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Clinical Study

PTEN Loss-Mediated Akt Activation Increases the Properties of Cancer Stem-Like Cell Populations in Prostate Cancer

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Key Words
Prostate · Prostate neoplasm · Stem cell · PTEN

Abstract
Objective: To demonstrate that the PTEN/PI3K/Akt/NF-κB pathway plays an important role in regulating the prostate cancer stem-like cell population by upregulating ABCG2. ***Methods:*** Targeted PTEN knockdown in human prostate DU145 and 22Rv1 cells using a small interfering RNA were confirmed by immunoblot analysis using antibodies of PTEN, phospho-Akt, Akt, and α-tubulin. Knockdown PTEN DU145 and 22Rv1 cells were augmented, and the stem cell-like properties were examined by cell viability and tumor sphere formation and treated by Akt IV inhibitor to provide the signal transduction pathway. Luciferase activity assays were performed. ***Results:*** The knockdown of PTEN in prostate cancer cell lines increased the stem-like properties of the cells, including their sphere-forming ability, stem cell population number, epithelial-mesenchymal transition-related gene expression, and ABCG2 expression. Additionally, PTEN expression was highly associated with elevated expression of phospho-Akt. Treatment with an Akt inhibitor suppressed the PTEN-mediated effects on the properties of these stem-like cells as well as drug resistance, ABCG2 expression, and the NF-κB pathway. ***Conclusion:*** The loss of PTEN in prostate cancer cells resulted in an increased PI3K/Akt pathway. Due to the Akt activation, PTEN loss may play an important role in prostate cancer by promoting cancer stemness through a mechanism that involves enhanced NF-κB signaling.

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Introduction

Prostate cancer is the most common malignant disease in men worldwide [1]. Early detection and initial treatment increase patient survival. However, cancer relapse, metastasis, and therapeutic resistance are the most common causes of mortality in prostate cancer patients [2–4].

Although the exact causes of recurrence and metastasis in prostate cancer remain unclear, the cancer stem cells (CSCs) or tumor-initiating cells hypothesis suggests that CSCs are not only a renewable source of tumor cells, but are also a cause of antitumor drug resistance that leads to cancer recurrence, metastasis and cancer progression [5, 6]. Therefore, CSC-targeted therapies may prevent cancer relapse and provide a more effective treatment.

Phosphatase and tensin homologue deleted on chromosome-10 (PTEN) plays a role as a tumor suppressor gene, mainly through its negative regulation of the phosphatidylinositol-3-OH kinase (PI3K)-Akt pathway. Absent or decreased PTEN protein expression is associated with resistance to conventional therapy and relapse following initial treatment in many different types of cancer [7, 8]. Deletion of PTEN results in the expansion of the prostate stem/progenitor cell population and tumor-initiating cells [9, 10]. However, whether the loss of PTEN is related to prostate CSCs remains poorly defined. Recent reports have shown that the loss of PTEN resulted in increased ABCG2 expression and side populations (SP) in glioma tumors and acute leukemia [11, 12]. Furthermore, Mosaffa et al. [13] showed that the nuclear factor-κB (NF-κB) signal transduction pathway is involved in the upregulation of ABCG2. Together, these data suggest a possible link between the PTEN-mediated Akt pathway and ABCG2 expression through the NF-κB signaling pathway in prostate CSCs. In the present study, we demonstrate that the PTEN/PI3K/Akt/NF-κB pathway plays an important role in regulating the prostate cancer stem-like cell population by upregulating ABCG2. Furthermore, this study suggests that an Akt inhibitor targeting the cancer stem-like cell population could be beneficial for the treatment of prostate cancer.

Materials and Methods

Cell Culture and Reagents

Human prostate DU145 and 22Rv1 cells were cultured in RPMI-1640 medium (Welgene, Daegu, Korea) containing 10% FBS and 1% penicillin/streptomycin (Welgene), as previously described [14]. Akt inhibitor IV was purchased from Calbiochem/EMD Chemicals Inc. (San Diego, Calif., USA), and docetaxel was purchased from LC Laboratories (Woburn, Mass., USA).

