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EHMT2 promotes the development of prostate cancer by inhibiting PI3K/AKT/mTOR pathway

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Abstract. – OBJECTIVE: To investigate the expression characteristics and the potential role of euchromatic histone-lysine N-methyltransferase 2 (EHMT2) in the clinical pathology and prognosis of prostate cancer (PCa).

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to detect the expression of EHMT2 in 55 pairs of PCa tissues and adjacent normal tissues. The relationship between EHMT2 expression and the pathological features, as well as the prognosis of PCa patients was analyzed. EHMT2 expression in PCa cells was determined by qRT-PCR as well. In addition, EHMT2 knockdown model was constructed by transfection of the small interfering RNA in PCa cell lines PC-3 and DU-145. The regulatory effects of EHMT2 on the behaviors of PCa cells were evaluated through cell counting kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU), colony formation assay and flow cytometry. Finally, we detected the protein levels of relative genes in PI3K/AKT/mTOR pathway through Western blot.

RESULTS: QRT-PCR results showed that the expression level of EHMT2 in PCa tissues was markedly higher than that in adjacent normal tissues. Compared with PCa patients with low expression of EHMT2, those with high expression of EHMT2 had higher pathological grade and lower overall survival. EHMT2 was also highly expressed in PCa cell lines. Knockdown of EHMT2 inhibited the proliferative potential and induced apoptosis of PCa cells. Western blot results revealed that PCa cells with EHMT2 knockdown presented downregulated p-PI3K, p-AKT and p-mTOR.

CONCLUSIONS: EHMT2 was highly expressed in PCa, and its expression is closely correlated with tumor stage and poor prognosis of PCa patients. EHMT2 promoted the malignant progression of PCa by inhibiting PI3K/AKT/mTOR pathway.

Key Words:
EHMT2, PI3K/AKT, Prostate cancer, Malignant progression.

Introduction

Prostate cancer (PCa), as a common tumor that threatens male health, shows an increasing trend of morbidity and mortality in recent years[1-3]. Globally, the incidence of PCa ranks second in all male malignant tumors, and the mortality rate ranks fifth[4,5]. Although the incidence and mortality of PCa in China are much lower than that in the other developed countries, it still poses a severe influence on the physical health of men in China[6,7]. So far, diagnostic approaches of PCa, including prostate specific antigen (PSA) determination and ultrasonography have been greatly advanced. However, the clinical outcomes of advanced PCA are still far away from satisfaction[8,9]. The specific pathogenesis of PCa has not been fully elucidated. Hence, it is of great significance to search for novel therapeutic targets with good specificity and sensitivity, thus improving the clinical outcomes of PCa patients[10,11]. Euchromatic histone-lysine N-methyltransferase 2 (EHMT2), also known as G9a, is an important histone methyltransferase belonging to the SET domain family[12,13]. EHMT2 was reported to abnormally express in some tumors, such as breast cancer and pancreatic cancer[12-14]. Recent studies[12-14] have shown that the increase of EHMT2 expression was closely related to tumor cell proliferation. Besides, EHMT2 was also involved in the regulation of multiple cellular behaviors, as well as tumor development and progression[12-14]. PI3K is a class of phosphatidylinositide 3-kinases, called the PI3Ks family[15]. Among them, class I PI3K, a heterodimer composed of catalytic subunit p110 and ligation/regulation subunit p85, exerts an essential role in tumor development[15,16]. The amino terminus of p85 contains a Src homology domain 3 (SH3) and a proline-rich region capable of binding to the SH3 domain, whereas

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the carboxy terminus of p85 contains two Src homology domains (Src homology domain 2, SH2) and a region combined with p110[16]. Akt is a serine/threonine protein kinase with 57 kD in molecular weight. It is also called protein kinase B (PKB) since its amino acid sequence of the kinase active region is highly similar to protein kinase A and protein kinase C[17]. The PH domain of Akt is composed of about 100 amino acids, and its structure is highly similar to the PH domain of other molecules that bind to PIP3. Therefore, the PH domain is considered to regulate the binding of Akt to PIP3 and promotes the translocation of Akt from cytoplasm to cell membrane. Subsequently, a conformational change in Akt exposes the phosphorylation sites in threonine at position 308 and in tryptophan (Ser473) at position 473[17,18]. As a downstream effector of PI3K, activated Akt participated in regulating tumor cell proliferation, apoptosis, metabolism, cell cycle and tumor angiogenesis, thus promoting the development of PCa[18, 19]. Based on the above characteristics, we speculated whether EHMT2 could act as a serological marker for early diagnosis and a therapeutic target of PCa. Our study elucidated that EHMT2 participated in the proliferation and apoptosis of PCa cells through PI3K/AKT/mTOR pathway.

