KLF9 suppresses cell growth and induces apoptosis via the AR pathway in androgen-dependent prostate cancer cells

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**ABSTRACT**

Kruppel-like factors (KLFs) play an important role in many biological processes including cell proliferation, differentiation and development. Our study showed that the level of KLF9 is lower in PCa cell lines compared to a benign prostate cell line; the androgen-independent cell line PC3 expresses significantly lower KLF9 than the androgen-dependent cell line, LNCaP. Forced overexpression of KLF9 suppressed cell growth, colony formation, and induced cell apoptosis in LNCaP cells. We also found that KLF9 expression was induced in response to apoptosis caused by flutamide, and further addition of dihydrotestosterone antagonized the action of flutamide and significantly decreased KLF9 expression. Furthermore, activation of the androgen receptor (AR) was inhibited by the overexpression of KLF9. Our research shows that KLF9 is lower in androgen-independent cell lines than in androgen-dependent cell lines; Overexpression of KLF9 dramatically suppresses the proliferation, anchorage-independent growth, and induces apoptosis in androgen-dependent cells; KLF9 inhibition on prostate cancer cell growth may be acting through the AR pathway. Our results therefore suggest that KLF9 may play a significant role in the transition from androgen-dependent to androgen-independent prostate cancer and is a potential target of prevention and therapy.

1. Introduction

Prostate cancer (PCa) is an important health issue in males worldwide, particularly in developed countries, where it is the second leading cause of cancer related deaths in men; 191,930 new prostate cancer cases and 33,330 deaths are projected to occur in America in 2020 \cite{1}, and the incidence of PCa in recent years has also increased in China \cite{2}. PCa growth and progression is initially dependent on androgens, which formed the basis for endocrine therapy for PCa; however, most PCa after endocrine therapy will eventually develop independent of androgens and become resistant to anti-androgen therapy and referred to as castration-resistant prostate cancer (CRPC) \cite{3-5}. Multiple potential mechanisms for the development CRPC have been reported including but not limited to androgen receptor (AR) amplification, increased AR sensitivity, AR mutations, co-regulator alterations and the outlaw pathway \cite{5}. Nevertheless, the detailed mechanisms underlying the proliferation, migration and invasion in the development of CRPC has been proven to be extremely complex with much still unknown.

To better understand this transition to CRPC, it is important to explore gene products that are involved in the transition from androgen-dependent to CRPC to further understand the PCa progression and develop effective treatments. Kruppel-like factor 9 (KLF9), also known as basic transcription element-binding protein 1 (BTEB1), is one of the 17 mammalian KLF family numbers, which have been reported to regulate diverse biological processes, including development, differentiation and programmed cell death \cite{6,7}. Some KLFs have been shown to be involved in the carcinogenesis, progression and cell apoptosis of PCa \cite{8-10}. For instance, KLF6 is often mutated or deleted in PCa and can induce apoptosis and inhibit tumor growth in PCa cells \cite{8,9}; KLF8 was also found to show differential expression in tumor and non-tumor
tissues and be an AR co-activator in human PCa [10]; we have also found that KLF9 can act as a tumor suppressor gene in androgen-independent PCa cells [11]. Previous studies have found that KLF9 is downregulated in endocrine-related cancers of the female reproductive system, and reactivation of KLF9 can suppress cancer cell proliferation through estrogen receptor (ER) or progesterone receptor (PR) signaling in ovarian cancer and endometrial cancer [12,13]. However, the role of KLF9 in androgen-dependent PCa cell, especially in the transition from androgen-dependent to androgen-independent PCa remains unclear. Thus, we hypothesize that KLF9 may play a role in the progression of CRPC.

This current study has revealed that ectopic expression of KLF9 suppresses proliferation, anchorage-independent growth, and induces apoptosis in LNCaP cells, and KLF9 inhibition on LNCaP cell growth may be acting through the AR pathway. We speculate that KLF9 may participate in the transition from androgen-dependent to androgen-independent PCa.