Tumor Sphere Culture

Single cells were resuspended in serum-free DMEM (Invitrogen, Carlsbad, Calif., USA) containing B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (Peprotech, Rocky Hill, N.J., USA) and 4 μg/ml heparin (Sigma-Aldrich, St. Louis, Mo., USA), as previously described [15].

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Cell viability and tumor sphere formation were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [16].

Flow Cytometry

FACS and SP analyses were performed using the FACS Calibur and LSR Fortessa machines (Becton Dickinson, Palo Alto, Calif., USA), respectively. Previous reports showed that CD44, CD133 and aldehyde dehydrogenase (ALDH) could be used as markers for prostate CSCs [10, 17]. FACS data were analyzed using the Flowjo software (Tree Star, Ashland, Oreg., USA). Antibodies against the following proteins were used: PE-conjugated CD133 (Miltenyi Biotec, Germany) and APC-conjugated CD44 (BD Pharmingen, San Diego, Calif., USA). The Aldefluor kit (Stem Cell Technologies, Vancouver, B.C., Canada) was used to isolate the popu-

lation of cells with high ALDH enzymatic activity. The cells were stained for ALDH using the Aldefluor reagent according to the manufacturer's instructions.

Quantitative Reverse-Transcription-PCR

Quantitative reverse-transcription-PCR (qRT-PCR) was performed on the ABI ViiA-7 Real-Time PCR system using the SYBR green dye (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions. qRT-PCR reactions were performed in triplicate. The gene expression levels were normalized to the expression of 18S rRNA. Primer sequences are provided in online supplementary table 1 (www.karger.com/doi/10.1159/000363186).

RNA Interference

A small interfering RNA (siRNA) specific to PTEN (GenBank Accession Nos. 000314, 1123885, 1123878 and 11233889) and a nontargeting siRNA (SN-1012) were purchased from Bioneer Co. (Daejeon, Korea). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were treated for 48 h with a final concentration of 100 nM siRNA. We selected the siRNA that resulted in the greatest inhibition of the target gene (fig. 1a).

Immunoblot Analysis

Immunoblot analysis was performed as previously described [15]. Immunoblot bands were detected and scanned using the luminescent image analyzer Image Quant LAS-4000 (GE Healthcare, Tokyo, Japan). Antibodies against the following proteins were used: PTEN, Akt, phospho-Akt (Cell Signaling Technology, Beverly, Mass., USA), and α-tubulin (Sigma-Aldrich).

Promoter Reporter Assay

Luciferase activity assays were performed using the Luciferase Assay (Promega, Madison, Wis., USA), as previously described [15]. Briefly, cells in 12-well flat-bottomed plates were transfected with a total of 500 ng/well reporter plasmid and 250 ng/well β-gal plasmid DNA (for normalization) using Lipofectamine 2000 according to the manufacturer's instructions. After transfection, the cells were incubated for 24 h, and the cell lysates were harvested. Luminescence was measured using a SpectraMax L plate reader (Molecular Devices, Sunnyvale, Calif., USA). All measurements were performed at least three times.

Statistical Analysis

All experiments were conducted with a minimum of three samples, and the results are expressed as the mean ± SD. Statistical analyses were performed using an unpaired parametric Student's t test, unless otherwise indicated in the text.

Results

PTEN Knockdown Affects the Stem Cell-Like Properties of Prostate Cancer Cells

Before determining the effect of PTEN on the stem cell-like properties, we compared PTEN expression in DU145 and 22Rv1 prostate cancer cell lines following transfection with siPTEN or siCON. As shown in figure 1a, PTEN levels were knocked down using the validated siPTEN#1–#3 in the PTEN-positive DU145 and 22Rv1 prostate cancer cells. As expected, an increase in Akt phosphorylation (>80% for siPTEN#1; 1123885) (fig. 1a) was observed following the depletion of PTEN.

