Pathol. Oncol. Res. (2013) 19:275–280
DOI 10.1007/s12253-012-9579-8

RESEARCH

Toll-like Receptor 4 Gene Polymorphisms and Susceptibility to Bladder Cancer

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Received: 11 August 2012 / Accepted: 24 September 2012 / Published online: 12 October 2012

Abstract Bladder cancer is one of the most common malignancies in the world. Toll-like receptor 4 (TLR4) plays important roles in regulating innate immunity and may affect the development of cancers. Polymorphisms in *TLR4* gene have been shown to be associated with impaired immune responses. Here, we investigated the association of *TLR4* polymorphisms with bladder cancer. Four *TLR4* polymorphisms (-2242T/C, Asp299Gly, Thr399Il3, and +3725G/C) were genotyped in a total number of 436 bladder cancer patients and 522 healthy controls. Data were analyzed using the Chi-square test. Results showed that the prevalence of *TLR4* +3725GC and CC genotypes were significantly increased in bladder cancer cases than in controls (odds ratio [OR]=1.58, 95 % confidence interval [CI]=1.19–2.10, p= 0.0015, and OR=2.33, 95%CI=1.52–3.58, p<0.0001, respectively). Also, the frequency of *TLR4* +3725C allele was significantly higher in bladder cancer patients (p<0.0001). The -2242T/C, Asp299Gly and Thr399Il3 polymorphisms did not reveal any significant differences between cases and controls. Stratification analysis of the clinical features in the patients demonstrated that cases with invasive cancer were correlated with higher numbers of +3725GC and CC genotype (p=0.0004 and p=0.0231). In conclusion, these results indicate that *TLR4* +3725G/C polymorphism may be a novel risk factor for bladder cancer in the Chinese population.

Keywords Toll-like receptor 4 · Polymorphism · Bladder cancer

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Introduction

Bladder cancer is the ninth most frequent malignancies in the world and accounts for more than 20,000 deaths in China in 2009. The occurrence of this disease has dramatically increased between 1991 and 2009 [1, 2]. Incidence and mortality rate of bladder cancer are the highest among urinary system tumors, with the majority (about 70 %) of cases occurring in men [1]. Associated with the risk of bladder cancer are tobacco smoking, industrially related aromatic amines and amides (e.g. 4-aminobiphenyl, benzidine, and 2-naphthylamine), and some anticancer drugs, such as phosphoramide mustards [3]. Although many people are exposed to these risk factors, only a fraction of exposed individuals develop bladder cancer, suggesting that there are individual variations in susceptibility to exposure-related bladder carcinogenesis.

Toll-like receptor 4 (TLR4) plays important roles in defense against microbial infection [4]. This protein is expressed on macrophages, airway epithelia, adipose tissue, skeletal muscle and vascular endothelial and smooth muscle cells and is the key receptor for the lipopolysaccharide (LPS) component of Gram-negative bacteria [4, 5]. Toll-like receptor 4 signaling in immune cells is critical for regulation of innate and adaptive immune responses, such as DC maturation and antigen presentation as well as CD8+ T-cell cytotoxicity, all of which are critical factors in anti-tumor immunity [6]. On the other hand, TLR stimulation can also result in enhanced regulatory T cell proliferation which may up-regulate tumor development [7–9]. TLR expression is not limited to immune cells, and indeed many tumor cells have been found to express TLRs, signaling through which can promote tumor growth and immune evasion [10, 11]. Genetic single-nucleotide polymorphisms (SNPs) may affect the development of human diseases. Two

SNPs in *TLR4* gene are largely studied. The +896A/G polymorphism causes an amino acid exchange (aspartate to glycine) at position 299 (Asp299Gly) and the +1196C/T causes a switch from threonine to isoleucine at position 399 (Thr399Ile) [12]. Researches have revealed that the two polymorphisms are associated with different diseases such as gastric cancer, pyelonephritis, and hepatitis C virus-induced hepatocellular carcinoma, etc [13–15]. However, no study has reported the correlation between *TLR4* polymorphisms and bladder cancer. In addition, two novel polymorphisms (-2242T/C and +3725G/C) have been identified in the Asian population and have been shown functional significance [16, 17]. Here, we have tested 4 *TLR4* polymorphisms (-2242T/C, Asp299Gly, Thr399Il3, and +3725G/C) and investigated their associations with bladder cancer in the Chinese population.

Materials and Methods

Study Subjects

The study population consisted of 436 bladder patients and 522 healthy controls recruited from the General Hospital of Jinan Military Command between May 2006 and June 2011 (Table 1). Every subject participating in the study was genetically unrelated, of Han Chinese ethnicity, and permanently resided in Shandong province in China. All cases were urology clinic patients with histological confirmed transitional cell carcinoma. The controls were genetically unrelated cancer-free individuals living in the same residential areas and frequency-matched to the cases on age, sex, smoking status, and alcohol use. Individuals that smoked once a day for over 1 year were defined as ever smokers. The study was approved by the Review Board of the General Hospital of Jinan Military Command. Written informed consent was obtained from each participant.

