

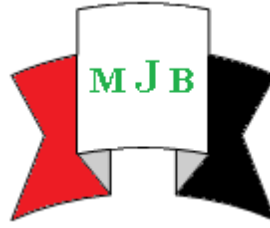
## Toll-Like Receptor 4 Gene Polymorphisms and Bladder Cancer

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**Received 3 February 2014**

**Accepted 3 March 2014**

### **Abstract**

**Background:** Bladder cancer (BCa) is one of the most common cancer diagnosed worldwide with multiple risk factors.

**Aims:** this study aimed to investigate the association between two single nucleotide polymorphisms (SNPs) in the toll-like receptor 4 (*Tlr4*) gene (*Asp299Gly* and *Thr399Ile*) and the incidence of BCa.

**Subjects and Methods:** A total of 48 BCa patients and 36 healthy controls were enrolled in this study. DNA was extracted from the blood samples taken from these participants. *Tlr4* gene was amplified with polymerase chain reaction (PCR) using specific primers. Genotyping of the SNPs of interest was done by restriction fragment length polymorphism (RFLP).

**Results:** Overall, there were no significant association of BCa with neither SNPs, however, the mutant allele (G) of the SNP *Asp299Gly* had higher frequency among patients compared with control (P=0.05).

**Conclusion:** allele G of the SNP *Asp299Gly* may be considered as a risk factor for BCa.

**Key words:** Bladder cancer, single nucleotide polymorphism, toll-like receptor 4

### **الخلاصة**

**الخلفية:** يعد سرطان المثانة من اكثر السرطانات المشخصة في العالم حيث هناك العديد من العوامل التي تساعد على الاصابه بهذا المرض.

**الاهداف:** هدفت الدراسة ببيان دور نوعين من التغيرات الوراثي في جين *Tlr4*(*Asp299Gly* and *Thr399Ile*) كعامل مساعد للاصابه بمرض سرطان المثانة.

**الاشخاص وطرق العمل:** شملت الدراسة ٤٨ شخصا مصابا بسرطان المثانة من الذكور والاناث وكذلك ٣٦ من الاشخاص الاصحاء. تم جمع عينات الدم من جميع المتطوعين لهذه الدراسة حيث تم استخلاص الحامض النووي من جميع عينات الدم ومن ثم استعملت تقنية ال PCR لمضاعفة جين *Tlr4* باستخدام بواقي خاصة. استعملت تقنية RFLP لمعرفة نوع التغيرات الجيني لكل من المرضى والاصحاء.

**النتائج:** هناك ارتباط وثيق بين سرطان المثانة مع الاليل الطافر (G) للتغيرات الوراثي في جين *Tlr4*(*Asp299Gly*) عند مقارنة المرضى مع الاشخاص الاصحاء.

**الاستنتاج:** الاليل G للتغيرات الوراثي *Asp299Gly* من المحتمل ان يلعب دورا كعامل مساعد للاصابة بسرطان المثانة.

### **Introduction**

**B**ladder cancer is the ninth most common cancer diagnosis worldwide, with more than

330000 new cases each year and more than 130000 deaths per year [1]. In Iraq, this malignancy represents about 6.65% from all malignancies with 10%

in males and 3.16% in females [2]. Many factors, such as tobacco smoking [3] occupational exposure to chemicals [4], bladder schistosomiasis and chronic infection [5] can be associated with the incidence of this disease. Although many people expose to these risk factors, the disease develops only in a small proportion suggesting that there are individual variation in the susceptibility to BCa.

Toll-like receptors (TLRs) are transmembranous signaling receptors which play a key role in the innate and adaptive immune response, since they are involved in the regulation of inflammatory reactions and activation of the adaptive immune cells to eliminate infectious pathogens and cancer cells [6]. To date, ten different kinds of TLRs have been described in human which are capable of specifically recognized different pathogens and/or endogenous damage molecules [7].

TLR4 is one of the most prominent members of TLRs which is present in immune and non-immune cells. TLR4 signaling in immune cells affects many aspects of immune responses, such as dendritic cell (DC) maturation and antigen presentation as well as CD8+ T-cell cytotoxicity, all of which are critical factors in anti-tumor immunity [8].