Patients and Methods

Patients and PCa Samples

A total of 55 PCa tissues and paired adjacent tissues were obtained from biopsy or surgical resection at Urology and Oncology Department in our hospital. Tissues were stored in a -80°C refrigerator. The enrolled 55 PCa patients aged from 56.3-78.4 years, with an average of 64.2 years. All cases were diagnosed by two senior pathologists independently. This study was approved by the Ethics committee of the Second Affiliated Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study.

Cell Lines

Four human PCa cells (PC-3, DU-145, 22RV1 and Lncap) and one human normal prostate matrix immortalized cell (WPMY-1) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA) in a 37°C, 5% CO_2 incubator. The cells were digested and passaged with trypsin supplemented with EDTA (Ethylene Diamine Tetraacetic Acid) when grown to 80-90% confluence.

Transfection

Negative control (si-RNA) and siRNA containing the EHMT2 interference sequence (si-EHMT2-1 and si-EHMT2-2) were purchased from GenePharma (Shanghai, China). Cells were plated into 6-well plates and grown to a cell density of 70%, followed by siRNA transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. Cells were harvested 48 h after transfection for quantitative Real-time polymerase chain reaction (qRT-PCR) analysis and cell function measurement.

Cell Proliferation Assay

The cells were harvested 48 h after transfection and seeded into the 96-well plate with 2000 cells per well. At 24 h, 48 h, 72 h and 96 h, cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan) was supplied in each well. After another 2 h of culture, absorbance of each well at 450 nm was recorded using a microplate reader.

Colony Formation Assay

After transfection for 48 h, the cells collected and were seeded into a 6-well plate with 200 cells per well and incubated at 37°C for 2 weeks. Subsequently, cells were fixed with methanol for 15 min and then stained with 0.1% crystal violet for another 20 min. The number of colonies in each well was counted and photographed.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

After transfection for 48 h, the cells were inoculated into 96-well plates, and 100 μL of EdU reagent (50 μM) (Solarbio, Beijing, China) were added to each well for 2 hour of incubation. After washing with phosphate-buffered saline (PBS), the cells were treated with 50 μL of fixation buffer, decolored with 2 mg/mL glycine and permeated with 100 μL of penetrant. After washing with PBS once, cells were then stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) (Southern Biotech, Birmingham, AL, USA) in dark for 30 min. EdU-positive rate was determined under a fluorescent microscope.

Flow Cytometry Analysis of the Cell Apoptosis

After transfection for 48 h, the cells were washed with PBS twice and digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin. After being adjusted to 1×10^6 /mL, the cells were transferred to a flow cytometry tube, and incubated wit 1.25 μL of Annexin V-FITC (fluorescein isothiocyanate) for 15 min in dark. After centrifugation at 1000×g for 5 min, the precipitate was incubated with 10 μL of Propidium Iodide (PI). Apoptosis was determined within 1 hour by flow cytometry.

QRT-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into the complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR reaction was performed using SYBR® Premix Ex TaqTM (TaKaRa Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: EHMT2, forward: 5'-GCTGGTTGTGGGTTACTCTC-3', reverse: 5'-GCCCTCTGTGCTACTTACTC-3'; β-actin, forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blot

Total protein was extracted using the cell lysate for determining protein expression. The concentration of protein samples was measured by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA), After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and blocked with 5% skim milk, the protein samples were then transfer into nitrocellulose membranes. The membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was utilized for statistical analysis. The quantitative data were presented as mean ± standard deviation ($\overline{x} \pm s$). Differences between two groups were analyzed by the *t*-test, whereas those among multiple groups were analyzed by one-way ANOVA followed by Post-Hoc Test LSD (Least Significant Difference). Chi-square test was performed to evaluate the relationship between EHMT2 expression with pathological features of PCa patients. Kaplan-Meier was introduced for survival analysis. $p<0.05$ was considered statistically significant.

