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ORIGINAL RESEARCH

RETRACTED ARTICLE: Effect of *SMYD3* on biological behavior and H3K4 methylation in bladder cancer

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Xiang Wu[1,2]
Qingjiang Xu[1,2]
Pingzhou Chen[1,2]
Chenbo Yu[1,2]
Liefu Ye[1,2]
Chen Huang[1,3]
Tao Li[1,2]

[1]Shengli Clinical Medical College of Fujian Medical University, Fuzhou 350001, People's Republic of China; [2]Department of Urology, Fujian Provincial Hospital, Fuzhou 350001, People's Republic of China; [3]Department of Thoracic Surgery, Fujian Provincial Hospital, Fuzhou 350001, People's Republic of China

Purpose: Our goal was to investigate the effect of *SMYD3* on the biological behavior and histone 3 lysine-4 (H3K4) methylation of bladder cancer (BLAC).

Patients and methods: qRT-PCR identified the *SMYD3* expression level in BLAC cell lines (T24, 5637, BUI-87 and J-82) and human normal uroepithelial cell line SV-HUC1. We also constructed green fluorescence protein lentiviral vector using the gene short hairpin RNA (shRNA) system. We used Western blot to analyze the *SMYD3,* H3K4me1, H3K4me2 and H3K4me3 expression levels in shRNA transfection lines. We also performed a colony-forming assay to determine colony-forming activity, cell counting kit-8 for cell proliferation detection, Transwell assay to determine cell migration and invasion and Annexin V-FITC/PI double staining to analyze cell apoptosis.

Results: The *SMYD3* expression level was significantly higher in BLAC cell lines (T24, 5637, BUI-87 and J-82) than in human normal uroepithelial cell line SV-HUC1, and exhibited the highest expression level in T24 cells, among the cell lines tested. qRT-PCR and Western blot analysis data showed that *SMYD3* was successfully suppressed in shRNA transfection lines, and identified that *SMYD3* suppression resulted inhibited H3K4me2 and H3K4me3 but not H3K4me1. *SMYD3* knockdown cells accelerated cell apoptosis and exhibited low cell colony-forming ability, proliferation ability, inhibition of cell migration and invasion compared with normal cells.

Conclusion: *SMYD3* may be activated in BLAC cells to increase H3K4 activity to modulate cell proliferation, migration and invasion ability. The data will be a useful source for future therapy.

Keywords: SMYD3, H3K4 methylation, bladder cancer, cell proliferation

Introduction

Bladder cancer has been listed as common cancer, and nearly 60% of the incident cases are reported from the developed western countries.[1] The etiology of BLAC is unclear, but is a good etiological model of genetic susceptibility and interactions between genetic and epigenetic factors in cancer.[2] Smoking is the most relative risk factor of bladder cancer, and the duration and intensity of smoking are correlated with morbidity and mortality, respectively.[3] Approximately, 20% of BLAC incidence is caused by occupational carcinogenic substances such as paints and hair dyes.[4] Few oncogenes, tumor, tumor suppressor genes and cell cycle regulators, including epidermal growth factor receptors and erb-b2 receptor tyrosine kinase 2 were identified in BLAC cells, thereby facilitating the development of targeted therapy in therapeutics.[5–7]

Almost 90% of the BLAC began from urothelial cells which prevent water and toxin exchanges between blood and urine.[8] Nonmuscle-invasive bladder cancer

Correspondence: Tao Li
Department of Urology, Fujian Provincial Hospital, Fuzhou 350001, People's Republic of China
Tel +86 1 370 507 8075
Email 80205105@qq.com

(NMIBC) accounts for 80% of newly diagnosed BLAC, thereby showing favorable prognosis, while the remaining 10–20% of patients with MIBC shows poor outcome of approximately 50% of 5-year disease-free survival, because of the invaded muscle layer and/or lymphatic spread or distant organ metastasis.[9,10] However, patients with NMIBC need needed lifelong follow-up and evaluation despite aggressive interventions because they are of high risk of recurrence and progression to MIBC.[11] Thus, increasing numbers of investigations have focused on carcinogenesis by genetic and epigenetic modifications, including chromatin structure change and histone modifications.[12,13]

Histone modification is crucial in chromatin structure change which regulates the DNA replication and following gene expression.[14,15] Histone modification is post-translational modification on the terminal tails of histones through methylation, acetylation, and phosphorylation, in which the various subtypes of modification perform its distinct functions.[16,17] Among the many types of histone modifications, lysine methylation deregulation is the most important in carcinogenesis.[18,19] Therefore, the identification of histone methyltransferase inhibitors allowed the development of new epigenetic drug development.[20,21]

