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Investigation of TLR4 Gene Expression in Prostate Tumor Tissues

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Abstract | **Original Research Article**

TLR4 is expressed on normal and malignant prostate epithelial cells. TLR4 activation signaling in prostate cells initiates innate immune responses to invading pathogens. However, long-term activation of TLR4 cell signaling pathway in prostate epithelial cells may promote tumor cell activation, proliferation, survival, and tumor transformation. Current study was involved 16 samples from prostate cancer tissues and 25 samples from prostate benign tumor tissues, compared with eight free cancer samples tissues as control group. For isolation total RNA from formalin-fixed paraffin-embedded (FFPE) samples, total RNA of all samples was extracted using the AccuZol reagent. Total RNA was reversely transcribed to complementary DNA (cDNA). Synthesized cDNA was immediately used as a template for real-time PCR. Our results showed that the TLR4 expression in prostate cancer cases higher than prostate benign hyperplasia tissues, while cancer tissues and benign tissues showed higher TLR4 expression than control group. The folding of TLR4 gene expression showed 2.21 folds in prostate cancer tissues, while the TLR4 expression in benign hyperplasia tissues showed 1.33 fold. We concluded that the over expression of TLR4 in prostate cancer may be due to the chronic inflammation in prostate cancer patients.

Keywords: Prostate, Cancer, Benign, TLR4, Tumor.

INTRODUCTION

Prostate cancer is one of the most common causes of morbidity and mortality in men, PCs is the second most common cancer in men globally, as approximately 1.3 million men were diagnosed with the disease in 2018, accounting for 13.5 % of the cancers diagnosed in men during that year with 6.7% mortality rate [1]. Toll-like receptors are a family of trans-membrane receptors that play a key role in the innate immunity [2]. It can also recognize endogenous damage-associated molecular patterns (DAMPs) in different disorders and diseases such as cancer [2]. Ten of TLRs have been identified in human. TLR1s, TLR2, TLR4, TLR5, and TLR6 are expressed on cell surface; however, TLR3, TLR7, TLR8, and TLR9 are found exclusively within endosomes [3]. Activation of TLRs leads to the activation of innate immunity and results in the production of pro-inflammatory cytokines, chemokines, as well as adhesion molecules, and then facilitates the activation of adaptive immunity [4]. TLR activation in tumor cells and its activation in tumor microenvironment such as in typical innate immune cells lead to a complex scenario; therefore, TLRs activation might play a “double-edged sword” role in the influence of tumor progression [3]. An endogenous TLRs ligand, DAMPs released from damaged and/or necrotic tissues, might play a pivotal role [3]. In term of endogenous TLR ligands in cancer, HMGB1 can activate TLR2 and TLR4 [5]. Peroxiredoxin 1 (Prx1) appears to be an agonist of TLR4 in prostate cancer development [6]. Perhaps, the activation of some TLRs might prevent the tumor growth of prostate cancer [3]. Stimulation of TLR4 occur through microbial molecules (e.g., LPS), as well as endogenous ligands (e.g., HSPs, fibrinogen, and HMGB1), induces pro-inflammatory cytokine production, such as IL-6, TGF-β1, TNF-α, IL-1β, inducible nitric oxide synthase, and antiapoptotic protein expression [7]. Notably, TLR4 is expressed on normal and malignant prostate epithelial cells [8]. TLR4 activation signaling in prostate cells initiates innate immune responses to invading pathogens. However, long-term activation of TLR4 cell signaling pathway in prostate epithelial cells may promote tumor cell activation, proliferation, survival, and tumor transformation [9]. In addition, TLRs downstream cytokines IL-6, IL-8, and IL-10 mediate prostate cancer development and disease progression [10]. Moreover, upon TLR4 ligand stimulation, prostate epithelial cells upregulate NF-κB, TGF-β1, and VEGF through increased TLR4 expression and induction of pro-inflammatory mediators [9]. Higher levels of some TLR4 downstream cytokines (e.g., IL-8) have been observed in prostate cancer tissues compared with

control non-tumor tissues, suggesting that prostate cells experience persistent elevated inflammation presumably in response to bacterial products such as LPS as well as endogenous TLR4 ligands released from injured cells [11].

