RESEARCH ARTICLE / *Araştırma Makalesi*

The Role of MEG3 in the Activation of Toll Like Receptor 3 in Prostate Cancer Cells

MEG3'ün Prostat Kanseri Hücrelerindeki Toll Benzeri Reseptör 3 Aktivasyonundaki Rolü

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Abstract

Objective	Prostate cancer accounts for approximately 10% of new cases diagnosed in men worldwide. Toll like receptors (TLRs) play a crucial role in the progression of cancer. Furthermore, the expression level of TLRs is mediated by different transcription factors and non-coding RNAs. Therefore, the aim of this study was to investigate the potential regulatory role of MEG3 and the interaction of TLR3 with MEG3 in the prostate cancer cells.
Materials and Methods	In this study, PC-3, LNCaP and HUVEC cells were used. To stimulate TLR3 expression, Poly I:C was used for a ligand of TLR3 and the less cytotoxic concentration of Poly I:C was determined by WST-1 analysis. The relative gene expression levels of TLR3 and MEG3 were analyzed by RT-PCR.
Results	According to the results, 5 μM of Poly I:C was chosen as a less cytotoxic concentration for the stimulation of TLR3 activity. The mRNA level of MEG3 (3.19-, 1.90-, and 1.90-fold) and TLR3 (6.17-, 5.75- and 2.27-fold) was significantly increased in PC-3, LNCaP and HUVEC cells, respectively after Poly I:C stimulation ($p<0.05$). Additionally, the expression level of MEG3 was 2.33- and 10.93-fold for PC-3 and LNCaP cells respectively, compared to HUVEC cells ($p<0.05$).
Conclusion	In conclusion, the activation of the TLR3 signaling pathway through Poly I:C promoted the level of MEG3 expression especially in castration-resistant prostate cancer cells. Thus, our preliminary data suggests that MEG3 could modulate TLR3 signaling pathway in prostate cancer cells.
Keywords	Prostate cancer; TLR3; MEG3; Poly I:C

Öz

Amaç	*Dünya genelinde erkeklerdeki yeni kanser tanılarının yaklaşık %10'unu prostat kanseri oluşturmaktadır. Toll-benzeri reseptörler (TLR), prostat kanseri gelişiminde önemli rol oynamaktadır. Ayrıca TLR'lerin ekspresyon seviyesi çeşitli transkripsiyon faktörleri ve kodlanmayan RNA'lar ile düzenlenmektedir. Bu nedenle bu çalışmada MEG3'ün potansiyel düzenleyici rolünün ve TLR3-MEG3 ilişkisinin belirlenmesi amaçlanmaktadır.*
Gereç ve Yöntemle	*Bu çalışmada PC-3, LNCaP ve HUVEC hücreleri kullanılmıştır. Poli I:C; bir TLR3 ligandı olarak TLR3 ekspresyonunu uyarmak için kullanılmıştır. Poli I:C' nin toksik olmayan konsantrasyonu WST-1 analizi ile belirlenmiştir. TLR3 ve MEG3'ün rölatif gen ekspresyon seviyeleri RT-PCR ile analiz edilmiştir.*
Bulgular	*Sonuçlara göre, 5 μM Poli I:C TLR3'ü aktive edilmesi için toksik olmayan konsantrasyon olarak seçilmiştir. MEG3 (3.19-, 1.90- ve 1.90-kat) ve TLR3 (6.17-, 5.75- ve 2.27-kat) mRNA seviyelerinin Poli I:C uygulamasından sonra sırasıyla PC-3, LNCaP ve HUVEC hücrelerinde anlamlı bir şekilde arttığı belirlenmiştir ($p<0.05$). Ayrıca, MEG3'ün mRNA seviyesi, HUVEC hücrelerine kıyasla PC-3 ve LNCaP hücrelerinde sırasıyla 2.33- ve 10.93- kat olarak tespit edilmiştir ($p<0.05$).*
Sonuç	*Sonuç olarak, Poli I:C aracılığıyla uyarılan TLR3 sinyal yolunun aktivitesi özellikle kastrasyon dirençli prostat kanseri hücrelerinde, MEG3 ekspresyon seviyesini arttırmıştır. Ön verilerimiz prostat kanserinde MEG3'ün TLR3 sinyal yolağını düzenleyebildiğine dair kanıtlar sunmaktadır.*
Anahtar Kelimeler	*Prostat kanseri; TLR3; MEG3; Poli I:C*

INTRODUCTION

Prostate cancer is one of the most common types of cancer in males and takes the second place in cancer-related deaths.[1-3] Nearly one million of people diagnoses with prostate cancer annually, which accounts for 10% of all new cancer diagnoses in males worldwide.[4-5] Prostate cancer cells require androgens for proliferation. Therefore, the primary aim in the treatment of prostate cancer has been to reduce the levels of androgens in the blood to prevent the effects of the hormone on the cancer cells. However, nearly a third of all hormone-based treatments fail within 10 years due to metastasis and the stage of the disease.[6] Therefore, the understanding the carcinogenesis and growth mechanisms of androgen-dependent/independent, and metastatic prostate cancer is of importance in the development of new effective treatments.

