

Original Article

Impact of E6-associated protein on the proliferation and invasion of prostate cancer cells in bone metastasis

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Abstract: Purpose: To understand E6 associated protein (E6-AP)'s influence on prostate cancer cell proliferation and infiltration, thus providing the theoretical basis for developing therapeutic drugs for prostate cancer metastasis to the bone. Methods: Electroporation was performed to introduce linear regulatory plasmid PrevTet-off-in and conjugative plasmid PrevTRE2-flag-E6AP into prostate cancer cell line to establish wild-type E6-AP over-expressing transgenic LNCaP cell line; Western blot assay was adopted to examine expression levels of E6-AP, mammalian target of rapamycin (mTOR), protein kinase B (Akt), and phosphoinositide 3-kinase (PI3K); PI3K inhibitor LY294002 was applied to all the cells and MTT assay was used to measure cell proliferation; Matrigel invasion chamber assay was adopted to detect cancer cell migration and invasion. Results: Stably transfected LNCaP cells that over expressed E6-AP had higher expression levels of PI3K, Akt, and mTOR than control LNCaP cells; MTT assay showed that E6-AP-LNCaP cells were more responsive to the inhibitory effect of LY294002; Matrigel invasion chamber assay revealed increased cell crawling and adhesiveness of E6-AP-LNCaP cells. Conclusion: Stable over-expression of E6-AP increases the proliferation and invasion of LNCaP cells.

Keywords: Prostate cancer, E6-AP, proliferation, invasion

Introduction

Prostate cancer is a malignant tumor commonly seen among elderly males. China has witnessed an increased prevalence of this disease in recent years. Prostate cancer metastasis to the bone occurs in over 80% cases [1, 2]. It is of great clinical significance for treating bone metastasis of prostate cancer to study its pathogenesis, migration and invasion of cancer cells. E6-AP as a member of the E3 class of ubiquitin-protein ligases could be expressed in the tissue of prostate gland, uterus, and mammary gland etc. [3, 4]. However, the correlation between E6-AP and prostate cancer cell migration and invasion has been rarely reported. Therefore, we established E6-AP over-expressing prostate cancer cell line and carried out in vitro study to see what influence it has on cell migration and adhesion, and the expression of proteins which are functionally related to cell division and proliferation, when the cancer cells are treated by Dox. We hope to explore how to inhibit the signaling pathway related

with prostate cancer metastasis to the bone and seek potentially effective treatment for the disease.

Materials and methods

Materials

LNCaP cells, PrevTet-off-in plasmid and PrevTRE2 plasmid were all donated by Institute of Neurology of Capital Medical University. Akt antibody, mTOR antibody and PI3K antibody were purchased from Cell Signalling Technology (CST). Matrigel Invasion Chamber was purchased from BD Biosciences Corp. Reagents were purchased from Dingguo Biotechnology Co. Ltd (Changchun).

Major equipments included: high-speed centrifuge (TGL-16G, Shanghai), CO₂ constant temperature incubator (VWR), constant temperature concussion incubator (WM-KD02, Beijing), UV Transilluminator (UV-1, Beijing), nucleic acid analyzer (geneQuant), and fluorescence scanning electron microscope (ZEISS).

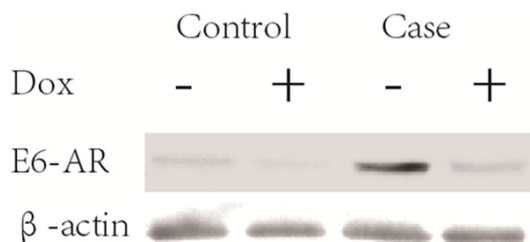


Figure 1. Expression levels of E6-AP, and mTOR in both treatment group and control group, assayed by Western blot analysis. Cells in treatment group over-express E6-AP. tTA in response to the presence of Dox deactivates downstream gene, thus leading to decreased expression of E6-AP.

