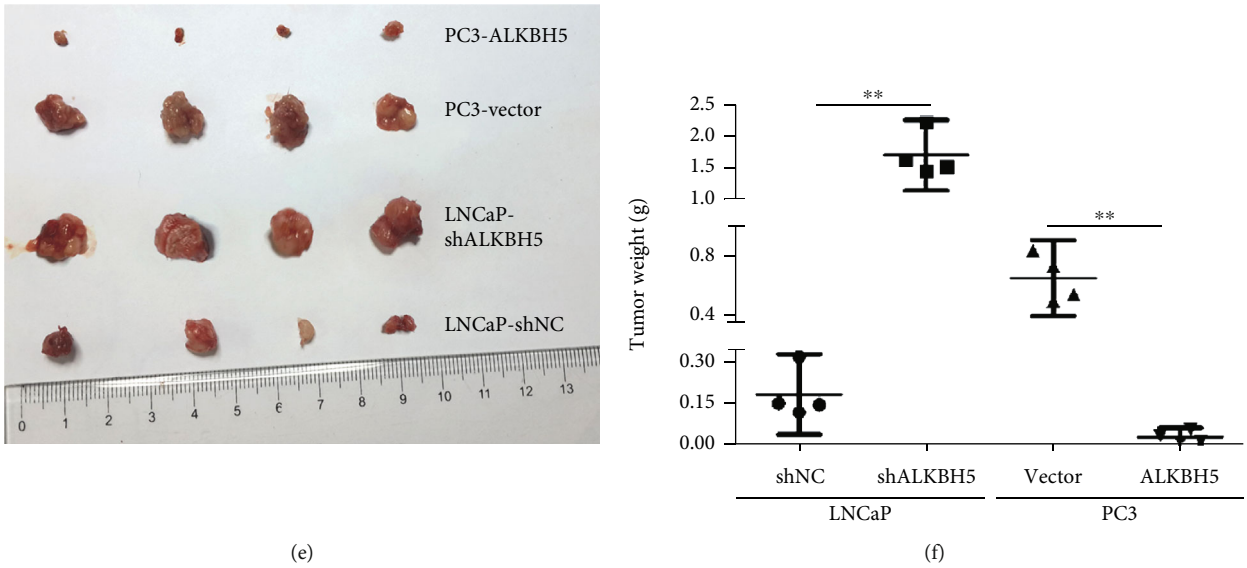


Figure 2: ALKBH5 silencing suppressed the proliferation of PCa cells. (a, b) The effect of ALKBH5 on stem cell markers CD44 and CD133 expressions were detected by western blotting and qRT-PCR. (c) The effect of ALKBH5 on PCa cells proliferation ability was measured by MTS assay. (d) Plate clonality assays were used to measure cell clonality. (e) A representation of the size of the xenotransplanted tumors. (f) Comparison of transplanted tumor weight in different groups. $^{*}P < 0.05$ and $^{**}P < 0.01$.

overnight. After washing the RNA-protein complexes 6 times, the protein A/G complex was resuspended with proteinase K buffer. RNA bound to the YTHDF1 protein was finally extracted by TRIzol reagent, and the amount of KLF4 mRNA bound to YTHDF1 was evaluated by qPCR.

2.10. mRNA Stability Assay. qRT-PCR was used to assess mRNA stability after actinomycin D treatment. Briefly, actinomycin (5 μg/mL) was added to the cell medium; then, total RNA was extracted at 30-minute intervals, and the residual RNA content at different time points was detected by qRT-PCR. The half-life of mRNA was calculated according to the following equation: $\ln(C/C_0) = -K_{decay}t$. C_0 represents the initial mRNA level, t represents the treatment time of actinomycin, and C is the mRNA level at time T.

