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Award Number: DAMD17-03-1-0090

TITLE: PTEN Regulates Beta-Catenin in Androgen Signaling: Implication in Prostate Cancer Progression

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REPORT DATE: March 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Annual (1 Mar 2003 - 28 Feb 2004)

4. TITLE AND SUBTITLE

PTEN Regulates Beta-Catenin in Androgen Signaling: Implication in Prostate Cancer Progression

5. FUNDING NUMBERS

DAMD17-03-1-0090

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Observations from androgen ablation treatment of prostate cancer have shown that the androgen-signaling pathway is important in the growth and progression of prostate cancer. The growth-promoting effects of androgen are mediated mostly through the androgen receptor (AR). PI3K/Akt plays a critical role in prostate cancer cell growth and survival. It has been shown that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling. The PI3K inhibitor, LY294002, and a tumor suppressor, PTEN, negatively regulate the PI3K/Akt pathway and repress AR activity. However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. In this study, we will use several biological relevant experiments to test whether β–catenin, an AR coactivator, is a major downstream effector of the PI3K/Akt and PTEN pathways in androgen-mediated prostate cell growth and survival. Successful completion of the proposed study should provide fresh insight into the pathogenesis of prostate cancer that may help us to identify new pathways that can be targeted for prostate cancer treatment.

14. SUBJECT TERMS

PTEN, Akt, PI3K, androgen receptor, prostate cancer

15. NUMBER OF PAGES

9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

Prostate cancer is the most commonly diagnosed malignancy among males in the United State (Landis et al., 1999). However, in contrast to other tumors, the molecular events involved in the development and progression of prostate cancer remain largely unknown. Observations from androgen ablation treatment of prostate cancer have shown that the androgen-signaling pathway is important in the growth and progression of prostate cancer (Kyprianou and Isaacs, 1988). The growth-promoting effects of androgens are mediated mostly through the androgen receptor (AR). PI3K/Akt pathway has been implicated in the androgen-mediated prostate cell growth and survival (Li et al., 2001). Recent studies have shown that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling (Wen et al., 2000). PTEN tumor suppressor gene acts as an inhibitor of the PI3K to hydrolyzes the lipid products of PI3K (Cantley and Neel, 1999). Loss of PTEN in prostate cancer cells results in constitutive activation of enzymes downstream of PI3K, including the Akt protein-Ser/Thr kinase (Li et al., 1997). Reintroduction of PTEN or addition of PI3K inhibitors can block this pathway and leads to decreased cell and increased apoptosis. The PI3K inhibitor, LY294002, and PTEN negatively regulate the PI3K/Akt pathway in prostate cancer cells and repress AR activity (Li et al., 2001). However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. Recently, we demonstrate that blocking the PI3K/Akt pathway reduces the expression of an endogenous AR target gene, and that the repression of AR activity by LY294002 is mediated through phosphorylation and inactivation of GSK3β, a downstream substrate of PI3K/Akt (Sharma et al., 2002). Given the recent evidence that β-catenin acts as a coactivator of AR (Yang et al., 2002), our findings suggest a novel mechanism by which PI3K/Akt modulates androgen signaling. In this study, we proposed several biological relevant approaches to test whether β-catenin is a major downstream effecter of the PI3K/Akt and PTEN pathways in androgen-mediated prostate cell growth and survival. Further study of the regulation of AR co-factor, β-catenin, by PI3K/Akt in prostate cancer cells should provide fresh insight into the pathogenesis of prostate cancer that may help us to identify new pathways that can be targeted for prostate cancer treatment.

BODY:

The PI3/Akt pathway plays a critical role in prostate cell proliferation and survival (Cantley and Neel, 1999). PTEN, which is frequently mutated in prostate cancer cells, negatively regulates this process by blocking the PI3K/Akt pathway. Recently, several lines of evidence showed that PI3K/Akt and PTEN can modulate androgen-induced cell growth and AR-mediated transcription in prostate cancer cells (Li et al., 2001; Wen et al., 2000), suggesting a potential link between the PI3K/Akt and androgen pathways. However, the precise molecular basis by which PI3K/AKT and PTEN regulate AR-mediated transcription is currently unclear. Under normal conditions, the free β-catenins are tightly regulated by the destruction complex, which includes APC, GSK3β, and Axin. When the levels of these components are changed, such as by mutation, aberrant expression of the proteins, or inactivation by protein modulation, cellular levels of β-catenins are increased and the proteins can be translocated into the nucleus, where they specifically interact with the AR to induce androgen mediated cell growth or survival. Recently, we demonstrated the crosstalk between the androgen and PI3K/Akt pathways, which delineates a novel mechanism by which PI3K/Akt and PTEN regulate the androgen pathway in the pathogenesis of prostate cancer. Four specific objectives were proposed in the original proposal. Substantial progresses have been made in this funding year and described as followed.

