DOI: 10.4274/forbes.galenos.2022.35229
Forbes J Med 2022;3(2):197-202

Original Article / Orijinal Araştırma

Different mRNA and Protein Expression of Versican in TGF-β1-treated Prostate Cancer Cells

TGF-β1 Uygulaması Sonrasında Prostat Kanseri Hücrelerinde Versicanın Farklı mRNA ve Protein Ekspresyonu

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Cite as: Soner B, Açıkgöz E, Caggia S, Khan S, Taşkıran A, Öktem G. Different mRNA and Protein Expression of Versican in TGF-β1-treated Prostate Cancer Cells. Forbes J Med 2022;3(2):197-202

ABSTRACT

Objective: Cellular heterogeneity of tumors stems from cell populations expressing a unique set of proteins defective genetic and epigenetic networks. It is shown that versican (VCAN) is upregulated by transforming growth factor-beta (TGF-β1) in several types of cancer cell types. Our prior studies revealed that TGF-β1 expression is elevated in cancer stem cell (CSCs) monolayer cultures and VCAN expression was also significantly increased in spheroid formed CSCs.

Methods: Within the scope of these results, it was hypothesized that high TGF-β1 expression in the monolayer might trigger the three-dimensional architectural organization by inducing VCAN expression. This study investigated the expression profiling of VCAN in primary and secondary tumor-derived cell lines and the effect of TGF-β1 signaling in these cells.

Results: The findings showed that the *VCAN* gene expression level in the PC3 human prostate cell line was correlated with VCAN protein expression as analyzed by western blot. There was no inducing or suppressing effect of TGF-β1 on VCAN protein expression.

Conclusion: TGF-β1 has neither stimulatory nor inhibitory effects on VCAN expression in prostate cancer cells at the doses used in this study. In the metastatic, high VCAN protein levels were observed while no mRNA was detected which could have resulted from post-transcriptional changes such as increased protein stability and/or expression of different VCAN isoforms that may play an important role in the secondary tumor cells in prostate carcinoma.

Keywords: Versican, TGF-beta, prostate cancer

ÖZ

Amaç: Tümörün hücresel heterojenliği, kusurlu genetik ve epigenetik ağlar nedeniyle bir dizi eşsiz protein eksprese eden hücre popülasyonlarından kaynaklanır. Versicanın (VCAN), çeşitli kanser hücre tiplerinde transforme edici büyüme faktör-beta (TGF-β1) tarafından up regüle edildiği gösterilmiştir. Daha önceki çalışmalarımızda, kanser kök hücre (CSC'ler) tek katmanlı kültürlerinde TGF-β1 ekspresyonunun yükseldiğini ve CSC'lerden oluşan sferoidlerde VCAN ekspresyonlarının da önemli ölçüde arttığını ortaya koydu.

Yöntem: Bu sonuçlar dahilinde monolayer yüksek TGF-β1 ekspresyonunun VCAN ekspresyonunu uyararak üç boyutlu yapılanmasını tetikleyebileceği varsayılmıştır. Bu çalışma, primer ve sekonder tümör derive hücre dizilerinde VCAN'ın ekspresyon profilini ve bu hücrelerde TGF-β1 sinyalleşmesinin etkisini araştırdı.

Received/Geliş: 28.06.2022
Accepted/Kabul: 07.07.2022

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Bulgular: PC3 insan prostat hücre dizisindeki *VCAN* gen ekspresyon düzeyinin western blot analizinde VCAN protein ekspresyonu ile korele olduğunu gösterdi. TGF-β1'in VCAN protein ekspresyonu üzerinde hiçbir indükleyici veya baskılayıcı etkisi yoktu.

Sonuç: TGF-β1'in bu çalışmada kullanılan dozlarda prostat kanseri hücrelerinde VCAN ekspresyonu üzerinde uyarıcı ya da engelleyici etkileri yoktur. Metastatik yüksek VCAN protein seviyeleri gözlenirken, artmış protein stabilitesi ve/veya prostat karsinomunda sekonder tümör hücrelerinde önemli bir rol oynayabilecek farklı VCAN izoformlarının ekspresyonu gibi transkripsiyon sonrası değişikliklerden kaynaklanabilecek hiçbir mRNA tespit edilmedi.