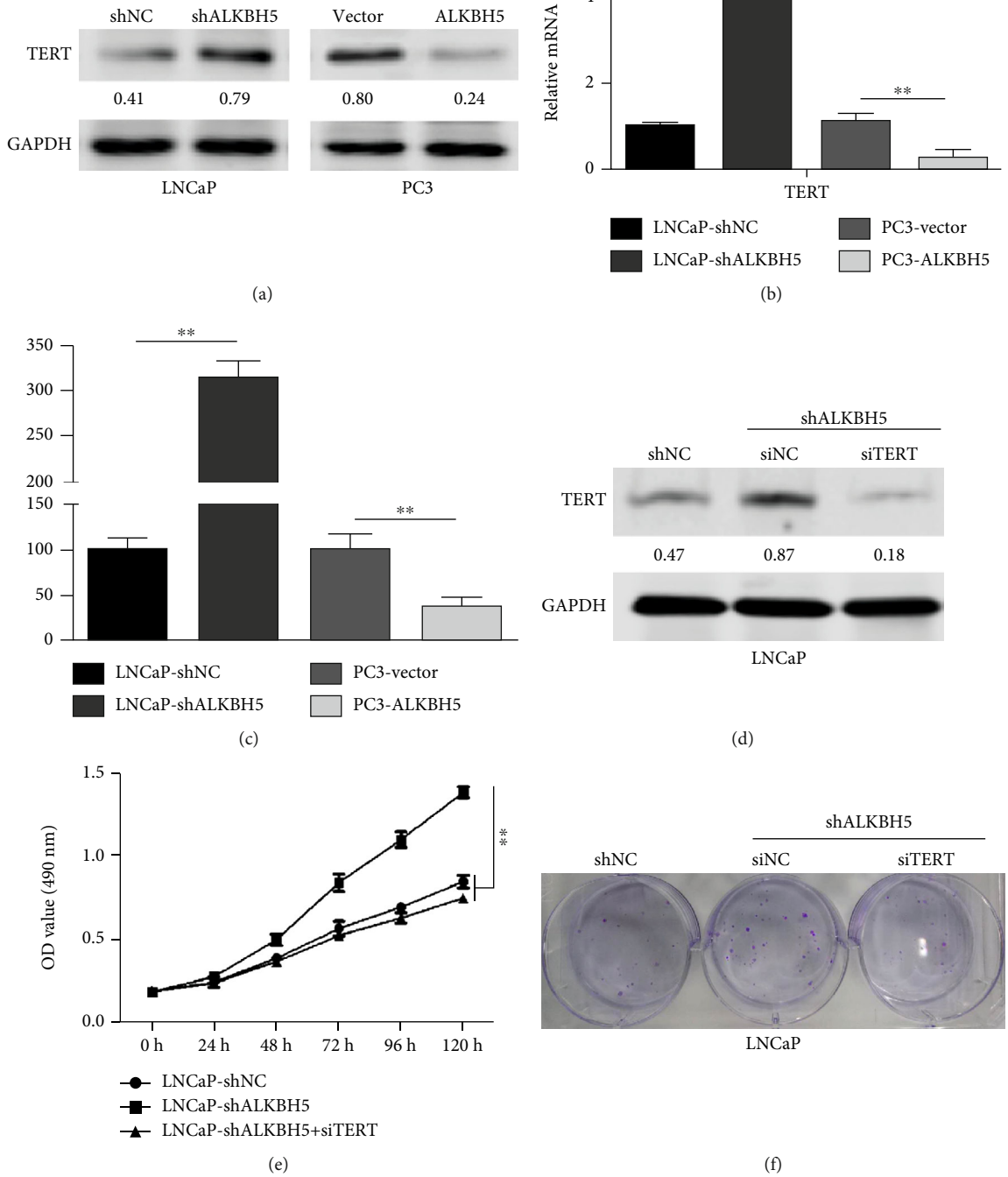
2.11. Statistics. The measurement data were presented as $\bar{X} \pm S$, two independent t-test was used for comparison between two groups, paired t-test was used for paired data, and statistical SPSS18.0 software was used for data analysis $P < 0.05$ indicating statistical significance. The above experiments were repeated three times.

3. Results

3.1. Expression Level and Clinical Significance of ALKBH5 in PCa. First, the expression levels of ALKBH5 in normal prostate tissues and prostate tumor tissues were analyzed based on TCGA database (GSE21032, GSE35988, and GSE3325). The results showed that ALKBH5 mRNA levels were decreased in prostate tumor tissues in comparison with normal prostate tissues (Figures 1(a)–1(c)). Moreover, the expression level of ALKBH5 in clinical PCa tissues was measured by IHC (Figure 1(d)). The mean IRS of ALKBH5 in PCa tissues was significantly lower than normal adjacent tissue (4.82 ± 2.44 vs. 6.83 ± 2.76, $P = 0.001$, Figure 1(e)). Lower ALKBH5 expression was significantly associated with poor prognosis and lower survival rate in PCa (Figure 1(f), $P = 0.006$). Consistent with the IHC results, the expression level of ALKBH5 in PCa cell lines was lower than that in RWPE-1 (Figures 1(g) and 1(h), $P < 0.01$). These results suggest that the occurrence of PCa may be related to the abnormal expression of ALKBH5.

3.2. ALKBH5 Overexpression Inhibits PCa Cell Proliferation. We first established stable cell lines PC3-ALKBH5 and LNCaP-shALKBH5 after lentivector transduction. Western blot and qRT-PCR analyses confirmed that the stable cell lines were successfully established (Figures 2(a) and 2(b)). ALKBH5 significantly inhibited CD44/CD133 expression in PC3 cells; conversely, ALKBH5 knockdown increased CD44/CD133 expression in LNCaP cells (Figures 2(a) and 2(b)). Flow analysis also showed that the level of CD44/CD133 in LNCaP-shALKBH5 was lower than that in LNCaP-shNC (Supplementary Figure S1A-1B). We also investigated the effect of ALKBH5 on the expression of some proliferation-related proteins. The results showed that ALKBH5 overexpression in PC3 cells inhibited the expression levels of c-Myc and Bcl-2 and upregulated apoptosis-related protein Bax expression (Supplementary Figure S1C-1E). The effect of ALKBH5 interference was opposite to that of ALKBH5 overexpression. Furthermore, ALKBH5 silencing significantly accelerated, while ALKBH5 overexpression significantly inhibited PCa cell proliferation and colony-forming ability (Figures 2(c) and 2(d), $P < 0.01$).