Results

EHMT2 was Increased in PCa Tissues and Cell Lines

To determine the role of EHMT2 in PCa, we collected 55 pairs of PCa tissues and adjacent non-tumor tissues. The expression of EHMT2 in PCa tumor tissues and adjacent non-tumor tissues was detected by qRT-PCR. The expression of EHMT2 in the PCa tissues was significantly higher than that in the adjacent normal tissues (Figure 1A). Identically, EHMT2 also showed an increase in PCa cell lines at both protein and mRNA levels (Figure 1B and 1C). Among them, PC-3 and DU-145 cells were utilized for the subsequent *in vitro* experiments.

EHMT2 Expression was Correlated With Clinical Stage and Overall Survival of PCa Patients

Based on the EHMT2 expression, PCa patients were divided into high expression and low expression group, and the number of each group was counted. Chi-square test was used to analyze the relationship between EHMT2 expression with age, sex, clinical stage, lymph node metastasis and distant metastasis of PCa patients. The results suggested that high expression of EHMT2 was positively correlated with clinical stage of PCa (Table I). In addition, the follow-up data of enrolled PCa patients were collected for analyzing the relationship between EHMT2 expression and prognosis. Kaplan-Meier survival curves showed that high expression of EHMT2 was correlated with poor prognosis of PCa. The higher the expression level of EHMT2, the worse the prognosis of PCa ($p<0.05$, Figure 1D).

Knockdown of EHMT2 Inhibited Proliferative Potential of PCa Cells

To clarify the potential effect of EHMT2 on the proliferative potential of PCa cells, we first constructed si-EHMT2-1 and si-EHMT2-2.