Objective 1. Determine the roles of PI3K pathway in β-catenin mediated cell growth.

Our preliminary studies have shown that PI3K/Akt enhances AR-mediated transcription through GSK3β and β-catenin. One aspect of the hypothesis being examined in this proposal is whether the augmentation of AR transcriptional activity by β-catenin is regulated by the PI3K inhibitor. We proposed to generate several inducible prostate cancer cell lines, in which the wild type or the mutant β-catenin gene

expressed. In this study, we plan to use the retrovirus-based tetracycline-off system. Fragments containing the Flag-tagged full-length β-catenin or a mutant of β-catenin (S33F) containing a single point mutation within the GSK3β phosphorylation site have been subcloned into the retroviral vector, pRev-TRE (Clontech, Palo Alto, CA). Currently, we are in the process of production of the viruses by transfecting the constructs into a packaging cell line, PT67. Once obtaining high titer retroviruses, we will infect the viruses into LNCaP and other prostate cancer cell lines. Plates containing large numbers of individual colonies can be expanded for the selection of pools of clones. Since using pools and colonies both have advantages and disadvantages, we will use both in order to allow us to identify the effects of the genes on the overall population.

Objective 2. To study the correlation of PTEN and β-catenin expressions during prostate cancer progression.

We proposed a systematic analysis of expression of PTEN and β-catenin in the different stages of prostate cancers. Using immunohistochemical techniques, we attempt to define a correlation between loss or reduction of PTEN expression and increased cytoplasmic and nuclear staining of β-catenin. Recently, we tested the β-catenin antibody. The samples used in our pilot experiments are from a prostate tissue bank in the Department of Urology at Stanford Medical Center. Four tissue samples from normal and different grades of prostate cancers were used. All tissues were fixed intact for 20 hr in 10% zinc formalin. Following fixation, samples were sectioned at 3mm intervals in planes perpendicular to the rectal surface as previously described (McNeal et al., 1995; McNeal and Yemoto, 1996). The human β-catenin antibody (Transduction Lab. KY)

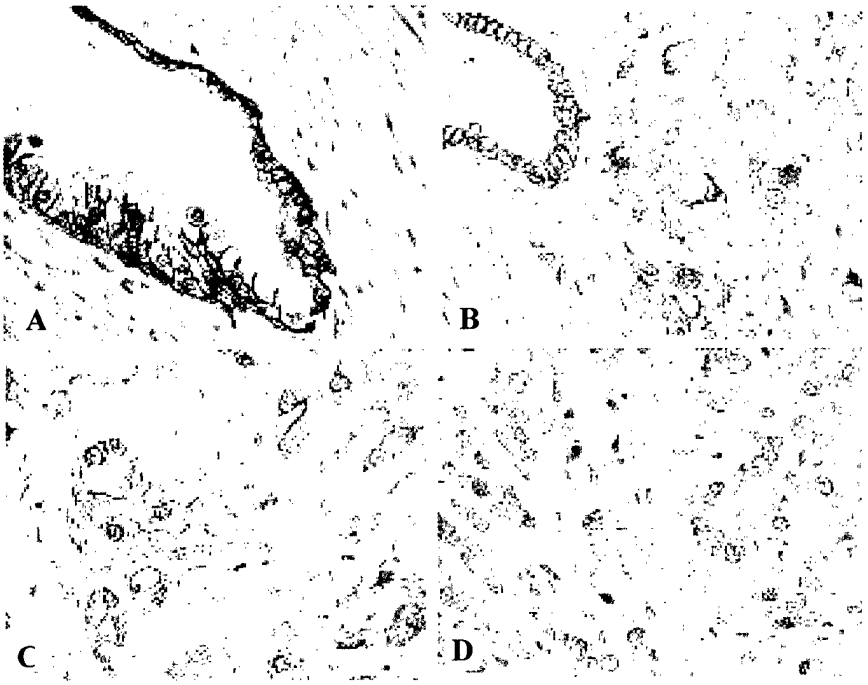


Figure 1. Immunohistochemistry of human prostate tissues with the β-catenin antibody. Four human prostate tissue samples (A-D) were stained with the β-catenin antibody (Transduction Lab). Color was developed with Immunocruz Staining System (Santa Cruz Inc). Samples were lightly counterstained with hematoxylin. Positive staining appears as brown color in the samples.