2. Materials and methods

2.1. Cell culture and reagents

The human prostate RWPE-1, LNCaP and PC3 cell lines were all purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); PC3 and RWPE-1 cells were cultured in DMEM (HyClone, Logan, UT, USA) and LNCaP cells maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), cells were grown in a humidified atmosphere of 5% CO2 at 37 °C. The drugs (flutamide, doxycycline, dihydrotestosterone and puromycin) used in the experiment were obtain from Sigma (Sigma-Aldrich, St Louis, MO, USA). They were dissolved and used in the experiment following the manufacturer’s protocols.

2.2. Lentivirus infection

For establishing the KLF9-overexpressing sublines, a FLAG-tagged human KLF9 cDNA was cloned into the lentiviral vectors pTRIPZ (Open Biosystems, Huntsville, AL, USA), which had a tetracycline (Tet)-induced expression vector. The cell transfection with Lipofectamine 2000 reagent (Invitrogen) was performed according to the manufacturer’s instructions and the HEK293T cell was used for lentiviral transfection. The lentivirus-infected cells were screened with puromycin (2 μg/ml) (Sigma-Aldrich, St Louis, MO, USA). They were dissolved and used in the experiment following the manufacturer’s protocols.

2.3. Cell viability assay

In this study, MTT assay was used to detect the growth ability of the cells. The cells at a density of 1000 cells/well were inoculated in 96-well plate at the final volume of 200 μl, and then reagents or inducers were added after 24 h. After that, cells were cultured in a medium containing MTT (5 mg/ml in PBS) (Sigma-Aldrich, St Louis, MO, USA) for 4 h, and the dimethylsulfoxide (DMSO) (150 μl) was added to dissolve the crystals in each well, and then continued to incubate at 37.0 °C for 10 min. The absorbance (OD) was tested at 540 nm wavelength. The reagent (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT)) used in this experiment was acquired from Sigma (Sigma-Aldrich, St Louis, MO, USA).

2.4. Colony formation assay

The cells were inoculated in 6-well ultra-low attachment culture dishes (Costar, Corning, NY) at a density of 1000 cells/well overnight and medium was replaced with medium containing doxycycline (0.5 μg/ml), and continued to culture with 5% CO2 at 37.0 °C for 10 days. The culture medium was removed, washed with PBS and fixed with methanol, then stained with crystal violet solution at room temperature for 3 h. The images were collected by a scanner and the number of colonies (>100 μm diameter) was calculated after washing 3 times and open-air drying.

2.5. Flow cytometric assay

The cell apoptosis was tested by flow cytometry with Annexin V/7-AAD staining (BD Biosciences, San Jose, CA, USA). Cells were inoculated in 12-well plates at a density of 2 × 104 cells/well overnight, and then medium was replaced by that containing reagents or inducers. After treatment for the indicated times, the cells were collected, washed with PBS and resuspended in binding buffer, and then were added by Annexin V (2.5 μl/100 μl) and 7-AAD (1 μl/ml), and cells were continued to culture for 20 min at room temperature. The cells were tested by flow cytometry (BD Biosciences, San Jose, CA) at last.

2.6. Real-time PCR analysis

The total RNA was isolated from cell lines as well as cultured cells using the RNeasy Reagent (TaKaRa, Dalian, China) after the specified treatment, and then reverse transcribed to cDNA with PrimeScript RT Master kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. SYBR Green PCR Master Mix (TaKaRa, Dalian, China) was used to carry out quantitative real-time PCR (qRT-PCR) on 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The fold changes were calculated using 2−ΔΔCt method in gene expression. Human primers sequence used were: KLF9, 5′- CCCCCATCTGGCGCTG TGGGAAATGC-3′ (Forward) and 5′- TTTTTAAGGCACTGTACCAGGT GC-3′ (Reverse); GAPDH, 5′- TCCTGTTCAGACGTACCGCGA -3′ (Forward) and 5′- ACCAGGGCCAATACGACCAA-3′ (Reverse). Human GAPDH was measured as an internal control to normalize the samples.