*Tlr4* gene is highly polymorphic, and to date, 15 polymorphisms in its coding sequence have been identified [9]. Among many SNPs, this gene has two co-segregated SNPs; *Asp299Gly* and *Thr399Ile*. The association of these SNPs with cancer risk has been widely investigated, including breast cancer [10], gastric cancer [11], Prostate cancer [12], hepatocellular cancer [13], nasopharyngeal cancer [14], leukemia [15], gall bladder cancer [16], cervical cancer [17], and colorectal cancer [18]. However, the

results were inconsistent. This study aimed to investigate the association of *Asp299Gly* and *Thr399Ile* SNPs in *Tlr4* gene with incidence of bladder cancer in Iraqi patients.

## **Subjects and Methods**

### **Subjects**

The study population consisted of 48 (41-76 years old, mean  $63 \pm 6.18$ , 31 males and 17 females) histologically confirmed transitional cell carcinoma of the urinary bladder, and 36 age matched (23 males and 13 females) healthy controls who were unrelated cancer-free individuals living in the same residential areas. All participants were recruited from Al-kadhimiya Teaching Hospital and Al-Yarmook Hospital/Baghdad.

Five milliliters of venous blood was taken from each participant in EDTA tubes which kept at -20 until be used for DNA extraction.

### **DNA extraction and genotyping of *TLR4* gene**

DNA was extracted from blood samples using ready kit (gSYNC™ DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea) according to the manufacturer's instructions. The primer used for amplification of *TLR4* gene (Bioneer/Korea) are shown in table 1.

Template DNA (10  $\mu$ L) from each sample and primers (5  $\mu$ L from each) were added to each master-mix tube (50  $\mu$ L PCR master-mix, Bioneer/Korea). The mixture then put in shaker and spinner for 10 cycles for better mixing. After mixing, the master-mix tubes were transferred to the thermo cycler (MyGenie 32 thermal block/Bioneer/Korea) which is previously programmed with certain protocol according to gene to be amplified.

For *Asp299gly* SNP, cycling conditions were an initial denaturation for 5 min at 95 °C, followed by 28

cycles of denaturation at 95 °C for 40 sec , annealing at 58 °C for 30 sec, extension at 72 °C for 50 sec, followed by final extension at 72 °C for 10 min. For *Thr399Ile* SNP, cycling conditions were an initial denaturation for 5 min at 95 °C, followed by 28 cycles of denaturation at 95 °C for 40 sec, annealing at 62 °C for 40 sec, extension at 72 °C for 50 sec, followed by final extension at 72 °C for 10 min. One µg amount DNA from *Asp299Gly* and *Thr399Ile* PCR products was mixed with a 5µl 10X NEB buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl<sub>2</sub>, 1mM dithiothreitol, pH 7.9), and 1µl of *Nco* I and *Hinf* I (10U) restriction enzyme ((New England

Biolabs Inc./USA) respectively. Deionized sterile H<sub>2</sub>O was used to adjust the volume to 50 µl. The mixture was then incubated at 37°C for 60 min.

**Agrose gel electrophoresis**

A 2% gel was prepared, and 10 µL aliquot of digested PCR product from each SNP was mixed with 2 µL loading dye and loaded into the wells. After 1 hour of electrophoresis , the gel was stained with ethidium bromide (Biobasic/Canada) (0.5 µg/mL) for 20 min and examined using U. V. transilluminator with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA).

**Table 1** Specific polymerase chain reaction primers and restriction enzymes for the two SNPs

SNP	Primers (5'→3')	Product (bp)	Enzymes
<i>Asp299Gly</i>	F:GATTAGCATACTTAGACTACTACCTCCATGR :GATCAACTTCTGAAAAAGCATTCCCAC	249	<i>Nco</i> I
<i>Thr399Ile</i>	F:GGTTCGTGTTCTCAAAGTGATTTTGGGAGAA R:ACCTAAGACTGGAGAGTGAGTTAAATGCT	406	<i>Hinf</i> I

**Statistical Analysis**

The Statistical Package for the Social sciences version 14.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. The polymorphisms were tested for deviation from Hardy-Weinberg Equilibrium (HWE)by comparing the observed and expected frequencies (Chi-square test). The association between genotype and risk of BCa was estimated by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression analysis adjusted for age, gender, and smoking status. Statistical significance was set at a p value≤ 0.05.