MATERIALS AND METHODS

Patients study

Patients were confirmed as a prostate cancer by pathologists from AL Karamah and AL Zahrah Teaching Hospitals in Wasit Province with no other disease such as autoimmune and other chronic diseases. Current study was involved 16 samples from prostate cancer, 25 samples from prostate benign tumor and eight samples from tumor free individuals as control group. For isolation total RNA from formalin-fixed paraffin-embedded (FFPE) samples, the samples were cutting into small pieces with 4-8 cm thick, 25-40 µgm of samples were treated by xylene 3 times in 1.5 eppindrofe tube and then washing by absolute ethanol,70% and 50% for 2 times and centrifuged at 2,000 rpm for 5 minutes. Samples were incubated with 10µ proteinase K at 56°C and 100 µl D.W overnight.

Quantification of TLR4 gene

TLR4 gene expression is carried out using reverse transcription quantitative Real-time polymerase chain reaction

Total RNA extraction with *AccuZol* (TRIzol)

Prostate Cancer tissues in Formalin-Fixed Paraffin-Embedded were used to extraction RNA. Total RNA of all samples was extracted using the *AccuZo*l reagent following the protocol provided by the manufacturer.

Total RNA directly converted to cDNA, or also can be in 100 % formamide (deionized) and store at -70C. RNA concentration was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop – Promega USA), RNA concentration more than 1.6 n.ml was used in reverse transcription reactions .The extracted RNA was reversely transcribed to complementary DNA (cDNA) using (СИНТОЛ Company / Russia). The procedure was carried out in a reaction volume of 25 µl according to the manufacturer's instructions. Three main steps were needed to conversion.mRNA-TLR4 was quantified using Real time PCR.GAPDH was used as reference gene. Lyophilized primers were diluted in a D.W.as mentioned in manufacturer's instructions. TLR4 and GAPDH primer sequences obtained from references (12, 13) were amplified using the following sequences 5-TGG ACC TGC GAT TTA ATC CC-3 for TLR4 forward and 5-GTC TGG ATT TCA GAG CAG GA-3 for TLR4 reverse, 5-ATG GCT ATG ATG GAG GTC CAG-3 for GAPDH forward and 5-TTG TCC TGC ATC TGC TTC AGC-3 for GAPDH reverse. Components of reaction volume used RT-qPCR and program and cycling condition for amplifying TLR4 gene by RT-qPCR were seen in table (1 and 2).

Table-1: Components of reaction volume used RT-qPCR

Components	Reaction volume
cDNA	2 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Eva Green	10 µl
H2O	5.8 µl
Mgcl2	0.2 µl
Total	20 µl

Table-2: Program and cycling condition for amplifying TLR4 gene by RT-qPCR

Cycling Step	Temperature	Time	Cycles
Enzyme activation	95 C°	5 min	1
Denaturation	95C°	30 sec	X 35
Annealing	59 C°	30 sec	
Signal stabilization	72 C°	40 sec	
Melting	25 C°	6 sec	

Evaluation of gene expression variations were calculated in term of fold respect to the genes gene target for patients and control group by the $2^{-}\Delta\Delta CT$ method [14].

RESULTS AND DISCUSSION

Quantitative Real- Time PCR results

Real time PCR quantitative was applied in this study by using EVA green. The fluorescent dye recognized any double stranded DNA including cDNA and the amplification was recorded as a cycle threshold (Ct) value. The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate a low gene

expression and a low Ct values indicates a high gene expression [15, 16]

TLR4 Gene Expression

TLR4 was overexpressed in prostate cancer cases and benign hyperplasia tissues ,the expression of TLR4 was ranged between 0.09 and 9.13 folds with mean 2.21folding in prostate cancer tissues, while the TLR4 expression in benign hyperplasia tissues was ranged from 0.33 to 7.64 fold with mean 1.33 folding as shown in table (3) and Figure (1-3) .Our results showed the TLR4 expression in prostate cancer cases higher than prostate benign hyperplasia tissues ,while cancer tissues and benign tissues showed higher TLR4 expression than control group.