For the catalytic mechanism of histone lysine methylatio a group of proteins which contains the evolutionarily conserved Suppressor of variegation, Enhancer o and Trithorax (SET) domain are involved.[22] Rea al[23] ide ified SET domains as methyltransferases for the firs ime of the mammalian proteins contain a ET doma SET and (Myeloid-Nervy-DEAF1) MYND (SM D) domain- ntaining proteins are defined as a SET domain t is divided into two segments by an MYN domain, and a T domain is followed by cysteine-ri post-SE domain.[24] SMYD family has five members (SMYD SMYD5 and SMYD1-3 has been characteri d we whe SMYD4-5 is unclear compared with MYD1-3 For exa ple, *SMYD3* is often upregulated in can s and plays an oncogenic role in different types of cancer ce including colorectal carcinoma, hepatocellular carcinoma and prostate and breast cancers.[26–30] *SMYD3* upregulation accelerates cancer progression by promoting cancer cell biological function.[13,31,32] *SMYD3* is a pivotal player of human carcinogenesis through methylation of histone 3 lysine-4 (H3K4) and histone 4 lysine-5 (H4K5) methyltransferase.[33,34] Generally, H3K4, H3K36, and H3K79 methylation promote gene activation, whereas that of H3K9, H3K27 and H4K20 represses gene modifications.[35] Previous studies revealed that *SMYD3* induced di- and trimethylation of H3K4 in cancer.[33] However, the mechanism of *SMYD3* in BLAC cell progression is still elusive.

SMYD3 oncogenic function has been described well in the previous studies. However, the molecular mechanism of *SMYD3* regulating BLAC cell apoptosis, migration and invasion has not been described. Thus, in this study, we aimed to elucidate the epigenetic regulations of *SMYD3* particularly on H3K4 methylation regulating biological behavior of BLAC cells.

Materials and methods

Cell culture

The human urothelial carcinoma ells lines T 4, 5637, BUI-87, and J8 were pu hased f m BeN , Beijing, China Culture Collecti (BNC 10217 NCC100680, BNCC100982, BNCC 656 respectively), cultured in DMEM with 10 inactiv d FBS and antibiotics. All cells were c ltu d in a hum d ed atmosphere of 5% CO_2 at 37°C. Am g the cell lines, T24 cells were selecte as the main ce lines because of the maximally exp ssed *SMYD3* detected by RT-PCR.

Len ivirus v ctor construction and cell transfe n

Th t hairpin RNA (shRNA) sequences of SMYD3 gene shRNA-SMYD3-1,-2,-3/NC) were designed and synthesized by Sangon (Shanghai, China). Then, these sequences were all loned into the pLVX-shRNA-Puro reporter vector. The Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA) was used to shRNA-1,-2,-3 reporter plasmid (50 nmol/L) when 293T cells reached 80–90% confluence. At 24 hrs after transfection, transfection efficiency was measured by qRT-PCR. The primer sequences are as follows: shRNA-SMYD3-1 forward: 5′-GATCCGTGATGAAAGTTGGCAAACTTCAAGAGAGTTTGCCAACTTTCATCACTTTTTG-3′ and reverse: 5′-AATTCAAAAAGTGATGAAAGTTGGCAAACTCTCTTGAAGTTTGCCAACTTTCATCACG-3′; shRNA-SMYD3-2 forward: 5′-GATCCGCCTTGTTCTATGGTACTCTTCAAGAGAGAGTACCATAGAACAAGGCTTTTTG-3′ and reverse: 5′-AATTCAAAAAGCCTTGTTCTATGGTACTCTCTCTTGAAGAGTACCATAGAACAAGGCG-3′; shRNA-SMYD3-3 forward: 5′-GATCCGTATGGAAGGAAGTTCAAGTTCAAGAGACTTGAACTTCCTTCCATACTTTTTG-3′ and reverse: 5′-AATTCAAAAAGTATGGAAGGAAGTTCAAGTCTCTTGAACTTGAACTTCCTTCCATACG-3′; shRNA-NC forward: 5′-GATCCCAGAACTCGTAATGACATTTGCCAATTCAAGAGATTGGCAAATGTCATTACGAGTTCTGTTTTTG-3′ and

reverse: 5′-AATTCAAAAACAGAACTCGTAATGACATT TGCCAATCTCTTGAATTGGCAAATGTCATTACGAGTT CTGG-3′. We collected the supernatant of cells rich in lentivirus particles and then obtained the high titer-concentrated lentivirus solution. Virus titers were measured and calibrated in 293T cells.

For transfection, three different shRNA-SMYD3 virus transfections were performed when T24 cells reached 80–90% confluence. We selected the multiplicity of infection (MOI) value of 5 for lentivirus infection. At 72 hrs after transfection, we added the puromycin (5 μg/mL) after 1 week and collected the cells and detected via qPCR and Western blot. The bright field and fluorescence images were captured by a fluorescence microscope at ×100 magnification.