To examine the functional role of PTEN in prostate CSCs, we measured the effect of PTEN knockdown on tumor sphere formation using an MTT assay. We found that the knockdown of PTEN significantly increased the efficiency of tumor sphere formation in DU145 and 22Rv1 prostate cancer cells (fig. 1b). FACS analysis showed that the siPTEN-transfected DU 145 cells had a 1.5- to approximately 2-fold higher stem cell population with CD44+, CD133+ and ALDH-positive cells compared to the siCON-transfected cells (fig. 1c). Since the epithelial-mesenchymal transition (EMT) has been linked to the CSC phenotype [18], we examined whether the increase in PTEN loss resulted in an induction of the EMT markers. qRT-PCR

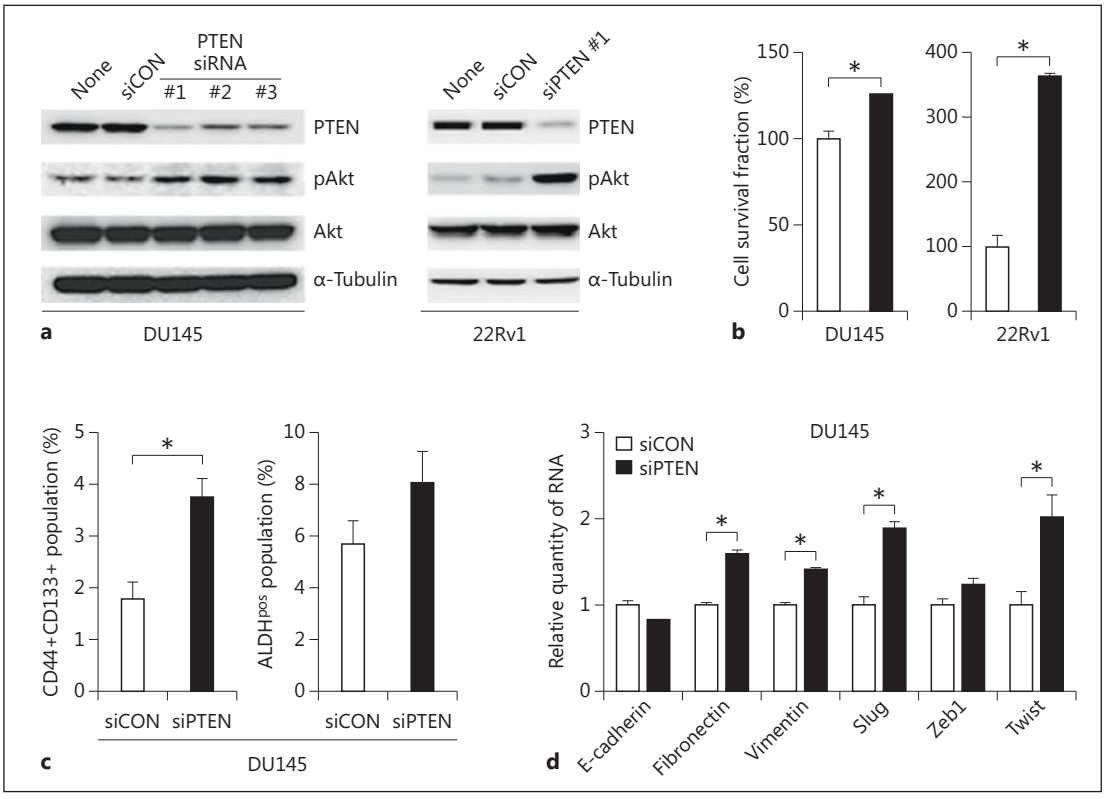

Fig. 1. Effect of PTEN expression on tumor sphere formation and stem marker expression in prostate cancer cells. **a** Immunoblot analysis of PTEN and Akt signaling in prostate cancer cell lysates from control cells (None = non-transfected) and cells transfected with a nonspecific control siRNA (siCON) or a PTEN-targeted siRNA (siPTEN). α-Tubulin was used as a loading control. **b** Analysis of tumor sphere formation in prostate cancer cells following knockdown of PTEN expression. After transfection with the PTEN-targeted siRNA under adherent conditions, the cells were detached and cultured in suspension under