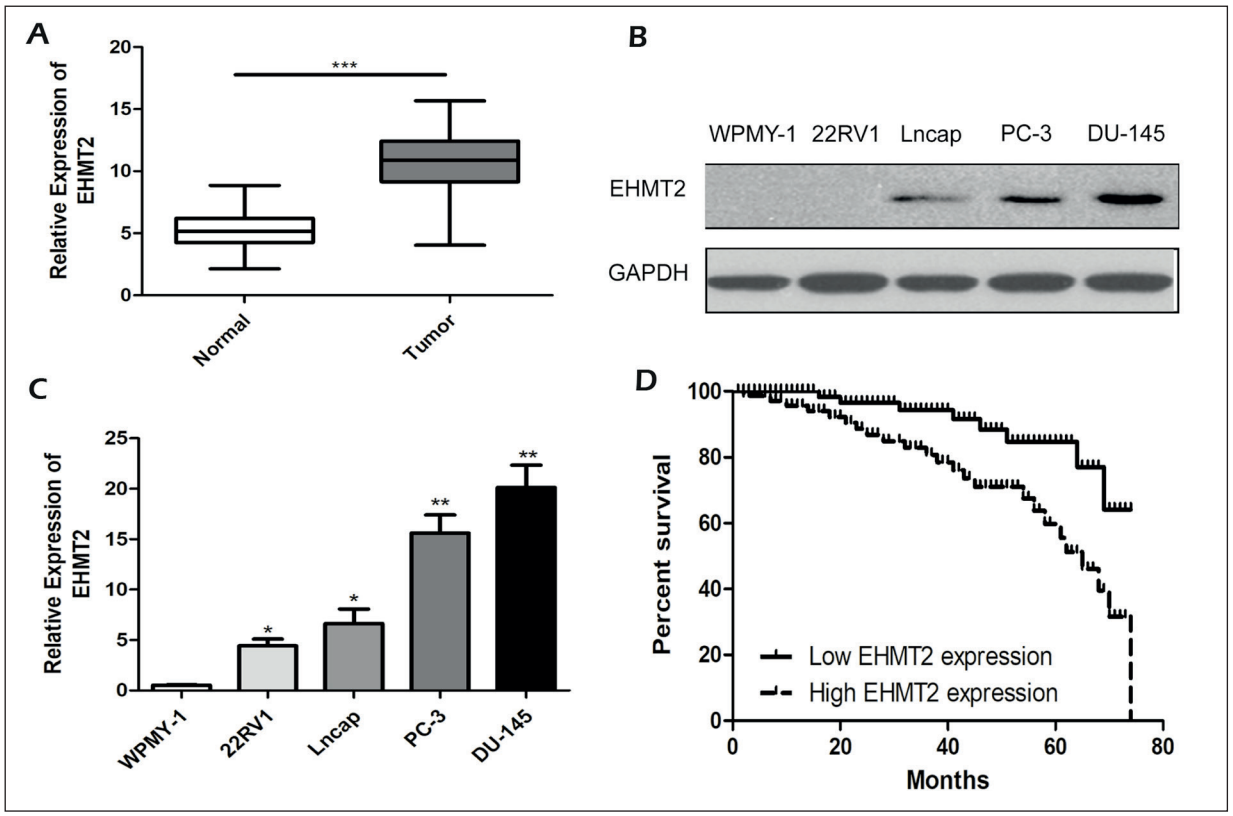

Figure 1. EHMT2 was highly expressed in PCa tissues and cell lines. **A,** EHMT2 was highly expressed in PCa tissues compared to the adjacent normal tissues, which was detected by qRT-PCR. **B, C,** EHMT2 was highly expressed in PCa cell lines at both protein and mRNA levels. **D,** Survival analysis of PCa patients based on their expressions of EHMT2. *p<0.05, **p<0.01, ***p<0.001.

Transfection efficacy of these two siRNAs in PC-3 and DU-145 cells was verified at both mRNA and protein levels (Figure 2A and 2B). CCK-8 assay showed that PCa cells transfected with si-EHMT2-1 or si-EHMT2-2 had a lower proliferation rate than the controls (Figure 2C). Identically, colony formation and EdU assay both confirmed that PCa cells with EHMT2 knockdown presented an inhibited proliferative potential (Figure 2D and 3A).

Table I. Correlation between EHMT2 expression with pathological characteristics of prostate cancer.

Parameters	Number of cases	EHMT2 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.592
< 60	20	12	8	
≥ 60	25	13	12	
T stage				0.023
T1-T2	22	16	6	
T3-T4	23	9	14	
Lymph node metastasis				0.540
No	27	16	11	
Yes	18	9	9	
Distance metastasis				0.577
No	29	17	12	
Yes	16	8	8	