Toll-like receptors (TLRs) are involved in the innate immune response. Although TLRs are predominantly expressed in immune system cells such as dendritic cells, macrophages, and natural killer cells (NK), recent studies have been indicated that TLRs are also expressed in cancer cells.[7] The expression levels of TLRs are regulated by various transcription factors, such as miRNAs, LncRNAs, and other cellular signaling pathways.[8] In prostate cancer, the activation of TLR3 can result in the inhibition of prostate cancer progression.[9] On the other hand, TLR3 expression levels are higher in 85% of prostate cancer patients compared to healthy individuals.[10] Furthermore, the TLR3 signaling pathway initiates apoptosis through PI3K/Akt suppression and inhibits LNCaP cell proliferation.[11] Therefore, the activation of TLR3 signaling pathways and its association with apoptosis need further investigation.

Long non-coding RNA's (LncRNAs) are DNA transcripts longer than 200 nucleotides with a low chance of coding for proteins.[12-16] There are many studies showings that LncRNAs have an important role in determining tumor behavior in carcinogenesis.[17-19] In different cancer types, including prostate cancer, changes in the expression levels of LncRNA's have been linked with the clinical prognosis of cancer.[20] For instance, SNHG1 has been associated with the cell proliferation in prostate cancer.[21] On the other hand, MEG3 has inhibited the cell proliferation and metastasis in gastric cancer through the p53 signaling pathway.[22] Additionally, recent studies have suggested that MEG3 has an inhibitory effect on cell growth and metastasis in prostate cancer and a significant decrease in the MEG3 expression level is detected in prostate cancer patients.[23] Zhang et.al[24] show that MEG3 inhibits the growth of breast cancer cell through NF-κB and p53 activation. MEG3 also inhibits the progression of prostate cancer by modulating the miR-9-5p/QKI-5 axis involved in prostate cancer cell proliferation, migration and invasion.[25] However, the molecular role of MEG3 in the development and progression of prostate cancer has not been fully elucidated.

Therefore, the aim of this study was to determine the changes in MEG3 and TLR3 expression levels after TLR3 activation through Poly I:C stimulation in androgen dependent and metastatic prostate cancer cells.

MATERIALS and METHODS

Cell Culture

This study was conducted in Cancer Research Laboratory of Sakarya University Faculty of Medicine in 2019. In this study, LNCaP (androgen-dependent/sensitive) and PC-3 (androgen-independent/metastatic) prostate cancer cell lines and human umbilical vein endothelial cells (HUVEC, control group) were used and obtained from ATCC (Manassas, VA). PC-3 and LNCaP cell lines were cultured in Roswell Park Memorial Institute Medium (RPMI-1640, Gibco), and HUVEC cell lines in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) medium with 100 Units/mL penicillin/streptomycin at 37°C in a humidity atmosphere with 5% CO2.

Cell Viability Assay

To determine the less cytotoxic concentration of Poly I:C, the cells were cultured into 96-well plates at 2x104 cells/

mL in per-well. The cells were incubated with different (1, 2.5, 5 and 10 μM) Poly I:C concentrations for 24 and 48 hours. Then, 10 μL of WST-1 dye was added to the each well and further incubated for 1-4 hours at 37 °C. After that, the absorbance was measured at 460-620 nm wavelengths using the Elisa Reader.

RT-PCR Gene Expression Analysis

To determine the expression levels of the TLR3 and MEG3, RT-PCR was conducted. Total RNA was isolated according to the Total RNA Isolation Kit protocol. After the quality and concentration of RNA was measured at 260 nm in a spectrophotometer, the total RNA was converted into the cDNA. The obtained cDNA was diluted with nuclease-free distilled water. The TLR3 and MEG3 expression levels were determined by RT-PCR. Actin-β was used as a control gene.

Statistical Analyses

The data was evaluated using the "SPSS 22.0" statistical program and $p<0.05$ was considered statistically significant. Differences between cell viability percentages were evaluated by one-way analysis of variance (Post-Hoc Tukey). Differences in mRNA expression levels, were analyzed by online software.

