Original Article

Androgen receptor functions as a negative transcriptional regulator of DEPTOR, mTOR inhibitor

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ABSTRACT — It has been noticed that crosstalk between androgen receptor (AR) and mammalian target of rapamycin (mTOR) signaling pathways plays a crucial role in the proliferation of prostate cancer cells. To clarify this mechanism, we focused on DEPTOR, a naturally occurring inhibitor of mTOR. The treatment of a human AR-positive prostate cancer cell line, LNCaP, with the AR-agonist dihydrotestosterone (DHT) repressed DEPTOR mRNA expression in a time-dependent manner. This repression was abrogated by treatment with the AR-antagonist bicalutamide. Knockdown of DEPTOR mRNA by siRNA resulted in the increased phosphorylation of 70 kDa ribosomal protein S6 kinase 1 (S6K), a substrate of mTORC1, accompanied by the elevated expression of cyclin D1, a positive regulator of cell proliferation. Furthermore, the ChIP assay demonstrated that AR could bind to AR-responsible element-like region within the 4th intron of the *DEPTOR* gene. The amount of acetylated histone H3 (Lys9, Lys14) was reduced by the DHT treatment in this region. Taken together, these results propose that AR-dependent prostate cancer cell proliferation requires decreased DEPTOR transcription directly controlled by AR.

Key words: Androgen receptor, mTOR, DEPTOR, Prostate cancer

INTRODUCTION

Androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily of ligand-regulated transactivation factors. AR mediates various biological effects such as the development of male reproductive tissues, sexual development, and spermatogenesis (Matsumoto *et al.*, 2003, 2005, 2008; Lee and Chang, 2003). AR also plays a central role in prostate cancer growth and development (Heinlein and Chang, 2004; Dehm and Tindall, 2007; Bennett *et al.*, 2010). In the absence of ligand, AR is localized in the cytoplasm, where it forms a complex with chaperones. Upon binding of ligands, such as testosterone and 5- α -dihydrotestosterone (DHT), AR translocates into the nucleus. Following nuclear translocation, AR homodimer binds to androgen responsive elements (AREs) in the promoter regions of its target genes.

The mammalian target of rapamycin (mTOR) is an atypical serine/threonine protein kinase, interacting with several proteins to form two distinct complexes (mTORC1 and mTORC2) (Laplanche and Sabatini, 2012).

mTORC1 mainly regulates mRNA translation, cell size and cell proliferation by phosphorylation of 70 kDa ribosomal protein S6 kinase 1 (S6K) and eukaryotic translation initiation factor 4E (eIF4E), while mTORC2 regulates cell survival by activation of AKT and the serum and glucocorticoid inducible kinase 1 (SGK1) (Guertin and Sabatini, 2007).

Recently, it was reported that the crosstalk between AR and mTOR played an important role in prostate cancer cell proliferation (Xu *et al.*, 2006). DHT activated mTORC1 and subsequently increased translation of cell cycle positive regulator, cyclin D1, in LNCaP prostate cancer cells. However, the detailed mechanism of AR-dependent regulatory mechanism of mTOR is not yet clear. DEP domain-containing mTOR-interacting protein (DEPTOR) was identified as a naturally occurring inhibitor of mTOR in both mTORC1 and mTORC2 (van Duin *et al.*, 2005; Peterson *et al.*, 2009). DEPTOR is suggested to be a tumor suppressor due to the inhibitory effect on mTORC1 and mTORC2 pathways. Recently, the post-translational regulation of DEPTOR was reported

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(Peterson *et al.*, 2009; Li *et al.*, 2014; Zhao *et al.*, 2011; Gao *et al.*, 2011). DEPTOR was phosphorylated by casein kinase 1 alpha, facilitating ubiquitylation by the F box protein SCF (β TrCP) (Duan *et al.*, 2011). Although post-translational regulation of DEPTOR expression has been investigated, transcriptional regulation of DEPTOR remains unclear.

In this study, we examined whether the *DEPTOR* gene was regulated by AR. We show that DHT down-regulates the transcription of the *DEPTOR* gene by promoting the binding of AR to intronic ARE in prostate cancer cells. This AR-mediated transrepression of the *DEPTOR* gene is a novel finding to explain the mechanism of the cross-talk between AR and mTOR signaling pathways.

MATERIAL AND METHODS

Chemicals

Dihydrotestosterone (DHT), bicalutamide and cycloheximide (CHX) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

Androgen-receptor-negative and -positive prostate cancer cell lines, PC-3 and LNCaP, respectively, and bone-derived cell line, vertebral-cancer of the prostate (VCaP), were cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were treated as described in the figure legends.

