Cancer Letters 330 (2013) 217–224

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

HoxB3 promotes prostate cancer cell progression by transactivating CDCA3

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ARTICLE INFO

Article history:
Received 29 August 2012
Received in revised form 30 October 2012
Accepted 28 November 2012

Keywords:
HoxB3
CDCA3
Transactivation
Prostate cancer
Prognosis

ABSTRACT

Homeobox (Hox) genes encode homeodomain-containing transcription factors critical to development, differentiation, and homeostasis. Their dysregulation has been implicated in various cancers. In the present study, we show that HoxB3 mRNA and protein are overexpressed in primary prostate cancer tissues compared to the adjacent normal prostate tissues. Moreover, HoxB3 overexpression is associated with higher Gleason grade ($\geqslant 7$) ($P = 0.002$), clinical stage ($P < 0.001$) and PSA level ($\geqslant 10$) ($P = 0.013$). The Kaplan and Meier analysis showed that HoxB3 overexpression predicts poor survival outcome. Overexpression of HoxB3 promotes LNCaP cells proliferation and migration *in vitro*. Furthermore, depletion of HoxB3 in PC-3 cells decreased the capacity of proliferation in a cell division cycle associated 3 (CDCA3)-dependent manner both *in vitro* and *in vivo*. The ChIP analysis indicates that HoxB3 can bind to the CDCA3 promoter region and transactivate the CDCA3 expression. These data suggested that HoxB3 promote prostate cancer progression by upregulating CDCA3 expression and may serve as a potential therapeutic target for human prostate cancer.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of death in males in the US world [1]. There are currently no effective pharmacologic therapies for primary or recurrent prostate cancer. While androgen deprivation therapy provides a temporary inhibition of the cancer growth before castration-resistant prostate cancer (CRPC), the cancer quickly progresses to CRPC for which no known effective therapy is currently available. Moreover, no effective pharmacologic treatments exist for elimination of residual cancer cells after prostate cancer surgery. Therefore, understanding the molecular basis of the disease is highly desirable for developing newer strategies for prevention and treatment of prostate cancer.

The homeobox genes, the first identified for their role in axial patterning, encode a family of highly conserved transcription factors that play fundamental roles during embryonic development [2–4]. These genes contain a highly conserved homeobox sequence, which encodes a 60 amino acid homeodomain. Structural analyses have shown that the homeodomain consists of an evolutionarily conserved helix-turn-helix motif that binds to the DNA [5]. Hox genes are also important in hematopoiesis, suggested by their characteristic expression profiles in hematopoietic cells of different lineages or at different stages of maturation [6,7]. A gradual decrease of Hox expression is a characteristic feature of hematopoiesis, evidenced by a high level of Hox expression in hematopoietic stem cells and undetectable expression in differentiated cells. The impact of individual Hox genes on self-renewal, proliferation, and lineage differentiation in hematopoietic cells at different stages of maturation has been studied in overexpression and knock-out models [8–11]. There is indication that the abnormal expression and function of Hox expression plays a key role in the development or progression of many kinds of human cancers [11–19]. Cell division cycle associated 3 (CDCA3) contains an F box motif and bind to Skp1 and cullin, a component of Skp1-cullin-F-box. Previous microarray analysis indicated that CDCA3, referred to as a trigger of mitotic entry, mediates destruction of mitosis and the inhibitory kinase by the E3 ligase, SCF [20–22]. Furthermore, CDCA3 was upregulation in the oral squamous cell carcinoma-derived cells [23]. However, the HoxB3 and CDCA3 expression in prostate cancer are not well known.

In the present study, we evaluated the expression level of HoxB3 in prostate cancer and normal tissues. We also carried out retrospective follow-up analysis to explore the correlation between HoxB3 expression and survival outcome of patients with prostate cancer. Molecularly, our results indicated HoxB3 promote prostate cancer progression by upregulation of CDCA3 expression. Thus, HoxB3 may serve as a potential therapeutic target for human prostate cancer.

2. Materials and methods

2.1. Cell culture and human samples

LNCaP and PC-3 prostate cancer cells were obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences and maintained in RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 2 mmol/L L-glutamine (Gibco), and 1% penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere.