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes using a commercially available kit according to the manufacturer's instructions (Blood genomic DNA miniprep kit; Axygen Biosciences). Single nucleotide polymorphisms (SNPs) of *TLR4* were detected using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) procedure. The PCR primers are shown in Table 2. Genomic DNA (300 ng) was amplified in a final volume of 30ul using the following conditions: initial denaturation at 95 °C for 6 min followed by 35 cycles of 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension step was at 72 °C for 10 min. The PCR products (10ul) were digested overnight at 37 °C with 5 U of restriction enzymes, Ear I for -2242T/C and +3725G/C, NcoI for the Asp299Gly and Hinf I for the Thr399Ile polymorphisms. Digestion products were separated by electrophoresis through 2.5 % agarose gel. Fragment sizes after digestion are shown in Table 2. To confirm the genotyping results, more than 20 % of PCR-amplified DNA samples were examined by DNA sequencing. Results between PCR and DNA sequencing analysis were 100 % concordant.

Table 1 Distribution of selected demographic variables and risk factors among the cancer cases and healthy controls

Characteristic	Cancer patients (n=436)	Controls (n=522)	p value
Age (years)	62.6±11.5	61.5±12.3	0.532
Sex no. (%)			0.608
Male	339 (77.8)	413 (79.1)	
Female	97 (22.2)	109 (20.9)	
BMI (kg/m^2)	23.7±9.8	23.1±10.5	0.561
Hematological parameters			
Hemoglobin (g/L)	137±38	133±42	0.733
White blood cells (10^9/L)	5.49±2.41	5.72±2.01	0.328
Red blood cells (10^{12}/L)	4.81±0.52	4.79±0.93	0.664
Platelets (10^9/L)	267±111	249±156	0.233
Smoking status			<0.001
Never	143 (32.8)	314 (60.2)	
Ever	293 (67.2)	208 (39.8)	
Drinking status			<0.001
Never	152 (34.9)	286 (54.8)	
Ever	284 (65.1)	236 (45.2)	
Primary tumor stage			
Superficial tumor (pTa-pT1)	245 (56.2)		
Invasive tumor (pT2-pT4)	191 (43.8)		
Histo-pathological grade			
G1/G2	300 (68.8)		
G3	136 (31.2)		
Tumor number			
Single	284 (65.1)		
Multiple	152 (34.9)		
Tumor size (cm)			
<1	135 (31.0)		
1–3	133 (30.5)		
>3	168 (38.5)		

Statistical Methods

The SPSS statistical software package ver.13.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Demographic data between the study groups were compared by the chi-square test and by the Student *t*-test. The polymorphisms were tested for deviation from Hardy-Weinberg equilibrium (HWE) by comparing the observed and

Table 2 Primers and enzymes for detecting the TLR4 SNPs

SNPs	Primers	Enzymes	Amplicon size (bp)
TLR4 (-2242T/C)	F: 5′- CTGTCTCTAGTTGTCTGGTA -3′	Ear I	T: 196
	R: 5′- GGTGGAAATCTCTTTGAC-3′		C: 311
TLR4 (Asp299Gly)	F: 5′- GATTAGCATACTTAGACTACTACCTCCATG-3′	Nco I	A: 249
	R: 5′- GATCAACTTCTGAAAAAGCATTCCCAC-3′		G: 233
TLR4 (Thr399Il3)	F: 5′- GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′	Hinf I	C: 406
	R: 5′- CCTGAAGACTGGAGAGTGAGTTAAATGCT-3′		T: 377
TLR4 (+3725G/C)	F: 5′- ACAAGTGATGTTTGATGGAC -3′	Ear I	G: 163
	R: 5′- GCCATTCTACCTGGTATAAG-3′		C: 361

expected genotype frequencies using the chi-square test. For SNP analysis, genotype and allele frequencies of *TLR4* polymorphisms were compared between groups using the chi-square test, and odds ratios (OR) and 95 % confidence intervals (CIs) were calculated using unconditional logistic regression. *P* values less than 0.05 were considered significant.

