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Original Article

Androgen-deprivation therapy with enzalutamide enhances prostate cancer metastasis *via* decreasing the EPHB6 suppressor expression

Jiaqi Chen [a, b, 1], Lei Li [a, b, **, 1], Zhao Yang [a, b, 1], Jie Luo [b], Shuyuan Yeh [b], Chawnshang Chang [b, c, *]

[a] Sex Hormone Research Center, Department of Urology, The First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, 710061, China
[b] George Whipple Lab for Cancer Research, Departments of Pathology and Urology, The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, 14642, USA
[c] Sex Hormone Research Center, China Medical University/Hospital, Taichung, 404, Taiwan

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ABSTRACT

Early studies suggested that using ADT with the recently developed anti-androgen Enzalutamide (Enz, also named as MDV3100 could extent castration resistant prostate cancer (CRPC) patients' survival an extra 4.8 months. Yet the therapy in most patients might eventually fail due to development of Enz-resistance. Here we found Enz might also increase some unwanted side-effects *via* increasing the CRPC cell invasion that might involve altering the Enz-mediated androgen receptor (AR)/EPHB6 suppressor/JNK signaling. Results from multiple clinical surveys also indicated that EPHP6 might function as a suppressor of PCa metastasis. Mechanism dissection revealed that Enz-mediated AR might function *via* binding to the androgen-response-element (ARE) on the EPHB6 promoter to decrease EPHB6 suppressor expression, which might then activate the phosphorylation of JNK signals to increase the CRPC cell invasion. Targeting this newly identified AR/EPHB6/JNK signaling with JNK inhibitor (SP600125) may then block/reverse the Enz-increased CRPC cell invasion. Collectively, our results suggest that Enz may increase CRPC cell invasion *via* altering the AR/EPHB6/JNK/MMP9 signaling and targeting this newly identified signaling may help us to increase the Enz efficacy to better suppress the CRPC at the later metastatic stage.

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Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer death in men [1,2], which may involve the development of PCa metastasis [3]. Since the androgen receptor (AR), a classical nuclear receptor that binds androgens to activate gene transcription, plays very important roles in PCa progress [4], targeting these androgen/AR signals with the androgen deprivation therapy (ADT) with various antiandrogens remains as an effective therapy to suppress the PCa progression. However, most of PCa patients will progress into metastatic castration resistant prostate cancer (CRPC) inevitably after an average treatment of 12–18 months [5,6].

Enzalutamide (Enz, also named MDV3100) is a recently developed powerful antiandrogen with a better capacity to prevent the androgens from binding to the AR and the AR nuclear translocation that results in better suppression of the AR target genes [7]. Enz has been approved recently by the FDA after demonstration of improving the overall survival of 4.8 months in men with metastatic CRPC [8,9]. However, recent preclinical studies using multiple CRPC cells and *in vivo* mouse models also indicated that ADT with Enz (ADT-Enz) might have the unwanted side-effects of increasing PCa cell invasion that involves multiple mechanisms [10–12]. The tumor suppressor EPHB6, is a member of erythropoietin-producing hepatocyte (Eph) tyrosine kinase receptor family that involves migration during embryonic development [13,14] and cancer invasiveness [15,16]. However, the linkage of Enz-increased CRPC cell invasion to altering the expression of tumor suppressors, remains unclear.

* Corresponding author. Dr. Chawnshang Chang, George Whipple Lab for Cancer Research, Departments of Pathology and Urology, The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, 14642, USA.
** Corresponding author. Dr. Lei Li, Sex Hormone Research Center, Department of Urology, The First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, 710061, China.
E-mail addresses: lilydr@163.com (L. Li), chang@urmc.rochester.edu (C. Chang).
[1] Contributed equally.

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Recent studies indicated that EPHB6 downregulation was consistently correlated with enhanced aggressiveness and invasiveness in colorectal cancers, non-small cell lung carcinoma, melanoma, and breast cancers [17–21]. EPHB6 could inhibit the JNK activation following the stimulation with ephrinB1 [22], and EPHB6 could also suppress cancer cells' aggressiveness through interacting with EPHB4 [23]. However, the role of EPHB6 in the progression of CRPC, especially the Enz-increased CRPC cell invasion, has been poorly investigated.

Here, we found Enz could promote CRPC cell invasion *via* regulating the AR/EPHB6/JNK signals, and targeting this newly identified signaling with the small molecule JNK inhibitor may help us to increase the Enz efficacy to better suppress the metastatic PCa at the castration-resistant stage.

Material and methods

Cell culture

The C4-2 cell line was from Dr. Jer-Tsong Hsieh, UT Southwestern Medical Center, Dallas and CWR22Rv1 cell lines were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 media containing 10% fetal bovine serum (FBS), antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin), and 2 mM glutamine (Invitrogen, Grand Island, NY) in a humidified 5% CO_2 environment at 37 °C.

Reagents and materials

GAPDH (6c5), AR (N-20) antibodies were purchased from Santa Cruz Biotechnology. EPHB6 antibody were purchased from One World Lab. JNK and P-JNK (81E11) antibodies were purchased from Cell Signaling Technology Company (Boston, MA), MMP-9 (ab38898) antibody was from Abcam Company (San Diego, CA). Anti-mouse/rabbit secondary antibody for Western Blot was from Invitrogen. Normal rabbit IgG was also from Santa Cruz Biotechnology.