Anahtar Kelimeler: Versican, TGF-beta, prostat kanseri

INTRODUCTION

Prostate cancer ranks second among the causes of death due to cancer in men, and patients with prostate cancer usually develop drug resistance and metastasis.[1] Cancer progression from a small primary tumor to a large invasive and/or disseminated metastatic disease is affected by the tissue-specific microenvironment and particularly extracellular matrix (ECM) proteins. The presence of specific ECM proteins with a different plethora in the microenvironment may result from transcriptional or translational control mechanisms, altered secretion, and the assembly/stability of ECM proteins in the tissue microenvironment.[2] Several studies have determined that mRNA levels of a gene may not predict its protein levels. These differences can be specified by high throughput RNA sequencing and mass spectrometry. Post-transcriptional and post-translational regulation of gene expression can be determined using probe transcripts and proteins.[3] Although mRNA profiling has high sensitivity, regulatory processes and post-transcriptional modifications cannot be detected, directly affecting the total active protein amounts.[4]

Versican (VCAN), a chondroitin sulfate proteoglycan, constitutes a major part of the ECM. VCAN spatiotemporal expression differs in various cells and under different developmental and pathological conditions.[5] Although higher VCAN expression in epithelial cells has been correlated with the long-term survival of patients, high VCAN levels can be indicative of tumor stroma.[6]

Transforming growth factor-beta (TGF-β) signaling plays an important role in regulations characterized by its roles in normal prostate development and cancer.[7] The effects of TGF-β are like a double-edged sword. At the early stages of prostate cancer, it acts as a tumor suppressor. However, at advanced stages, TGF-β can induce malignity and metastasis via its effects on tumor cells and the microenvironment.[7,8] The study carried out by Tu et al.[9] (2003) revealed that the impairment of TGF-β signaling was associated with the induction of prostate cancer metastasis. Likewise, it was reported that the disruption of TGF-β via dominant-negative TGF-type 2 receptor in the prostate epithelium of transgenic adenocarcinomas accelerated the progression of prostate cancer by altering the prostate growth rate and inducing epithelial-mesenchymal transition.[10]

Undoubtedly, exploring the mysterious role of TGF-β in the prostate cancer process has contributed to discovering new perspectives in treatment. In various cell types, it has been shown that VCAN is been upregulated by TGF-β.[11-14] Stroma-specific mediator genes related to TGF-β signaling, such as collagen type 5 alpha 1 chain, collagen type 9 chain, tissue inhibitor of metalloproteinase 3 (TIMP3), and VCAN in the tumor microenvironment can be modulated, and this modulation indirectly affects tumor growth, migration, and invasion.[15,16] It has been reported that VCAN-V1 (one of the predominant isoforms in cancer tissues in addition to VCAN-V0) mRNA and protein expression can be dose-dependently modulated by TGF-β.[17] It has been revealed that TGF-ß1 increases VCAN accumulation in the prostatic fibroblast culture medium, and the addition of neutralizing antibodies to TGF-β1 or exogenous TGF-β1 prevents the increase in VCAN accumulation in the PC3, LNCaP, and DU145 cell lines.[18]

Cancer stem cells (CSCs) in prostate cancer represent quite a small population responsible for drug resistance, invasion, and metastasis.[19] Increased VCAN expression seems to be related to the metastatic potential and poor patient outcomes in prostate cancer.[20,21] In our previous study, we showed that TGF-β1 was significantly upregulated in monolayer prostate cancer cells, while high expression levels of VCAN, Col7A1, and ITGβ3 were detected in 3D cancer cells in spheroids. Upregulated VCAN was detected only in the prostate CSCs.[20] Within the scope of these results, it was hypothesized that high TGF-β1 expression in the monolayer might trigger the three-dimensional architectural organization by inducing VCAN expression.[20] Our study investigated the role of TGF-β1 in VCAN expression via mRNA and protein levels in different prostate cancer cell lines.