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http://dx.doi.org/10.1016/j.canlet.2012.11.051

Prostate cancer tissue samples were obtained from patients who had undergone radical prostatectomy for prostate cancer. None of the patients included in this study had received preoperative hormonal therapy, chemotherapy, or radiation therapy. 100 primary cancers and 30 paired normal adjacent tissues were examined. Disease-free survival (DFS) was defined as the time interval between primary surgery and any relapse (local–regional, contra-lateral and/or distant), or terminal time of follow-up without any relapse events. Collection of tissue and use for this study were approved according to standard guidelines by the ethics committee of the Secondary Hospital of Tianjin Medical University and written consent was obtained from all participants. After radical prostatectomy, tissues were flash-frozen in liquid nitrogen and stored at −80 °C.

2.2. Plasmids construction and generation of stable cell lines

The full-length HoxB3 and CDCA3 cDNA were reverse transcriptase-polymerase chain reaction using total RNA from PC-3 cell line. The primer sequences were as following: HoxB3, 5′-GAATTCATGCAGAAAGCCACCTACTAC-3′ (forward) and 5′-CTCGAGTCACAGGTGTGTTAATTTG-3′ (reverse); CDCA3, 5′-GAATTCGGTTGAGATGGGCTCAGCCAA-3′ (forward) and 5′-TCTAGAGCCCTGGGTGACTGCATTGCT-3′ (reverse). The PCR products were cloned into EcoR I and Xho I sites of the mammalian expression vector pcDNA3.1 (+) (Invitrogen) (pcDNA3.1-HoxB3) or EcoR I and Xba I sites of pcDNA3.1 (+) (pcDNA3.1-CDCA3). The HoxB3 shRNA plasmid was purchased from Santa-Cruz biotechnology. *CDCA*3 promoter region (−260 to −1) was amplified from genomic DNA PC-3 cells and the fragment was cloned into the Bgl II and Kpn I restriction sites in the luciferase reporter plasmids pGL3-basic vector (Promega) (pGL3-CDCA3). All constructs were fully sequenced. Following the manufacturer's instructions, two independent LNCaP stable transfectants were screened for overexpressed HoxB3 and two independent PC-3 stable transfectants were screened for depleted HoxB3 after about three weeks selection in 800 μg/ml G418 (Calbiochem). Transfectants were routinely cultured under selection. The phenotype of selected clones and parental cells were showed in Fig. S1.

2.3. Tissue RNA isolation and reverse transcription quantitative real-time PCR

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions. 5 μg of total RNA was used to perform reverse transcribed by using SuperScript II and oligo dT following the manufacturer recommendations (Invitrogen). The reverse transcription quantitative real-time PCR (RT-qPCR) analysis was performed using the Fast SYBR Green MasterMix System (Invitrogen) according to the manufacturer's instructions. Primers for HoxB3, CDCA3, and GAPDH were as follows: HoxB3, 5′-GGCAAACGTCCAAGCTGAA-3′ (forward), 5′-CTCCAGCTCCACCAGCTGCG-3′ (reverse); CDCA3, 5′- TGGTATTGCACGGACACCTA-3′ (forward), 5′-TGTTTCACCAGTGGGCTTG-3′ (reverse); GAPDH, 5′-GAAATCCCATCACCATCTTCCAGG-3′ (forward), 5′- GAGCCCCAGCCTTCTCCATG-3′ (reverse). The PCR conditions were as follows: 95 °C for 20 s; 95 °C for 3 min; 62 °C for 30 s; for 40 cycles by using ABI 7500 (Applied Biosystems). The relative quantification was given by the CT values, determined by triplicate reactions for all of the samples for HoxB3, CDCA3 and GAPDH. The triplicate CT values of detectable gene were averaged, and the CT value of GAPDH was subtracted to obtain ΔCT. The relative mRNA expression level of HoxB3 and CDCA3 was determined as $2^{-\Delta C_T}$.

2.4. Immunoblotting analysis

A quantity of 30 μg of lysates per sample was separated by SDS–PAGE using 10% polyacrylamide gels and transferred to PVDF membrane which was subsequently incubated with rabbit polyclonal antibody to HoxB3 (1:2000, Abcam) or CDCA3 (1:2000, Abcam) for 4C overnight, and corresponding were immunodetected by incubation with HRP (horseradish peroxidase)-linked anti rabbit secondary antibody (1:3000, sigma) using an ECL detection kit (Pierce Biotechnology). Rabbit polyclonal antibody to β-actin (1:5000, Sigma) was used as gel loading control.