To further corroborate the observation that ALKBH5 inhibited tumor growth in vivo, nude mouse xenograft tumor model assays were conducted. As shown in Figure 2(e), the volumes of the transplanted tumor in LNCaP-shALKBH5 group were significantly enlarged



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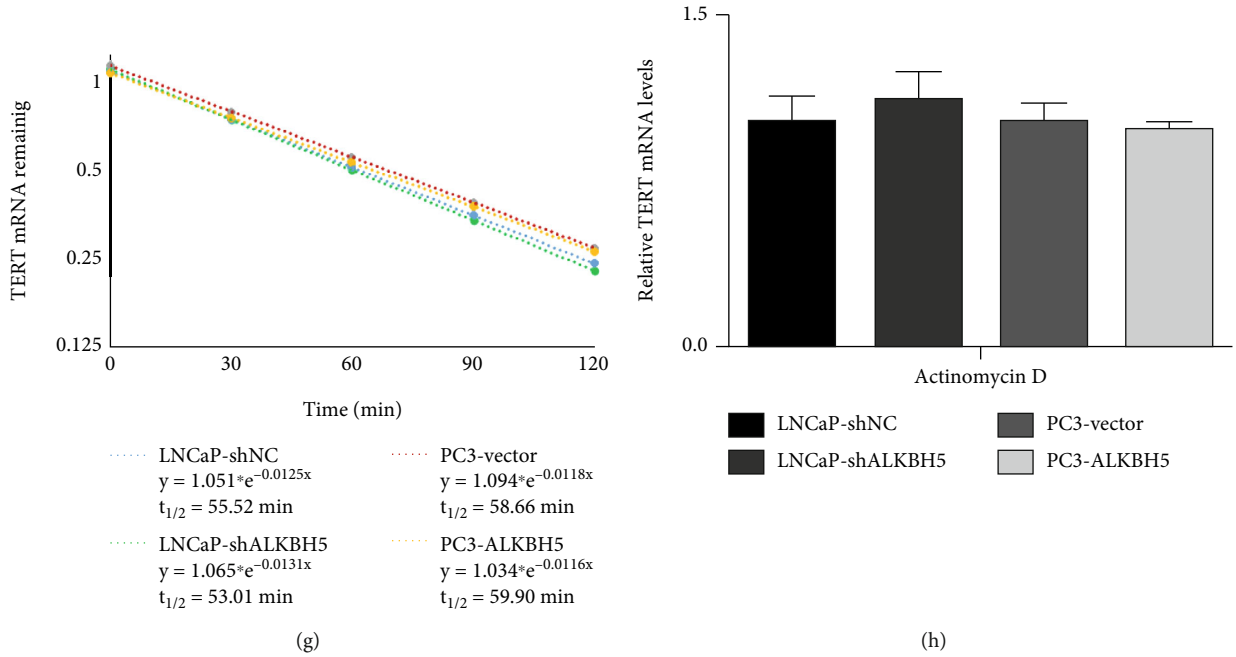
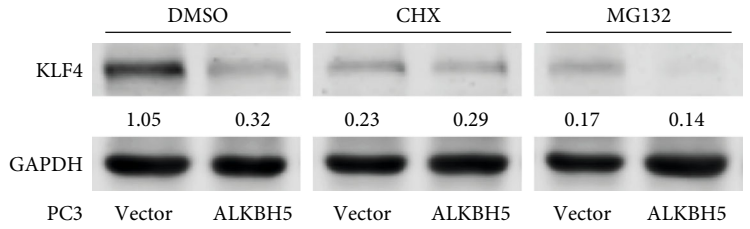


Figure 3: ALKBH5 inhibited carcinoma growth by downregulating TERT expression and telomerase activity. (a, b) The effect of ALKBH5 on TERT expression. (c) Q-TRAP assay was used to evaluate the effect of ALKBH5 on telomerase activity. (d–f) Following the transfection of LNCaP-shALKBH5 cells with siRNA targeting TERT, the expression of TERT was detected by western blotting (d). MTS assay was conducted to evaluate cell proliferation ability (e). Plate clonality assays were used to detect the colony-forming ability (f). (g) Effects of ALKBH5 expression on TERT mRNA stability. (h) Effects of ALKBH5 expression on TERT mRNA half-life. $^{**}P < 0.01$.

compared with control ($P < 0.01$), while ALKBH5 overexpression in PC3 cells could deflate xenograft tumor volume clearly ($P < 0.01$). Similarly, compared with control, the mean weight of the transplanted tumor in LNCaP-shALKBH5 was significantly increased (0.168 ± 0.157 g vs. 0.974 ± 0.238 g, $P < 0.01$, Figure 2(f)). The mean weight of PC3-ALKBH5 was reduced compared with PC3 vector (0.022 ± 0.014 g vs. 0.594 ± 0.133 g, $P < 0.01$, Figure 2(f)). Furthermore, we used IHC to detect the expression levels of ALKBH5, YTHDF1, KLF4, TERT, CD44, and CD133 in xenograft tumors. The expression of KLF4, TERT, CD44, and CD133 in the LNCaP-shALKBH5 group was remarkably higher than in the LNCaP-shNC group, and in the PC3-ALKBH5 group, the expression was significantly lower than in the PC3-vector group (Supplementary Figure S2, $P < 0.01$).