METHODS

Cell Line and Cell Culture Conditions

Normal prostate epithelial cells (RWPE1) and human prostate cancer cell lines (LNCaP, DU145, and PC3) were purchased from the American Type Cell Culture Collection (Manassas, VA). PC3 cells were established from a grade 4 human prostate adenocarcinoma patient. The DU145 cell line was isolated from a brain metastasis of a prostate cancer patient. All cells were cultured in the recommended growth medium at 37 °C with 5% CO_2 and 100% humidity, as described previously.[20,22,23]

RNA Isolation, cDNA Synthesis, and Reverse Transcription-Polymerase Chain Reaction

Cells were seeded at a density of 1×10^6/well in a 10 cm^2 dish (for basal expression) and 2.5×10^5/well into a 6-well plate (for TGF-β1 treatments), overnight at 37 °C. For the basal expression, after 24 h, RWPE1, LNCaP, DU145, and PC3 cells were harvested in Trizol (Life Technologies), and RNA was isolated. For TGF-β1 PeproTech (Rocky Hill, NJ) treatments, after 24 h, the culture media of DU145 and PC3 were changed to 1% FBS, and the cells were treated with TGF-β1 (5 ng/mL) for 0, 1, 4, and 24 h. Total RNA was isolated using Trizol, as described previously.[24] 2 µg of total RNA were reverse-transcribed, and 3-µl cDNA was used to amplify the gene of interest. The following primers were used: VCAN forward, 5′-AAGTGATGCGGGTCTTTACC-3′; VCAN reverse, 5′-CTCCTGCCTTTCCCATCTTATC-3′ (amplify Exon 4 of the V0 isoform); L19 forward, 5′-GAAATCGCCAATGCCAACTC-3′; L19 reverse 5′-TCTTAGACCTGCGAGCCTCA-3′ (Integrated DNA Technologies, Skokie, IL). The polymerase chain reaction products were visualized on 1% agarose gels stained with ethidium bromide (Amresco, Solon, OH).

Western Blotting Analysis

The cells obtained from different experimental setups were trypsinized, collected and washed with cold PBS. The cells were lysed in the cell lysis buffer containing 1 mM Na_3VO_4, 20 mM Tris-HCl (pH 7.5), 1 mM β-glycerophosphate, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton-100, 2.5 mM sodium pyrophosphate, 1 Î¼g leupeptin, and a protease inhibitor mixture. The protein concentration in the samples was measured as previously described.[24] The cell lysate was mixed with Laemmli's buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol. Total protein (60 µg of proteins) in each individual sample was subjected to SDS/PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Thereafter, the membranes were blocked in 1xTBST containing 5% fat-free skim milk at room temperature for 1 h. The blots were incubated with anti-VCAN antibodies (dilution 1:1000, ab177480, Abcam, Cambridge, MA) overnight at 4 °C. The next day, after the washing steps, the blots were incubated with an HRP-conjugated secondary antibody. The Western blots were visualized with the Syngene PXI 6 imaging system (Syngene, Frederick, MD) using Millipore Luminata Forte (EMD Millipore, Billerica, MA). β-actin was used as a control in the study. The relative protein expression levels were determined using ImageJ (version 1.48, National Institutes of Health).

Statistical Analyses

The relative protein expression levels were determined by ImageJ (version 1.48, National Institutes of Health). Total protein levels between groups were compared.

RESULTS

1. Increased *VCAN* gene expression was determined in the normal prostate epithelium and PC3 cell line

In this assay, RWPE1 positivity can be considered a positive control. Furthermore, although *VCAN* gene expression levels of the followed cells on monolayer cultures in DU145 prostate cancer cells were negative, PC3 prostate cancer cell results were positive (Figure 1).

