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Association of Toll-like receptor (TLR) 2, 3 and 9 genes polymorphism with prostate cancer risk in North Indian population

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Abstract Prostate cancer (PCa) is the most common cancer among men. It has been suggested that toll like receptors (TLRs) may contribute to PCa pathogenesis by stimulating prostate epithelial cell proliferation in response to infectious stimuli. We performed case control study to analyze the genetic variants of TLR2, 3 and 9 gene polymorphisms with PCa risk in a North Indian population. For this study we genotyped age matched, unrelated 195 PCa patients and 250 healthy controls of similar ethnicity in a case–control study. They were genotyped for TLR2 (−196 to −174 Del), TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) [rs352140] gene polymorphisms using polymerase chain reaction and restriction fragment length polymorphism method. Variant allele Del (D) carriers i.e. (ID + DD) of TLR2 (−196 to −174 Del) SNP, demonstrated 1.57 fold increased risk ($p = 0.040$; OR = 1.57, 95% CI = 1.02–2.24) as compared to Ins (I) allele, suggesting a dominant effect model involved in the risk of this polymorphism in PCa. However, variants of TLR3 and 9 gene polymorphisms were not associated with PCa risk. Our results suggested the low penetrance variant of TLR2 (−196 to −174 Del) to be at increased PCa risk in North Indian population. Functional studies in ethnically diverse populations may provide a more comprehensive involvement of innate immunity in identifying the disease-associated variants for PCa etiology.

Keywords Toll-like receptors · Polymorphism · Prostate cancer

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Introduction

Prostate cancer (PCa) is the second most commonly diagnosed and second leading cause of cancer related death among men [1]. The incidence rate of PCa in India is low compared to Western countries, and PCa is the sixth most commonly diagnosed cancer among men [2]. It is well established that genetic factors also play a significant role in pathogenesis of PCa [3].

Inflammatory process is implicated in cancer incidence and progression. Mounting evidence observed that specific RNA is frequently present in cases of clinical prostatitis [4], as well as the isolation of infectious agents including human papillomavirus (HPV) and herpes simplex virus (HSV) in PCa tissue [5, 6], which would support a role for toll-like receptors (TLRs) in PCa pathogenesis.

TLRs recognize a wide variety of pathogen associated molecular patterns (PAMPs) from bacteria, viruses, and fungi, as well as some host molecules [7]; to play a key role in the pathophysiology of a range of human diseases, including cancer. Although a vast number of TLR polymorphisms have been identified, several variant alleles within the TLR gene are reported to be associated with PCa risk, including various genotypes that have been associated with both an increase [8] and decrease [9] in PCa risk. A study has demonstrated that TLRs, specifically TLR subclass 4 (TLR4) and TLR9, may contribute to PCa pathogenesis by stimulating prostate epithelial cell proliferation in response to infectious stimuli [10].

TLR2 gene located on chromosome 4 is involved in the innate immune system and recognizes conserved molecular patterns (e.g. lipopolysaccharide (LPS)) on these bacteria, which are major inducers of the inflammatory response [11]. A 22 bp nucleotide deletion polymorphism (−196 to −174 Del) on TLR2 alters the promoter activity of TLR2.

TLR3 and 9 are localized intracellularly mostly in the endocytic compartments; TLR3 recognizes dsRNA from viruses as well as poly (I:C), a synthetic dsRNA analogue, while TLR9 recognizes unmethylated CpG motifs present in bacteria and viruses [12]. TLR3 mediates its functional effects via the myeloid differentiation primary response protein 88 (MyD88) independent pathways, alternatively, TLR9 functions through the MyD88 dependent pathway leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. Various polymorphisms in TLR genes have been identified and implicated in susceptibility of many cancers including PCa [13, 14]. Therefore, polymorphisms in TLR genes of the inflammatory pathway could influence disease susceptibility and progression by altering the response to infection and downstream inflammatory effects.

The aim of the present study was to further clarify the role of TLR 2 (−196 to −174 Del), TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) [rs352140] polymorphism with association of PCa risk in North Indian men.

Materials and methods

Study subjects

PCa patients were treated in Department of Urology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, between January 2007 to June 2010. Histologically proven, age matched 195 PCa cases were only included in this study. Tumor grade was evaluated in PCa samples by the Gleason scoring system [15]. All the participants in the study were unrelated individuals of similar ethnicity from Lucknow and other adjoining cities of Northern India. Information on demographic features was obtained through personal interview using a standard clinical proforma.