Western blotting

Total protein was extracted with SDS sample buffer supplemented with protease inhibitor cocktail and the protein concentration was measured with the Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Hudson, NH, USA). The protein samples were separated by SDS-PAGE. Western blotting was performed with rabbit anti-DEPTOR, anti-pS6K, anti-S6K and anti-cyclin D1 antibodies (Cell Signaling Technology, New York, NY, USA) as primary antibodies and horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling) as a secondary antibody. Anti- α -tubulin pAb-HRP-Direct (MBL, Aichi, Japan) antibody was used as loading control. Protein bands were visualized using the Immobilon Western Detection Reagent (Millipore, Billerica, MA, USA) and then the signals were captured by Typhoon 9500 (GE Healthcare, Piscataway, NJ, USA). Band density was measured by ImageJ software.

Realtime RT-PCR

The cells were seeded in 12-well plates in phenol red-free RPMI containing 5% charcoal-stripped FBS (csFBS; GIBCO, Grand Island, NY, USA). On the next day, the cells were treated with DHT for 24 hr. Total RNA was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative RT-PCR (qRT-PCR) was conducted using KOD SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol and using the 7500 Fast Real-Time PCR System Software (Applied Biosystems, Foster City, CA, USA). The following pairs of primers were used for FKBP51 (forward 5'-TCCCTCGAATGCAACTCTCT-3', reverse 5'-GCCACATCTCTGCAGTCAAA-3'), DEPTOR (forward 5'-AAGCTGATGAGCCCTGAAAA-3', reverse 5'-TTGTTGGACACATGCTGGAT-3') and β -actin (forward 5'-TCCTCCTGAGCGCAAGTACTC-3', reverse 5'-CTGCTTGCTGATCCACATCTG-3').

siRNA-induced mRNA knockdown

LNCaP cells were transfected with DEPTOR-targeting or control small interfering RNAs (siRNAs; Sigma-Aldrich) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

The LNCaP cells were seeded in 100 mm dishes in phenol red-free RPMI containing 5% csFBS. On the next day, cells were treated with DHT or solvent for 6 hr. Chromatin immunoprecipitation (ChIP) assay was carried out using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to manufacturer's protocol. Cells were fixed with 1% formaldehyde (Sigma Aldrich) for 15 min at 37°C and nuclei were prepared. Chromatins were digested with micrococcal nuclease to achieve a DNA ladder of 300-1,000 bp. The chromatins were immunoprecipitated with anti-AR (Abcam, Cambridge, MA, USA), anti-Histone H3, and anti-Acetyl-Histone H3 (Lys9/Lys14) (Cell Signaling Technology) antibodies overnight at 4°C. The precipitated DNA was used for PCR amplification with the following specific primer pairs to ARE regions: FKBP5 ARE (5'-GGAGCCTCTTTCTCAGTTTTG-3' and 5'-CAATCGGAGTGTAACCACATC-3'), DEPTOR promoter (5'-CCATAGAAAAAGATACAGGC-3' and 5'-TCATTCCATGAAATTTGAATG-3'), DEPTOR ARE1 (5'-TCCCCTCTTAATCTGGTTA-3' and 5'-CTAATGGCATACGGGCTTGT-3') and DEPTOR ARE2 (5'-TGAAGACGTTGAGGCATTTG-3' and 5'-ACAG-

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GGCCAAAGCATATGAG-3'). Band density was measured by ImageJ software.

Statistical analysis

Statistical comparisons were performed with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test as the post hoc test, and differences were considered statistically significant.

RESULTS AND DISCUSSION

Androgen receptor dependent repression of DEPTOR mRNA expression in prostate cancer cell lines

Crosstalk between AR and mTOR signaling pathways has been expected. DEPTOR is known as a natu-

rally occurring inhibitor of mTOR via direct binding to both mTORC1 and mTORC2 (Peterson *et al.*, 2009). Therefore, we investigated whether the expression of DEPTOR mRNA was regulated by AR in prostate cancer cell lines. LNCaP cells were treated with DHT for 3, 6 and 24 hr. The amount of DEPTOR mRNA was reduced by the DHT treatment in a time-dependent manner (Fig. 1A), whereas time-dependent transactivation of *FKBP5* gene, one of AR-target genes, was observed. The DHT-dependent repression in DEPTOR mRNA was attenuated by co-treatment with the AR antagonist bicalutamide (Fig. 1B). Similar results were obtained in AR-positive prostate cancer VCaP cells (Fig. 1C). On the other hand, DHT-dependent alternation of DEPTOR mRNA was not observed in AR-negative prostate cancer PC-3 cells (Fig. 1D). The results suggest that negative regulation

Fig. 1. Suppression of *DEPTOR* gene transcription by DHT in an AR-dependent manner. (A) LNCaP cells were treated with 10 nM DHT for the indicated times. (B) LNCaP cells were treated with 10 nM DHT with or without 10 μ M bicalutamide. (C, D) VCaP and PC-3 cells were individually treated with 10 nM DHT for 24 hr. Cells were harvested and the mRNA levels of *DEPTOR* were measured by qRT-PCR, simultaneously with those of known AR-target gene *FKBP51*. Results normalized against those of β -actin are expressed as averaged fold activation over the solvent control of the four individual experiments (mean $\pm$ S.D., $n = 4$); * $P < 0.05$, ** $P < 0.01$.

of DEPTOR mRNA expression by DHT requires AR in prostate cancer cells.