RNA extraction and quantitative RT-PCR

The collected cells were lysed in 0.5 mL of NucleoZol reagent (Gene Company Ltd., HongKong, China) and vortexed for 1 min. We added 200 μL of RNase-free water to each sample, vortexed for 15 s, stood for 15 mins at room temperature and centrifuged at 12,000 rpm for 15 mins. We obtained the supernatant, added 500 μL of lysopropylation and 500 μL of isopropylene glycol mixed well and [illegible] fuged at 12,000 rpm for 10 mins. After centrifugation we displaced the alcohol cleaning steps by using 75% ethanol. Total RNA was measured using NanoDrop Thermo Shanghai, China) and preserved at −80°C until [illegible] cDNA synthesis and qRT-PCR were performed using qPCR mix reverse transcription kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The primer sequences are as follows: SMYD3 forward: 5′-GAAAAGTTCGCAGCCGCCAA-3′ and reverse: 5′-TGAGAGCATCGGATCAGCTT-3′ and GAPDH forward: 5′-GTCAAGGCTGAGAACGGGAA-3′ and reverse: 5′-AAATGAGCCCCAGCCTTCTC-3′. The expression levels of the target genes were calculated by comparative delta-delta CT method ($2^{-\Delta\Delta CT}$).

Cell migration and invasion assay

Transwell assay was used to detect cell migration and invasion ability. All cells were seeded at a density of 1×10^5/mL at the transwell chamber (Corning Incorporated, Corning, NY, USA). The chamber was filled with serum-free cell culture medium containing 10% FBS and cultured at 37°C for 24 h. After culture, the cells were dyed with a crystal violet solution and observed. Matrigel was melted and uniformly placed in a chamber for invasion assay. The cells were inoculated at 1×10^5/mL at the transwell chamber. The procedures were the same as described above.

Cell proliferation assay

Cell proliferation was measured using cell counting kit-8 (Dojindo, Jiuzhou, Japan) assay. The cells were inoculated at 1×10^5/mL in 96-well plates and each group was detected at 0, 24, 48, 72, and 96 hrs. The OD of each plate and their cell proliferation ability were measured. For colony formation assay, the cells were inoculated at 200 per well into 6-well plates and cultured at 37°C for 2 weeks. The cell plates were stained with crystal violet solution and observed.

Flow cytometry

To detect cell apoptosis we performed flow cytometry. The cells were transfected with shRNA-NC, shRNA-SMYD3-3, and control. Each group was stained using Annexin V Alexa Fluor 488/PI apoptotic test kit (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) according to the manufacturer's protocol and analyzed by fluorescence activated cell sorting (FACS; BD, New York, USA).

Western blot analysis

Cells were harvested in an ice-cold lysis buffer (7 M urea, 2 M thiourea, 2% 3-[(3-Chloamidopropyl)dimethylammonio] propanesulfonate (CHAP)S, 40 mM Tris base, 40 mM dithiothreitol, and 1% protease inhibitor) to obtain whole-cell extracts. The membranes were incubated with the following primary antibodies (all antibodies from Abcam, Cambridge, MA, USA): anti-SMYD3, anti-H3K4me1, anti-H3K4me2, anti-H3K4me3, and anti-β-actin. The membranes were washed two times with PBS and incubated with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000; Cell Signalling Technology, Danvers, MA, USA) for 1 hr.

Statistical analysis

The data of each group were presented as the mean ± SD. All data were analyzed using SPSS 22.0 software (SPSS, Chicago, IL, USA). Statistical analysis was conducted using one-way ANOVA. Quantitative data were compared by the one-sample *t*-test. $P<0.05$ was considered statistically significant.

Results

SMYD3 is upregulated in BLAC cell lines

To investigate *SMYD3* function in BLAC, we examined the endogenous expression level of *SMYD3* in BLAC cells. qRT-PCR results showed that *SMYD3* level was higher in the BLAC cell lines T24, 5637, BUI-87, and J-82 than in human normal uroepithelial cell line SV-HUC1. The expression levels of T24, 5637, BUI-87 and J-82 were 3.66±0.451, 2.09±0.337, 1.18±0.132 and 2.50±0.214, respectively. T24 cell line showed the highest *SMYD3* expression level compare to the other cell lines (Figure 1). Thus, T24 cell line was selected for further experiments.

SMYD3 was efficiently knocked down in bladder cancer cells by shRNA transfection

To analyze *SMYD3* function in BLAC, we generated the cells knockdown of *SMYD3* by using shRNA to demonstrate the role of SMYD3 in BLAC cells. The results showed that shRNA-NC, hRNA-*SMYD3-1*, shRNA-*SMYD3-2*, and shRNA-*SMYD3-3* were successfully generated which was verified by green fluorescence protein (GFP) signal detection (Figure 2A). Consequently, the *SMYD3* expression level was examined by qRT-PCR. The result showed that *SMYD3* expression was effectively suppressed by shRNA-*SMYD3-1*, −3, and the inhibition rates of the normal control expression levels were 0.62% and 37%, respectively (Figure 2A). Western blot analysis was performed to verify the qRT-PCR results. The data indicate that Western blot results were similar with qRT-PCR data with best suppression in shRNA-*SMYD3-3* cells (Figure 2B).