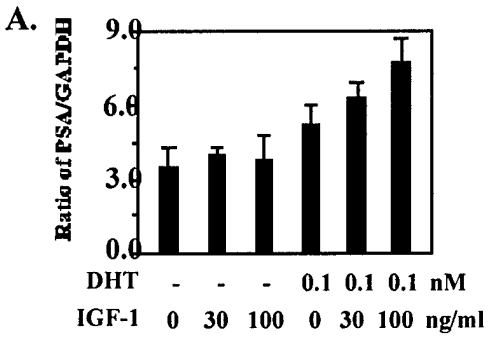
was used in this study. As shown in Figure 1, a clear cell membrane staining with the β-catenin antibody was shown in normal prostatic epithelial cells (A and B). No staining of the stromal elements was observed. In comparison to the normal epithelial cells, β-catenin shows a weak (B and C) or no staining (D) on the cell membrane, but a simultaneous increase in cytoplasmic and nuclear staining in the tumor cells. Due to technical difficulties, we were not able to make the double staining with both PTEN and β-catenin antibodies in the above samples. Currently, we are working on the experimental conditions and hope to overcome these technical difficulties.

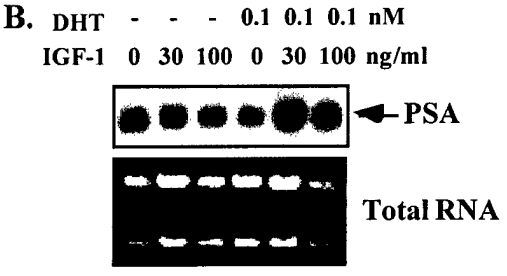
Objective 3: To determine whether LY294002 and PTEN regulate Tcf/LEF activity.

The signaling activity of β-catenin is mediated through its interaction with Tcf/LEF family members and subsequent activation of target genes (Polakis, 2000). We have shown that LY294002 and PTEN can regulate β-catenin mediated augmentation of AR activity (Sharma et al., 2002). However, it is currently unknown whether Tcf/LEF transcription factors are active in prostate cancer cells. The armadillo domain of β-catenin is involved in a protein-protein interaction with both the Tcf/LEF and AR (Yang et al., 2002). In this study, we examined whether PI3K and PTEN can crosstalk with Tcf/LEF and β-catenin mediated transactivation. Our initial experiments were to test whether the Tcf/LEF complex functions in prostate cancer cells. Luciferase reporters driven by an optimal LEF-binding site (TOPFlash) or mutated LEF-binding sites (FOPFlash) (Morin et al., 1997) were transfected alone or cotransfected with a β-catenin expression vector into either AR-positive (LNCaP, LAPC4) or AR-negative (PC3, DU145) prostate cancer cell lines. Interestingly, there was no significant induction in the samples transfected with the TOPFlash reporter in LNCaP, PC3, and DU145 cells, which are consistent with the previous observation by others (Truica et al., 2001). The non-canonical pathway has been suggested in the Wnt signaling, which functions through the activation of JNK and Ca+ mediated pathways (Pandur et al., 2002). Based on the above observations, we adjusted our research direction and started to investigate whether the non-canonical pathway plays a role in prostate cancer cells.

Objective 4: Determine whether IGF is involved in b-catenin mediated enhancement of AR activity.

Previous experiments showed that AR can be activated by treatment of cells with growth factors in the absence of, or at low levels of ligand (Culig et al., 1994). IGF-1 is the most efficient growth factor capable of ligand-independent activation of AR. However, the mechanisms by which IGF-1 or other growth factors regulate AR activity in prostate cells are still unclear. Recently, Playford *et al.* reported that in human colorectal cancer cells, IGF-1 enhances tyrosine phosphorylation of β-catenin, which results in dissociation of β-catenin from E-cadherin complexes at the cell membrane and relocation to the cytoplasm (Playford et al., 2000). In addition, IGF-1 also increases the stability of the β-catenin protein itself. This result suggests a new function for IGF-1 in cell growth and transformation. Based on this observation, and our recent results showing that β-catenin functions as a coactivator of AR in prostate cells, we hypothesized that the mechanism by which IGF-1 induces activation of AR involves an increase in the cellular level of β-catenin. Recently, we performed a series of experiments to test this hypothesis.