Age and ethnicity matched healthy individuals ($n = 250$) were recruited as controls from men participating in PCa screening programs of the hospital. The total PSA (Prostate Specific Antigen) levels were determined in PCa, and healthy controls using CanAg EIA kits (Sweden). Control individuals with PSA > 4.0 ng/ml were excluded. All subjects gave informed consent for the study, which was reviewed and approved by the Ethical review board of the Institute.

DNA extraction

Blood samples (4.0 ml) from PCa patients and control subjects were collected in EDTA vials and stored at −20°C until required. Genomic DNA was extracted from peripheral blood leukocytes using salting-out method [16].

Genotyping

TLR2 (−196 to −174 del) genotypes were determined by a polymerase chain reaction (PCR) method, while TLR3 (c.1377C/T; rs3775290) and TLR9 (G2848A; rs352140) were genotyped by PCR–Restriction Fragment Length Polymorphism (RFLP) The PCR product of TLR3 and 9 were digested with the restriction enzyme *Taq*I and *Bst*UI respectively (MBI Fermentas, USA) at 65 and 60°C overnight respectively, and then analyzed on 10% polyacrylamide gel electrophoresis. Details of primers and cycle conditions were referred as described earlier [17, 18]. Positive and negative controls were used in each genotyping assay and 5% of randomly selected samples were re-genotyped by other lab personal with 100% concordance.

Statistical analysis

The sample size was calculated and was found to be adequate using QUANTO software, version 1.0 (http://hydra.usc.edu/gxe) for the genetic marker. Sample size achieved >80% of the statistical power. A two-tailed p value < 0.05 was considered statistically significant. Chi-square ($\chi 2$) analysis was used to assess deviation from Hardy–Weinberg equilibrium (HWE) and to compare the genotype and allele frequency between patients and controls. Odds ratio (OR) were obtained by unconditional logistic regression analysis and adjusted for age and smoking as a continuous variable. PCa patients with different Gleason grades, bone metastasis were identified using the same statistics as mentioned. All the statistical analyses were conducted using the SPSS software, version 11.5 (SPSS, Chicago, IL, USA).

Results

Demographical and clinical details of study subjects

A total of 445 individuals (195 PCa and 250 controls) were analyzed in the study. There was no statistical difference between age of the PCa patients (66.6 ± 5.46 years) and healthy controls (64.7 ± 5.71 years) ($p = 0.286$) and smoking habits ($p = 0.136$). As expected there was high degree of statistical difference between serum PSA of PCa patients (221 ± 57.4 ng/ml) and controls (2.3 ± 0.8 ng/ml). Majority of the patients had high Gleason grade at the time of diagnosis Gleason score 7 in 29.2%, Gleason score >7 in 43.1%) and 41.8% cases with bone metastasis were also documented (Table 1).

Table 1 Clinical and demographic details of study subjects

	Controls ($n = 250$)	PCa ($n = 195$)	p value
Age (years ± SD)	64.7 ± 5.71	66.0 ± 5.46	0.286
Total PSA (mean ± SD) (ng/ml)	2.3 ± 0.8	221 ± 57.4	<0.0001δ
Demographic details	*n* (%)	*n* (%)	
Cigarette/bidi smoking[a]			
Non smokers	174 (71.6)	126 (64.9)	
Smokers	69 (28.4)	68 (35.1)	0.136
Clinical details			
Bone metastasis			
Bone Mets (−)	–	91 (47.3)	
Bone Mets (+)	–	80 (41.8)	
Bone Scan not done	–	24 (10.9)	
Gleason grade			
<7	–	54 (27.7)	
7	–	57 (29.2)	
>7	–	84 (43.1)	

δ-Student *t* test was used to determine the *p* value

PCa Prostate cancer, *PSA* prostate-specific antigen

[a] Numbers may not add to the total because of some missing data

Table 2 TLR2, TLR3 and TLR9 gene variants and susceptibility to PCa risk

	Controls $n = 250$ (%)	Patients $n = 195$ (%)	OR (95% CI)	p value
TLR2 (−196 to −174 del)				
I/I	193(77.2)	135(69.2)	Reference	
I/D	52(20.8)	54(27.7)	1.54(0.98–2.41)	0.059
D/D	5(2.0)	6(3.1)	1.88(0.55–6.41)	0.309
I/D + DD	57(22.8)	60(30.8)	**1.57(1.02–2.42)**	**0.040**
Allele I	438(87.6)	324(85.1)	Reference	
Allele D	62(12.4)	66(14.9)	1.43(0.98–2.09)	0.057
TLR3 (c.1377C/T) rs3775290				
CC	157(62.7)	115(59.0)	Reference	
CT	84(33.7)	68(34.9)	1.07(0.70–1.62)	0.751
TT	9(3.6)	12(6.1)	1.64(0.65–4.1)	0.289
CT + TT	93(37.3)	80(41.0)	1.15(0.78–1.70)	0.467
Allele C	398(79.6)	298(76.4)	Reference	
Allele T	102(20.4)	92(23.6)	1.20(0.87–1.65)	0.253
TLR9 (G2848A) rs352140				
GG	79(31.6)	54(27.7)	Reference	
GA	142(56.8)	118(60.5)	1.17(0.76–1.80)	0.473
AA	29(11.6)	23(11.8)	1.05(0.54–2.05)	0.865
GA + AA	171(68.4)	141(72.3)	1.27(0.83–1.94)	0.260
Allele G	300(60.0)	226(57.9)	Reference	
Allele A	200(40.0)	164(42.1)	1.08(0.83–1.42)	0.537