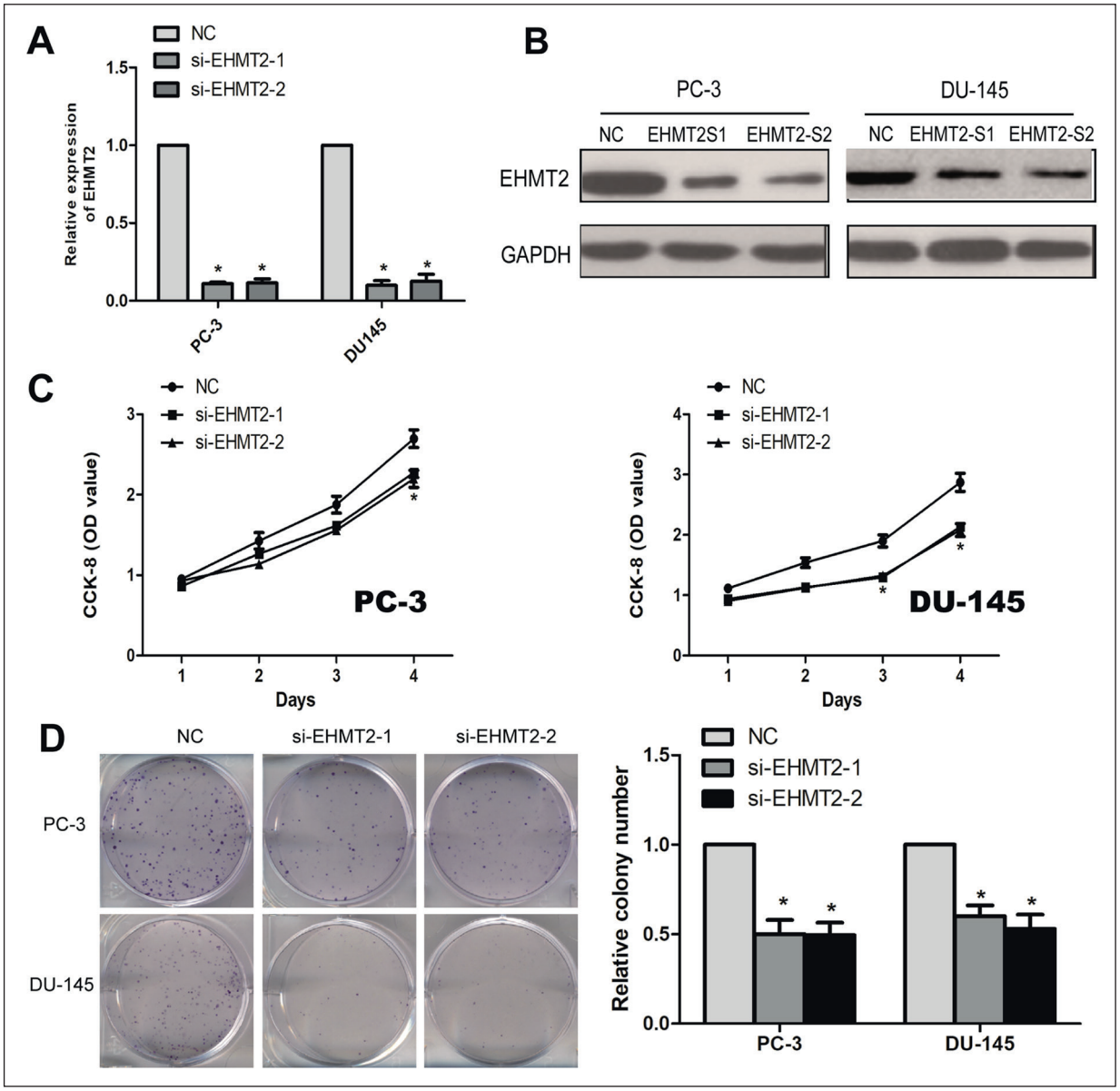

Figure 2. Knockdown of EHMT2 inhibited the proliferative potential of PCa cells. **A, B,** Transfection efficacy of si-EHMT2-1 and si-EHMT2-2 in PC-3 and DU-145 cells at both mRNA and protein levels. **C,** CCK-8 assay showed that PC-3 and DU-145 cells transfected with si-EHMT2-1 or si-EHMT2-2 had decreased proliferative rate. **D,** Colony formation assay showed that PC-3 and DU-145 cells transfected with si-EHMT2-1 or si-EHMT2-2 had fewer colonies. *$p<0.05$.

Knockdown of EHMT2 Induced Apoptosis of PCa Cells

Subsequently, we wondered whether EHMT2 could regulate the apoptosis of PCa cells. The results of flow cytometry demonstrated that PC-3 and DU-145 cells transfected with si-EHMT2-1 had a significant increase of apoptosis rate compared to the controls (Figure 3B). Above results suggested that EHMT2 knockdown could induce apoptosis of PCa cells.

Knockdown of EHMT2 Suppressed PI3K/AKT/mTOR Pathway in PCa

To further elucidate the mechanisms of EHMT2 regulating the malignant progression of PCa, we determined the relative expression of proteins in PI3K/AKT/mTOR pathway by Western blot. Downregulation of p-PI3K, p-AKT and p-mTOR were observed in PCa cells transfected with si-EHMT2-1 or si-EHMT2-2 (Figure 4B). Hence, our data indicated that EHMT2 knockdown inhibited PI3K/AKT/mTOR pathway in PCa.