serum-free conditions for 7 days. **c** Characterization of the CSC population in the PTEN siRNA-treated DU145 cells. **d** Expression profile of the EMT-related genes in the siPTEN-transfected DU145 cells. The values represent the mean ± SD for three independent experiments. pAkt = Phospho-Akt. * $p < 0.05$.

analysis showed that the mRNA expression of EMT markers, including fibronectin, vimentin, slug, and twist, was increased by ~2-fold in the siPTEN-transfected DU145 cells (fig. 1d).

PTEN Loss Enhances the SP Fractions and ABCG2 Expression

The SP phenotype has been shown to be associated with the properties of CSCs [19]. To determine whether PTEN loss affects the SP fraction, the cells were stained with Hoechst 33342, which is actively exported by verapamil-sensitive ATP-binding cassette (ABC) transporters. Flow cytometry analysis showed that the siPTEN-transfected DU145 and 22Rv1 cells have a ~1.2–1.5-fold larger SP fraction (fig. 2a). Recent evidence suggests that the SP fraction highly expresses the ABC transporter [20]. Next, we examined whether PTEN loss influences the expression of ABC transporters in prostate cancer cells. qRT-PCR analysis showed a ~2-fold increase in ABCA1 and ABCG2 gene expression, but no significant change was observed in the expression of the other ABC transporters in the siPTEN-transfected DU145 cells (fig. 2b).