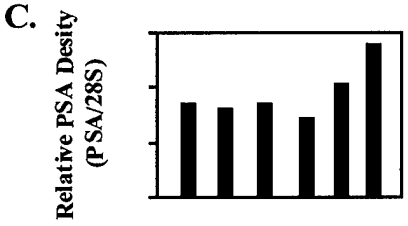


Figure 2. IGF-1 enhances the transcription of an endogenous AR target gene, *PSA*. (A) An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium with 10% fetal calf serum. For androgen induction experiments, the cells were cultured in T-medium with IGF-1 or vehicle in the presence and absence of 0.1 nM DHT for 16 hr. Total RNAs were isolated. Expression levels of PSA mRNA were quantitated using quantitative fluorescent real-time PCR. The levels of PSA mRNA were normalized by co-amplification of GAPDH mRNA. Densities of DNA bands were measured by densitometry. The relative ratios of PSA and GAPDH are reported. (B) Northern blotting assays were performed as described previously. Expression of the endogenous *PSA* gene was detected by a cDNA probe in LNCaP cells treated with IGF-1 as described above. Total RNAs were used to confirm equal loading. (C) Densitometry of the Northern blot was performed, and the relative numbers are reported as PSA/28S total RNA.

To evaluate the effect of IGF-1 in a biologically relevant setting, we tested whether IGF-1 regulates expression of the PSA gene, an endogenous AR target, in the AR-positive prostate cancer cell line, LNCaP. Using real-time PCR, we first measured transcripts of PSA in LNCaP cells treated with different amounts of IGF-1. In the presence of 0.1 nM DHT, PSA expression was increased approximately 20 or 35% in LNCaP cells treated with 30 or 100 ng/ml of IGF-1, respectively, over that found in cells not treated with IGF-1 (Fig. 2A). However, in the absence of DHT, the expression of PSA was not significantly affected by IGF-1. To confirm this finding, we examined the expression of PSA by conventional Northern blotting. As observed in the real-time PCR assays, an increase of PSA transcripts was found in the cells treated with 30 or 100 ng/ml of IGF-1 (Fig. 2B). Using total RNA samples as an internal control, we showed an approximately 20 to 50% increase in PSA transcripts in the cells treated with 30 or 100 ng/ml of IGF-1, respectively (Fig. 2C). These results provide the first line of evidence that IGF-1 is able to enhance endogenous AR-mediated transcription in prostate cancer cells, which also confirms previous studies showing that IGF-1 enhances AR-mediated transcription in transient transfection experiments (Culig et al., 1994). However, we observed that IGF-1 showed a clear effect on AR-mediated transcription at low concentrations of DHT rather than in the absence of ligand. Since the transactivation mediated by AR is a nuclear effect, it requires that AR binds to the androgen and translocates from the cytoplasma into the nucleus (Zhou et al., 1994). Our results indicate that the enhancement of AR by IGF-1 may be modulated through the recruitment of other transcriptional cofactors on the target promoters.