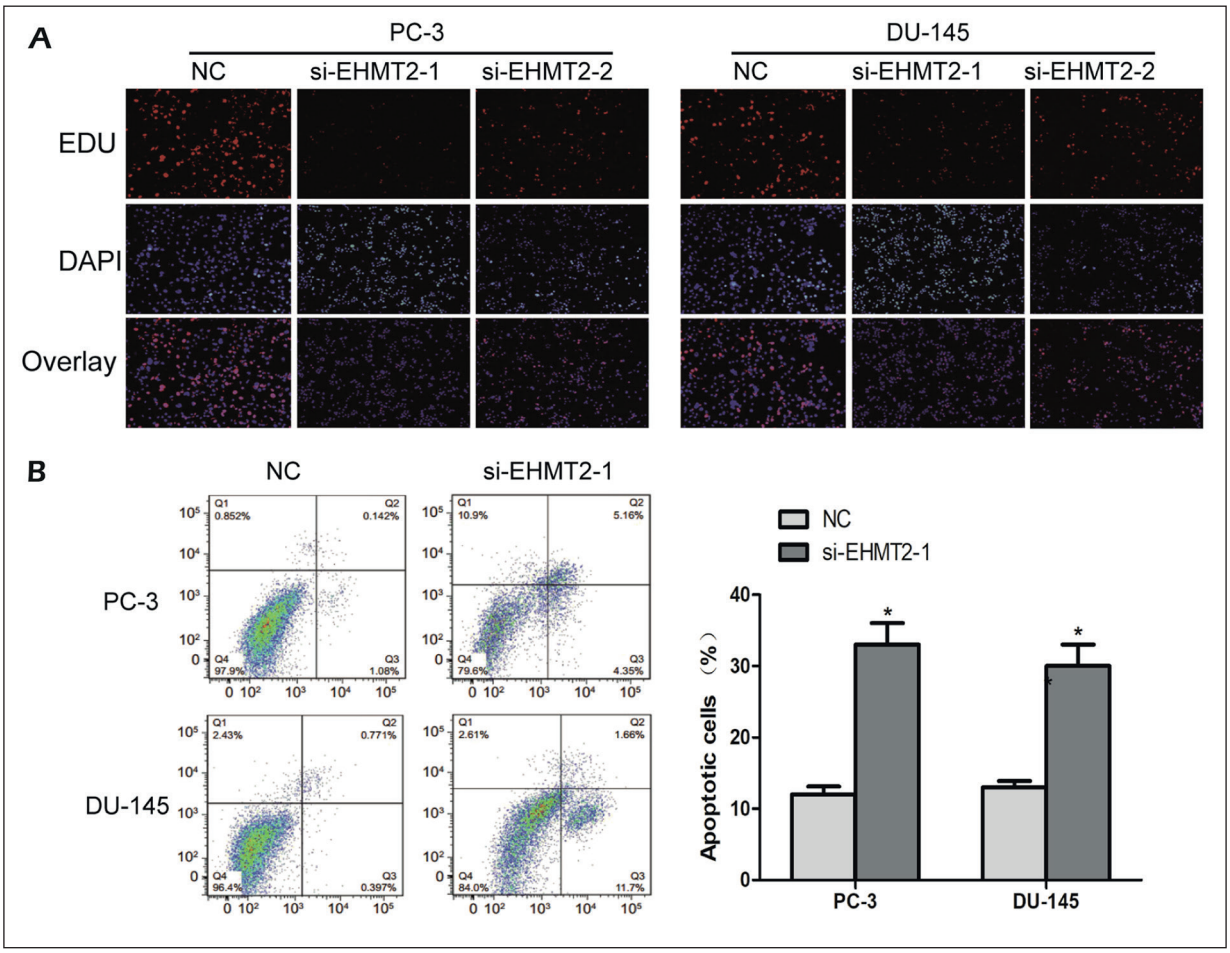

Figure 3. Knockdown of EHMT2 inhibited the proliferative potential but induced the apoptosis of PCa cells. **A,** EdU assay showed that PC-3 and DU-145 cells transfected with si-EHMT2-1 or si-EHMT2-2 had a decreased proliferative rate. **B,** Flow cytometry showed that PC-3 and DU-145 cells transfected with si-EHMT2-1 had accelerated apoptosis. *$p<0.05$.