**Results**

Chi-square test revealed that alleles' distribution in both SNPs is within Hardy-Weinberg equilibrium.

***Asp299Gly*PCR-RFLP**

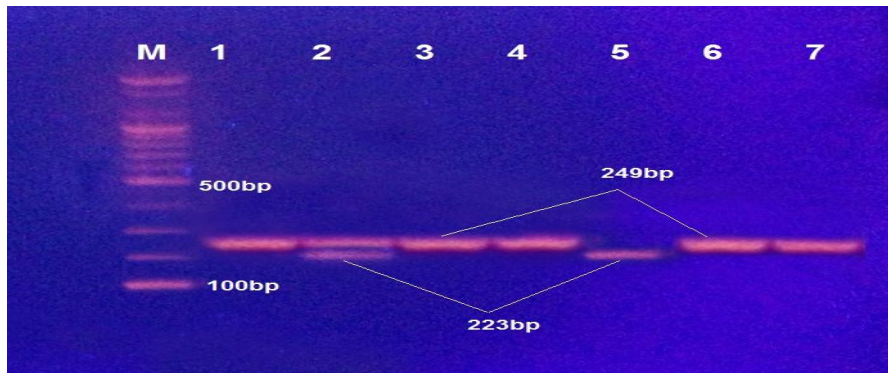
*Nco*I enzyme identifies the sequence CCATGG[19] whenever it presents in the nucleic acid and cuts exactly between the two Cs'. For homozygous wild type (AA), the enzyme does not work, and there will be a single band with 249 bp. For homozygous mutant genotype (GG), the enzyme cuts the two alleles, and the PCR product will appear as double bands of 223 and 26 bp; whereas the heterozygous genotype (AG) will appear as three bands of 249, 223, and 23 bp.Unfortunately, the smallest band (23 bp) was not visible on the gel because its small size (Figure 1).

There were three genotypes for this SNP among BCa patients; AA, AG and GG with frequency of 79.17%, 18.75% and 2,08% respectively, whereas, there were only two genotypes among control group; AA and AG respectively with frequency of 94.45% and 5.55% respectively with no significance differences between patients and control (OR=3.81, 95%CI= 0.745-19.47). However, the frequency of allele G (mutant) was higher among BCa group (11.45%) than control (2.77%) with significant difference (p=0.05).

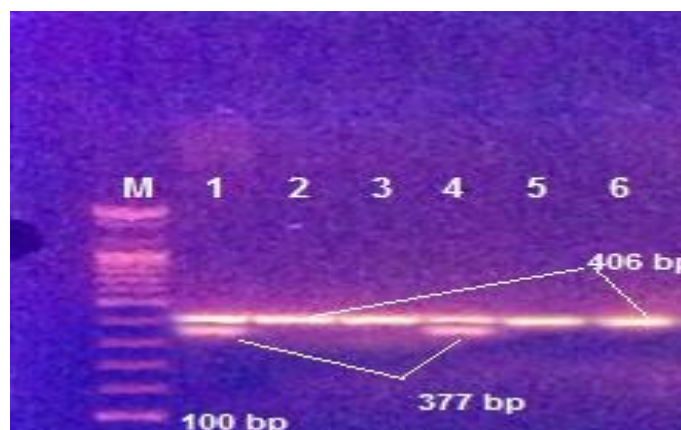
**Thr399Ile PCR-RFLP**

The enzyme *HinfI* recognizes the sequence GANTC [19], and accordingly, it cuts PCR product of homozygous mutant genotype (TT)

into two bands (377 and 29 bp), while heterozygous genotype (CT) is cut into three bands (406, 377, and 29 bp), whereas, homozygous wild genotype is not affected (the band size is 406 bp) (figure 2). This SNP had only two genotypes in patients and control with frequencies of 83.34% and 11.66% respectively among patients, and 94.45% and 5.55% respectively among control with no significant differences (OR=3.479, 95%CI= 0.67-18.046). Similarly, there was no significant difference in allele frequency between patients and control as the frequency of the mutant allele (C) among patients was 8.33% compared to 2.77% among control (OR= 3.182, 95%CI=0.655-15.46).