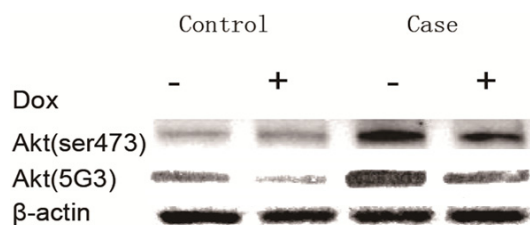


Figure 2. Dox's effect on expression levels of Akt and its phosphorylated forms in both treatment group and control group, assayed by Western blot analysis. The result shows that in the absence of Dox, expression levels of Akt and its phosphorylated forms increase, indicating that Akt may have mediated the signaling pathway of E6-AP.

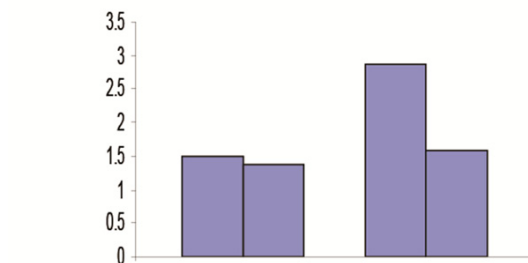
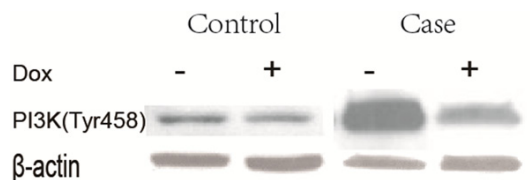


Figure 3. PI3K/β-actin. The expression of PI3K has been decreased as Dox inhibits the expression of E6-AP, which indicates that PI3K may have mediated the signaling pathway of E6-AP.

Establishment of wild-type E6-AP over-expressing transfected cell line

1 to 10 μ g of linear plasmid PrevTet-off (containing tetracycline-controlled transactivator

(tTA) which could be regulated by Doxycycline) was resolved in 40 μ l of water/TE buffer and pipetted into an electroporation cuvette. 400 μ l of LNCaP cell suspension was added into the cuvette and mixed with the plasmid. An electrical field (1050 μ F, 220-300 V) was applied to the mixture. Immediately after the electroporation, the cell suspension at $(2-5) \times 10^6$ /ml was placed in complete medium. G418 was used for selecting positive clones which were expanded afterwards.

Recombinant pTRE-flag-E6-AP was obtained by combination of pRevTRE2 plasmid (containing Tet-response element (TRE), which responds to tTA and activate transcription of downstream gene) and E6-AP cDNA. The recombinant was then introduced into G418 positive clones and resistant cells were selected with Hygromycin. Afterwards Hygromycin-resistant cells were screened to establish E6-AP over-expressing treatment group.

Western blot analysis

Cells were treated with or without Doxycycline and cultured at 37°C in 5% CO₂ for 24 hours. Then cells were lysed and the supernatants were collected by centrifugation. The protein concentration of lysates was measured. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes which afterwards were probed with relevant anti-bodies to measure target proteins.

MTT assay

TACS MTT was adopted to confirm that PI3K and Akt in response to E6-AP would lead to prostate cell proliferation and growth. E6-AP-LNCaP cells and control LNCaP cells were grown in 96-well cell culture plates at a density of 2×10^3 cells/well in 100 μ l of culture solution for 24 hours in 5% CO₂ at 37°C. Afterwards LY294002 was added to each well. The absorbance at 570 nm was measured using a microplate reader for 7 consecutive days.

Cell migration and adhesion assay

A 24-well culture plate with complete medium and 500 μ l of rehydrated matrigel inserts was adopted. Cell suspension was incubated in 5% CO₂ at 37°C with a medium change after 2 hours. Inserts were added into cell suspension