Bold values represent significant risk

OR Odds ratio adjusted with age and smoking, *CI* confidence interval

Association of TLR2 (−196 to −174 Del), TLR3 (c.1377C/T) and TLR9 (G2848A) gene variants with PCa risk

The observed genotype frequencies in control subjects were in agreement with HWE. To evaluate the association between genetic variant with risk of PCa, we compared genotype frequency distribution of TLR2, 3 and 9 in PCa and control group shown in Table 2. The del/del genotype inclined to be more frequent among patients than control subjects (3.1% vs. 2.0%, $p = 0.309$). The variant allele (D) carrier (ID + DD) was higher in cases (30.8%) than in control (22.8%), the result showed statistically significant risk ($p = 0.040$; OR = 1.57) with PCa. The TLR3 and 9

Table 3 Joint effect of TLR2 (−196 to −174 Del) and TLR3 (c.1377C/T) genes polymorphisms in PCa patients and healthy controls

	Controls $n = 250$ (%)	Patients $n = 195$ (%)	OR (95% CI)	p value
TLR2 (−196 to −174 del) and TLR3 (c.1377C/T) rs3775290				
I/I-CC	122(48.8)	81(41.5)	Reference	
I/I-CT	63(25.2)	46(23.6)	1.10(0.68–1.76)	0.693
I/I-TT	8(3.2)	8(4.1)	1.50(0.54–4.17)	0.431
I/D-CC	34(13.6)	30(15.4)	1.32(0.75–2.34)	0.324
I/D-CT	17(6.8)	20(10.3)	1.77(0.87–3.58)	0.112
I/D-TT	1(0.4)	4(2.1)	6.02(0.66–54.87)	0.111
D/D-CC	1(0.4)	4(2.1)	6.02(0.66–54.87)	0.111
D/D-CT	4(1.6)	2(1)	0.75(0.13–4.20)	0.747
D/D-TT	0	0	NC	

NC Not calculated

Table 4 Joint effect of TLR2 (−196 to −174 Del) and TLR9 (G2848A)) genes polymorphisms in PCa patients and healthy controls

	Controls $n = 250$ (%)	Patients $n = 195$ (%)	OR (95%CI)	p value
TLR2 (−196 to −174 del) and TLR9 (G2848A) rs352140				
I/I-GG	59(23.6)	38(19.5)	Reference	
I/I-GA	111(44.4)	87(44.6)	1.21(0.74–1.99)	0.437
I/I-AA	23(9.2)	18(9.2)	1.21(0.58–2.54)	0.606
I/D-GG	19(7.6)	13(6.7)	1.06(0.47–2.39)	0.884
I/D-GA	28(11.2)	28(14.4)	1.55(0.79–3.01)	0.194
I/D-AA	5(2)	5(2.6)	1.55(0.42–0.57)	0.509
D/D-GG	1(0.4)	3(1.5)	4.65(0.46–46.44)	0.190
D/D-GA	3(1.2)	3(1.5)	1.55(0.29–8.09)	0.602
D/D-AA	1(0.2)	0	NC	

NC Not calculated

genotypic and allelic frequency distributions between cases and healthy controls were similar demonstrating no association for the risk of PCa.

Joint effect between TLR2 (−196 to −174), TLR3 (c.1377C/T) and TLR9 (G2848A) genes polymorphisms with PCa risk

As the selected genes are in inflammatory pathway, we hypothesized that combined effect (gene–gene interactions) might project a robust explanation than main effects only, which might have a synergistic interaction effect on modulating risk of PCa. We performed all possible interactions of TLR2 with TLR3 and TLR9 gene polymorphisms. However, combination of these genes did not show multiplicative effect on risk of PCa (Tables 3,4).