Table 3 TLR4 gene polymorphisms in bladder cancer patients and controls

Polymorphisms	Bladder cancer n=436 (%)	Controls n=522 (%)	OR (95 % CI)	*P* value
TLR4 (-2242T/C)				
Genotype				
TT	149 (34.2)	174 (33.3)	Referent	
TC	266 (61.0)	313 (60.0)	0.99 (0.76–1.30)	0.957
CC	21 (4.8)	35 (6.7)	0.70 (0.39–1.26)	0.231
Allele				
T	564 (64.7)	661 (63.3)	Referent	
C	308 (35.3)	383 (36.7)	0.94 (0.78–1.14)	0.536
TLR4 (Asp299Gly)				
Genotype				
AA	431 (98.9)	519 (99.4)	Referent	
AG	2 (0.5)	1 (0.2)	2.41 (0.22–26.67)	0.460
GG	3 (0.6)	2 (0.4)	1.81 (0.30–10.86)	0.512
Allele				
A	864 (99.1)	1039 (99.5)	Referent	
G	8 (0.9)	5 (0.5)	1.92 (0.63–5.90)	0.244
TLR4 (Thr399Il3)				
Genotype				
CC	433 (99.3)	517 (99.0)	Referent	
CT	1 (0.2)	3 (0.6)	0.40 (0.04–3.84)	0.410
TT	2 (0.5)	2 (0.4)	1.19 (0.17–8.52)	0.859
Allele				
C	867 (99.4)	1037 (99.3)	Referent	
T	5 (0.6)	7 (0.7)	0.85 (0.27–2.70)	0.789
TLR4 (+3725G/C)				
Genotype				
GG	220 (50.5)	334 (63.9)	Referent	
GC	153 (35.1)	147 (28.2)	1.58 (1.19–2.10)	0.0015*
CC	63 (14.4)	41 (7.9)	2.33 (1.52–3.58)	<0.0001*
Allele				
G	593 (68.0)	815 (78.1)	Referent	
C	279 (32.0)	229 (21.9)	1.67 (1.37–2.05)	<0.0001*

**P* value <0.05

Results

Characteristics of the Study Population

Selected characteristics of the cases and controls are presented in Table 1. Bladder cancer cases and controls did not show a significant difference in regard to age (p=0.532), sex (p=0.608), body mass index (BMI) (p=0.561), or hematological parameters. As expected, there were more ever smokers (67.2 % vs. 39.8 %, p<0.001) and ever alcohol users (65.1 % vs. 45.2 %, p<0.001) in the cases than in the controls. Among the 346 patients, 245 (56.2 %) were superficial tumor; 300 (68.8 %) were in histo-pathological grade 1 or 2; 284 (65.1 %) had single tumor number; and 268 (61.5 %) had tumor size less than 3 cm (Table 1).

Genotype and Allele Distributions of *TLR4* Polymorphisms Among the Cases and Controls

Genotype and allele frequencies of the four *TLR4* polymorphisms in bladder cancer cases and healthy controls are summarized in Table 3. The genotype distributions of the *TLR4* polymorphisms among the controls were in agreement with the Hardy–Weinberg equilibrium. Frequencies of the +3725GG, GC, and CC genotype were 50.5 %, 35.1 %, and 14.4 % among the cases and 63.9 %, 28.2 %, and 7.9 % among the controls. Prevalence of the *TLR4* +3725GC and CC genotypes were significantly increased in patients than in controls (OR=1.58, 95%CI=1.19–2.10, p=0.0015, and OR=2.33, 95%CI=1.52–3.58, p<0.0001, respectively). Also, bladder cancer cases showed higher numbers of +3725C allele than healthy subjects (OR=1.67, 95%CI: 1.37–2.05, p<0.0001). Frequencies of the Asp299Gly and Thr399Il3 SNPs were very low in our study population. We did not reveal any correlation between bladder cancer and *TLR4* -2242T/C, Asp299Gly or Thr399Il3 SNPs (Table 3). These data suggest that the *TLR4* +3725GC genotype, CC genotype and C allele were associated with an increased susceptibility to bladder cancer in Chinese population.

Stratification Analysis Between *TLR4* -330 Polymorphism and Bladder Cancer Risk

In order to further understand the association between *TLR4* +3725G/C polymorphism and bladder cancer, we compared the *TLR4* +3725G/C polymorphism in patients based on different clinical-pathological factors. The stratification analysis, including age, gender, primary tumor stage, and

