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

<table>
<thead>
<tr>
<th>Table-1: Components of reaction volume used RT-qPCR</th>
</tr>
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<tbody>
<tr>
<td>Components</td>
</tr>
<tr>
<td>cDNA</td>
</tr>
<tr>
<td>Forward Primer</td>
</tr>
<tr>
<td>Reverse Primer</td>
</tr>
<tr>
<td>Eva Green</td>
</tr>
<tr>
<td>H2O</td>
</tr>
<tr>
<td>Mgcl2</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table-2: Program and cycling condition for amplifying TLR4 gene by RT-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycling Step</td>
</tr>
<tr>
<td>Enzyme activation</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Annealing</td>
</tr>
<tr>
<td>Signal stabilization</td>
</tr>
<tr>
<td>Melting</td>
</tr>
</tbody>
</table>

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^ΔΔCT 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 2.21 folding 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-∆∆Ct method, normalized with GAPDH gene**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Ct GAPDH Mean</th>
<th>Ct target gene mean</th>
<th>Folding of TLR4 mean</th>
<th>Folding range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancer patients</td>
<td>24</td>
<td>22.32</td>
<td>2.21</td>
<td>0.09 to 9.13</td>
</tr>
<tr>
<td>Benign prostate patients</td>
<td>26</td>
<td>25.21</td>
<td>1.33</td>
<td>0.33 to 7.64</td>
</tr>
</tbody>
</table>

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 morphogenetic 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 defenses 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 perox 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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