RESULTS

The Results of Cell Viability Assay

To determine the less cytotoxic concentration of Poly I:C, PC-3, LNCaP, and HUVEC cells were treated with different concentrations (1μM, 2.5μM, 5μM and 10μM) of Poly I:C for 24 and 48 hours, respectively (Figure 1). While the viability of PC-3 was 94%, 93%, 81% and 70%, the viability of LNCaP cells was reduced to 94%; 91%, 83% and 71% at concentrations of 1, 2.5, 5 and 10 μM Poly I:C for 48 h, respectively ($p<0.05$). Additionally, the viability of HUVEC cells was found to 96%; 91%, 90% and 87% at different concentrations of Poly I:C (1, 2.5, 5 and 10 μM) for 48 h, respectively ($p<0.05$). According to our results, the less cytotoxic concentration of Poly I:C in all the cells was determined as 5μM for 48 hours.

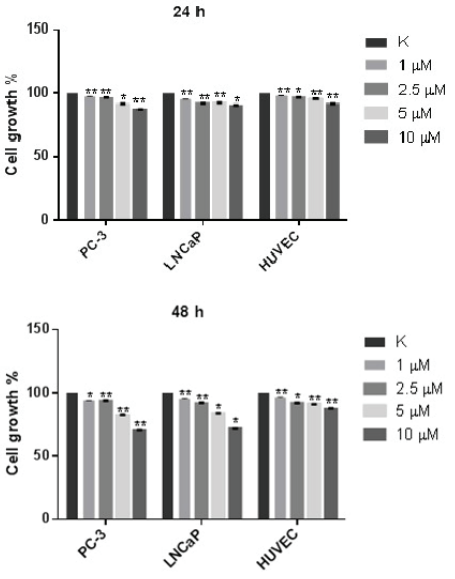

Figure 1. The effect of various Poly I:C concentrations on the cell viability of PC-3, LNCaP and HUVEC cell lines at (A) 24 and (B) 48 hours (; $p<0.05$, **; $p<0.001$, μM: micromolar, %; percentage).*

The Results of Gene Expression Assay

The relative expression levels of TLR3 and MEG3 in PC-3, LNCaP and HUVEC cells were shown in Figure 2 and 3. While the TLR3 mRNA level increased 3.97- and 1.56-fold, the MEG3 expression levels increased 2.33- and 10.93- fold, while in PC-3 and LNCaP cells, respectively compared to HUVEC cells ($p<0.05$) as shown in Figure 2. Furthermore, the mRNA level of TLR3 was up-regulated 6.17-, 4.75- and 2.27-fold, whereas there was a 3.19-, 1.90- and 1.90-fold increase in the expression of MEG3 in PC-3, LNCaP, and HUVEC cells, respectively ($p<0.05$) after stimulation with 5μM Poly I:C in Figure 3. Therefore, both TLR3 and MEG3 mRNA levels were higher in PC-3 cells than LNCaP cells upon stimulation with Poly I:C.

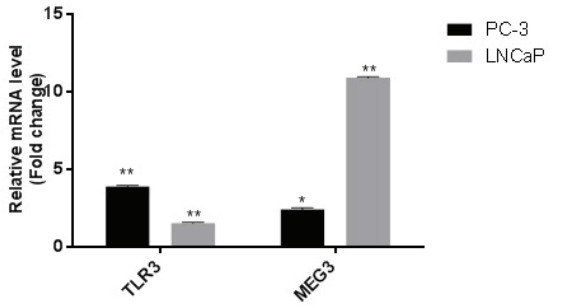

Figure 2. Fold changes in TLR3 and MEG3 mRNA expression levels in PC-3 and LNCaP cell lines in comparison to the control cells (HUVEC) (; p<0.05, **; p<0.001, µM: micromolar, %; percentage).*

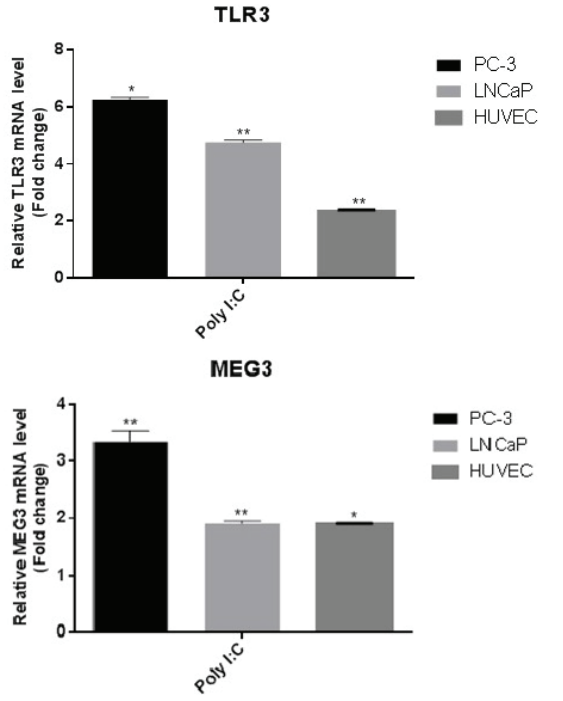

Figure 3. The relative mRNA expression levels of (A) TLR3 and (B) MEG3 in Poly I:C (5 µM) stimulated PC-3, LNCaP and HUVEC cells (; p<0.05, **; p<0.001, µM: micromolar, %; percentage).*