**Figure 1** the 2% agarose gel electrophoresis showing the restriction digestion patterns of *Asp299Gly* polymorphisms of *TLR4* gene using *Nco* I enzyme. M: DNA marker. Lanes 1,3,4,6,7: homozygous wild type (AA). Lane 2: heterozygous genotype (AG). Lanes 5: homozygous mutant genotype (GG).



**Figure 2** the 2% agarose gel electrophoresis showing the restriction digestion patterns of *Thr399Ile* polymorphisms of *TLR4* gene using *Hinf* I enzyme. M: DNA marker. Lanes 1,4: heterozygous genotype (CT). Lane 2,3,5,6: homozygous wild type (CC).

**Table 2** Genotypes and allele frequencies of *Asp299Gly* and *Thr399Ile* in patients and control

Variables	Cases N=48	Control N=36	P-value	OR(95%CI)
<i>Asp299Ile</i>				
AA	38 (79.17%)	34 (94.45%)	0.11	1.0
AG	9 (18.75%)	2 (5.55%)		3.81(0.745-19.47)
GG	1 (2.08%)	0 (0%)		
Alleles				
A	85 (88.55%)	70 (97.23%)	0.05	1.0
G	11 (11.45%)	2 (2.77%)		4.53 (0.997-21.12)
<i>Thr399Ile</i>				
CC	40 (83.34%)	34 (94.45%)	0.138	1.0
CT	8 (16.66%)	2 (5.55%)		3.479(0.67-18.046)
TT	0	0		
Alleles				
C	88 (91.67%)	70 (97.23%)	0.151	1.0
T	8 (8.33%)	2 (2.77%)		3.182(0.655-15.46)

### Discussion

Single nucleotide polymorphisms of immune genes not only increase the susceptibility to infectious diseases [9], but also affect the susceptibility to cancers [12] and anticancer immune response-induced by chemotherapy [20]. Therefore, the study of these SNPs has a significant clinical importance. The results of this study indicate the association of allele G of the SNP *Asp299Gly* as a risk factor for bladder cancer. This result is not in

accordance with that obtained by Shenet *al.* [21] who found that the SNP +3725G/C but not *Asp299Gly* nor *Thr399Ile* in the *TLR4* gene may be considered as a risk factor for BCa in Chinese population. It is fair to say that this study enrolled insufficient participants neither for BCa cases nor for control subjects, which does not allow a generalization for the current result. In addition, this study did not include the SNP +3725G/C which is one of two novel polymorphisms in

Asian population, and has shown functional significant [22]. However, in a meta-analysis involved 14627 cases and 17438 controls from 34 publications, Zhu *et al.* [23] found that the two SNPs *Asp299Gly* and *Thr399Ile* were significantly associated with increased risk of overall cancers. The presence of these two SNPs is responsible for blunt immune response [24], compromised recognition of apoptosis signals during anti-cancer therapy, or the presence of decreased functional TLR4 levels [25]. That is supposed to be a result of conformational changes in the receptor. Such changes and disrupting in the ligand docking will alter the signaling pathways of the mutant *Tlr4*. The study of Davoodi and co-workers [26] revealed that the activity of NF- $\kappa$ B in the mutant *Tlr4* cells was higher than that of wild type in response to lipopolysaccharide (LPS), a component of cell wall of gram negative bacteria. Besides, there were high levels of interleukin-1 receptor associated kinase (IRAK) accompanied with rapid degradation of this factor upon LPS treatment in wild type compared with mutant *Tlr4*. This implies reduced signaling and less cytokine genes transcription because degradation of IRAK serves as negative feedback mechanism. The positive association of G allele and the incidence of BCa may be referred to reduced immune response to bladder infection and subsequent chronic inflammation as the latter condition is known as predisposing factor for cancer [27].

### **Conclusion**

The results discussed previously showing that direct association of allele G of the SNP *Asp299Gly* with BCa that may play a role in the induction for bladder cancer.

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