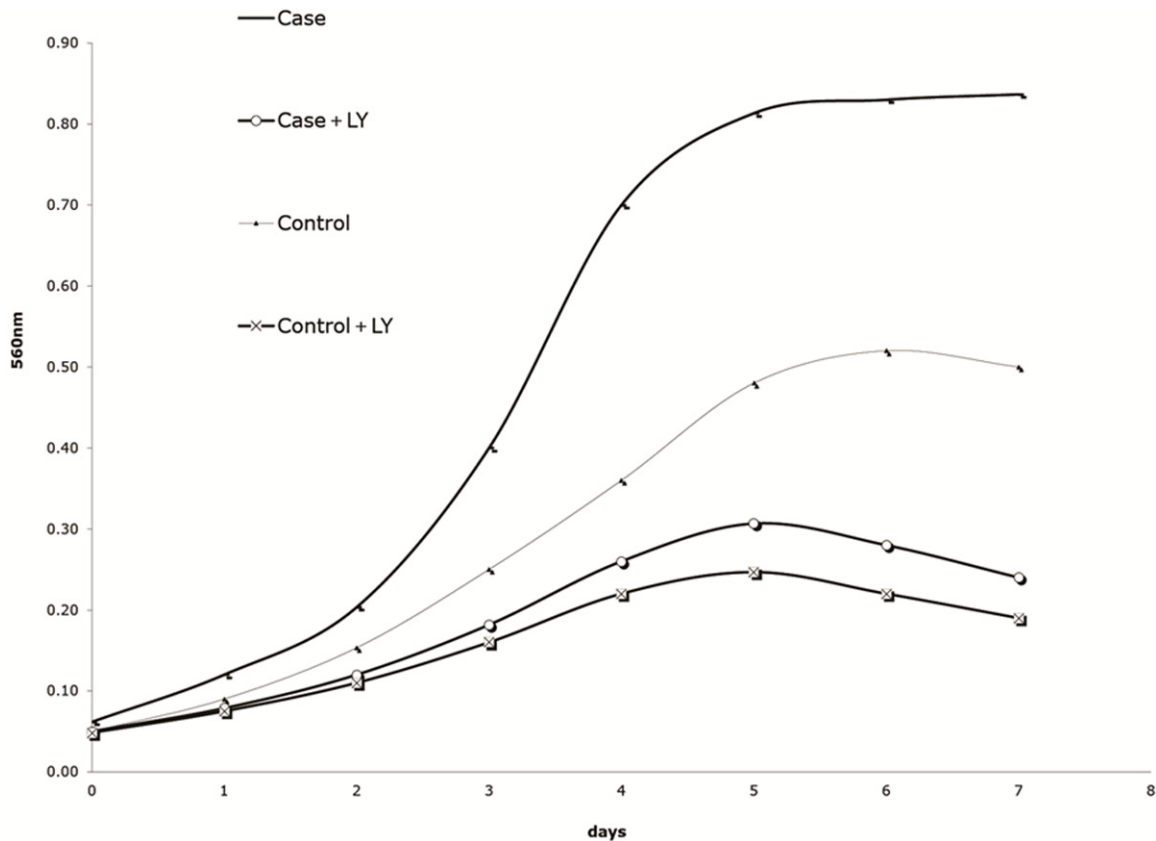


Figure 4. Cell proliferation measured by MTT assay. Cells of treatment group has more evident proliferation than control cells and are sensitive to prohibition effect of LY294002, indicating that PI3K has mediated signaling pathway of E6-AP and led to accelerated cell proliferation.

which was at a density of 2.5×10^5 cells/ml and was incubated in 5% CO₂ at 37°C for 22 hours. Then cells were washed and the adherent ones were fixed and stained to be observed by fluorescence scanning electron microscope.

Results

The following figures show expression levels of E6-AP, AR and its phosphorylated forms, Akt and its phosphorylated forms, and PI3K. As shown in **Figure 1**, in the presence of Dox to which tTA responds, LNCaP cells of treatment group has decreased level of E6-AP and mTOR. **Figure 2** shows that in the absence of Dox, transfected cell line had a remarkably higher expression level of phosphorylated Akt than control LNCaP cells, which indicated that increased E6-AP could promote the expression and activity of phosphorylated Akt. **Figure 3** shows that in the absence of Dox, LNCaP cells of the treatment group have higher expression level of PI3K than control LNCaP cells. **Figure 4** shows that as assayed by MTT, LNCaP cells of

the treatment group that over express E6-AP are more sensitive to the cell proliferation inhibition effect of PI3K inhibitor LY294002. **Figure 5** shows that according to cell migration and adhesion assay, transfected cells that over express E6-AP have higher ability to adhere and migrate, compared with control LNCaP cells.