DISCUSSION

TLRs play an important role in cancer progression and have a potential to become therapeutic targets according to studies. The expression levels of TLR3 in prostate cancer cells are controversial in the literature. The study of Gonzalez-Reyes et al.[10] states that higher TLR3 expression level is associated with poor prognosis in 85% of prostate cancer patients. On the other hand, the activation of TLR3 signaling pathway induces apoptosis through PI3K/Akt signaling inhibition in LNCaP prostate cancer cells.11 Furthermore, some lncRNAs regulate the activation of different TLRs[26-28]. Engrailed-2 (EN-2) of the HOX gene family has oncogenic function due to promoting the proliferation and migration of prostate cancer cells.[29] Zhou et.al[30] suggest that MEG3 suppressed EN2 expression and increased MEG3 expression level inhibits the proliferation, viability, invasiveness, and metastasis of PC-3 cells xenograft model, in vivo. Additionally, the over expression of EN-2 treatment has increased PSA production as well as the proliferation of LNCaP cells through the modulation of cancer-related genes, PI3K/AKT signaling pathway and androgen receptor phosphorylation.[29] On the other hand, Ding et al.[31] indicate that the treatment of polyinosine–polycytidylic acid (PIC) as TLR3 agonist inhibits drug transporters and enhances the low-dose cisplatin-induced cell death in TLR3- and caspase-3–dependent manner in OSCCC cell lines. Additionally, activated TLR3 suppresses the inflammation-related long noncoding RNA lnc-IL7R, which is upregulated during this chemotherapy. Therefore, different lncRNAs regulates TLR3 activation. On the other hand, MEG3 regulates the secretion of proinflammatory cytokines as well as inflammatory response through TLR4–NF-κB signaling pathway.[32,33]

However, there is no study exploring the interaction of TLR3 and MEG3 in cancer cells. In the present study, the expression level of both MEG3 and TLR3 investigated in prostate cancer cells. Furthermore, we focused on the changes in the expression levels of MEG3 and TLR3 after Poly I: C stimulation to further explore the link between

TLR3 and MEG3 expression. Before the stimulation of TLR3 signaling through Poly I:C, MEG3 expression level was lower in PC-3 cells than LNCaP cells compared with HUVEC cells. However, TLR3 mRNA expression was higher in PC-3 cells than LNCaP cells. On the other hand, Poly I:C treatment changed the expression level of both TLR3 and MEG3. We concluded that the expression levels of MEG3 was significantly increased after the stimulation of TLR3 with Poly I:C in PC-3 cells. Additionally, a higher increase in both TLR3 and MEG3 expression at mRNA levels were observed in PC-3 cells compared with LNCaP cells. However, the MEG3 mRNA level was reduced after Poly I:C stimulation in LNCaP cells despite of increased TLR3 expression level. Therefore, our preliminary findings demonstrated that the expression of MEG3 level could regulate the activation of TLR3 signaling pathways in especially metastatic prostate cancer cells. However, further MEG3 targeted genes should be elucidate for the identification of the tumor suppressive role of MEG3 in prostate cancer cells.

In the literature, metastatic castration resistant prostate cancer cells exhibit aggressive behaviors than hormone-dependent prostate cancer cells.[34] On the other hand, Lee et al.[35] show that increased androgen receptor expression leads to lower survival and worse prognosis in prostate cancer patients. Therefore, different molecular features of prostate cancer cells could lead to changes in the TLR3 and MEG3 expression levels in this study in response to Poly I:C treatment. However, further molecular experiments are required to identify the molecular interaction of MEG3 with TLR3 mediated signaling pathways in prostate cancer cells.

In conclusion, we determined that changes in the expression level of the MEG3 and TLR3 in response to the activation of the TLR3 signaling pathway through Poly I:C, for the first time. Furthermore, higher expressions of MEG3 and TLR3 were detected in the castration resistant prostate cancer cell line (PC-3) compared to the androgen dependent cell line (LNCaP). However, further comprehensive studies are required to understand the molecular mechanisms of MEG3 mediated TLR3 signaling pathway in prostate cancer cells.

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