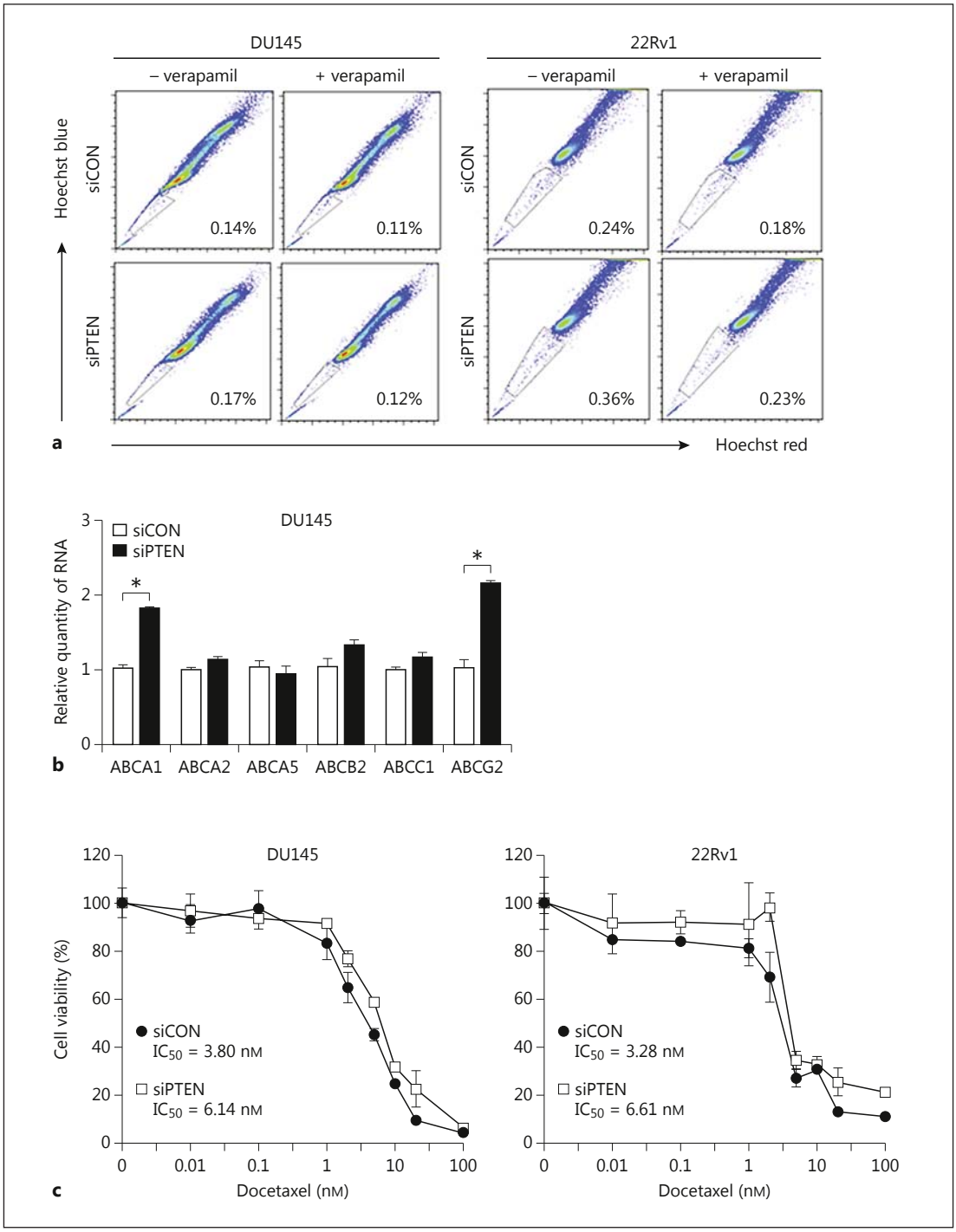

Fig. 2. Effect of PTEN expression on the SP phenotype and ABC transporter expression in prostate cancer cells. **a** Representative SP phenotype from the siCON or siPTEN-transfected prostate cancer cells. Each sample was incubated with 50 μM verapamil, which was used as a control, and the population was gated to analyze only the PI-negative (live cells). **b** Expression profile of the ABC transporters in the siPTEN-transfected DU145 cells. The values represent the mean ± SD for three independent experiments. **c** Cell proliferation analysis of the siCON or siPTEN-transfected prostate cancer cells after incubation with docetaxel for 48 h. * $p < 0.05$.