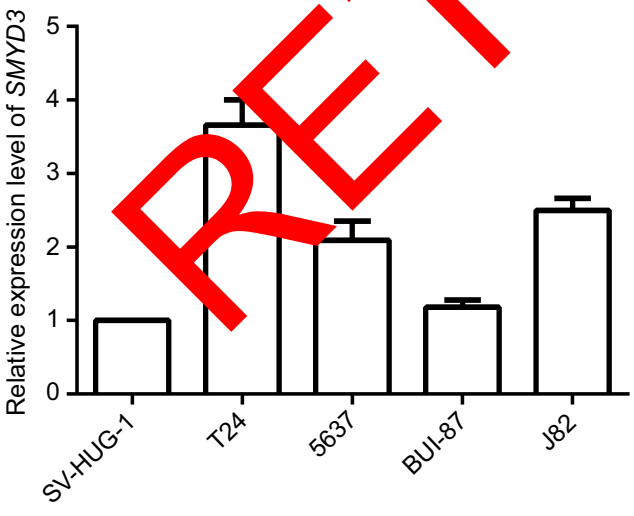

Figure 1 Expression level of endogenous SMYD3 in bladder cancer cell lines analyzed by qRT-PCR. SV-HUC1, 1.00±0.084; T24, 3.66±0.451; 5637, 2.09±0.337; BUI-87, 1.18±0.132; J82, 2.50±0.214. U6 was used as the control. Statistical analysis was calculated with unpaired Student's *t*-test, n≥3.

SMYD3-mediated H3K4 di- or trimethylation in BLAC

To analyze *SMYD3* function in H3K4 methylation status, we performed Western blot analysis to analyze H3K4me1, H3K4me2 and H3K4me3 levels when silencing *SMYD3*. The results indicated that *SMYD3* silencing induced significant change in H3K4me2 and H3K4me3 but not H3K4me1, thereby showing H3K4me2 and H3K4me3 expression level downregulation (Figure 3).

SMYD3 inhibited T24 cell proliferation and colony formation. Proliferation and colony formation are important factors for cancer cell development. Therefore, *SMYD3* effects on T24 cell proliferation and colony formation were investigated. The shRNA-*SMYD3-3* group exhibited significantly lower proliferation rate and was negatively correlated with time than the control and shRNA-NC groups. The cell proliferation rates in shRNA-NC were 91.64%±4.76, 79.44%±3.46, 70.45%±3.63, and 67.22%±7.10, respectively, after 24, 48, 72 and 96 hrs, respectively, compared with control or shRNA-NC (Figure 4A). The cell proliferation rate in the control and shRNA-NC groups was also similar (Figure 4A). Next, colony formation was examined in three groups. The colony numbers in the control, shRNA-NC, and shRNA-*SMYD3-3* groups were 280±30, 268±25, and 160±17, respectively, thereby indicating that SMYD3 suppression significantly inhibited T24 cell colony formation (Figure 4B).

SMYD3 inhibited T24 cell migration and invasion. We also examined the *SMYD3* effects on BLAC cell migration and invasion. Transwell assay was used to analyze T24 cell migration and invasion after 48 hrs of virus transfection. The migration assay showed that *SMYD3* suppression inhibited cell migration by 38.6% compared with the control and shRNA-NC groups (Figure 5A). Cell invasion ability was similar to migration, and *SMYD3* suppression significantly inhibited cell invasion. The inhibition rate in the shRNA-*SMYD3-3* group was 48.83% compared with the control and shRNA-NC groups (Figure 5B).

SMYD3-induced T24 cell apoptosis

Given that *SMYD3* suppression inhibited cell proliferation, colony formation, cell migration and invasion, apoptosis rate in control, shRNA-NC, and shRNA-SMYD3-3 groups were