Lentivirus packaging

The pLKO.1-sh-EPHB6, pPWI-EPHB6, pWPI-AR or pLKO.1-shAR, the psAX2 packaging plasmid, and pMD2G envelope plasmid, were transfected into 293T cells using the standard calcium chloride transfection method for 48 h to get the lentivirus supernatant. The lentivirus supernatants were collected and concentrated by density gradient centrifugation, then used immediately or frozen in −80 °C for later use.

RNA extraction and qRT-PCR analysis

For RNA extraction, total RNAs were isolated using Trizol reagent (Invitrogen). One μg of RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen). Quantitative real-time PCR (Q-RT-PCR) was conducted using a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression level of a gene of interest. Expression levels were normalized to the expression of GAPDH RNA.

Protein extraction and western blot

Cells were lysed in RIPA buffer and proteins (30 μg) were separated on 10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking membranes, they were incubated with appropriate dilutions of specific primary antibodies, the blots were incubated with HRP-conjugated secondary antibodies, and then visualized using ECL system (Thermo Fisher Scientific, Rochester, NY).

Cell invasion assay

PCa cells were treated with Enz and incubated for 3 days. For inhibitor studies, the appropriate inhibitors were added into the culture. Cells (1×10^5) were then placed into the upper chamber of transwell plates (8 μm) with membranes precoated with 20% Matrigel. Each sample was assayed in triplicate. The bottom chamber contained 800 μl of media supplemented with 10% FBS. The cells that invaded into the bottom were fixed and stained using 1% toluidine blue, and the numbers were averaged after counting 6 randomly selected fields. Each experiment was repeated at least twice.

Chromatin immunoprecipitation assay (ChIP)

Cell lysates were precleared sequentially with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and protein A-agarose. Anti-AR antibody (2.0 μg) was added to the cell lysates and incubated at 4 °C overnight. For the negative control, IgG was used in the reaction. PCR products were identified by agarose gel electrophoresis.

Luciferase assay

The human promoter region of EPHB6 was constructed into pGL3-basic vector (Promega, Madison, WI, USA). Cells were plated in 24-well plates and the cDNAs were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The pRL-TK was used as internal control. Luciferase activity was measured by Dual-Luciferase Assay (Promega) according to the manufacturer's manual.

3D invasion assay

The 3D invasion assay was modified from the previous publication [24]. Briefly, 1×10^4 cells in 500 μl media containing 2.5% matrigel and 5 ng/ml were plated into the collagen/matrigel mixture coated 24-well plates. The spheres with protrusion were considered as positive for cell invasion. After 2 weeks, the spheres with/without protrusions were recorded in each well under the Olympus IX70 inverted microscope. Each experiment was repeated twice and each experiment was done in triplicate.

Statistics

All statistical analyses were carried out with SPSS 19.0 (SPSS Inc, Chicago, IL). The data values were presented as the mean ± SD. Differences in mean values between two groups were analyzed by two-tailed Student's *t*-test, and the means of more than two groups were compared with one way ANOVA. The P values of <0.05 were considered significant.

Results

Clinical surveys indicated that EPHB6 expression is negatively correlated with PCa metastasis and progression

Early studies of EPHP6 suggested that it might function as suppressor in several cancers [17–20]. The role of EPHB6 in PCa metastasis, especially the impacts on ADT, however, remains unclear. We first performed clinical surveys using the TCGA databases to analyze the linkage of EPHP6 expression to the PCa progression. The results revealed that EPHP6 expression was negatively correlated with PCa Gleason score showing lower EPHP6 expression in patients with higher Gleason score (Fig. 1A). Using online databases in PubMed, GEO profiles GDS1439 and GDS2545, we also found that EPHP6 expression was lower in metastatic PCa than primary PCa and benign patients (Fig. 1B-C). Results from the Kaplan-Meier plot in the TCGA dataset (http://cancergenome.nih.gov/) also indicated that in the PCa patients, higher EPHP6 expression was linked to the better survival than the lower EPHP6 expression (Fig. 1D).

Together, results from multiple clinical surveys (Fig. 1A–D) all suggest EPHP6 may function as a suppressor of PCa metastasis.

EPHB6 functions as suppressor to inhibit PCa cell invasion in vitro

To confirm above multiple clinical survey results, we applied the *in vitro* chamber transwell invasion assay [24] to determine EPHP6 expression on the PCa cell invasion. The results revealed that increasing EPHP6 expression (Fig. 2A) significantly suppressed CRPC C4-2 cell invasion (Fig. 2B). Similar results were also obtained when we replaced C4-2 cells with the androgen sensitive CWR22Rv1 cells (Fig. 2B).

Importantly, transwell invasion assay results from the rescue approach with EPHP6-shRNA to knock down EPHP6 expression, also showed that reduced EPHP6 expression (Fig. 2C) led to increase PCa cell invasion in both C4-2 cells and CWR22Rv1 cells (Fig. 2D).

Together, results from Fig. 2A–D confirm above clinical surveys showing EPHP6 can function as suppressor to inhibit the PCa cell invasion *in vitro*.

EPHB6 reverses the Enz-increased PCa cell invasion

Next, we examined EPHP6 expression impacts on the Enz-increased CRPC cell invasion and found Enz could suppress

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