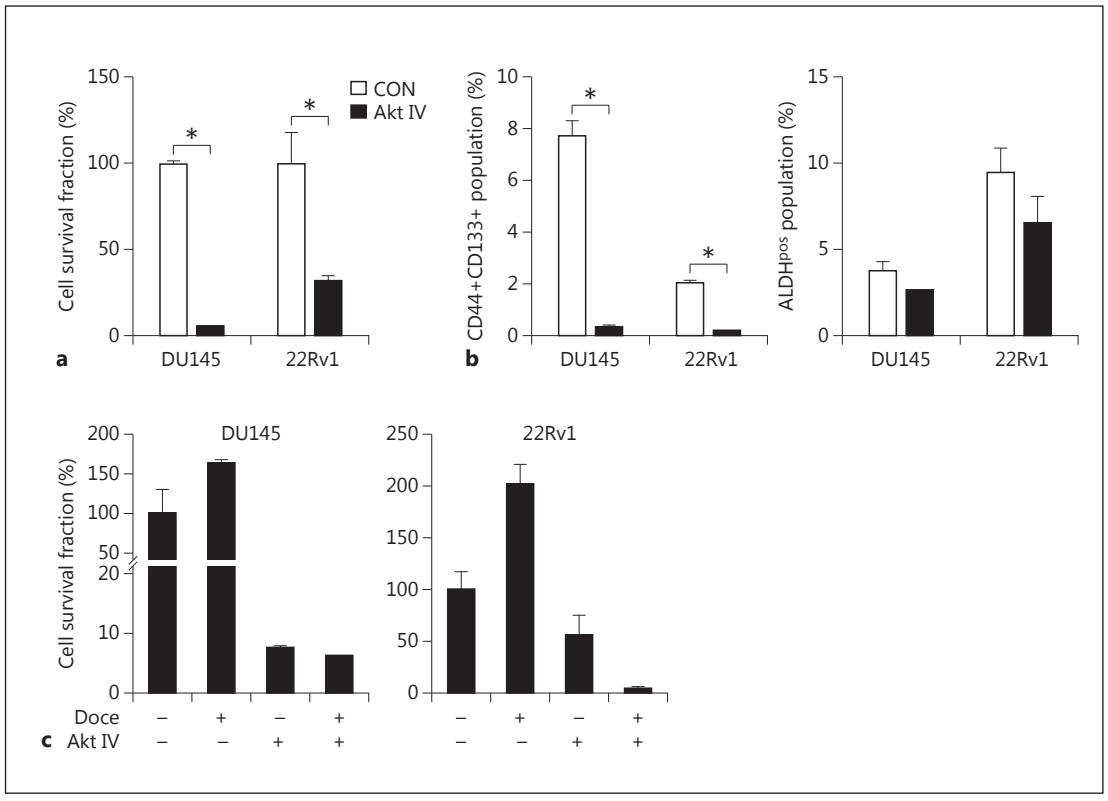

Fig. 3. Effect of the Akt inhibitor on stem cell-like properties. **a** Effect of the Akt inhibitor on tumor sphere formation in prostate cancer cells. DU145 and 22Rv1 cells were cultured in low attachment dishes with 0.5 μM Akt IV for 7 days, and the ability to form a tumor sphere was determined as described in the Materials and Methods section. **b** Characterization of the CSC population in the Akt inhibitor-treated DU145 cells. **c** Effect of combining the Akt inhibitor with docetaxel on the tumor sphere formation of prostate cancer cells. DU145 and 22Rv1 cells were cultured in low attachment dishes with 0.5 μM Akt IV or 0.5 nM docetaxel for 7 days. The values represent the mean ± SD for three independent experiments. CON = Control; Doce = docetaxel. * $p < 0.05$.

PTEN expression has been associated with resistance to chemotherapy [21]. To investigate the correlation between PTEN expression and anticancer drug resistance, we examined the sensitivity of the siPTEN-transfected DU145 and 22Rv1 prostate cancer cells to docetaxel. The half-maximal inhibitory concentration of docetaxel (IC_{50}) was ~1.6–2-fold higher in the siPTEN-transfected cells than in their counterparts (fig. 2c).

Inhibition of Akt Signaling Suppresses the Characteristics of CSCs

To provide evidence that the Akt signaling pathway is important for CSC-like cell expansion and maintenance, we tested the effect of the Akt pathway modulator Akt IV. As demonstrated in figure 3a, treating the DU145 and 22Rv1 prostate cancer cells with Akt IV reduced tumor sphere formation. Consistent with its effects on tumor sphere formation, Akt IV significantly reduced the proportion of the CD44+/CD133+ and ALDH-positive cell population in DU145 and 22Rv1 prostate cancer cells (fig. 3b). Next, we compared the effects of Akt IV alone or in combination with docetaxel, a chemotherapeutic agent that is commonly used to treat metastatic prostate cancer. As shown in figure 3c, treating the cells with docetaxel had no effect on tumor sphere formation. In contrast, Akt IV treatment without docetaxel