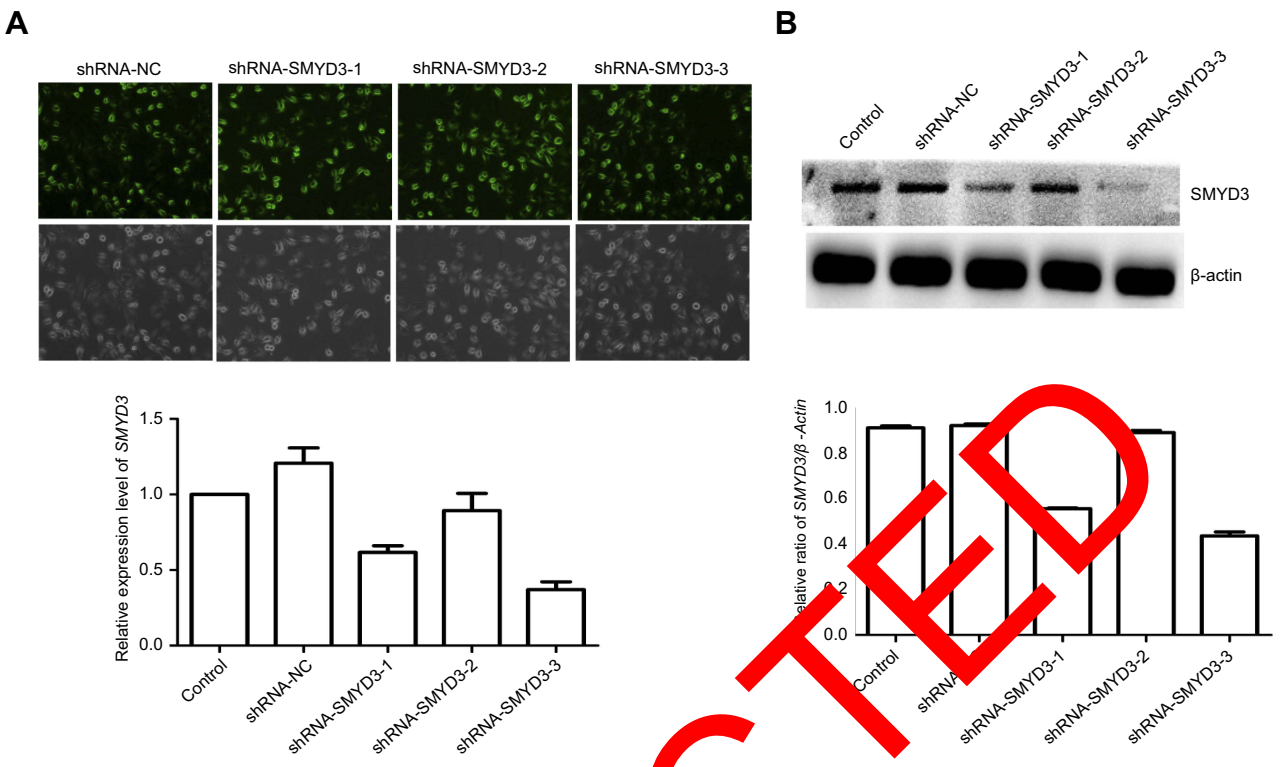

Figure 2 SMYD3 was efficiently suppressed in bladder cancer cells. Bladder cancer cell line T24 was infected with three shRNA-SMYD3 plasmids. **(A)** Bright field and image and fluorescence images of the GFP reporter were captured by a fluorescent microscope (magnification, ×200). mRNA level was measured by qRT-PCR. **(B)** Densitometric analysis and Western blot analysis compared with β-actin. Statistical analysis was calculated with unpaired Student's *t*-test, n≥3.
Abbreviations: shRNA, short hairpin RNA; GFP, green fluorescence protein.

evaluated by FACS. The FACS result showed that cell death rate was significantly higher in shRNA-SMYD3-3 cells than in control and shRNA-NC cells. The cell apoptosis was 4.65%, 4.78%, and 16.84%, in the control, shRNA-NC and shRNA-SMYD3-3 groups, respectively (Figure 6).

Discussion

BLAC is one of the types of cancer that severely affect male patients worldwide. *SMYD3* is a methyltransferase member that induced H3K4 di- and trimethylation in cancer.[33] *SMYD3* upregulation was identified in many types of cancer cells including colorectal carcinoma, hepatocellular carcinoma, prostate cancer and breast cancer and plays an oncogenic role.[26–30] *SMYD3* upregulation attenuates cancer progression by promoting cancer cell biological function.[13,31,32] In this study, qRT-PCR was performed to analyze *SMYD3* expression level in the BLAC cell lines T24, 5637, BUI-87, and J-82 and in human normal uroepithelial cell line SV-HUC1.

qRT-PCR was also used to investigate *SMYD3*role in BLAC cells. Similar to other cancer types, the accumulation of *SMYD3* was high in BLAC cell lines, especially in T24 line compared with that in normal uroepithelial cell line. This result suggested that *SMYD3* may play an important role in cancer cell development. Further experiments by using the tumor and adjacent tissues will be conducted to examine *SMYD3* expression further.