2.7. Western blot analysis

Cells were collected and lysed in RIPA lysis buffer (Beyotime, Nan tong, China) with a mixture of Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Monza, Italy). The protein concentration was detected by BCA method to standardize the number of samples isolated in SDS-PAGE. The total RNA was isolated from cell lines as well as cultured cells using the RNAiso Reagent (TaKaRa, Dalian, China) after the specified treatment, and then reverse transcribed to cDNA with PrimeScript RT Master kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. SYBR Green PCR Master Mix (TaKaRa, Dalian, China) was used to carry out quantitative real-time PCR (qRT-PCR) on 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The fold changes were calculated using 2−ΔΔCt method in gene expression. Human primers sequence used were: KLF9, 5′- CCCCCATCTGGCGCTG TGGGAAATGC-3′ (Forward) and 5′- TTTTTAAGGCACTGTACCAGGT GC-3′ (Reverse); GAPDH, 5′- TCCTGTTCAGACGTACCGCGA -3′ (Forward) and 5′- ACCAGGGCCAATACGACCAA-3′ (Reverse). Human GAPDH was measured as an internal control to normalize the samples.

2.8. Statistical analysis

Statistical analysis was applied on SPSS software (Version 16.0). Differences between groups were analyzed using Student’s t-test or one-way ANOVA. The data were shown as mean ± SD of three independent experiments. A P-value of <0.05 was defined as statistically significant.

3. Results

3.1. KLF9 is downregulated in androgen-dependent prostate cancer LNCaP cells

In order to determine the expression of KLF9 in variable human
prostate cell lines, we compared the expression levels in three representative prostate lines; normal prostate epithelial cell line RWPE-1, androgen-dependent PCA cell line LNCaP and androgen-independent PCA cell line PC3 by qRT-PCR and Western Blot. Interestingly, we discovered that the levels of KLF9 were distinctly lower in PCA cell lines LNCaP and PC3 than those in normal prostate epithelial cell line RWPE-1 (P < 0.01 and P < 0.001, Fig. 1A). Additionally, we found that the mRNA expression level of KLF9 was notably lower in androgen-independent PCA cell line PC3 when compared with androgen-dependent PCA cell line LNCaP (P < 0.001, Fig. 1A). We also confirmed these findings at the protein level by Western blot (Fig. 1B). These data show that the expression of KLF9 is low in androgen-independent prostate cancer cells, and even lower in androgen-independent prostate cancer cells.

3.2. Forced expression of KLF9 suppresses proliferation of androgen-dependent LNCaP cells

To further investigate the biological function of KLF9 in androgen-dependent LNCaP cells, lentiviral vectors carrying a tetracycline (Tet)-induced-KLF9 expression cassette (Tet-KLF9) and the control (Tet-empty) were transduced into LNCaP cells, and established stable doxycycline-induced KLF9-overexpressing cell lines (Fig. 2A). We next compared proliferation of LNCaP cells with KLF9 overexpression to control cells by MTT assay; Forced expression of KLF9 (Tet +) significantly decreased proliferation of LNCaP cells (P < 0.05, Fig. 2B), and showed a more pronounced difference over time (P < 0.01, Fig. 2B); KLF9 overexpression completely abolished evidence of proliferation by day 7 (P < 0.001, Fig. 2B).

3.3. Overexpression of KLF9 inhibits colony formation in LNCaP cells

We next measured the effects of KLF9 overexpression on LNCaP cells ability to form colonies by colony formation assay, to assess the long-term effects of KLF9 induction on cell growth and proliferation capacity. After 10 day incubation, LNCaP/KLF9 cells in the presence of doxycycline showed a strong visible reduction in colony formation compared to control cells (Fig. 2C). Doxycycline was confirmed to have no effect on empty control vector cells or native LNCaP cells (Fig. 2C). Quantification of total colonies formed in the LNCaP cells with KLF9 induction (Tet +) were significantly reduced by approximately 80%, compared with the cells without induction (Tet -) (P < 0.001, Fig. 2D). Overall, these results confirm that KLF9 overexpression strongly inhibits proliferation and colony formation of androgen-dependent LNCaP cells.

