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Restoration of cyclin D2 has an inhibitory potential on the proliferation of LNCaP cells

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ABSTRACT

Despite well known oncogenic function of G1–S cell-cycle progression, *cyclin D2* (*CCND2*) is often silenced epigenetically in prostate cancers. Here we show that CCND2 has an inhibitory potential on the proliferation of androgen receptor (AR)-dependent prostate cancer LNCaP cells. Forced expression of CCND2 suppressed the proliferative ability and induced cell death in LNCaP cells in a cdk-independent manner. Knocking down CCND2 restored the proliferation of LNCaP subclones with relatively high CCND2 expression and low proliferative profiles. Immunoprecipitation using deletion mutants of CCND2 indicated that a central domain of CCND2 is required for binding to AR. A deletion mutant lacking the central domain failed to hinder LNCaP cells. Collectively, our results indicated that CCND2 inhibits cell proliferation of AR-dependent prostate cancer through the interaction with AR. Our study suggests that restoration of CCND2 expression potentially prevents the carcinogenesis of prostate cancer, which is mostly AR-dependent in the initial settings.

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy among men in industrialized countries and the second leading cause of cancer related death in the United States [1,2]. Gene methylation and subsequent silencing of tumor suppressor genes are considered to play an important role in the molecular mechanisms for carcinogenesis of prostate cancer [3]. It is reported that certain epigenetic modulators including histone deacetylases (HDAC) and DNA methyltransferases (DNMT) are aberrantly activated in the early step of prostate carcinogenesis [4]. The absence of tumor suppressor genes including *GSTP1* [5], *PTEN* [6], *CDKN2* [7] and *E-cadherin* [8] which are frequently down-regulated in prostate cancer and adjacent precursor lesions, can be attributed at least partially to hypermethylation of the CpG island sequences encompassing the regulatory region resulting in prevention of the transcription of the genes, as well as allelic loss of the genes.

Cyclin D2 (CCND2) gene, a member of type-D cyclin family located at 12p, consists of five exons, holds CpG islands around exon 1, and is one of the common genes which are silenced by epigenetic modulation in prostate cancer [9,10]. Padar and colleagues examined 101 prostate cancer and 32 nonmalignant prostate tissue samples by methylation-specific PCR and reported that the frequency of methylation was significantly higher (32%) in prostate cancers than in nonmalignant prostate tissues [9]. Indeed, expression of CCND2 is progressively suppressed according to development and progression of prostate cancer opposite to that of CCND1 [11].

Well known function of D-type cyclins includes binding to and activating cyclin-dependent kinase (cdk) 4/6 [12]. Activated CCND2-cdk4/6 complex phosphorylates *retinoblastoma* (Rb) protein resulting in releasing and up-regulating transcription factor E2F, which targets following cell-cycle accelerators including cdk2, cdc25, cyclin A and cyclin E. This series of action is essential for cell-cycle progression from G1- to S-phase, and therefore, CCND2 is a potentially oncogenic protein.

There is an unexplained gap that *CCND2* is an oncogene but is frequently silenced in prostate cancer. Prostatic tissue developing adenocarcinoma is frequently in hypermethylation status and DNA methylation seems to be accumulated as it develops adenocarcinoma [13,14]. Therefore, CCND2 gene silencing can be just a result of hypermethylated tendency and has no significant role driving prostate carcinogenesis. On the other hand, it can be oncogenic and drive prostatic epithelial cells for carcinogenesis, if CCND2 has any anti-oncogenic function. Here we address this issue and demonstrate that CCND2 has a novel inhibitory function on

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androgen-dependent proliferation through biding to the androgen receptor.

Materials and methods

Antibody and reagents. Anti-HA antibody was purchased from Covance (Berkeley, CA), and anti-CCND2 (M-20, sc-593), anti-androgen receptor (C-19, sc-815), anti-PSA (C-19, sc-7638) and anti-β-tubulin (D-10, sc-5274) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin (ab6276) was purchased from Abcam (Cambridge, MA). Anti-phospho-pRb (Ser 780, #9307) antibody was obtained from Cell Signaling Technology (Beverly, MA). R1881 (methyltrienolone) was purchased from DuPont Merck Pharmaceutical (Boston, MA), 5-Aza-2′-deoxycytidine (5-aza-DC) and trichostatin A (TCA), from Sigma, and BD Matrigel, from BD Bioscience (Bedford, MA).

Cell culture, cell counting, colony-forming assay, apoptosis assay. Methods for cell culture are described elsewhere [15]. For cell count assay, cells were inoculated to 6- or 10-cm dishes at 5.0×10^5 cells/plate. After 24 h incubation for adhesion, the number of cells was counted for baseline (day 0) and on the days 2, 4, 6 and 8. Cells were collected by trypsinization and counted using hemocytometer. In colony-forming assays, cells (1×10^5) were seeded on 6-cm dishes and cultured in puromycin-containing (2 μg/ML) medium for 2 weeks. The cells were washed with phosphate-buffer saline (PBS), fixed with neutral-buffered formaldehyde (10%), stained with 0.1% of crystal violet-H_2O. Apoptosis assays were done as described previously [16].