Effect of DEPTOR mRNA knockdown on mTOR signaling pathway in prostate cancer cells

DEPTOR protein expression was suppressed by the DHT treatment for 24 hr in LNCaP cells (Fig. 2A). To confirm that S6K was activated by downregulation of DEPTOR, siRNA for DEPTOR was transfected in LNCaP cells. The siRNA-induced knockdown of DEPTOR mRNA raised not only the phosphorylation level of S6K but also the level of cyclin D1 protein, a positive regulator of cell proliferation (Fig. 2B). Various functions of DEPTOR in cancer progression have been reported. Ectopic expression of the *DEPTOR* gene is known to reduce mTORC1 activity and cell growth in pancreatic cancer cells (Li *et al.*, 2014). Here, we showed that knockdown of DEPTOR mRNA enhanced phosphorylation degree of S6K and protein level of cyclin D1 in prostate cancer LNCaP cells. These observations suggest that downregulation of DEPTOR increases cell proliferation by DHT in AR-positive prostate cancer cells.

Binding of AR to intronic ARE of *DEPTOR* gene

Next, we investigated whether the repression of DEPTOR mRNA by DHT was regulated by *de novo* protein

synthesis. The repressed expression of DEPTOR mRNA by DHT was observed even in the presence of cycloheximide (CHX) (Fig. 3A). This result suggests that the transrepression of the *DEPTOR* gene by DHT does not require the novel protein synthesis. By *in silico*, using published dataset of ChIP-on-chip analysis in LNCaP cells (Wang *et al.*, 2009), we identified two putative AR binding regions, which were named ARE1 and ARE2, within the 3rd and 4th introns, respectively, of the *DEPTOR* gene (Fig. 3B). In order to estimate the binding potential of AR to ARE1 and ARE2, ChIP assay was carried out in the absence or presence of DHT in LNCaP cells (Fig. 3C). It was demonstrated that ARE2 was immunoprecipitated by anti-AR antibody, but not the region or ARE1. Furthermore, the extent of acetylated histone H3 (Lys9, Lys14), which enhanced gene transactivation, was examined by ChIP assay. Whereas total histone H3 level remained unchanged, acetylated histone H3 level was reduced by the DHT treatment in ARE2 region (Fig. 3D). In contrast, acetylated histone H3 level at ARE of *FKBP5* gene was enhanced. It is known that the histone acetylation leads to transcriptional activation. Especially, histone acetylation is dynamically controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Various negatively AR-regulated genes have been reported. In the case of E-cadherin (Liu *et al.*, 2008), activated AR down-

Fig. 2. Reduction in DEPTOR protein level by the DHT treatment and elevation in cyclin D1 protein level by knockdown of *DEPTOR* gene. (A) LNCaP cells were treated with solvent control (sol.) or 10 nM DHT for 24 hr. Whole-cell lysates were separated by SDS-PAGE, and proteins were detected by immunoblotting using antibodies against DEPTOR, pS6K(T389), S6K and tubulin used as a loading control. (B) LNCaP cells were transfected with DEPTOR-targeting or control siRNA. After 48 hr, cells were treated with DHT or sol. for an additional 24 hr. Whole-cell lysates were separated by SDS-PAGE, and proteins were detected by immunoblotting using antibodies against Cyclin D1, DEPTOR, pS6K(T389) and tubulin used as a loading control.

AR downregulates DEPTOR mRNA expression

Fig. 3. Binding of AR to intronic ARE of *DEPTOR* gene. (A) LNCaP cells were treated with 10 μ M CHX. After 30 min, cells were treated with solvent control (sol.) or 10 nM DHT for 6 hr. Cells were harvested and the mRNA level of *DEPTOR* was measured by qRT-PCR. Results normalized against those of β -actin are expressed as averages of fold activation over the solvent control of four individual experiments (mean $\pm$ S.D., $n = 4$); $*P < 0.05$. (B) Schematic representation of *DEPTOR* gene locus. Black boxes designate the exons. Two putative AR-binding regions (ARE1 and ARE2) were indicated by arrows. (C, D) LNCaP cells were treated with sol. or 10 nM DHT for 24 hr. ChIP-experiment was performed using individual antibodies against AR, histone H3 and acetylated histone H3 as well as control ones. The precipitated DNA was used for PCR amplification with the specific primers for ARE regions of *DEPTOR* gene and ARE region of *FKBP5* gene used as a positive control. N.D., not detected.

regulates *E-cadherin* gene expression via recruitment of HDACs to ARE. Taken together, our observations suggest that downregulation of *DEPTOR* gene transcription was provoked by decreasing acetylated histone H3 levels.

In conclusions, we showed that *DEPTOR* gene expression was downregulated directly by AR and consequently activated mTOR/S6K-mediated cyclin D1 accumulation. The AR-DEPTOR-mTOR-S6K-cyclin D1 might be essential for the androgen-dependent cell proliferation in prostate cancer cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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