Association of TLR2 (−196 to −174 Del), TLR3 (c.1377C/T) and TLR9 (G2848A) genes polymorphism with tumor/grade of PCa

We performed a case-only analysis to investigate whether any possible association existed between TLR2, 3 and 9 gene polymorphisms and clinical stages of PCa. For this comparison the patients with different Gleason grades were sub categorized into three group (Low grade < 7, Intermediate grade 7, High grade > 7) based on degree of differentiation of cells. Our result did not observe any significant association with any tumor grade (Table 5).

Association of TLR2 (−196 to −174 Del), TLR3 (c.1377C/T) and TLR9 (G2848A) genes polymorphism with risk of bone metastasis

We also studied TLR2, 3 and 9 genes variants and their risk associated with bone metastasis. The PCa patients were stratified into two groups, one with positive and the other with negative bone metastasis. None of the two groups analyzed for risk of susceptibility for bone metastasis with these SNPs, revealed any significant association (Table 6).

Association of TLR2 (−196 to −174 Del), TLR3 (c.1377C/T) and TLR9 (G2848A) genes polymorphism with smoking habit in PCa

We evaluated the gene–smoking interaction to study the modulation of PCa risk with respect to TLR2, 3 and 9 gene polymorphisms. We grouped the PCa patients into two

Table 5 Genotype frequency and odds ratio (OR) of the TLR2, 3 and 9 genes polymorphism in PCa patients with different gleason grade

	Gleason < 7 (low grade)	Gleason 7 (intermediate grade)	Gleason > 7 (high grade)	Between low and intermediate grades		Between low and high grades	
	$n = 54$(%)	$n = 57$(%)	$n = 84$(%)	OR (95% CI)	p value	OR (95% CI)	p value
TLR2 (−196 to −174 del)							
II	40(74.1)	39(98.4)	64(76.2)	Reference		Reference	
I/D	11(20.4)	18(31.6)	17(20.2)	1.63(0.67–3.93)	0.276	0.96(0.40–2.32)	0.940
D/D	3(5.5)	0(0)	3(3.6)	0.00	0.999	0.60(0.11–3.29)	0.561
ID + DD	14(25.9)	18(31.6)	20(23.8)	0.86(0.35–2.15)	0.760	1.49(0.67–3.30)	0.321
TLR3 (c.1377C/T) rs3775290							
CC	32(59.3)	33(57.9)	50(59.5)	Reference		Reference	
CT	21(38.9)	21(36.8)	26(31.0)	0.95(0.43–2.07)	0.901	0.79(0.38–1.65)	0.504
TT	1(1.8)	3(5.3)	8(9.5)	3.07(0.29–31.89)	0.346	5.09(0.60–42.91)	0.134
CT + TT	22(40.7)	24(42.1)	34(40.5)	1.81(0.81–4.03)	0.148	0.98(0.49–1.98)	0.975
TLR9 (G2848A) rs352140							
GG	13(24.0)	18(31.6)	23(27.4)	Reference		Reference	
GA	36(66.7)	28(49.1)	54(64.3)	0.51(0.21–1.26)	0.150	0.84(0.37–1.90)	0.676
AA	5(9.3)	11(19.3)	7(8.3)	1.57(0.43–5.67)	0.490	0.67(0.17–2.64)	0.568
GA + AA	41(76.0)	39(68.4)	61(72.6)	0.85(0.36–2.01)	0.720	1.18(0.53–2.62)	0.676

OR Odds ratio adjusted with age and smoking, *CI* confidence interval

Table 6 Genotype frequency and odds ratio (OR) of TLR2, 3 and 9 genes polymorphism in PCa patients with bone metastasis status

	Metastasis (−ve) $n = 91$(%)	Metastasis (+ve) $n = 80$(%)	Age adjusted OR (95% CI)	p value
TLR2 (−196 to −174 del)				
I/I	69(75.8)	55(68.8)	Reference	
ID	20(22.0)	22(27.4)	1.40(0.69–2.84)	0.350
DD	2(2.2)	3(3.8)	2.05(0.32–13.0)	0.445
ID + DD	22(24.2)	25(31.2)	0.66(0.31–1.36)	0.264
TLR3 (c.1377C/T) rs3775290				
CC	56(61.5)	44(55.0)	Reference	
CT	28(30.8)	33(41.2)	1.45(0.76–2.76)	0.255
TT	7(7.7)	3(3.8)	0.53(0.12–2.19)	0.383
CT + TT	35(38.5)	36(45.0)	0.74(0.38–1.43)	0.380
TLR9 (G2848A) rs352140				
GG	31(34.1)	19(23.8)	Reference	
GA	48(52.7)	53(66.2)	1.74(0.85–3.53)	0.126
AA	12(13.2)	8(10.0)	1.18(0.40–3.46)	0.762
GA + AA	60(65.9)	61(76.2)	0.81(0.41–1.58)	0.540

OR Odds ratio adjusted with age and smoking, *CI* confidence interval

groups, one non smoker (never smoked) and the other as smokers (smoking more than 5 years). On analyzing the genotype frequency between these two groups for susceptibility to PCa, none of these polymorphisms demonstrated association (data not shown).