To test *SMYD3* function in BLAC cells, we determined lentivirus-mediated knockdown in bladder cell lines. In T24 cells, *SMYD3* successfully was suppressed by shRNA system, and transfection efficiency was visually analyzed by GFP detection in the construct. GFP images suggested that almost all the cells in each line were transfected. *SMYD3* expression level was definitely suppressed in three independent shRNA-*SMYD3* lines in different grades. *SMYD3* played an important role in histone lysine methylation and subsequently regulates downstream target gene transcription by changing the chromatin status. The data indicated that *SMYD3* suppression inhibited H3K4me2 and H3K4me3 levels but not H3K4me1, thereby suggesting that *SMYD3* may regulate H3K4 di- and trimethylation to control the downstream gene expression levels. The subsequent examination revealed that *SMYD3* suppression inhibited cell proliferation, colony formation, cell migration and invasion. The cell proliferation, colony formation, migration and

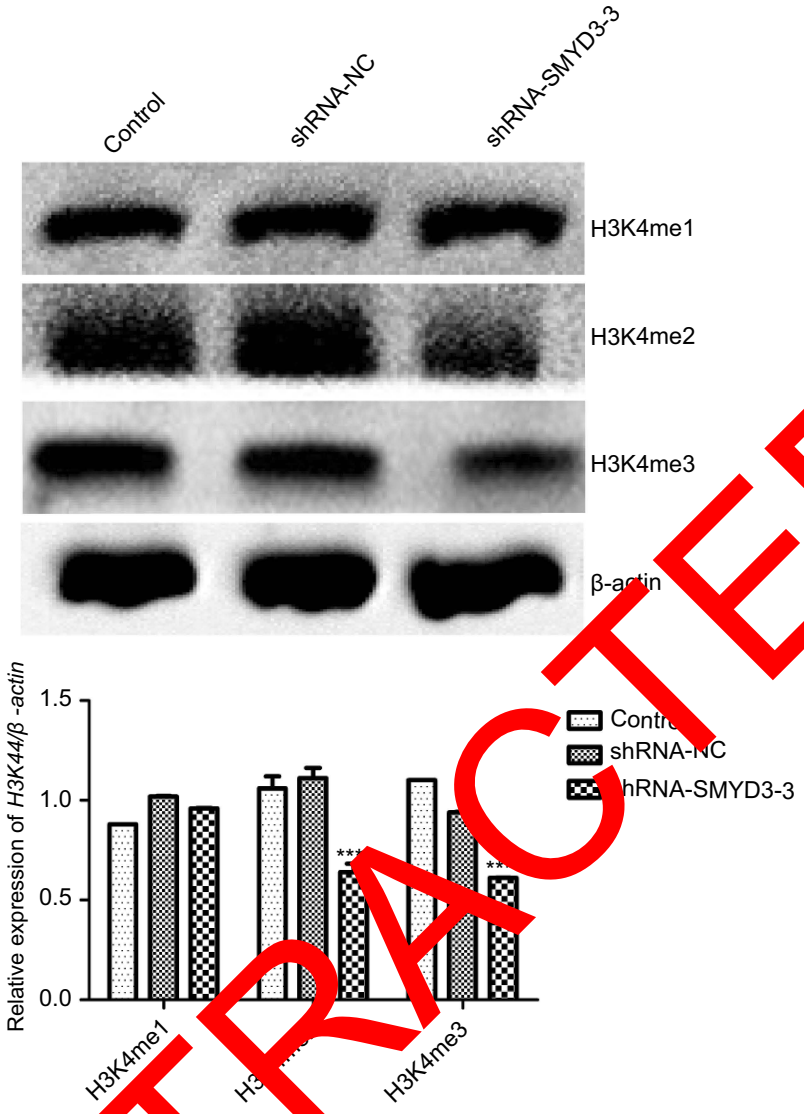

Figure 3 SMYD3 effects on H3K4 level. Western blot analysis was performed to analyze H3K4me1, H3K4me2, and H3K4me3 levels. β-Actin was used as the loading control. The relative H3K4me1, H3K4me2, and H3K4me3 levels against β-actin shown were calculated. Significant differences between the control and shRNA-SMYD3-3 groups were shown. Statistical analysis was calculated with unpaired Student's *t*-test; n≥3, ***$P \leq 0.001$.
Abbreviations: H3K4, histone lysine-4; shRNA, short hairpin RNA.

invasion are key steps of cancer cells, and the inhibition rates were all >30%, thereby suggesting these steps are tightly controlled by *SMYD3*. We also analyzed whether the *SMYD3* suppression-mediated inhibition of the T24 cells is associated with cell death/apoptosis. The FACS results clearly indicated that *SMYD3* suppression induced T24 cell apoptosis which may inhibit BLAC proliferation, migration, invasion, and colony formation cell line T24. These results suggested that *SMYD3* was negatively regulated cell apoptosis which may alter cell migration, invasion, and proliferation.

SMYD3 is a member of the *SMYD* family and control the di- or trimethylation of histone H3K4 in cancer cells, and *SMYD3* is abundant in cancer cells compared with that in normal cells, thereby suggesting that *SMYD3*-mediated methylation at H34K significantly changed the downstream target gene expressions levels. In our analyses, cell proliferation, migration, invasion, and colony formation were positively regulated by *SMYD3*, and apoptosis was negatively regulated by SMYD3. Further study will be needed to analyze the target genes of *SMYD3* in cancer development, will be important to understand the regulatory basis of cancer cell behavior, and may be a key point to demonstrate that *SMYD3*-mediated apoptosis signaling regulates BLAC cell migration, proliferation, and invasion.