3.4. KLF9 induces cell apoptosis in androgen-dependent LNCaP cells

Next, we wanted to determine if the reduction of proliferating LNCaP cells due to KLF9 overexpression was affected by activation of cell apoptosis. We next performed Annexin V and 7-AAD staining in LNCaP cells with and without KLF9 overexpression (Fig. 3A). Staining showed evidence of a significant increase in apoptosis in LNCaP/KLF9cells (Tet +) (27% ± 1.65%) compared with control cells without doxycycline (8% ± 0.69%, P < 0.001, Fig. 3B) after 72 h incubation. Additionally, we showed overexpression of KLF9 in LNCaP cells significantly decreased the level of the anti-apoptotic protein, Bcl2 and increased the expression of the apoptosis-related protein, cleaved PARP by Western Blot analysis (Fig. 3C). Taken together, these findings distinctly demonstrate that KLF9 could induce cell apoptosis and negatively influence the survival of androgen-dependent LNCaP cells.

3.5. KLF9 overexpression inhibits AR activation in LNCaP cells

In a previous study, we found that the expression of KLF9 was especially low in androgen-independent PCA cell line PC3 compared with LNCaP cells; and also the androgen receptor (AR) antagonist flutamide could affect the expression of KLF9 in LNCaP cells [11]. Activation of the AR pathway has been extensively demonstrated to accelerate the proliferation of PCa cells [5, 14, 15]. Consequently, we next aimed to determine if there was an impact on AR expression by overexpression of KLF9. Western Blot analysis revealed that overexpression of KLF9 in LNCaP cells significantly decreased the expression of AR, decreased the level of Bcl2 and increased the expression of cleaved PARP in LNCaP/KLF9 cells compared to cells without induction (Tet -) (Fig. 3C). To further explore the connection between AR and KLF9, LNCaP cells were treated with flutamide with or without the AR agonist dihydrotestosterone (DHT) and lysesates were collected for Western Blot analysis. Treatment with flutamide alone induced cell apoptosis, increased the expression of cleaved PARP in LNCaP cells, decreased AR expression and increased KLF9 expression (P < 0.05, Fig. 4A and B). Furthermore, dihydrotestosterone (DHT) treatment significantly reduced KLF9 expression in the cells treated with flutamide and also elevated the expression of androgen receptor (AR) (P < 0.05, Fig. 4A and B). These results indicate a connection between the expression of AR and KLF9, and that the AR pathway may attribute to the action of KLF9 in androgen-dependent PCA cells.

4. Discussion

Krüppel-like factors (KLFs) are transcription factors containing an evolutionarily conserved zinc finger, which can bind GC-rich regions in DNA through three C2H2-type zinc fingers and regulate a variety of biological processes including proliferation, development, differentiation, growth, and cell death [6]; It is reported that dysfunction of KLFs are associated with many human diseases including obesity, cardiovascular disease, inflammatory and cancer [6,16]. As one of the 17 mammalian KLF family numbers, increasing evidence suggests that KLF9 plays a critical role in carcinogenesis and cancer progression [16–21]. In this study, we found that the expression of KLF9 is also low in androgen-dependent PCA cell line LNCaP compared to benign
prostate cells; Overexpression of KLF9 inhibited the growth of LNCaP cells and induced cell apoptosis, and these effects may be regulated via the AR pathway; we speculate that KLF9 may be participating in the transition of PCa cells from androgen-dependent to CRPC.

Previous studies have provided data on the effect of KLF9 on inducing apoptosis and reducing cell viability in several types of cancer cell lines [19,20]. It is demonstrated that KLF9 has been displayed to regulate keratinocytes proliferation in a daytime dependent way [22], and affect adipocyte differentiation through modulating PPARγ transactivation [23]. Recently, KLF9 was also reported to be a novel transcriptional regulator of bortezomib- and LBH589-induced apoptosis in multiple myeloma cells [18]. In our previous study, we have observed

Fig. 2. Effects of KLF9 on cell proliferation and colony formation of androgen-dependent LNCaP cells.