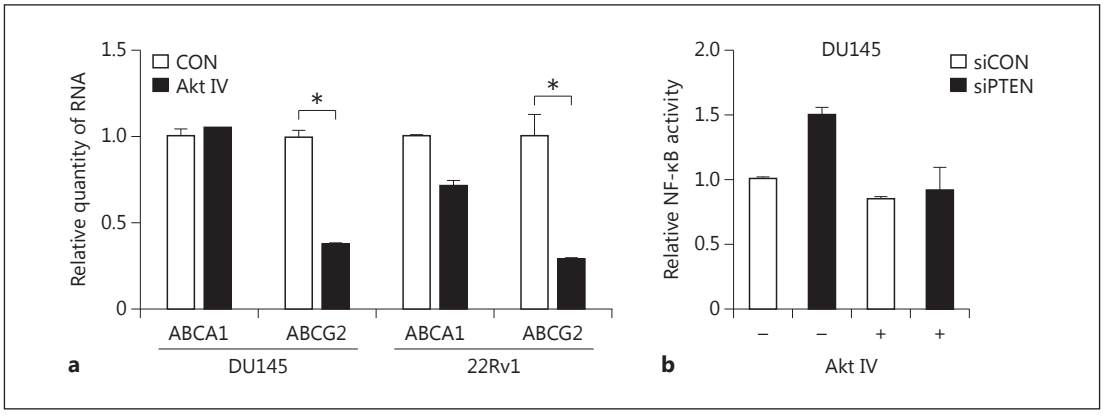

Fig. 4. Effect of the Akt inhibitor on PTEN-mediated ABCG2 expression and NF-κB activation. **a** Effect of the Akt inhibitor on ABCA1 and ABCG2 expression. **b** Effect of the Akt inhibitor on PTEN-mediated NF-κB pathway activation. The cells were transfected with NF-κB luciferase and β-gal plasmids after PTEN knockdown in DU145 cells. Luciferase activity was normalized to β-gal expression.

reduced the tumor sphere formation by over 80% compared to the untreated cells. Tumor sphere-forming efficiency was increased by treatment with docetaxel in DU145 and 22Rv1 prostate cancer cells. In contrast, Akt IV treatment without docetaxel reduced the tumor sphere formation by over 80% compared to the untreated cells (fig. 3c). These data suggest that Akt inhibitor can reduce docetaxel-induced expansion of CSCs.

Inhibition of Akt Signaling Suppresses PTEN-Mediated ABCG2 Expression and NF-κB Activation

Since PTEN loss increased the expression of ABCA1 and ABCG2, we used qRT-PCR analysis to determine whether Akt IV treatment affected the expression of ABCA1 and ABCG2. qRT-PCR analysis showed that Akt IV treatment caused a >2-fold reduction in the mRNA expression of the ABCG2 genes, but no significant change was observed in ABCA1 expression (fig. 4a). It has been reported that most drugs involved in ABCG2 upregulation are associated with NF-κB activation [22]. We found that PTEN loss upregulated the transcription from an NF-κB promoter reporter construct in DU145 cells. Additionally, the inhibition of Akt suppressed the PTEN-mediated activation of the NF-κB pathway (fig. 4b). The data suggest that PTEN loss enhances the activation of the NF-κB pathway, which leads to increased ABCG2 expression.

Discussion

The identification of target molecules involved in the expansion and maintenance of prostate CSCs could aid in the development of more effective cancer therapies. The PTEN/PI3K/Akt network has been recognized as an important player in the maintenance of prostate CSCs [10]. However, the signaling pathways downstream of PTEN in prostate CSCs remain largely uncharacterized. To the best of our knowledge, our study is the first experimental report showing that the PTEN/PI3K/Akt pathway may play an important role in the expansion and maintenance of prostate CSCs through a mechanism involving enhanced NF-κB signaling, which leads to the increased production of ABCG2. These results are supported by previous

findings that demonstrated that PTEN expression is highly correlated with the characteristics of stem cells [10].