Table-3: TLR4 gene expression in prostate cancer and benign **hyperplasia, depending on 2-ΔΔCt method, normalized with GAPDH gene**

Cases	Ct GAPDH Mean	Ct target gene mean	Folding of TLR4 mean	Folding range
Prostate cancer patients	24	22.32	2.21	0.09 to 9.13
Benign prostate patients	26	25.21	1.33	0.33 to 7.64

The result of this study is agreed with results of Zhang *et al.,* who reported the TLR4 gene was highly expressed in prostate cancer cells [17]. Recently it was reported that the TLR4 ligand peroxiredoxin-1 is over-expressed in human PCa specimens and that it regulates prostate tumor growth in a murine cancer experimental model through TLR4-dependent induction of prostate tumor vasculature [18].

On the other hand, study of He *et al.* showed that the mean TLR4 staining score was significantly higher in BPH tissues with inflammation compared with BPH tissues without inflammation (5.13±1.21 and 2.96±0.73, respectively; P<0.001), and reported that lipopolysaccharide LPS/TLR4 signaling induces down-regulation of the bone morphogenic protein and active in membrane-bound inhibitor (BAMBI), which enhances transforming growth factor TGF-β signaling in the epithelial mesenchymal transition EMT process during prostatic hyperplasia [19]. Moreover other studies have suggested that LPS/TLR4 signaling plays a key role in the process of chronic inflammation inducing organ fibrogenesis and cancer development [20, 21]. Furthermore, inhibition of LPS-mediated activation of TLR4 signaling pathway by selenium in human PCa cells results in decreased pro-inflammatory and likely anticancer activities [22].

In addition, TLR4 responses have been shown to confer antitumor activity. Recognition of TLR4 on antigen-presenting cells is able to enhance antigen-specific antitumor immunity [23]. So, Ou, T. *et al.* hypothesized that long-term low doses of microbial or endogenous TLR4 ligand simulation in local microenvironments (e.g., prostate) may promote cancer development; whereas one or two-time administration with high doses of TLR4 ligands (e.g., vaccine adjuvant) enhance antigen specific antitumor immune responses [24].

Also, Shcheblyakov *et al.* reported that TLRs have had the opposite effects on tumor progression [25]. On the one hand, TLR ligands can suppress tumor growth; while on the other hand, TLR agonists can promotes the survival of malignant cells and increase their resistance to chemotherapy [24]. In addition, TLRs recognize microbial molecules, which results in the development of inflammatory reactions caused by the activation of the NF-kB regulating expression of anti-inflammatory cytokines (TNF-α, IL-1, IL-6, etc.) and chemokines (MCP-1, MCP-3, GM-CSF, etc.) [26]. TLRs have been implicated in the transcriptional and posttranslational regulation (proteolytic cleavage and secretion) of antimicrobial factors, such as defensins α and β, phospholipase A2, lysozyme, and so on [27]. TLRs intensify the phagocytosis of microorganisms and optimize their inactivation by regulating the release of peroxy radicals and nitric oxide [28].

Some studies showed low or physiologic doses of TLR agonists may mediate cancer development [29, 30], as evidenced by the use of TLR inhibitors to treat certain cancers [31]. TLR4 expression and its attendant chronic inflammation (e.g., IL-6) are associated with faster progression and poorer treatment outcomes in prostate cancer [32]. Moreover, TLR4 is expressed on normal and malignant prostate epithelial cells [9]. Activation of TLR4 signaling in prostate cells initiates innate immune responses to invading pathogens. However, long-term activation of TLR4 cell signaling pathway in prostate epithelial cells may promote tumor cell activation, proliferation, survival, and tumor transformation [33]. In addition, TLR downstream cytokines IL-6, IL-8, and IL-10 mediate prostate cancer development and disease progression [34]. Higher levels of some TLR4 downstream cytokines (e.g., IL-8) have been observed in prostate cancer tissues compared with control non-tumor tissues [10].