2. TGF-β1 treatment did not affect the *VCAN* gene expression level of the DU145 and PC3 cell lines

These cancer cell lines were treated 5 ng/mL TGF-β1 at different time points (1 h, 4 h, and 24 h, respectively). The *VCAN* gene expression of the PC3 cell line was neither induced nor inhibited in the absence or presence of TGF-β1. The results of the assay were similar at all time points. In this assay, it was observed that the basal *VCAN* gene expression of the DU145 human prostate cancer cell line was negative, independently of TGF-β1. The presence or absence of TGF-β1 did not have an inducing effect on the gene expression in these cells (Figure 2).

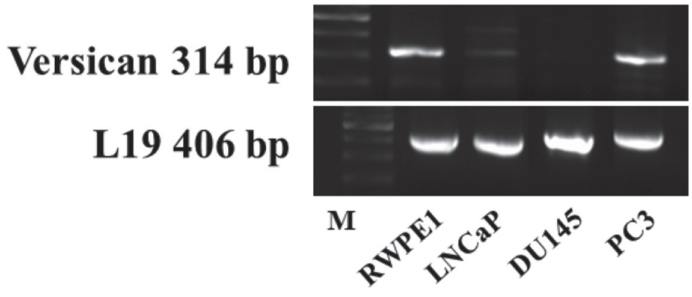

Figure 1. Reverse transcription-polymerase chain reaction for the basal expression of versican in four different prostate cell lines. RWPE1 (normal epithelial cells), LNCaP, DU145 and PC3 (prostate cancer cell lines)

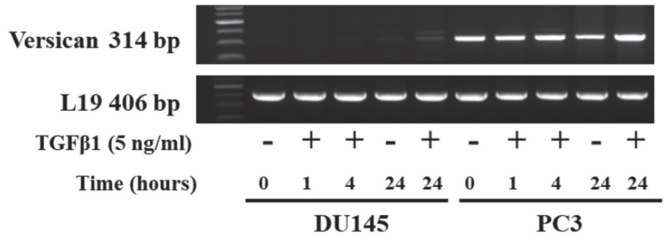

Figure 2. Reverse transcription-polymerase chain reaction for versican expression in DU145 and PC3 after treatments with transforming growth factor-β1 (5 ng/mL) at three different time points (1, 4 and 24 hours)

3. Increased VCAN protein levels in prostate cells with unrelated TGF-β1 expression

The results of the *VCAN* gene expression level in the PC3 human prostate cell line were correlated with Western blotting results. Increased protein levels were shown at different time points. Significantly low expression levels were observed at a 0-time point, while gradually high levels were detected at 24 h in the PC3 human prostate cell line. However, this time-dependent increase in 24 h was the same in treatment groups, and there was no inducing or suppressing effect of TGF-*β*1 in VCAN protein expression (Figure 3).

However, the results strikingly showed that VCAN protein expression increased in the DU145 human prostate cancer cell lines, while the results of the gene expression level were negative. The protein expression was not affected by the treatment of TGF-*β*1 positively or negatively at the current dose (5 ng/mL). While an increased VCAN level was observed at all time points in the DU145 cell line, a significantly high VCAN level was time-dependently detected in the PC3 cell line, especially at 24 h (Figure 4).

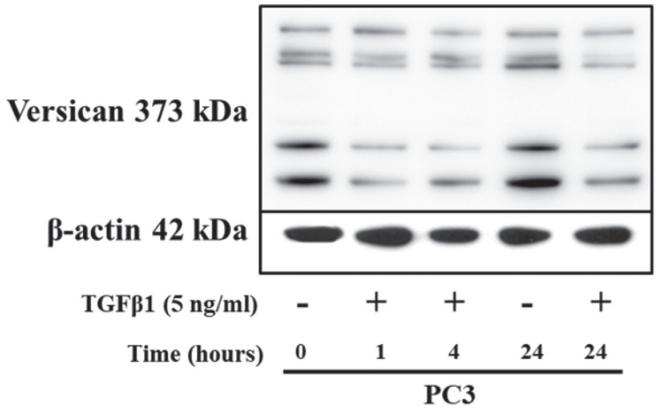

Figure 3. Western blot analysis for versican expression in PC3 after treatments with transforming growth factor-β1 (5 ng/mL) at three different time points (1, 4 and 24 hours)