Expression construct, transfection and retrovirus infection. Expression constructs including mammalian expression vectors and retrovirus vectors are described in Supplementary Information. Transfection of the plasmids was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. To establish stable expression subclones from PC3 cells, cells were cultured in RPMI supplemented with 10% FBS containing 1 mg/ML G418 three days after transfection. Colonies were picked up and expression of target gene was confirmed by Western blotting. Retrovirus vectors were transfected along with VSVG (kind gifts of Dr. T. Era, Kobe, Japan) and gag/pol plasmids to obtain the virus using Lipofectamine 2000 reagents (Invitrogen) according to manufacturer's instruction. More than 70% of infection efficiency was confirmed by counting GFP-positive cells with fluorescence microscopy three days after retrovirus infection. Cells were cultured in puromycin-containing (2 μg/ML) medium for additional three days, and then subjected to cell count assay, colony-forming assay, reverse transcriptase PCR (RT-PCR) or SDS–PAGE.

In vivo xenograft model for tumorigenesis. PC3 cells were collected by trypsinization and 5×10^6 cells were suspended in 100 μL RPMI/BD Matrigel (1:1) and injected subcutaneously to the right flank of BALB/c AnNCrj nude mice. Tumor volumes were

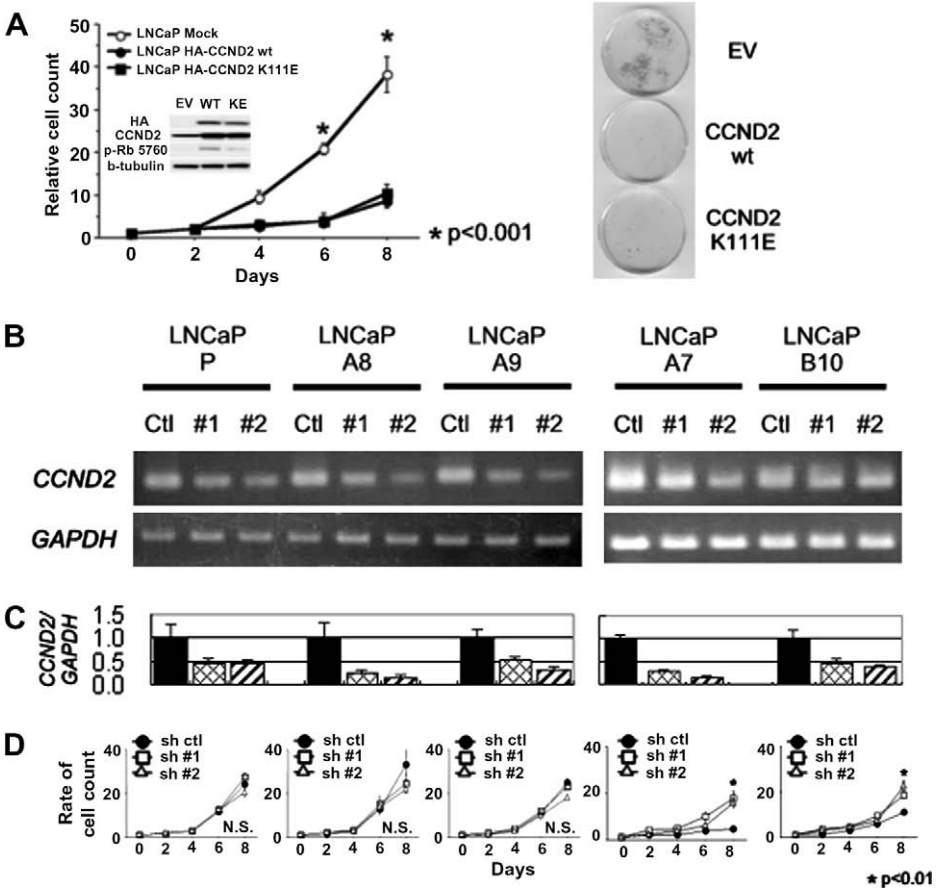

Fig. 1. Overexpression of CCND2 reduces the proliferation of LNCaP cells in culture (A). LNCaP cells were infected by retrovirus for empty vector (EV), HA-CCND2 wt (WT) and K111E mutant (KE) and protein expression was confirmed by Western blotting. For cell count assay, cells were inoculated to 6- or 10-cm dishes at 5.0×10^5 cells/plate and counted on the days 0, 2, 4, 6 and 8. Colony-forming ability of LNCaP cells expressing wild-type (CCND2 wt) or K111E mutant (CCND2 K111E) of CCND2 as well as control cells (EV) was also examined (right). Knocking down of CCND2 in LNCaP subclones with relatively higher expression of CCND2 potentiates their proliferation in culture (B, C and D). Clones A7 and B10 were selected as cells hyperexpressing *CCND2* (Supplementary Fig. 2), while parental LNCaP (LNCaP P) and subclones A8 and A9 served as controls. Results of semiquantitative (B) and quantitative (C) RT-PCR showed successful suppression of *CCND2* expressions by two short-hairpin RNAs. In cell count assays, the cell proliferation rates were significantly increased in clones A7 and B10 infected by shRNA expression retrovirus (D).