The PTEN tumor suppressor gene is one of the most frequently deregulated genes in many different types of cancer, such as breast, colon, prostate and lung tumors, and its loss is associated with excessive proliferation and reduced apoptosis [7, 8]. It has been shown that PTEN loss was significantly linked to advanced tumor stage. Therefore, PTEN loss was frequently associated with important biological and clinical features such as rapid tumor progression and recurrence [23, 24]. Increased activity of the PI3K/Akt pathway due to the loss of PTEN resulted in increased resistance to chemotherapy [12, 25]. In glioma cells and esophageal carcinoma cells, Akt regulates ABCG2 activity, and the loss of PTEN increases the SP [11, 26]. It has been recently reported that an Akt inhibitor directly inhibited the properties of breast CSCs [27]. Our findings are consistent with the published data, and we showed that Akt signaling plays an important role in prostate CSCs.

EMT is an embryonic program in which epithelial cells lose their characteristics and gain mesenchymal features. It has been shown that aberrant activation of the EMT cancer stemness program, which is characteristic of cancer cells at the invasive edge of a tumor, separates a few of the tumor cells from the primary lesion, exhibits stem cell properties, and enables migrating CSCs to enter the blood vessels [28–30]. Recently, molecular similarities between the CSC and EMT phenotypes have been reported. Moreover, cells with an EMT phenotype induced by different factors are rich sources for stem-like cancer cells [18, 31]. It has been reported that PTEN loss induces EMT in colon cancer cells [32]. In the present study, RTQ-PCR analysis showed that PTEN-loss enhanced EMT markers (fig. 1d). These findings support the possibility that PTEN loss might be involved in the regulation of EMT. Therefore, these molecular details are under investigation.

The development of chemoresistance is a serious problem in the treatment of many cancers. Anticancer drug resistance is recognized as the doorway for cancer recurrence and metastasis. Deletion or reduced expression of PTEN in a wide variety of human tumors is associated with resistance to conventional therapy [33, 34]. In our findings, tumor sphere formation was significantly different between the docetaxel alone group and the docetaxel group with Akt modulation; it might be a future treatment option in men with metastatic castration-resistant prostate cancer who relapsed after chemotherapy. We especially observed an increase in the SP fraction in the siPTEN-treated cells compared to their counterparts. The biological basis for the differential efflux of dyes in stem cell compartments has not yet been clearly shown, but recent studies have indicated that the capacity to pump the dye out of the cells is associated with transmembrane proteins, such as the ABC transporters [35]. Additionally, several transporters, such as ABCG2 and ABCA3, may contribute to the malignant SP phenotype [20, 36]. NF-κB transcriptional activity has been reported to inhibit apoptosis and to induce drug resistance in cancers [37]. In our promoter-reporter study, we showed that inhibition of Akt suppressed the PTEN-mediated activation of the NF-κB pathway. Although there is no evidence for the direct transcriptional regulation of ABCG2 through an NF-κB-binding site on the ABCG2 promoter, most drugs involved in ABCG2 upregulation have been shown to target the NF-κB signaling pathway by activating NF-κB [22]. Furthermore, a recent report showed that the NF-κB signal transduction pathway is involved in the upregulation of ABCG2 [13]. Therefore, additional studies are needed to determine whether the PTEN/PI3K/Akt pathway can control ABCG2 through the regulation of NF-κB transcriptional activity. However, our present report does not fully explain the molecular mechanism of PTEN loss-mediated Akt activation in cancer stemness. Thus, we are still investigating possible mechanisms.

Conclusion

Taken together, we have shown that Akt activation caused by PTEN loss may play an important role in prostate cancer by promoting cancer stemness through a mechanism that involves enhanced NF-κB signaling, which leads to increased expression of ABCG2. Therefore, targeting the Akt signaling pathway may be beneficial to the development of more effective prostate cancer therapies.

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Disclosure Statement

The authors have no conflicts of interest with any institutions or products. No financial support was received by any author.

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