Table 4 Stratification analysis between TLR4 SNP and bladder cancer risk

Age	Age ≤55 (N=134) (%)	Age >55 (N=302) (%)	OR (95 % CI)	P value
TLR4 (+3725G/C)				
Genotype				
GG	66 (49.3)	154 (51.0)	Referent	
GC	48 (35.8)	105 (34.8)	1.07 (0.68–1.67)	0.777
CC	20 (14.9)	43 (14.2)	1.09 (0.59–1.99)	0.791
Sex	Male (N=339) (%)	Female (N=97) (%)	OR (95 % CI)	P value
TLR4 (+3725G/C)				
Genotype				
GG	174 (51.3)	46 (47.5)	Referent	
GC	116 (34.2)	37 (38.1)	0.83 (0.51–1.36)	0.559
CC	49 (14.5)	14 (14.4)	0.93 (0.47–1.82)	0.822
Tumor stage	Invasive (N=191) (%)	Superficial (N=245) (%)	OR (95 % CI)	P value
TLR4 (+3725G/C)				
Genotype				
GG	77 (40.3)	143 (58.3)	Referent	
GC	82 (42.9)	71 (29.0)	2.15 (1.41–3.27)	0.0004*
CC	32 (16.8)	31 (12.7)	1.92 (1.09–3.38)	0.0231*
Tumor numbers	Single (N=284) (%)	Multiple (N=152) (%)	OR (95 % CI)	P value
TLR4 (+3725G/C)				
Genotype				
GG	147 (51.8)	73 (48.0)	Referent	
GC	99 (34.9)	54 (35.5)	0.91 (0.59–1.41)	0.672
CC	38 (13.3)	25 (16.5)	0.75 (0.42–1.35)	0.339

*p<0.05

tumor numbers are shown (Table 4). Data revealed that there were no significant differences in regard to age, sex and tumor numbers. However, prevalence of +3725GC and CC genotype were significantly increased in patients with invasive cancer than those with superficial cancer (OR=1.58, 95%CI=1.19–2.10, p=0.0015, and OR=2.33, 95%CI=1.52–3.58, p<0.0001, respectively). These data indicate that the *TLR4* +3725G/C polymorphism may be correlated with the prognosis of bladder cancer.

Discussion

TLR4 plays important roles in regulating a variety of inflammatory and immune disorders [12]. Polymorphisms in *TLR4* gene may have functional significance [13]. Here, we revealed that the *TLR4* +3725G/C polymorphism was associated with increased susceptibility to bladder cancer. It is the first study reporting that polymorphism in *TLR4* gene could be a risk factor for bladder cancer.

The role of +986A/G (Asp299Gly) and +1196C/T (Thr399Ile) SNPs in the pathogenesis of cancers remains diverse. Some study has shown that +986A/G (Asp299Gly) and +1196C/T (Thr399Ile) polymorphisms are associated with increased susceptibility to gastric cancer [3], whereas other report has revealed a correlation between these two SNPs and a decreased risk of hepatitis C virus-induced hepatocellular carcinoma [15]. *TLR4* +986A/G (Asp299Gly) and +1196C/T (Thr399Ile) mutant alleles seem to have originated in Africa in an environment where malaria represented a major evolutionary pressure: first the Asp299Gly and later on the Thr399Ile allele [16, 17]. The frequency of these alleles varies from 0 % in Polynesian and native South-American populations to 5–9 % in Indo-Europeans [16, 17]. In a Plasmodium spp.-infested environment, although the Asp299Gly increases susceptibility to infections as well as the risk of septic shock, it induced protection against malaria-associated mortality [16, 17]. However, the ‘addition’ of the Thr399Ile mutant allele, as in Indo-Europeans, reduced the risk for septic shock to rates similar to those observed in carriers of the wild-type alleles [16, 17]. This simultaneous presence– linkage disequilibrium – of both mutant alleles was a useful addition, as the presence of the Asp299Gly alone would result in greater susceptibility during the plague, typhoid fever and influenza outbreaks in Europe [16, 17]. Prevalence of the two SNPs in our study subjects was very low (Table 3). In this situation, it is extremely hard to investigate whether they can be associated with bladder cancer. A larger study population would be necessary.

The *TLR4* -2242T/C polymorphism is a recently identified SNP which is located in the promoter region of *TLR4* gene [18]. The polymorphism can increase the transcriptional activities of TLR4 promoter and cause up-regulation of necrosis factor-alpha [18]. Chen et al found that the -2242T/C was associated with higher sepsis morbidity rated and multiple organ dysfunction scores in patients with major trauma [18]. However, we did not observe any correlations between this SNP and bladder cancer (Table 3). The +3725G/C SNP is located in the 3′ untranslated region (UTR) of *TLR4* gene. Study has shown that the SNP may contribute to translational regulation of TLR4 by binding to microRNAs [19]. The +3725G/C SNP is associated with various diseases. For example, Hishida et al. have found that the GC or CC genotype is associated with severe gastric atrophy in H. pylori seropositive Japanese subjects [20]. Zhou et al. have found that the CC genotype was significantly associated with hepatitis type B virus (HBV) recurrence after liver transplantation [21]. Our results revealed that +3725 GC and CC genotypes were associated with increased risk of bladder cancer (Table 3). Epidemiologic investigation in other ethnicities or with larger number of subjects would be important to confirm our findings.

In summary, our study demonstrated for the first time that *TLR4* +3725G/C polymorphism could be a novel risk factor for bladder cancer in the Chinese population. These results may provide support for using TLR4 as a therapeutic target and a prognostic marker for bladder cancer in the future.

Author Disclosure Statement No competing financial interests exist.

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