3.3. ALKBH5 Downregulates TERT Expression and Telomerase Activity to Inhibit Carcinoma Growth. TERT-mediated telomerase activity plays an important role in the self-renewal of cancer stem cells and is an important target for tumor-targeted therapy. In this study, we found that ALKBH5 overexpression could significantly downregulate TERT expression and telomerase activity in PC3 cells, but there are opposite results when ALKBH5 knockdown in LNCaP cells (Figures 3(a)–3(c)). We then transfected LNCaP-shALKBH5 cells with siRNA targeting TERT (Figure 3(d)), which suppressed the proliferation and colony-forming capacity of LNCaP-shALKBH5 cells (Figures 3(e)–3(f)). These results showed that ALKBH5 might inhibit PCa cell proliferation by downregulating TERT expression. However, we also found that ALKBH5 had no effect on the mRNA stability of TERT (Figure 3(g), $P > 0.05$), and actinomycin D could offset the regulatory effects of ALKBH5 on TERT mRNA levels (Figure 3(h)). Therefore, we speculated that ALKBH5 might regulate TERT at the level of transcription by affecting the expression of its transcription factors.

3.4. ALKBH5 Downregulates the Expression Levels of KLF4 mRNA by Reducing Its Stability. KLF4 has been reported to bind sites in the TERT promoter, inducing TERT transcription [18, 19]. Therefore, we speculated that ALKBH5 downregulated the expression of TERT via suppressing KLF4. KLF4 expression in PC3 cells was dramatically downregulated after ALKBH5 overexpression (Figure 4(a), $P < 0.01$). Furthermore, we used CHX and the proteasome inhibitor MG132 to further study the regulatory mechanism underlying ALKBH5 on KLF4 expression. CHX, but not MG132, significantly abolished ALKBH5-induced downregulation of KLF4 protein expression in PC3 cells (Figure 4(a)). To figure out how ALKBH5 regulated the expression of KLF4, a luciferase reporter assay was conducted. ALKBH5 reduced, while ALKBH5 silencing increased luciferase activity in PC3 cells transfected with pGL3-KLF4-CDS wild type (WT) rather than pGL3-KLF4-CDS mutant type (MUT) (Figure 4(b)). Furthermore, both KLF4 mRNA stability and half-life in LNCaP-shALKBH5 cells were increased compared with LNCaP-shNC cells, while ALKBH5 overexpression in PC3 cells could reduce the stability of KLF4 mRNA and shorten its half-life compared with PC3 vector



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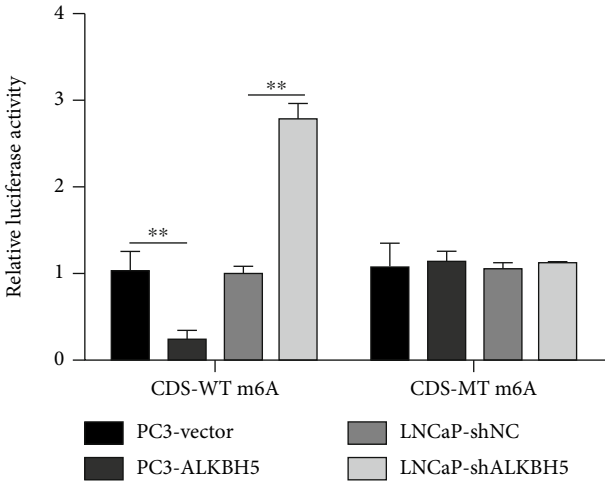

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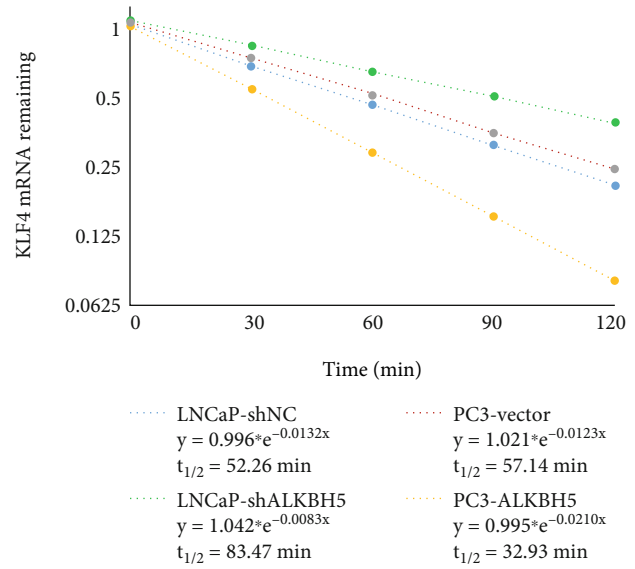

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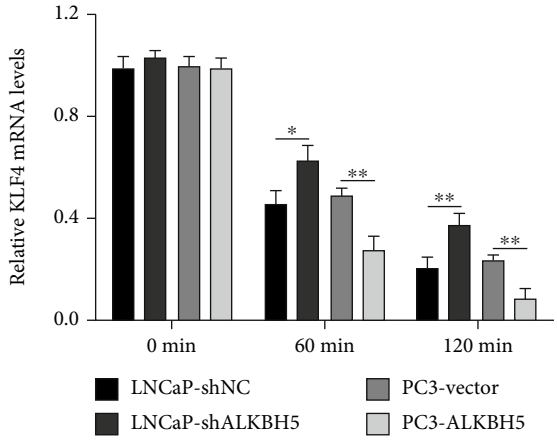