Discussion

An ubiquitin ligase, also called an E3 ubiquitin ligase, assists the transfer of ubiquitin which will be attached to a lysine on the target protein. In most cases, ubiquitin ligases could lead to polyubiquitination which means the target protein is modified by a chain of ubiquitins, and thereafter degradation of the target protein. Ubiquitin ligases could be classified into three groups based on their structure, and HECT family is one of the three. E6-AP comes first in this family [5, 6] and promotes the ubiquitination and degradation of cancer suppressor proteins. Given the fact that E6-AP is closely related with the onset and development of cancer, this pro-

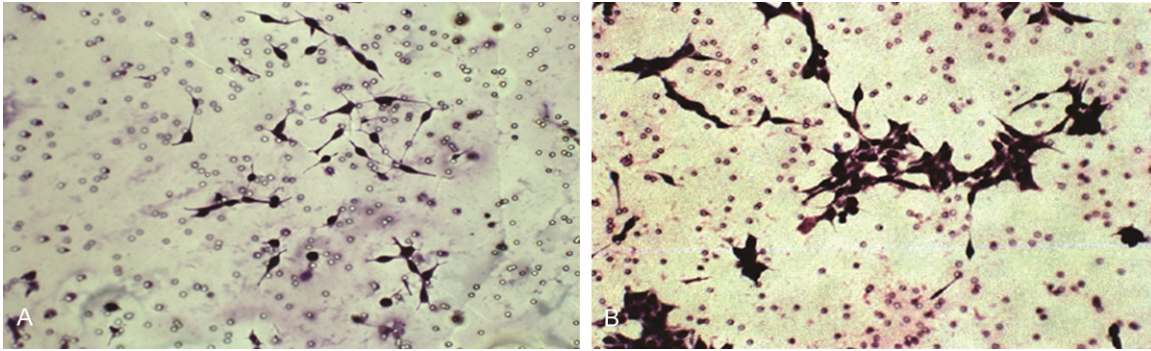


Figure 5. Cell migration and adhesion assay. Transfected E6-AP-LNCaP cell line has higher ability of migration and adhesion compared with control LNCaP cells.

tein could be used as an effective target for cancer treatment [7].

We established a LNCaP cell line that over expresses E6-AP to study the influence of E6-AP on cell proliferation, migration and adhesion in cases of prostate cancer metastasis to the bone. In this study Dox was considered to control the expression of E6-AP since tTA, the protein that activates the expression of E6-AP is regulated by Dox. After we found that in the absence of Dox, LNCaP cells of treatment group had increased expression level of E6-AP, we measured respectively from the treatment group and control group, the total level of proteins that are closely related with cell growth, including Akt and its phosphorylated forms, PI3K and mTOR. We used PI3K inhibitor LY294002 to treat cells from both groups to observe how sensitive they are to the inhibition effect. The result indicates that E6-AP has mediated the signaling pathway of PI3K/Akt/mTOR. Matrigel Invasion Chamber assay revealed that transfected cells that over express E6-AP had enlarged cell nucleus and body, and higher ability to adhere and migrate, compared to control cells.

One previous study by Xinhua [8] discovered increased expression of E6-AP in the tissue section of ductal carcinoma in situ. Both breast cancer and prostate cancer are hormone-dependent cancers and the two share similar mechanism of development and treatment principles [9]. Meanwhile, results of our study have also indicated that stable over-expression of E6-AP may accelerate invasion and migration of prostate cancer cells. Prostate cancer metastasis to the bone is a complicated progression. Recent studies have found that there

is the same type of adhesion molecule on the surface of both bone marrow endothelial cells and prostate cancer cells, which could function to modulate adhesion between bone marrow endothelial cells and prostate cancer cells. In this respect, the mechanism of bone metastasis of prostate cancer remains to be further studied [10].

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Disclosure of conflict of interest

None.

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