The findings of Croasdell *et al.* showed that the ability of specialized proresolving mediators (SPMs), including resolving D2 RvD2 to reduce TLR4 expression and attenuate LPS-induced inflammation , and explained that by in THP-1 cells, RvD2 reduced expression of TLR4, lymphocyte antigen (MD-2), and downstream signals (MyD88, TRIF, and TAK1) [35].

These effects were partially mediated through RvD2 induction of microRNA-146a, and RvD2's actions were blocked by microRNA-146a inhibition.

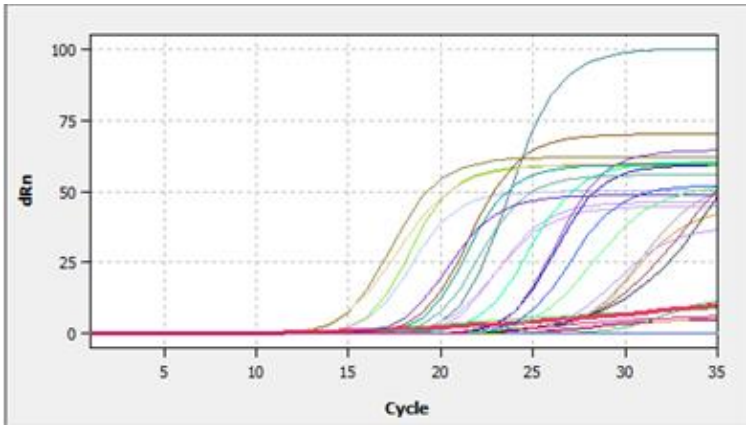

Fig-1: TLR4 gene amplification plots by qPCR. Normalized with GAPDH

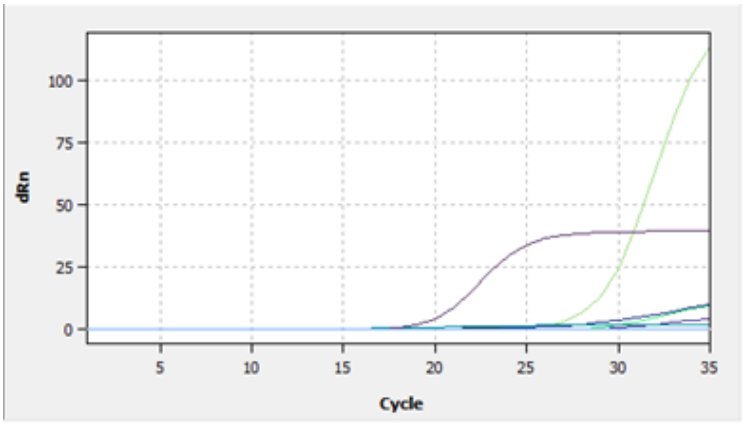

Fig-2: First test of TLR4 gene amplification plots by qPCR .

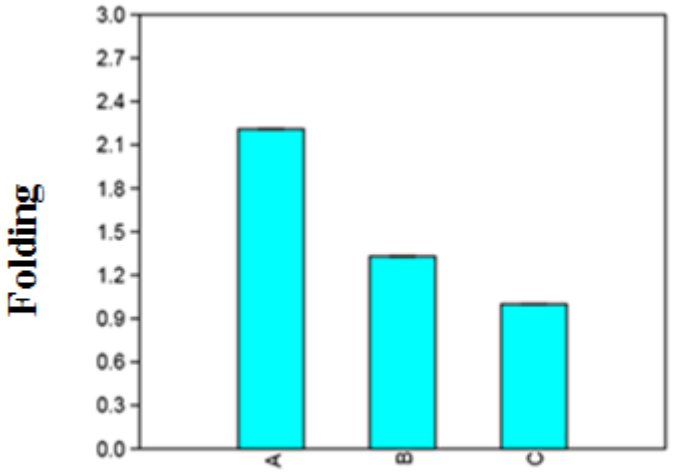

Fig-3:TLR4 Expression in prostate cancer, prostate benign hyperplasia and control group, normalized with GAPDH (A: Prostate cancer tissues B: Prostate benign tissues C: Control group)

CONCLUSION

Our results concluded that TLR4 amplification plays an important role in prostate hyperplasia and carcinogenesis.

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