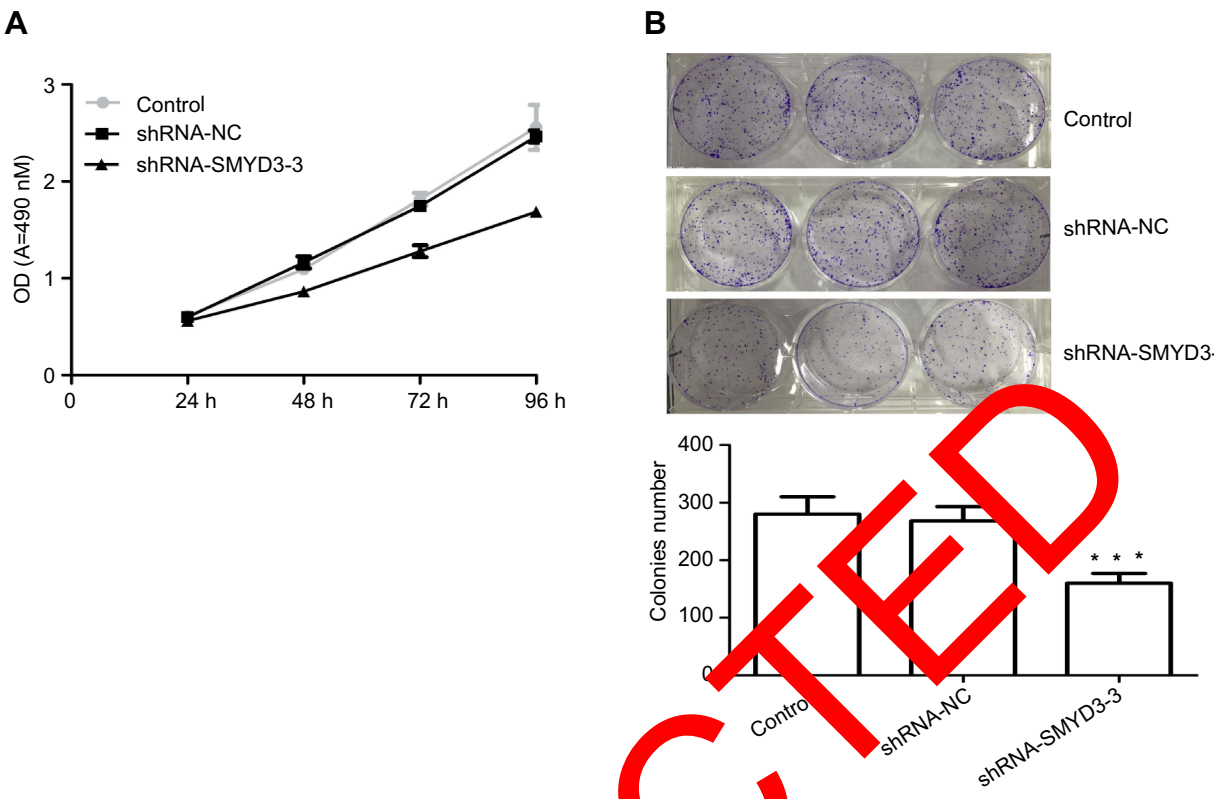

Figure 4 SMYD3 suppression inhibited T24 cell proliferation. (**A**) Cell proliferation was analyzed by measuring cell OD of the control, shRNA-NC, and shRNA-SMYD3-3 groups by the cell counting kit-8 assay. (**B**) Colony formation in control, shRNA-NC, and shRNA-SMYD3-3 was analyzed. Significant differences between the control group and shRNA-SMYD3-3 were shown. Statistical analysis was calculated with unpaired Student's *t*-test. n≥3, ***P*≤0.001.
Abbreviation: shRNA, short hairpin RNA.