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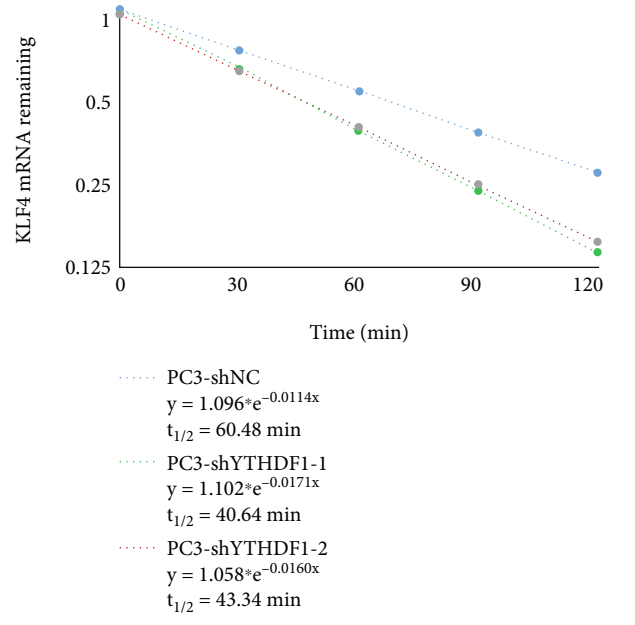

(e)

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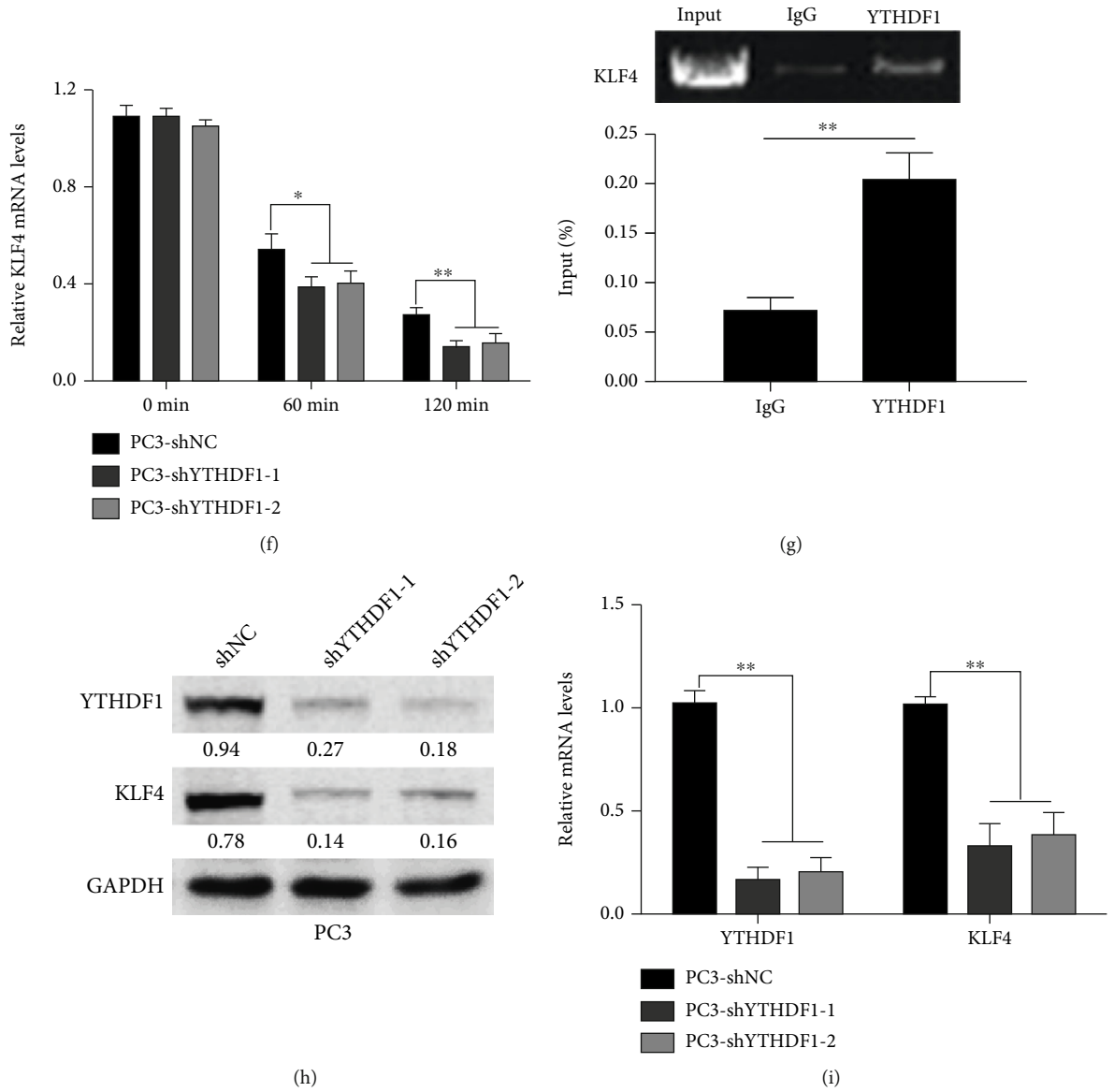
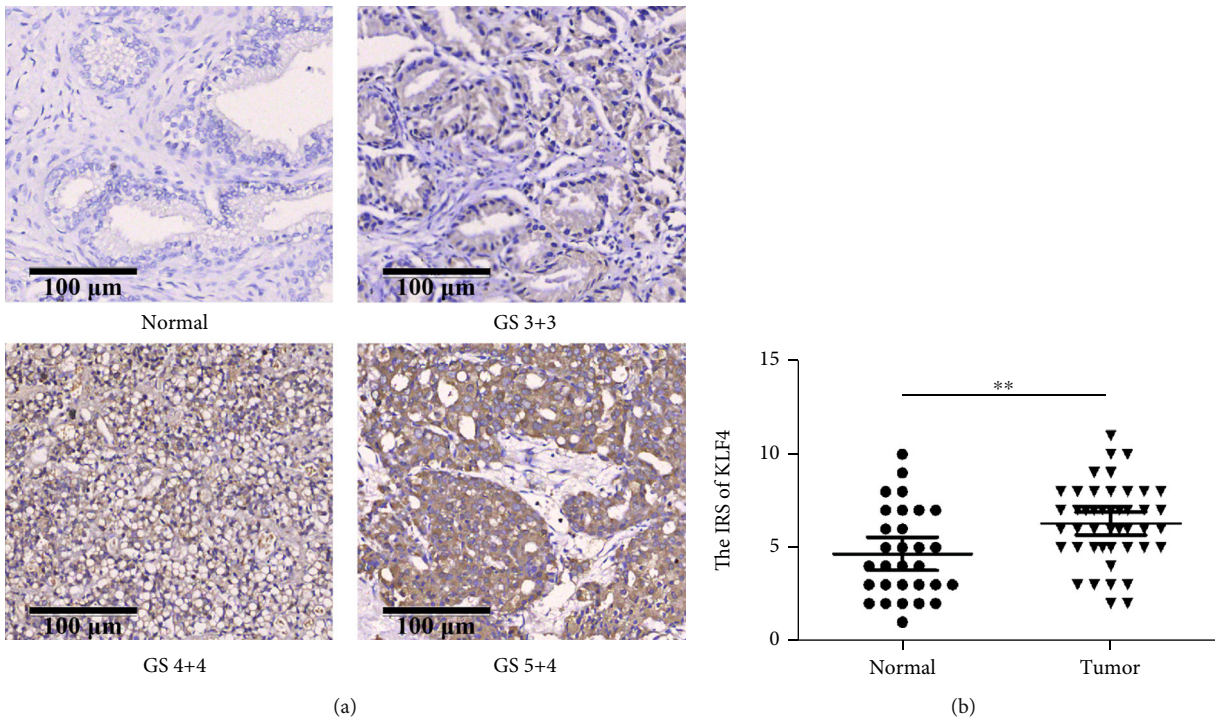