Discussion

Inflammation involves a complex interaction of gene networks and is largely self-regulating, thus it is reasonable to assume that certain combinations of alleles may contribute to an imbalanced immune response and increased PCa risk. It has been suggested that TLRs play a central role in resisting microbial infections by initiating most of the immune responses that occur during infection. Studies have shown that TLRs control multiple dendritic cells capable of sensitizing naïve T cell functions and activate signals that are critically involved in the initiation of adaptive immune response [19]. Mounting evidence suggest that bacterial inflammation is implicated in PCa [20]. Thus, genetic variations in TLR2, 3 and 9 genes could play a substantial

role in modifying the risk for PCa. The variant allele carrier (ID + DD) of TLR2 associated with an increased risk of PCa suggested a dominant effect model involved in the risk of this polymorphism on PCa. Our findings were compatible with the observations of Srivastava et al. [21] in Gall bladder cancer from India. Genetic studies have identified (−196 to −174 Ins/Del) polymorphism in the promoter region of the TLR2 gene to significantly alter the function of TLR2 promoter activity. The variant Del/Del genotype of this polymorphism has been shown a decreased transactivation of TLR2 gene promoters [22].

The non-synonymous 1377C>T polymorphism present in exon 4 of TLR3 gene on chromosome 4 affects the receptor–ligand interaction by altering the TLR3 ectodomain and thereby functionally impairing the receptor. The minor allele frequency (MAF) of TLR3 in our population was almost similar in controls and patients (20.4 vs. 23.6) and did not show significant association. TLR3 gene polymorphisms have also been implicated in nasopharyngeal carcinoma risk [23] though no association of TLR3 (c.1377C/T) gene polymorphism with breast cancer risk has been also reported [24].

The synonymous +2848G>A polymorphism in exon 2 of TLR9 gene affects the expression at the mRNA level. In this case–control study no evidence for an association between the TLR9 polymorphisms and PCa was observed. Similar results were observed in another study from India by Pandey et al. [18] in cervical cancer and Hold et al. [25] in gastric cancer in Caucasian population. Few studies with conflicting observations have also been reported as Mollaki et al. [26] observed the TLR9 polymorphisms to be associated with the development of Hodgkin's lymphoma. We also analyzed joint effect of genes by different combination to evaluate the synergistic effect on PCa. None of the combination of this polymorphism revealed significant association with PCa. However none of these polymorphisms were associated with bone metastasis. Further, we also investigated case only analysis with smoking status with these polymorphisms and observed no significant association.

Currently, it is not clear if altered TLR function should increase or decrease cancer risk. The discovery that TLRs mediate the enhanced immune responses induced by many immunoadjuvants has lead to the suggestion that increased TLR activation may stimulate anticancer immunity. Thus, enhanced TLR activity could inhibit carcinogenesis while decreased activity could allow cancer cells to escape immune detection and elimination. Alternatively, TLR activation may promote carcinogenesis by creating a proinflammatory environment that enhances tumor growth and chemo-resistance and/or result in chronic inflammation-induced immune suppression that allows cancer progression [27]. Further basic and clinical research into the effects of TLR activation will be necessary before making accurate predictions of how variation in TLR action will influence cancer risk.

Our study has several strengths like all the TLRs polymorphisms in our control subjects followed HWE, all PCa cases were histopathologically confirmed and the study subjects enrolled were of similar ethnicity; since both PCa patients as well as controls were of North Indian ethnicity, the possibility of population admixture was ruled out. Also stringent quality control and reproducible genotyping measures were used to minimize systematic errors. SNPs with a MAF of less than 5% were excluded from the study, as these were likely to be insufficiently powered to detect association. Limitation of the study is the small number of patients used due to low incident rate of PCa in our country. Though the sample numbers of our study was sufficient to achieve (>80%) power. Nevertheless, a higher number of samples are warranted for more modest results.

To further conclude, based on our results we suggest that the polymorphism (−196 to −174 Ins/Del) in TLR2 gene seems to be associated with an increased risk of PCa. Given the complex nature of PCa, further study of these SNPs in additional populations as well as exploration of the effect of TLR activation on carcinogenesis is needed to determine the role of TLR SNPs in PCa risk.

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Conflict of interest None.

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