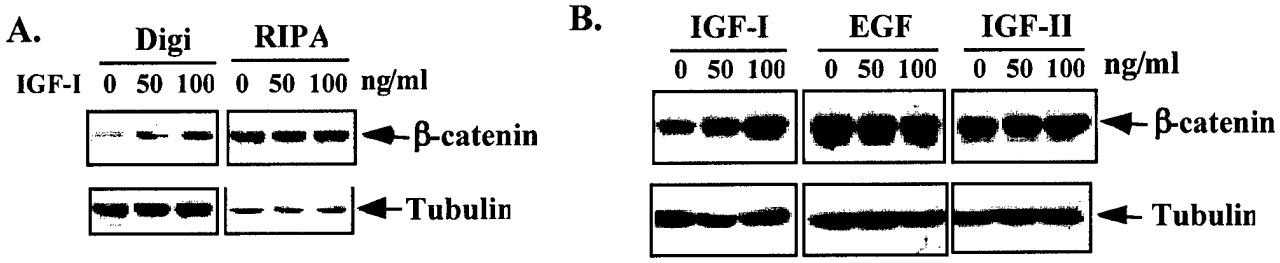


Figure 3. IGF-1 signaling enhances the level of free cytosolic β-catenin. (A) Both cytosolic (Digi) or cytoskeletal fractions (RIPA) were prepared in digitonin lysis buffer (1% digitonin, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM MgCl2) or in RIPA buffer (0.5% Nonidet P-40, 0.3% Triton X-100, 15mM MgCl2, 5mM EDTA, 150mM NaCl, 50mM Tris-Cl pH 7.8), respectively. Protein fractions for immunoblotting were boiled in SDS-sample buffer and then resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane, probed with a β-catenin antibody, and detected using the ECL kit. (B) The cytosolic fractions were isolated from LNCaP cells treated with different concentrations of growth factors. The levels of the β-catenin protein were analyzed by Western blotting as described above. Tubulin was detected to control for protein loading.

To determine whether β-catenin is involved in IGF-1 mediated AR transcription, we examined free cellular β-catenin in prostate cancer cells as described previously (Playford et al., 2000). As shown in Figure 3A, there was no significant change in the amount of β-catenin in the cytoskeletal compartment (RIPA) of cells treated with IGF-1. However, there was a 2- to 4-fold increase in cytosolic β-catenin in cells treated with 50 or 100 ng of IGF-1, respectively. In contrast, the level of cytosolic tubulin, used as a control, showed no significant difference in the treated and untreated cells. To further test whether the effect of IGF-1 on β-catenin is a specific event, we repeated the above experiments with LNCaP cells treated with different growth factors. As shown in the figure, a pronounced increase in cytosolic β-catenin was observed in the cells treated with IGF-I (Fig. 3B). Culig *et al* showed that IGF-I is one of the most efficient growth factors in modulating AR-mediated transcription (Culig et al., 1995), which is an agreement with our above results showing that IGF-I elevates the cellular level of β-catenin. In this study, we also confirmed the observation by Culig et al using the PSA-promoter/reporter (data not shown). In addition, the evidence that IGF-1 modulates the levels of cytosolic β-catenin in prostate cancer cells is also consistent with the earlier report showing that IGF-1 modulates β-catenin in human colorectal cancer cells (Playford et al., 2000).

KEY RESEARCH ACCOMPLISHNETS:

1) Demonstrate that the nuclear and cytoplasmic distribution of β-catenin in prostate cancer samples.
2) Demonstrate that TCF/LEF are transcriptionally inactive in prostate cancer cells.
3) Demonstrate that IGF-1 enhances the expression of PSA, a target gene of AR, in prostate cells.
4) Demonstrate that IGF-1 increases the level of cellular β-catenin in prostate cancer cells.

REPORTABLE OUTCOMES:

Publications:

Meletios Verras and Zijie Sun (2004). β-catenin Is Involved in Insulin-Like Growth Factor 1-Mediated Transcription of the Androgen Receptor. *Submitted.*

Funding applied for based on work supported by this award:

2003-2008 NIH/RO1DK61002, Principal Investigator: Zijie Sun
Beta-catenin and androgen signaling in prostate cancer

CONCLUSIONS:

The PI3/Akt pathway plays a critical role in prostate cell proliferation and survival. PTEN, which is frequently mutated in prostate cancer cells, negatively regulates this process by blocking the PI3K/Akt pathway. Recently, several lines of evidence showed that PI3K/Akt and PTEN can modulate androgen-induced cell growth and AR-mediated transcription in prostate cancer cells, suggesting a potential link between the PI3K/Akt and androgen pathways. The goal of this study is to determine the molecular mechanisms by which PI3K and PTEN regulate β-catenin in androgen signaling pathway in prostate cancer cells and the biological consequences of this regulation. In this funding year, we have performed several experiments in order to achieve our goals. We demonstrated the different cellular distribution of β-catenin in prostate cancer cells. We have also shown that TCF/LEF are transcriptionally inactive in several prostate cancer cell lines, which suggests that the other pathway(s) may play a role in the Wnt mediated cell growth. Based on the observations, we started to investigate the non-canonical pathway in prostate cancer cells. Moreover, we provided several lines of evidence that IGF-1 enhances AR-mediated transcription at low level of androgen and increases the level of cellular β-catenin in prostate cells. Currently, we are in the process of characterizing the molecular mechanism by which IGF1 induces the AR-mediated transcription. We hope that through these efforts we can gain more information on the interaction between PI3K/Akt, IGF1, and androgen signaling pathways.

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