The cells were cultured in the presence (Tet +) or absence (Tet -) of doxycycline at a concentration of 0.5 μg/ml for 72 h and the levels of KLF9 were determined by western blot (A). MTT assay was used to detect the growth ability of the cells at the specified time (B). The number of colonies (>100 μm diameter) was calculated after washing 3 times and open-air drying and the images were collected by a scanner. The left side (C) shows the representative plate, and the right side (D) shows the quantitative analysis of the number of colonies in each group. Data represents mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the control group.
that KLF9 was downregulated in prostate carcinoma cell lines compared to the benign cell lines, and could suppress tumor growth of androgen-independent PCa cells in vitro [11]. Meanwhile, it was also revealed that miR-141-3p promoted PCa cell proliferation through inhibiting KLF9 expression in PCa [24], suggesting that KLF9 may play a key role in the PCa progression [11, 24].

Consistent with the previous studies, our results showed that KLF9 had different expressions between androgen-dependent and -independent cell lines. Forced expression of KLF9 could significantly reduce cell viability and induce apoptosis in androgen-dependent LNCaP cells. This notion may be related to the AR pathway. Additionally, previous data showed that KLF9 was involved in carcinogenesis and progression of some endocrine-related cancers of the female reproductive system, including ovarian cancer, endometrial cancer and breast cancer [12, 16, 19, 25]. For instance, KLF9 may function at the node of progesterone receptor (PR) and estrogen receptor (ER) genomic pathways to influence cell proliferation and act as a transcriptional repressor of ER signaling [13], indicating that KLF9 can decrease endocrine-related cancer cell development via the ER or PR pathway.

Androgen has been elucidated to promote the occurrence of PCa, and it has been indicated that androgen requires AR to facilitate growth of PCa [26]. The previous studies showed that AR pathway was essential for the proliferation of the androgen-dependent PCa cells [14, 15, 27]. As one kind of endocrine-related cancer, we observed in this study that the expression of AR was significantly inhibited by overexpression of KLF9; meanwhile, dihydrotestosterone treatment elevated the expression of AR and also reduced KLF9 expression in the cells treated with flutamide. These data suggested that KLF9 can influence AR and suppress the
development and growth of androgen-dependent PCa cells and even induce cell apoptosis. Although more work is needed to elucidate the specific mechanism between KLF9 and the AR pathway, evidence based on our results suggests that KLF9 may play a role in the progression of PCa from the androgen-dependent to CRPC. Related issues remain to be elucidated for our further in-depth research in future, and our current explorations provide a foundation for the further investigation.

In summary, our results demonstrate new insight into the function of KLF9 in the development and progression of PCa. Forced expression of KLF9 dramatically suppresses growth of LNCaP cells and induces cell apoptosis. Additionally, these effects were associated with the inhibition of AR pathway. These results suggest that downregulation of KLF9 may play an important role in the progression of PCa from the androgen-dependent state to CRPC. Hence, KLF9 may be a promising therapeutic target for advanced PCa treatment in the future.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 4. KLF9 downregulates AR expression in the process of apoptosis in LNCaP cells.

The cells were cultured for indicated times after the specified treatment, and the lysates were analyzed by Western Blot with the antibodies designated on the left. Cells were grown in medium supplemented with 10% charcoal-stripped FBS with flutamide (Flu) (20 μM) or dihydrotestosterone (DHT) (5 nM) for 72 h. (A) The relative changes of KLF9 mRNA were detected by qRT-PCR. (B) The expression of related proteins and AR protein were measured by Western blot with the antibodies designated on the left and the quantification of protein expression was also analyzed. Tubulin was used as loading control. *P<0.05 and **P<0.01 compared with the control group. AR, androgen receptor; Flu, flutamide; DHT, dihydrotestosterone.
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