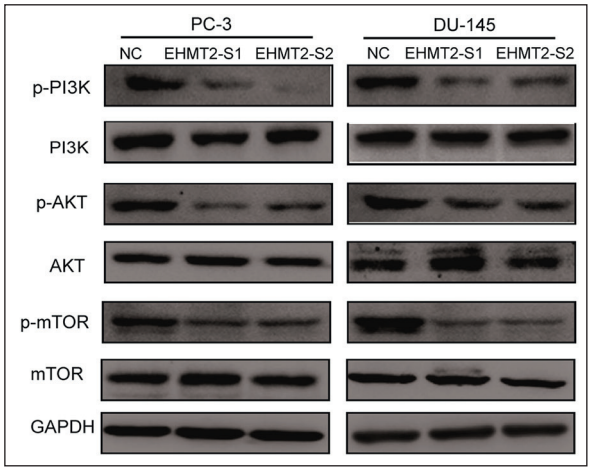

Figure 4. Knockdown of EHMT2 inhibited the PI3K/AKT/mTOR pathway in PCa. Western blot analyses was performed to detect the p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in PC-3 and DU-145 cells transfected with si-EHMT2-1 or si-EHMT2-2.

Discussion

Due to the infinite disorders of the proliferation and apoptosis in malignant tumors, the development of effective therapy is still a great challenge. Therefore, the mechanisms underlying the proliferation and apoptosis of malignant tumors have been widely explored[20]. Tumor is a complex genetic disease accompanied by the dysregulation of a variety of genes, manifesting as oncogene activation and tumor suppressor silence[21,22]. It is of great importance to elucidate the biological functions of tumor-related genes. EHMT2 can regulate the proliferation and apoptosis of various cancers, such as breast cancer and pancreatic cancer, by altering the expression of cell cycle and apoptotic proteins[12-14]. The specific role of EHMT2 in PCa, however, is rarely reported. In recent years, genetic factors, including loss

of heterozygosity, gene allelic mutations, etc., have been identified to participate in the occurrence and progression of PCa[22-24]. Thus, effective prevention and early diagnosis of PCa could help to control the incidence and improve the survival rate of PCa[25, 26]. Further researches on the etiology of PCa, especially the role of genetic genes in the pathogenic progression of PCa will greatly contribute to the development of novel diagnostic and therapeutic methods[25-27]. Abnormal expression of EHMT2 was found in different tumors of multiple organs previously, suggesting that it might be associated with malignant progression of tumors[12-14]. In our study, we determined mRNA and protein levels of EHMT2 in PCa tissues and cell lines. EHMT2 was highly expressed in PCa and exhibited a potential role in the malignant progression of PCa. To further clarify the biological function of EHMT2 in regulating the cellular behaviors of PCa cells, a series of function experiments were conducted. Knockdown of EHMT2 inhibited the proliferative potential and induced the apoptosis of PCa cells, as indicated in the results of the CCK-8, EdU, colony formation assay and flow cytometry. We proposed that EHMT2 accelerated the malignant development of PCa.

The PI3K/mTOR pathway is critical for cell metabolism, survival, and proliferation. Previous studies have shown that PTEN was mutated in a variety of primary cancers. Downregulation of PTEN and activation of mTOR signaling pathway have been observed in PCa. Mutations in PTEN activated the PI3K/Akt pathway and the downstream mTOR signal, thus regulating cellular functions. Therefore, mTOR and associated downstream signal molecules might also be markers for the prediction and diagnosis of PCa[15-19]. At present, both *in vivo* and *in vitro* experimental evidences indicated that the PI3K/AKT/mTOR pathway played a pivotal role in primary and secondary metastasis of cancers, such as gastric cancer, lung cancer, and pancreatic cancer[13, 15, 28]. We believed that PI3K/AKT/mTOR pathway is of great significance in tumor development, especially for tumor metastasis[16]. In this experiment, Western blot results showed that the expression levels of p-PI3K, p-AKT and p-mTOR markedly decreased after the knockdown of EHMT2, indicating that EHMT2 could regulate PI3K/AKT/mTOR pathway in PCa. To demonstrate whether EHMT2 promoted the development of PCa by regulating the PI3K/AKT/mTOR pathway, we examined expression changes of p-PI3K, p-AKT, and p-mTOR in the PI3K/AKT/mTOR pathway after knockdown of EHMT2 by Western Blot. Our results suggested that EHMT2 promoted the proliferation and apoptosis of PCa cells *via* the PI3K/AKT/mTOR pathway.

Conclusions

EHMT2 was highly expressed in PCa, and its expression was correlated with the tumor stage and poor prognosis of PCa patients. EHMT2 promoted the malignant progression of PCa by inhibiting PI3K/AKT/mTOR pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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