Figure 4: ALKBH5 downregulates the expression levels of KLF4 mRNA by reducing its stability. (a) After addition to 50 μg/mL CHX or 10 μM MG132 or DMSO, the impact of ALKBH5 on KLF4 protein expression was detected by western blotting. (b) Luciferase reporter was performed to verify whether ALKBH5 binds to KLF4 gene. (c, d) ALKBH5 silencing increased the stability and half-life of KLF4 mRNA. (e, f) YTHDF1 silencing decreased the stability and half-life of KLF4 mRNA. (g) RIP-qPCR was performed to verify interaction between YTHDF1 protein and KLF4 transcripts. (h, i) The effect of YTHDF1 on KLF4 expression was analyzed by western blotting and qRT-PCR. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(Figures 4(c) and 4(d)). These results suggest that ALKBH5 may regulate KLF4 expression by affecting its mRNA stability or translation.

To test this idea, we downregulated the expression of YTHDF1/2/3, some specific m^{6}A-binding proteins, in PC3 cells via transfection with shRNA. Interestingly, knockdown of YTHDF1 (Figures 4(e) and 4(f)), but not YTHDF2/3 (Supplementary Figure S3), decreased the mRNA stability of KLF4 and shortened its half-life. RIP assay also confirmed that YTHDF1 was the binding protein of KLF4 mRNA (Figure 4(g)). Consistently, knockdown of YTHDF1 decreased KLF4 protein and mRNA expressions (Figures 4(h) and 4(i)).

3.5. KLF4 Is a Critical Downstream Target of ALKBH5 to Suppress PCa Growth. We firstly examined KLF4 expression in clinical PCa tissues by IHC (Figure 5(a)). The result was opposed to ALKBH5, and the mean IRS of KLF4 in PCa tissues was significantly higher than that in normal epithelial tissue (6.29 ± 2.07 vs. 4.67 ± 2.37, $P = 0.002$, Figure 5(b)). Importantly, there was a significant negative correlation between KLF4 and ALKBH5 expressions (Figure 5(c)). Furthermore, we silenced KLF4 expression in LNCaP-shALKBH5 cells by transfection with siRNA targeting KLF4. Knockdown of KLF4 reduced the expression of TERT and CD44 and largely abolished ALKBH5 silencing-induced growth promoting action (Figures 5(d)–5(g), Supplementary