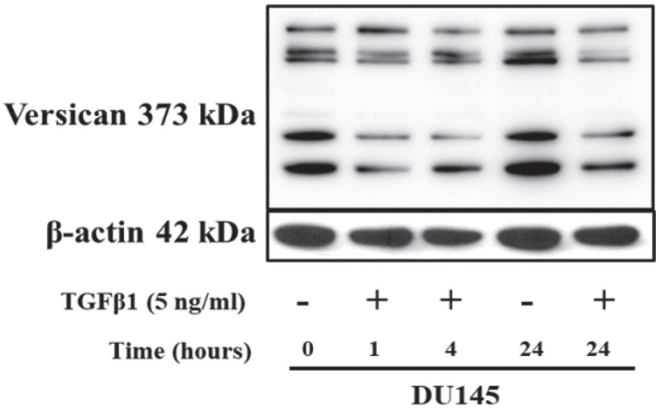

Figure 4. Western blot analysis for versican expression in DU145 after treatments with transforming growth factor-β1 (5 ng/mL) at three different time points (1, 4 and 24 hours)

DISCUSSION

In this study, we showed that high VCAN protein levels were independent of TGF-*β*1 with negative transcription results in the DU145 prostate cancer cell line. In our previous study, we demonstrated the increased expression in CSCs isolated from the DU145 cell line in a three-dimensional model. When considered, grade 4 human prostate adenocarcinoma may be more complicated in terms of cellular organization, and VCAN, a large ECM proteoglycan found in prostate tissue, may be expressed more. There are many studies showing that increased VCAN expression in different types of adenocarcinomas may be related to a patient's prognosis.[20,25-27] However, this study showed that VCAN protein expression was increased by different genetic mechanisms, especially in metastatic cancer cells. This probably reveals that cells in the metastatic region are effective molecules in the organization. Therefore, studies aiming at VCAN at the protein level can be the mechanism to slow or inhibit the progression of metastatic cancer.

It is logical to expect that DU145 cells will exhibit higher expression of VCAN since the DU145 cell line is derived from metastatic cancer and since CSCs play an important role in metastasis. The present study showed high VCAN protein levels, which were not associated with high mRNA levels in DU145 cells. These data indicate that prostate cancer brain metastasis cells may display different expression profiles compared with primary adenocarcinoma-derived cells in prostate cancer. The differential expression pattern in DU145 cells may also have resulted from other mechanisms. VCAN is encoded by the *VCAN* gene located on 5q12-14 in the human genome. It encodes 12 different mRNA transcripts produced by alternative splicing. Different transcripts are expressed during different developmental stages and different cell types. Four mRNA transcripts arise from alternative splicing, leading to V0, V1, V2, and V3 isoforms. During embryonic development, V0 and V1 isoforms are usually highly expressed, but their expression decreases after tissue differentiation.[28] Different transcripts produced by alternative splicing or other modifications may be a reason for different results of gene expression and protein expression in the primary and metastatic tumors and those observed in the present study. Post-transcriptional modifications of mRNA via miRNA or non-coding parts of mRNA are also a possible causes. 3-untranslated regions (3-UTRs) are non-coding parts of the mRNA. 3-UTRs determine protein levels by regulating mRNA stability and translation, which is mediated by AUC-

rich elements and miRNA. This region also enables local translation by regulating mRNA localization.[29-32]