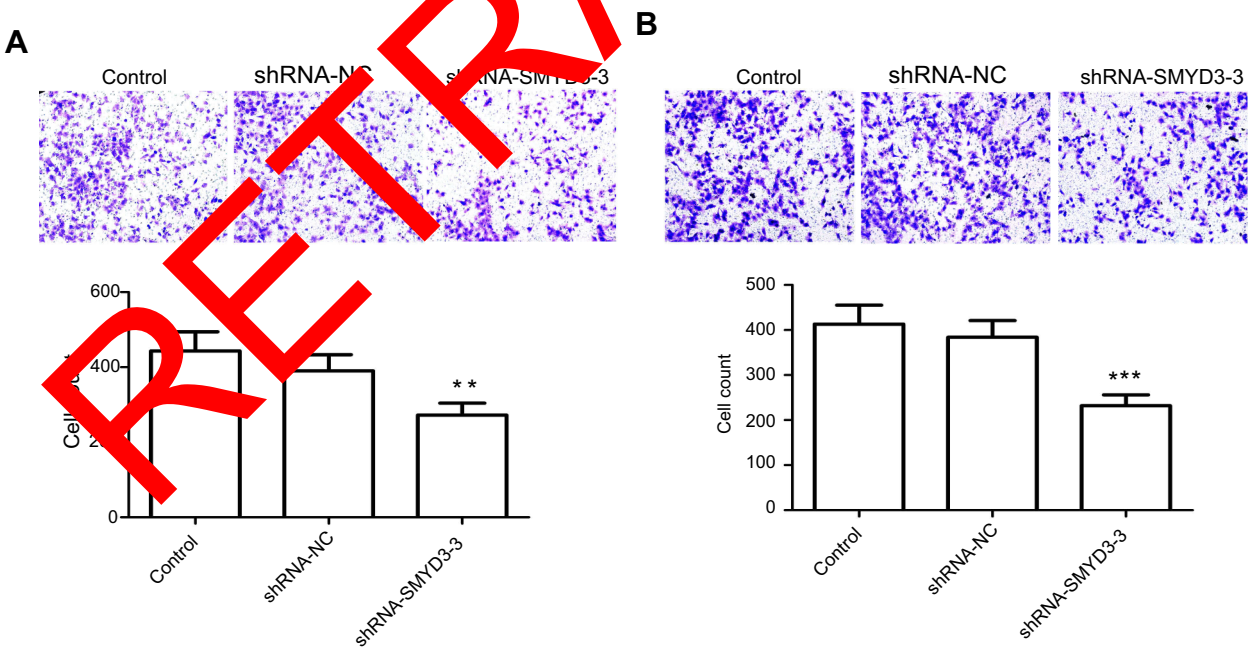

Figure 5 SMYD3 knockdown inhibited T24 cell migration and invasion. (**A**) The cells of the control, shRNA-NC, and shRNA-SMYD3-3 groups were photographed and cell migration counts shown were calculated. Significant differences between groups were shown (*P*<0.01). (**B**) The cells of the control, shRNA-NC, and shRNA-SMYD3-3 groups were photographed and cell invasion counts were calculated. Significant differences between groups were revealed. Statistical analysis was calculated with unpaired Student's *t*-test; n≥3, ***P*≤0.01, ****P*≤0.001.
Abbreviation: shRNA, short hairpin RNA.

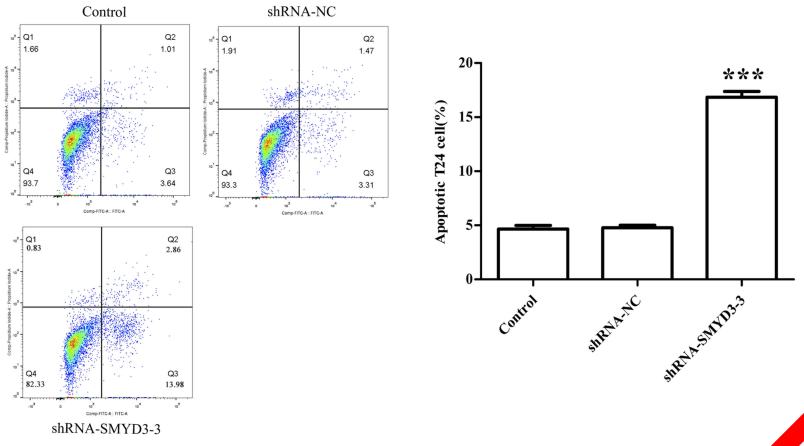

Figure 6 SMYD3 suppression promoted cell apoptosis. Cell apoptosis was analyzed by fluorescence-activated cell sorting assay in control, shRNA-NC, and shRNA-SMYD3-3 groups. The rate of cell apoptosis in the control, shRNA-NC, and shRNA-SMYD3-3 groups was calculated. Significant differences between the control, shRNA-NC, and shRNA-SMYD3-3 were shown. Statistical analysis was calculated with unpaired Student's *t*-test; n≥3, ***$P \leq 0.001$.
Abbreviation: shRNA, short hairpin RNA.

Also, *SMYD3* is also commonly induced in the type of tumor tissues, thereby indicating that *SMYD3* may be a considerable therapeutic target for cancer drug development.

Abbreviations

NMIBC, non-muscle-invasive bladder cancer; SMYD, SET and MYND domain-containing proteins; SMYD3, SMYD family members; H4K5, histone 4 lysine-5; H3K4, histone 3 lysine-4; shRNA, short hairpin RNA; GFP, green fluorescence protein.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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