2.5. ChIP and Dual-luciferase assays

Chromatin immunoprecipitaion (ChIP) analysis was carried out using ChIP Assay kit (Upstate). Briefly, about 2×10^7 PC-3 cells were crosslinked by 1% formaldehyde and resuspended, lysed in lysis buffer, then sonicated to shear the DNA to a range of 500 bp to 1000 bp. The chromatin fraction was immunoprecipitated by antibody against HoxB3 (Abcam), and the HoxB3-antibody/DNA complex was enriched by packed beads with protein A Agarose/Salmon sperm DNA. Beads were collected and washed serially by buffer with low salt, high salt, LiCl and TE. For a negative control, an equal amount sample of no-antibody immunoprecipation G (IgG) was also prepared. After reversal of crosslinking, chromosomal DNA was purified by using MinElute PCR Purification Kit (Qiagen) according to the manufacture's instruction.

For luciferase assay, 5×10^4 cells per well in 12-well plates were cultured without antibiotics overnight and then transfected with pGL3-CDCA3 and pcDNA3.1-HoxB3. After 24 h, cells were washed with phosphate-buffered saline (PBS), subjected to lysis, and their luciferase activities measured by using a dual luciferase assay kit (Promega). The results were normalized against *Renella* luciferase. All transfections were performed in triplicate.

2.6. Cell proliferation, migration and scratch assays

Both MTT and colony formation assays were used to observe and compare cell proliferation ability. Briefly, 2×103 cells in 200 μl culture medium were plated into a well of 96-well plates. After culturing cell for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and continued to culture for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a microplate autoreader (Bio-Rad, Hercules). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value. For colony formation assay, cell suspensions were seeded into 6-well plates, 300 cells per well. After incubated, cells were fixed in methyl hydrate for 10 min. Colonies were then stained and counted using an optical microscope.

Cell migration was assayed using the 8 μm pore polycarbonate membrane Transwellchambers (Costar). 1×10^5 cells were cultured in the upper chamber with serum-free medium. The lower chamber contained complete medium (10% fetal bovine serum). After incubation at 37 °C, 5% CO_2 for 12 h, cells adherent to top surface of the membrane were removed with a cotton applicator, whereas cells migrated to bottom surface were fixed with 70% methanol and stained with crystal violet. The migrated cells on the bottom surface of the membrane were photographed and counted on an inverted microscope.

LNCaP and transfectants were seeded in 6-well plates and cultured for 72 h to obtain 80% monolayer confluency. A scratch was created by scraping the cells using a plastic pipette tip, and the medium was replaced with fresh medium. Images were captured immediately (0 day) and every days for 4 days. Cell migration was qualitatively assessed by the size of the wounds at the end of the experiment.

2.7. Cell cycle analysis

Cell cycle phase distribution was assayed by flow cytometry [24]. Cells were incubated with propidium iodide (PI, 50 μg/ml) for 30 min the following day. Flow cytometric analysis was performed on a Beckman Coulter EPICS analyzer (Krefeld).

2.8. Xenografts assays in nude mice

PC-3 and transfectants were (1×10^6) suspended in 0.1 mL of serum-free RPMI 1640 was injected into the right subaxillary of each mouse. Seven weeks after inoculation, mice were killed, and the final volume of tumor tissues was determined.

2.9. Statistical analysis

Survival analysis was carried out according to the methods of Kaplan and Meier. All calculations were performed with the SPSS for Windows statistical software package (SPSS Inc.). Results of *in vitro* and *in vivo* experiments were depicted as mean ± SD. Student's two-sided *t*-test was used to compare values of test and control samples. HOxB3 expression was dichotomized at the median, which is the most conservative cutoff point for categorizing a continuous variable [25]. The level of significance was set to $P < 0.05$.

3. Results

3.1. HoxB3 is overexpression in primary prostate cancer and predicts poor outcome

To explore the HoxB3 expression in human carcinogenesis, we searched online ONCOMINE database. The microarray data showed increased expression of *HoxB*3 mRNA in a variety of cancer tissues compared to normal tissues (Fig. S2). To determine if HoxB3 has a role in human prostate carcinogenesis, we performed RT-qPCR to detect the *HoxB*3 mRNA expression in the primary prostate cancer tissues and the paired normal adjacent tissues. Our results revealed that expression levels of *HoxB*3 mRNA were upregulated in 28 of 30 primary prostate cancer tissues compared with the paired adjacent normal tissues ($P < 0.001$, Fig. 1A and B). To further investigate if HoxB3 protein levels were also upregulated in human prostate cancer, we examined expression levels of HoxB3 protein in 4 matched samples of human primary prostate tumor (T) and adjacent histologic normal tissues (N) using immunoblotting and immunochemistry. As shown in Fig. 1C, HoxB3 protein levels were upregulated in all primary prostate cancer tissues.