Significant VCAN expression was denoted in cancer-associated fibroblasts instead of cancer cells. One of the TGF-β regulated gene signatures in cancer-associated fibroblasts is VCAN. Exogenous VCAN can enhance the invasion of ovarian cancer cells, as demonstrated by a 3D culture model, Matrigel invasion assay, and confocal microscopy. The transcriptomic analysis showed different expression profiles between ovarian cancer cells (OVCA433) treated with VCAN and control cells. MMP9, HMMR, ADAMTS1, and CD44 were upregulated, whereas TIMP3 was downregulated. These genes are involved in ECM degradation and cell motility.[15]

VCAN has a close network not only with TGF-β1 but also TGF-β2, TIMP3, and the ADAMTS family. Prostatic stromal cells show higher expression levels of ADAMTS (except for ADAMTS-15) than prostate cancer cell lines LNCaP, DU145, and PC3. TGF-β1 showed a decreasing effect on ADAMTS-1, -5, -9, and -15 transcripts and an increasing effect on ADAMTS-4, VCAN, and TIMP3 inhibitor. TIMP3 is suggested to lead to VCAN accumulation in the prostate stroma in benign prostatic hyperplasia and prostate cancer.[33] However, exogenous TGF-β2 induces VCAN V1 isoform expression and cleavage, which is predicted to be mediated by ADAMTS-1 and ADAMTS-4. Moreover, the increased activity of metalloprotease-2 by TGF-β2 can induce V1 protein degradation.[34] VCAN expression is triggered by TGF-β2 in several cancer cells, which may play a key role in tumor development.[11,12,35] In prostate cancer, proteoglycan accumulation is a known process that supports the modulated transcript and protein expression levels of VCAN, ADAMTS proteoglycanase, and TIMP3 in prostatic stromal cells by TGF-β.[33] Moreover, LY2157299, an inhibitor of TGF-βR1, inhibits TGF-β signaling and reduces VCAN expression, an important stroma-derived protein. These findings suggest that tumor growth and ascites formation of ovarian cancer cells decrease with LY2157299.[16] Additionally, another study showed that prostatic fibroblasts were treated with the LNCaP-, PC3-, or DU145-conditioned medium. The conditioned medium had an increasing VCAN expression effect in fibroblastic cells compared with the control fibroblast medium culture. Secondly, prostatic fibroblasts were treated with TGF-β1, which increased VCAN accumulation in the culture medium, indicating that TGF-β1 modulated VCAN expression. This effect on fibroblasts can be reversed by TGF-β1-specific neutralizing antibody by inhibiting VCAN accumulation. However, the immunoblot analysis indicated that the PC3 and DU145 cancer cell lines had higher levels of VCAN expression compared to the LnCaP cancer cell line.[18]

Study Limitation

Only one dose has been administered in our study and this can be considered a limitation.

CONCLUSION

In conclusion, this study showed that TGF-β1 has neither an inducible nor inhibitory effect on prostate cancer cells. We can argue this at least for the dose we have used in this study. However, it can be inferred that the increase in protein expression independent of the TGF-β1 effect may be effective in metastatic tumors. According to the literature, inhibitory or stimulatory effects of TGF-β1 on VCAN have been shown in many studies.[12,16,33,36,37] The dose we have used in this study is the most appropriate dose in the literature for VCAN, but the effects of TGF-β1 at different doses can be examined in further studies. Inhibition therapy of the VCAN protein may influence the life span of patients with clinical diagnosis or relapse in a secondary tumor. From now on, it will be important to determine the mechanism by which the post-transcriptional effects of VCAN occur in secondary tumors. It can be thought that the metastatic tumor niche may also be effective in VCAN protein expression.

Ethics

Ethics Committee Approval: Our study was carried out in a cell culture line and there is no need for ethics committee approval.

Informed Consent: Cell culture study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.S., E.A., S.K., A.T., G.Ö., Design: B.S., E.A., G.Ö., Data Collection or Processing: B.S., E.A., S.C., S.K., G.Ö., Analysis or Interpretation: B.S., E.A., S.C., S.K., G.Ö., Literature Search: B.S., E.A., A.T., S.K., G.Ö., Writing: B.S., E.A.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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