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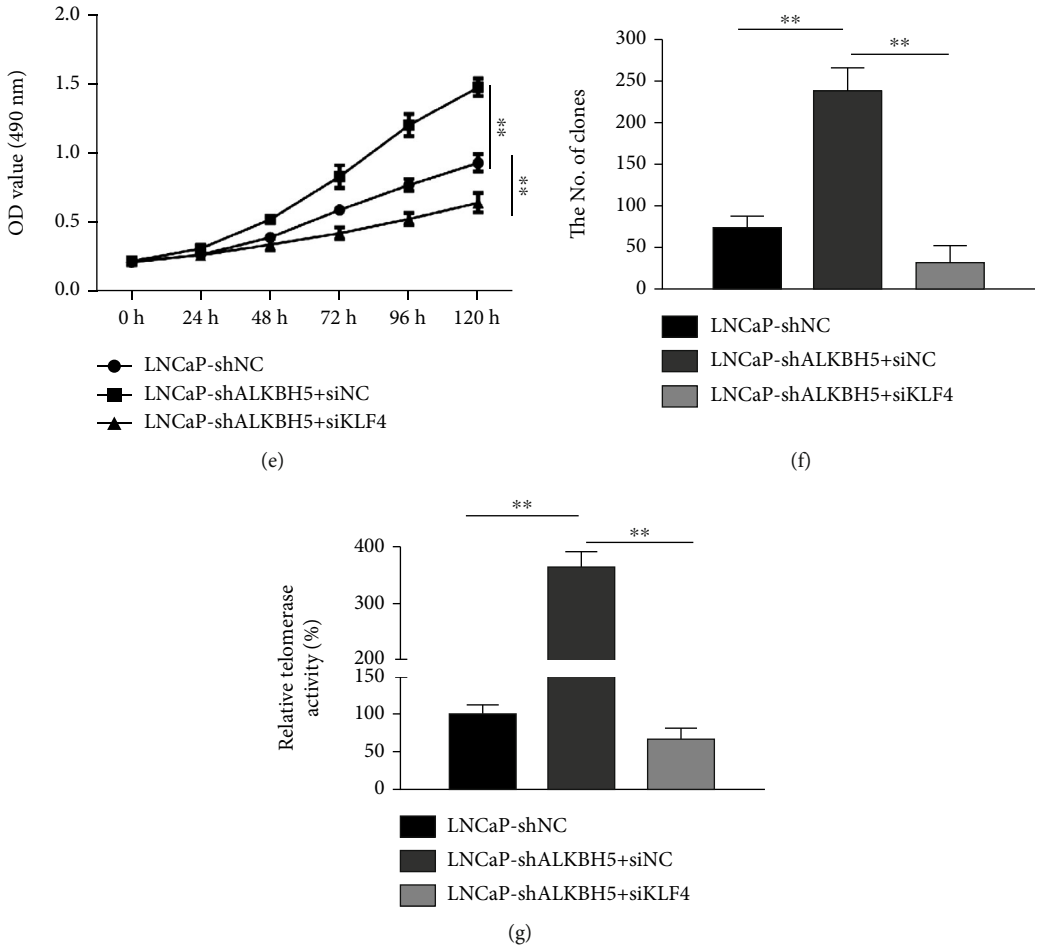

Figure 5: KLF4 is a critical downstream target of ALKBH5 to suppress PCa growth. (a) The expression of KLF4 in clinical PCa tissues was analyzed by IHC. (b) IRS of KLF4 in PCa tissues. (c) Correlations between KLF4 and ALKBH5 expression ($P = 0.006$). (d–g) Following the transfection of LNCaP-shALKBH5 cells with siRNA targeting KLF4, the expression of KLF4, TERT, and CD44 was detected by western blotting (d). The effect of KLF4 on PCa cell proliferation ability was measured by MTS assay (e). Plate clonality assays were used to measure cell clonality (f). Q-TRAP assay was used to evaluate telomerase activity (g). $^{**}P < 0.01$.

Figure S1F), indicating KLF4 is a critical downstream target of ALKBH5.

4. Discussion

Recently, with continued investigation of m^{6}A RNA methylation, we have acquired a comprehensive understanding of its functions and regulatory mechanisms [20]. Many studies directly focused on the role of m^{6}A modification in human diseases, especially in cancer [21–23]. Here, we found that ALKBH5 was at a low level in prostate carcinoma; lower ALKBH5 expression was significantly correlated with shorter OS in patients with PCa. ALKBH5 had a significant suppressive effect in tumor progression in vivo and in vitro, although the role of m^{6}A modification in cancer is still under debate. Yu et al. stated that ALKBH5 dramatically inhibited bladder cancer growth by downregulating CK2α in an m^{6}A-dependent manner [24], while the studies of Zhang et al. suggest some controversy. ALKBH5 is critical for the development and maintenance of renal cell carcinoma by stabilizing the mRNA targeting AURKB [25]. The dual role of m^{6}A modification in cancer may rely on the specific recognition of m^{6}A "readers." YTHDF2 localizes the target mRNA to processing bodies for accelerated degradation [26, 27], while YTHDF1/3 enhances mRNA stability and facilitates translation [28, 29]. In the present study, we found that ALKBH5 downregulated KLF4 expression and inhibited PCa proliferation via an m^{6}A-YTHDF1 mechanism, and thus, ALKBH5 is a promising biomarker for prognostic prediction and a potential therapeutic target in PCa.

Prostate cancer stem cells (PCSCs) are defined as a unique subpopulation expressing normal prostate stem cell markers ($\alpha 2\beta 1$/CD133/CD44) in PCa, which not only differentiate into ordinary tumor cells but also exhibit self-renewal ability [30]. ADT can destroy most tumor cells, but PCSCs do not express AR and its growth is independent of androgen. Thus, ADT is ineffective against PCSCs. Instead, ADT with enzalutamide could increase the PCa CD133+ stem/progenitor cell population, inducing the production of more malignant phenotypes with increased ability to resist ADT [31].

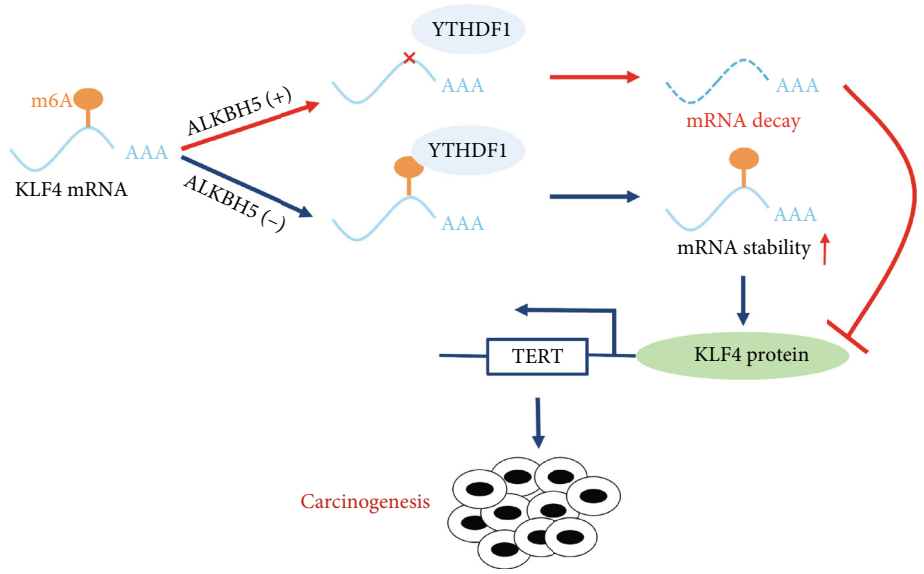

Figure 6: Schematic illustration of the ALKBH5 inhibited the pathogenesis of PCa through suppressing m^{6}A/KLF4/TERT signaling axis.

Luminal progenitor cells, characterized by long telomeres and telomerase activity, have been identified in PCa and implicated as a resistant cell population that survives ADT. Therefore, PCSCs are considered the main cause of ADT tolerance and recurrence of PCa.

Telomerase is a reverse transcriptase, composed of telomerase RNA (TR), TERT, and telomerase-associated protein (TEP), and can use an integrated RNA component as a template to synthesize DNA repeat fragments of telomeres (TTAGGG), which is associated with cell immortalization. Targeting TERT may help eliminate CSCs, thereby preventing cancer progression [32]. In this study, we demonstrated that ALKBH5 overexpression decreased the levels of CSC markers CD44/CD133 and inhibited PCa cell proliferation by downregulating TERT expression and telomerase activity. However, further studies are needed to determine whether m^{6}A modification is also involved in the regulation of PCSC self-renewal.

KLF4 has been reported to be able to maintain self-renewal of CSCs by activating expression of the human telomerase catalytic subunit TERT [33]. Huang et al. found that the downregulation of KLF4 gene m6A level is associated with differentiation of mouse embryonic stem cells in vitro [34]. In the present study, ALKBH5 silencing increased the expression and stability of KLF4 mRNA in an m^{6}A-dependent manner; KLF4 directly binds the TERT promoter, upregulating TERT expression and maintaining the PCa stemness phenotype. KLF4 knockdown by siRNA greatly reduced the expression of TERT and CD44 and largely abolished ALKBH5 silencing-induced growth promotion. In a word, our results demonstrated that KLF4 is an important downstream target of ALKBH5 and is essential for the promotion of PCa cell proliferation.

In conclusion, our study has revealed for the first time that m6A demethylase ALKBH5 has a suppressive effect on PCa growth, and ALKBH5 is expected to become a hopeful target in PCa therapy and prognosis. The ALKBH5-m6A-YTHDF1-KLF4 axis highlights novel regulatory mechanisms from an epigenetic perspective (Figure 6).

Data Availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table S1: description of the shRNA/siRNA sequences. Supplementary Table S2: description of the primer sequences for PCR or qRT-PCR. Supplementary Figure S1: the effect of ALKBH5 on the expression of CD44/CD133 and some proliferation-related proteins. (A, B) The expressions of CD44/CD133 in cell membrane were determined by flow cytometry. (C–E) Effects of ALKBH5 expression on the expression of some proliferation-related proteins (c-Myc, Bcl-2, and Bax). (F) KLF4 overexpression offsetted the inhibitory effect of ALKBH5 on c-Myc, Bcl-2, and Bax. $^*P < 0.05$ and $^{**}P < 0.01$. Supplementary Figure S2: the

expression levels of ALKBH5, YTHDF1, KLF4, TERT, CD44, and CD133 in the xenograft tumor were detected by IHC analysis. Supplementary Figure S3: (A, B) the effects of YTHDF2 on KLF4 mRNA stability and half-life. (C, D) The effects of YTHDF3 on KLF4 mRNA stability and half-life. *(Supplementary Materials)*

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